Cholesterol and Cholesterol Esters in Mycoplasma

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SUMMARY

Free and esterified cholesterol were determined in the lipid extract of nine parasitic and saprophytic *Mycoplasma* strains. The saprophytic strains were usually free from esterified cholesterol. Esterified cholesterol was found in the parasitic strains at a ratio of 1:8 to 1:3 to free cholesterol; in the growth medium the ratio was about 4:1. [4-¹⁴C]-cholesterol added to the growth medium was incorporated by all *Mycoplasma* strains without esterification or any other chemical change. [4-¹⁴C]-cholesteryl oleate was incorporated into the lipid of the parasitic strains and was not hydrolysed to yield free cholesterol. The small amounts of radioactive oleic acid incorporated by several *Mycoplasma* strains during growth was not detected in the cholesteryl ester fraction. Our results show that under the growth conditions examined, the *Mycoplasma* organisms tested were unable to esterify cholesterol or hydrolyse cholesteryl esters.

INTRODUCTION

Most *Mycoplasma* strains have been found to require cholesterol or closely related sterols for growth (Edward & Fitzgerald, 1951; Smith & Lynn, 1958; Rodwell & Abbot, 1961; Rodwell, 1963; Smith, 1964). Only the saprophytic *Mycoplasma laidlawii* strains do not have this requirement (Edward & Freundt, 1956; Razin & Knight, 1960; Rothblat & Smith, 1961; Razin & Cohen, 1963). So far no other group of bacteria is known to require sterols for growth (Razin, 1962). Cholesterol in the growth medium was found to be incorporated in appreciable quantities into the lipid fraction of the *Mycoplasma* cell (Smith & Rothblat, 1960; Rothblat & Smith, 1961; Smith, 1962; Smith, 1963*a*; Rodwell, 1963; Tourtellotte, Jensen, Gander & Morowitz, 1963). Most of the incorporated cholesterol was found in the cell membrane (Rothblat & Smith, 1961; Razin, Argaman & Avigan, 1963). When grown in a cholesterol-deficient medium *Mycoplasma mycoides* showed severe morphological changes, apparently due to faulty synthesis of cell membrane (Rodwell & Abbot, 1961). Thus, it is plausible that cholesterol is essential for the integrity and regular functioning of the delicate cell membrane of *Mycoplasma* (Razin, 1963*a*). Smith (1963*a*) suggested that cholesterol fulfils a bifunctional role: maintenance of the structural integrity of the cell membrane, and as a carrier of substrates and metabolic end-products across the cell membrane. To elucidate the role played by cholesterol in the physiology of *Mycoplasma*, it seemed worth while to investigate the fate of cholesterol incorporated by various *Mycoplasma* strains. The problems dealt with were: (a) is the incorporated

cholesterol esterified or metabolized in any way? (b) are *Mycoplasma* organisms able to incorporate cholesteryl esters from the growth medium? (c) are *Mycoplasma* organisms able to hydrolyse cholesteryl esters to yield free cholesterol?

METHODS

Organisms. *Mycoplasma mycoides* var. *mycoides* (PG 1), *M. mycoides* var. *capri* (PG 3), *M. agalactiae* (PG 2), *M. neurolyticum* (PG 28), *M. laidlawii* strain A (PG 8) and *M. laidlawii* strain B (PG 9) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). A strain of *M. gallisepticum* named (R) was provided by Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovoth, Israel). Strain s6 of *M. gallisepticum* was obtained from Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *M. laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity; this strain was serologically related to *M. laidlawii* strain A (Argaman, unpublished observations).

Growth medium and cultivation of organisms. The organisms were grown in a modified liquid Edward medium (Razin, 1963b) which contained 2% (v/v) Difco-PPLO serum fraction. The radioactive compounds incorporated into the growth medium included [4-¹⁴C]-cholesterol with a specific activity of 24 mC/mmmole; [4-¹⁴C]-cholesteryl oleate with a specific activity of 20 mC/mmmole and [U-¹⁴C]-oleic acid with a specific activity of 94 mc/mmmole (all products obtained from the Radiochemical Centre, Amersham, England). A benzene solution containing 1 μ C of the radioactive compound was evaporated to dryness under a stream of nitrogen and immediately re-dissolved in a few drops of ethanol. The ethanolic solution was then thoroughly mixed with 20 ml. Difco-PPLO serum fraction and added to 1 l. of growth medium. Fifty ml. of a young culture of the organisms in Edward medium were inoculated into 500 ml. medium in 1 l. Roux flasks, which were incubated at 37° for 24–48 hr in an inclined position to improve aeration. The organisms were harvested by centrifugation at 16,000 g for 5 min., washed twice in 0.25 M-NaCl and the sedimented packed organisms freeze-dried.

Extraction of lipids. Lipids were extracted from the freeze-dried organisms by chloroform + methanol (2 + 1, by vol.). Extraction was carried out at 60° for 30 min. under reflux in a nitrogen atmosphere. The extract was separated from the organisms by centrifugation and washed with water according to Folch, Lees & Sloane-Stanley (1957). The solvent was then evaporated to dryness by heating under a stream of nitrogen, and the lipid residue freeze-dried and weighed. To test the efficiency of the lipid-extraction procedure the extracted organisms were hydrolysed in 2 N-HCl at 90–100° for 15 hr in sealed ampoules. The hydrolysate was extracted three times with diethyl ether. The solvent was evaporated and radioactivity of the residue determined. Lipids were also extracted from freeze-dried Difco-PPLO serum fraction, and from the other dry constituents of Edward medium for the determination of their cholesterol content. Extraction was carried out by chloroform + methanol as described above.

Determination of cholesterol and its esters

Chromatographic separation. Cholesterol and cholesteryl esters in the lipids from the *Mycoplasma* organisms and in the constituents of the growth medium were separated by thin-layer chromatography (Razin *et al.* 1963; Avigan, Goodman & Steinberg, 1963). Chromatoplates were prepared by using a slurry containing 50 ml. of a 0.1% (w/v) aqueous solution of Rhodamine 6 G and 25 g. silica gel G (E. Merck A. G., Darmstadt, Germany). The lipid to be chromatographed was dissolved in a small volume of benzene and applied to the plates as bands. Chromatography was carried out at room temperature with developing solvent a mixture of benzene + ethyl acetate (5 + 1, by vol.). Running time was about 1 hr, during which the solvent front had advanced 12–15 cm. Cholesterol and cholesteryl ester zones were visible after chromatography under ultraviolet radiation as pink-yellow fluorescent areas on a pale green background. Cholesteryl esters migrated with the solvent front, whereas the R_f value of cholesterol was about 0.5.

The same chromatoplates were used for separation of free oleic acid from cholesteryl esters, but the solvent was *n*-hexane + diethyl ether + acetic acid + methanol (90 + 20 + 3 + 2, by vol.; Brown & Johnson, 1962). Oleic acid had a lower R_f value than cholesteryl esters.

The zones of cholesterol, cholesteryl esters and oleic acid to be recovered, and the zones in between were scraped off with a spatula into test tubes and the lipids eluted by extracting twice with 2 ml. chloroform. Two-thirds of the chloroform extract were taken for radioactivity measurements, while the remaining part was used for the colorimetric determination of cholesterol and its esters.

The purity of the cholesterol fraction separated on the chromatoplate by benzene + ethyl acetate (5 + 1, by vol.) was proved by re-chromatography of this fraction, with benzene + ethyl acetate (20 + 1, by vol.) as solvent (Avigan *et al.* 1963).

Colorimetric determination of cholesterol. The chloroform from the extracts of free and esterified cholesterol zones of the chromatoplates was evaporated under a stream of nitrogen and the dry material dissolved in glacial acetic acid. The amount of cholesterol in solution was determined by the FeCl_3 reaction according to Wycoff & Parsons (1957). By this colorimetric method the recoveries of chromatographically separated cholesterol and its esters averaged 90%. The non-polar lipids migrating with the cholesteryl esters had little influence on the results of the FeCl_3 reaction. This was proved by saponification of the lipid in the cholesteryl ester zone, re-chromatography and determination of the amount of free cholesterol produced. The liberated cholesterol produced practically the same colour intensity as the chromatographic fraction before saponification.

Radioactivity measurements. The chloroform extracts placed in scintillation vials were evaporated by heating under a stream of air. Seven ml. of 0.3% (w/v) 2,5-diphenyloxazole (PPO, scintillation grade) and 0.1% (w/v) 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP, scintillation grade, Packard Instrument Co., Inc., Illinois, U.S.A.) in toluene were added to each vial by use of a syringe. ^{14}C -counting was done in a liquid scintillation counter model 6012A (Isotope Developments Limited, Reading, England).

Determination of cholesterol esterase activity. A slight modification of the method described by Smith (1959) was used. *Mycoplasma* organisms grown in Edward

medium for 24–48 hr were harvested and washed twice with 0.25 M-NaCl as above. The washed organisms were suspended in 0.06 M-phosphate buffer (pH 6.5) to give cell protein 5–10 mg./ml. The organisms suspension was distributed in 2.5 ml. quantities into test tubes. Cholesteryl acetate (5 or 10 μ moles) dissolved in 0.1 ml. acetone was injected into the suspension of mycoplasmas. The resulting emulsion was stable for at least 24 hr at 37° without the aid of an emulsifying agent. Controls without substrate or organisms were run simultaneously. The reaction mixtures and controls were incubated at 37° and 1.2 ml. samples drawn after 5 and 24 hr of incubation. To each sample 4 ml. chloroform + methanol (2 + 1, by vol.) were added, well mixed and centrifuged. The lower chloroform phase was separated, evaporated to dryness under nitrogen, the residue dissolved in benzene and chromatographed. Free and esterified cholesterol appearing on the chromatoplate were quantitatively determined as described above.

RESULTS

Free and esterified cholesterol in Mycoplasma organisms

Free and esterified cholesterol in lipid extracts of various *Mycoplasma* strains were separated by thin-layer chromatography. The carotenoid pigments of *Mycoplasma laidlawii* migrated with the solvent front. The lipids of all three *M. laidlawii* strains contained less free cholesterol than the lipids of the parasitic strains and were usually free from esterified cholesterol (Table 1). The parasitic strains contained various amounts of cholesteryl esters, usually $\frac{1}{3}$ – $\frac{1}{4}$ of the total cholesterol, except *M. mycoides* var. *capri* which consistently contained more esterified than free cholesterol.

Table 1. *Free and esterified cholesterol in lipid of Mycoplasma*

Cholesterol and cholesteryl esters were separated by thin-layer chromatography (Avigan *et al.* 1963), and determined by the FeCl₃ reaction (Wycoff & Parsons, 1957).

Organism	% (w/w) of total lipid					
	Total cholesterol		Free cholesterol		Esterified cholesterol	
	Mean	Range	Mean	Range	Mean	Range
<i>Mycoplasma agalactiae</i>	30.2	24.1–36.0	26.1	21.4–31.5	3.4	2.7–4.5
<i>M. gallisepticum</i> (R)	19.5	13.8–23.0	14.7	13.2–16.0	3.5	0.6–7.0
<i>M. gallisepticum</i> (S6)	21.3	16.2–23.4	16.0	14.5–18.0	5.3	2.0–6.4
<i>M. neurolyticum</i>	12.2	9.7–12.8	9.3	5.8–10.5	3.4	2.3–3.9
<i>M. mycoides</i> var. <i>mycoides</i>	17.9	17.7–18.0	13.4	10.1–15.0	4.8	3.0–7.6
<i>M. mycoides</i> var. <i>capri</i>	19.7	18.1–20.8	6.2	5.0–8.1	14.4	10.0–15.8
<i>M. laidlawii</i> strain A	2.9	1.7–3.1	2.8	1.7–3.1	0.1	0–0.4
<i>M. laidlawii</i> strain B	3.5	2.2–4.6	3.4	2.2–4.6	0.1	0–0.4
<i>M. laidlawii</i> oral strain	3.6	2.4–4.4	3.4	2.4–4.4	0.2	0–0.7

The appearance of cholesteryl esters in the lipids of *Mycoplasma* prompted us to examine the amount of free and esterified cholesterol in the growth medium. The only component of Edward medium found to contain appreciable amounts of cholesterol and its esters was the Difco-PPLO serum fraction. In several analyses free cholesterol 90–150 μ g. and esterified cholesterol 360–600 μ g./ml. were found. In all other ingredients only negligible amounts of cholesterol were present.

Incorporation of [4-¹⁴C]-cholesterol into the lipids of Mycoplasma

Radioactive cholesterol added to Edward medium was incorporated by all *Mycoplasma* strains grown in this medium. Radioactivity appeared almost exclusively in the free-cholesterol fraction of the *Mycoplasma* lipids (Table 2). No appreciable amount of radioactivity was found in the cholesteryl ester zone. Essentially all radioactivity was removed from the organisms by chloroform + methanol extraction. The radioactivity of the defatted organisms, as determined by hydrolysis and ether extraction, was negligible.

Table 2. *Incorporation of [4-¹⁴C]-cholesterol into lipids of Mycoplasma*

The organisms were grown in Edward medium containing 1 μ C [4-¹⁴C]-cholesterol/l. The data given in the table represent typical results obtained in several experiments using different batches of organisms.

Organism	Quantity of lipid examined (mg.)	Radioactivity (counts/min $\times 10^{-3}$)		Total radioactivity in cholesterol fraction (%)
		Total lipid	Free cholesterol fraction	
<i>Mycoplasma agalactiae</i>	4.2	459.0	457.3	99.6
<i>M. gallisepticum</i> (R)	12.4	399.7	397.6	99.3
<i>M. gallisepticum</i> (S6)	9.6	490.0	488.0	99.4
<i>M. neurolyticum</i>	10.4	489.0	487.0	99.4
<i>M. mycoides</i> var. <i>mycoides</i>	12.0	686.2	683.6	99.6
<i>M. mycoides</i> var. <i>capri</i>	48.0	318.5	315.0	99.0
<i>M. laidlawii</i> strain A	11.1	263.0	261.0	99.2
<i>M. laidlawii</i> strain B	20.2	182.0	180.5	99.1
<i>M. laidlawii</i> oral strain	21.4	232.3	231.2	99.4
[4- ¹⁴ C]-cholesterol 0.1 μ C	—	204.0	202.0	99.0

Table 3. *Incorporation of [4-¹⁴C]-cholesteryl oleate into lipids of Mycoplasma*

The growth medium contained 1 μ C [4-¹⁴C]-cholesteryl oleate/l.

Organism	Quantity of lipid examined (mg.)	Radioactivity (counts/min $\times 10^{-3}$)			Total radioactivity in cholesteryl ester fraction (%)
		Total lipid	Cholesterol fraction	Cholesteryl ester fraction	
<i>Mycoplasma agalactiae</i>	4.0	72.4	0.7	71.0	98.0
<i>M. gallisepticum</i> (R)	6.0	107.9	0.93	106.6	98.7
<i>M. mycoides</i> var. <i>mycoides</i>	13.0	291.1	0.6	289.5	99.2
<i>M. mycoides</i> var. <i>capri</i>	13.7	153.7	1.0	152.2	99.0
<i>M. laidlawii</i> strain A	11.6	4.5	0.3	3.9	86.6
<i>M. laidlawii</i> strain B	12.6	4.8	0.0	4.7	98.3
<i>M. laidlawii</i> oral strain	11.5	0.9	0.0	0.5	62.2
[4- ¹⁴ C]-cholesteryl oleate, 0.1 μ C	—	239.4	0.6	238.0	99.4

Incorporation of [4-¹⁴C]-cholesteryl oleate into the lipids of Mycoplasma

Radioactive cholesteryl oleate, added to the growth medium, was incorporated into the lipids of the parasitic strains; the saprophytic strains incorporated only minute amounts of the radioactive compound. Radioactivity was limited to the cholesteryl ester fraction of the Mycoplasma lipids; no radioactivity was detected in the free-cholesterol fraction (Table 3). This showed the inability of the Mycoplasma strains tested to hydrolyse cholesteryl oleate. Washed suspensions of *Mycoplasma mycoides* var. *mycoides*, *M. mycoides* var. *capri*, *M. gallisepticum* (R) and *M. laidlawii* strains were unable to hydrolyse cholesteryl acetate. Extracts of *M. gallisepticum* strains did not hydrolyse cholesteryl acetate or cholesteryl stearate when tested at several values from pH 6.5 to 9.0 (see Rottem & Razin, 1964).

Table 4. *Incorporation of [U-¹⁴C]-oleic acid into lipids of Mycoplasma*

The growth medium contained 1 μ C [U-¹⁴C]-oleic acid/l.

Organism	Quantity of lipid examined (mg.)	Radioactivity (counts/min)			
		Total lipid	Chromatographic fractions		
			Cholesteryl ester fraction	Free oleic acid fraction	All other fractions
<i>Mycoplasma mycoides</i> var. <i>capri</i>	7.2	11,670	120	10,800	740
<i>M. laidlawii</i> oral strain	17.6	3,050	20	480	2,600
<i>M. laidlawii</i> oral strain*	23.0	8,150	100	2,850	5,200
[U- ¹⁴ C]-oleic acid 0.1 μ C	—	175,250	200	172,000	3,050

* Grown in Edward medium without Difco PPLO-serum fraction.

Incorporation of [U-¹⁴C]-oleic acid into the lipids of Mycoplasma

The saprophytic *Mycoplasma laidlawii* and the parasitic *M. mycoides* var. *capri* were tested for ability to incorporate radioactive oleic acid from the growth medium. Table 4 shows that these organisms incorporated small amounts of the radioactive compound. Radioactivity was not found in the cholesteryl ester fraction, indicating the inability of these strains to esterify cholesterol. A portion of the radioactive oleic acid incorporated by the organism was found at the starting line of the chromatoplates, indicating its presence in polar lipids, probably phospholipids. Omission of Difco-PPLO serum fraction from the growth medium of *M. laidlawii* did not have a significant effect on the incorporation of the radioactive oleic acid (Table 4).

DISCUSSION

The much lower cholesterol content of saprophytic than parasitic Mycoplasma strains noted previously (Razin *et al.* 1963) has been confirmed in the present study. The saprophytic mycoplasmas were also found to differ from the parasitic strains in being practically unable to incorporate cholesteryl esters from the growth medium. The saprophytic *Mycoplasma laidlawii* strains which do not require

sterols for growth (Razin & Knight, 1960; Rothblat & Smith, 1961; Razin & Cohen, 1963) synthesize appreciable amounts of carotenoids localized in the cell membrane, (Razin *et al.* 1963; Smith, 1963*b*). The hydrocarbon structure of the carotenoids bears some resemblance to the structure of cholesterol (Smith, 1963*a*). It is a reasonable speculation that the carotenoids occupy the same sites in the membranes of the saprophytic mycoplasmas as does cholesterol in the membranes of parasitic strains. The carotenoids may thus interfere with the uptake of cholesterol and its esters by the saprophytic strains (see also Smith, 1963*b*).

Our experiments with [4-¹⁴C]-cholesterol confirmed previous findings (Smith & Boughton, 1960; Rodwell, 1963; Tourtellotte *et al.* 1963) which suggested that the cholesterol found in *Mycoplasma* lipids originates in the growth medium. Our results show, however, that the incorporated cholesterol does not undergo esterification, glycosidation or any other chemical change. This is in accordance with the results reported by Rodwell (1963) for the incorporation of cholesterol by two *Mycoplasma mycoides* strains.

The cholesteryl esters found in the lipids of parasitic *Mycoplasma* are apparently incorporated as such from the growth medium and are not formed *de novo* by the organisms. The ability of mycoplasmas to incorporate cholesteryl esters is much lower than their ability to incorporate free cholesterol. Thus, in spite of the much higher concentration of cholesteryl esters in the growth medium, the amount of free cholesterol in the lipids of the parasitic *Mycoplasma*, except *Mycoplasma mycoides* var. *capri*, exceeded that of esterified cholesterol (see also Morowitz *et al.* 1962). The incorporated labelled-cholesteryl oleate was not hydrolysed by any of the *Mycoplasma* strains tested. Neither could we demonstrate cholesterol esterase activity in washed organisms or their extracts, by using cholesteryl acetate or stearate as substrates (see also Rottem & Razin, 1964). These results are in accordance with the inability of several sterol-requiring *Mycoplasma* strains to utilize the acetate, stearate or oleate esters of cholesterol for growth (Edward & Fitzgerald, 1951).

Smith (1959) described both cholesterol ester-hydrolysing and ester-synthesizing activities in several *Mycoplasma* strains which require cholesterol for growth. The enzymic formation of a cholesteryl glucoside by several strains capable of glucose utilization was also described (Rothblat & Smith, 1961). Smith (1963*a*) suggested that the function of these enzymic activities might be to mediate the transfer of glucose and short-chain fatty acids across the cell membrane. Our results indicate that cholesterol may fulfil its still unknown physiological function without being esterified or modified in any other way.

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