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Membrane Lipids of *Mycoplasma hominis*

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Essentially all of the lipids of *Mycoplasma hominis* (200 μg/mg of cell protein) were found to be located in the cell membrane. Over one-half were neutral lipids incorporated from the growth medium and consisting of 43% free cholesterol, 19% esterified cholesterol, 23% triglycerides, 10% free fatty acids, and small amounts of di- and monoglycerides. The polar lipids accounting for about 40% of the total were synthesized by the organisms. Phosphatidylglycerol was the predominant lipid of this fraction. The minor components, tentatively identified as lysophosphatidylglycerol and phosphatidic acid, seem to represent breakdown products of phosphatidylglycerol. No glycolipids were detected. Being unable to synthesize long-chain fatty acids, *M. hominis* utilized the fatty acids of the growth medium for polar lipid synthesis, preferentially the saturated ones, so that the polar lipids had highly saturated hydrocarbon chains. It is proposed that the large take up of unsaturated neutral lipids and cholesterol from the medium offsets the marked condensing effect of the saturated polar lipids, although electron paramagnetic resonance spectrometry of spin-labeled fatty acids incorporated into the *M. hominis* membrane indicated that the lipid region is still more rigid than that of the *Acholeplasma laidlawii* membrane.

The use of mycoplasma membranes for investigating the role of lipids in biological membranes has so far been largely confined to the cell membrane of the sterol-nonrequiring *Acholeplasma laidlawii* mainly because of the ease of its isolation (6, 11, 17, 26, 28; S. Razin. *In* A. H. Rose and D. W. Tempest, ed., Advances in microbial physiology, *in press*). Our recent finding that the cell membrane of the sterol-requiring *Mycoplasma hominis* can also be easily isolated by osmotic lysis or by digitonin treatment (20) offers an alternative which may be preferable for studies in which the role of cholesterol is to be examined. To this end, information on the lipid components of the *M. hominis* membrane is supplied by the present communication. A comparison of the *A. laidlawii* and *M. hominis* membrane lipids is also provided, indicating some differences in their composition and physical state.

MATERIALS AND METHODS

Organism and growth conditions. *M. hominis* (ATCC 15056) was grown in 2- to 5-liter volumes of Edward medium (13) adjusted to pH 6.5 and supplemented with 2% PPLO serum fraction (Difco) and 20 mM L-arginine. For labeling the membrane lipids, 0.5 μCi of oleic acid-*1*-^{14}C (59.7 mCi/mmole), 0.5 μCi of palmitic acid-*1*-^{14}C (55 mCi/mmole), or 20 μCi of cholesterol-*4*-^{14}C (55.8 mCi/mmole; The Radiochemical Centre, Amersham, England) was added to each liter of the growth medium. The organisms were harvested after 16 to 20 hr of incubation at 37 C and were washed twice in 0.25 M NaCl.

Isolation of cell membranes. Cell membranes were isolated by osmotic lysis of the organisms (20). The membranes were collected by centrifugation at 34,000 × *g* for 30 min, washed twice in deionized water and resuspended in β-buffer (12) diluted 1:20 with deionized water (dilute β-buffer).

Lipid extraction. Lipids were extracted from freeze-dried cells or membranes by two successive extractions with chloroform-methanol (2:1, v/v), the first at 45 C for 2 hr and the second at room temperature overnight. The extracts were combined and washed according to Folch et al. (7), dried under a stream of nitrogen, and redissolved in 1 ml of chloroform.

Separation of neutral from polar lipids. Neutral lipids were separated from polar lipids by silicic acid chromatography. The lipid extracts were applied to columns (6 by 100 mm) of activated silicic acid (100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.) prewashed with chloroform. Elution was carried out successively with 50 ml of chloroform, 50 ml of acetone, and 50 ml of chloroform-methanol (1:1, v/v) to separate the neutral lipids, glycolipids, and polar lipids (23). The lipid fractions were dried under a stream of nitrogen, weighed, and dissolved in 0.5 to 1.0 ml of chloroform-methanol (2:1).

Thin-layer chromatography. The neutral and polar lipid fractions were chromatographed on silica gel H plates (0.5 mm thick) containing 0.2% $NaHCO_3$. For neutral lipids, the developing systems were benzene-diethylether-ethanol-acetic acid (50:40:2:0.2, v/v) and hexane-diethylether (94:6, v/v); for polar lipids, chloroform-methanol-water (65:24:4, v/v) and chloroform-methanol-acetic acid-water (80:13:8:0.3, v/v) were used. The plates were washed with the developing solvent before activation. Lipid spots were detected by iodine vapor, phospholipid spots by the molybdate spray reagent (4), and α-glycol-containing lipids by the Schiff-periodate reagent (22). In some experiments, the lipid spots were scraped off the plate into scintillation vials containing 10 ml of a dioxane-toluene scintillation liquor (21), and radioactivity was measured in a Packard Tri-Carb scintillation spectrometer.

For the gravimetric determination of the various lipids, 50-mg samples were applied as bands on the chromatography plates. After chromatography, the separated bands were scraped off and the lipids were eluted by two or three extractions with chloroform-methanol (2:1). The extracts were filtered through glass wool, dried under nitrogen, and weighed. The data given in the Results represent an average of three analyses on different batches of lipids.

Gas-liquid chromatography. Methyl esters of the fatty acids were prepared by heating the lipid samples for 18 hr in anhydrous methanol containing 10% (w/w) HCl at 72 C in a sealed Pyrex ampoule. The resultant methyl esters were extracted with light petroleum ether (boiling point, 40 to 60 C) and subjected to gas-liquid chromatography in a Packard model 840 instrument equipped with a polar column (200 by 0.3 cm, 15% of diethylene glycol adipate on Chromosorb W). Fatty acids were identified by their retention time relative to that of standard methyl ester mixtures (Supelco, Inc. Bellefonte, Pa.).

Paramagnetic resonance spectroscopy. Membranes were spin-labeled with *N*-oxyl-4',4'-dimethyloxazolidine derivatives of 5-ketostearic acid and 12-ketostearic acid (Syva, Palo Alto, Calif.) by exchange from bovine serum albumin as previously described (17). Electron paramagnetic resonance spectra of the spin-labeled membranes were obtained by use of a Varian E-4 spectrometer. The hyperfine splitting (2Tm) was measured to within ± 0.5 gauss.

Analytical methods. Protein was determined according to Lowry et al. (9). Total phosphorus in the lipid fractions was determined by the method of Ames (1) after digestion of the sample with an ethanolic solution of $Mg(NO_3)_2$. Acyl ester bonds were determined according to Stern and Shapiro (27) with tripalmitin as standard. Total cholesterol was determined by the $FeCl_3$ method (29). Free cholesterol was separated from cholesterol esters by precipitation with digitonin (5).

RESULTS

Lipid classes of M. hominis. *M. hominis* contained about 200 μg of lipid per mg of cell protein. Analysis of the lipid content of the cell fractions obtained by osmotic lysis of the organisms showed the lipids to be located exclusively in the cell membrane. About 60% of the cell lipids subjected to silicic acid chromatography were eluted from the column with chloroform and can therefore be classified as neutral lipids; the residual lipids were eluted with chloroform-methanol (1:1) and hence constitute polar lipids. Acetone failed to elute any lipids from the silicic acid column, and thus no glycolipids were present.

Incorporation of labeled fatty acids into M. hominis lipids. Table 1 shows that *M. hominis* incorporated much more labeled palmitic acid than oleic acid from the growth medium. The difference was most pronounced in the polar lipid fraction, where the radioactivity resulting from ^{14}C-palmitic acid incorporation was over 10 times that due to ^{14}C-oleic acid incorporation. The percentage of labeled fatty acids incorporated into the neutral lipid fraction, though much smaller than that incorporated into the polar lipids, was still significant, especially the percentage of oleic acid (Table 1). In *A. laidlawii* or *M. arginini* under similar growth conditions, about 99% of either ^{14}C-palmitic acid or ^{14}C-oleic acid was incorporated into the polar lipid fraction. The possibility that the much higher degree of labeling with ^{14}C-palmitic acid was due to the lower free palmitic acid content of the growth medium could be ruled out by showing that the medium actually contained more palmitic than oleic acid (Table 6). The difference in the proportions of ^{14}C-palmitic acid and ^{14}C-oleic acid incorporated, moreover, persisted also when the organisms were grown in a medium supplemented with increasing concentrations (up to 10 μg/ml) of both unlabeled palmitate and unlabeled oleate.

The addition to the growth medium of 10 μCi of either ^{14}C-glucose (55 mCi/mmole) or ^{14}C-acetate (15.7 mCi/mmole) per liter did not result in any significant labeling of *M. hominis* lipids.

Neutral lipids of M. hominis. A quantitative analysis of the neutral lipid fraction of *M. hominis* is presented in Table 2. The unusually high content of free fatty acids and of glycerides should be noted. When the organisms were grown with radioactive fatty acids, the label was found almost exclusively in the free fatty acid fraction (Table 2). Free and esterified cholesterol constituted about 60% of the neutral lipid fraction, or about 40% of the total membrane lipids. The molar ratio of free to esterified cholesterol was 2:1. By decreasing the level of PPLO serum fraction in the growth

TABLE 1. *Incorporation of radioactive palmitic and oleic acids into M. hominis lipids*

^{14}C-fatty acid supplement[a]	Total lipid		Neutral lipid		Polar lipid	
	Amt (mg)	Radioactivity (counts/min)	Amt (mg)	Radioactivity (counts/min)	Amt (mg)	Radioactivity (counts/min)
Palmitic acid	17.3	630,000	10.1	25,200	6.7	600,000
Oleic acid	18.0	72,000	10.3	19,100	7.1	50,400

[a] The organisms were grown in Edward medium supplemented with 0.5 μCi of either palmitic acid-*1*-^{14}*C* (55 mCi/mmole) or oleic acid-*1*-^{14}*C* (59.7 mCi/mmole) per liter.

TABLE 2. *Incorporation of labeled palmitic and oleic acids into the various components of the neutral lipid fraction of M. hominis*

Component	Percentage of neutral lipid fraction (by wt)	Percentage of total radioactivity in neutral lipids	
		Cells grown with oleic acid-*1*-^{14}*C*	Cells grown with palmitic acid-*1*-^{14}*C*
Free fatty acids	10.3	89.8	96.7
Monoglycerides	1.1	1.0	0.2
Diglycerides	3.3	4.6	1.3
Triglycerides	23.3	2.5	0.6
Cholesterol	42.8	0.0	0.0
Cholesterol esters	19.2	1.8	0.0

TABLE 3. *Cholesterol content of M. hominis cells grown with various concentrations of serum fraction*

PPLO serum fraction in medium (%)	Cell yield[a]	Total cholesterol[b]
2.0	32.6	91.2
1.0	22.2	73.9
0.5	14.0	61.9
0.2	5.2	53.0

[a] Expressed as milligrams of cell protein per liter.
[b] Expressed as micrograms per milligram of cell protein.

TABLE 4. *Fatty acid composition of the esterified cholesterol fraction isolated from M. hominis and from PPLO serum fraction*

Cholesterol esters of	Fatty acid				
	14:0[a]	16:0	18:0	18:1	Others
M. hominis	0.5[b]	38.2	34.0	20.0	7.0
PPLO serum fraction	1.4	42.7	31.0	21.9	3.0

[a] The first number indicates chain length and the second indicates the number of double bonds.
[b] Percentage of total peak area.

medium, the cholesterol content of the cells could be brought down to 60% of its initial level without any change in the free to esterified cholesterol ratio (Table 3). The lowering of the PPLO serum fraction content was, however, accompanied by a marked decrease in cell yield. Growth was not improved by the addition of bovine serum albumin (10 mg/ml) together with palmitate (1 to 5 μg/ml) or oleate (1 to 5 μg/ml) or both.

The addition of ^{14}C-cholesterol (20 μCi/liter) to the growth medium resulted in intensive labeling of the cells (about 200,000 counts per min per mg of cell protein). Over 99% of the radioactivity was found in the free cholesterol fraction, so that the cholesterol incorporated from the growth medium was not esterified or otherwise changed by the organisms. Moreover, the fatty acid composition of the cholesterol ester fraction of *M. hominis* closely resembled that of the PPLO serum fraction (Table 4), supporting the thesis that the cholesterol esters found in *M. hominis* originate from the growth medium (14).

Polar lipids of M. hominis. The characteristics of the polar lipid components of *M. hominis* and their tentative identification are given in Table 5. Over 90% of the radioactivity in the polar lipid fraction derived from the ^{14}C-palmitic acid in the growth medium was recovered in a single lipid spot having an R_F of 0.58 on silica gel plates developed with chloroform-methanol-water (65:25:4) and an R_F of 0.38 when the plates were developed with chloroform - methanol - acetic acid - water (80:13:8:0.3). This lipid, which constituted over 80% of the total polar lipid fraction, could be identified as phosphatidylglycerol. The identification was based on its migration on the chromatography plates coinciding with that of known preparations of phosphatidylglycerol, the characteristic purple color appearing immediately on spraying with the periodate-Schiff reagent (22), and on the 2:1 fatty acid to phosphorus ratio.

Of the two other phospholipids of *M. hominis*, one (component A in Table 5) appeared to be a lysophosphatidylglycerol-like compound, and the other (component C) resembled phosphatidic acid. The labeling with ^{14}C-palmitic

acid of components A and C was about the same in lipid extracts from whole cells and freshly isolated membranes, but when the membrane preparation was incubated at room temperature for 24 hr the labeling of component C increased about 10-fold and that of component A by about 25% with a concomitant decrease in the labeling of phosphatidylglycerol. These observations suggest the presence of phospholipases in the membranes.

Fatty acid composition of M. hominis lipids. Marked differences were observed in the fatty acid composition of the neutral and polar lipid fractions (Table 6). Whereas the polar lipid fraction contained more saturated fatty acids, the ratio of saturated to unsaturated fatty acids amounting to 4.5, the reverse was true for the neutral lipid fraction. Both fractions, however, varied considerably from the growth medium in their fatty acid composition (Table 6).

Molecular motion of spin-labeled fatty acids in M. hominis membranes. The physical state of *M. hominis* membrane lipids was investigated by electron-paramagnetic resonance spectroscopy of spin-labeled fatty acids incorporated into the membrane. Figure 1 shows that the hyperfine splitting (2Tm) of the incorporated spin-labeled fatty acids depended on temperature and the position of the nitroxide group on the fatty acid hydrocarbon chain, decreasing as the nitroxide group moved from the polar end of the molecule. Thus, the *N*-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid [spin label I (12,3)] whose nitroxide radical is on carbon 5 of the fatty acid chain showed a much higher hyperfine splitting than did the *N*-oxyl-4',4'-dimethyloxazolidine derivative of 12-ketostearic acid, whose nitroxide radical is on carbon 12. These data support the suggestion (17) that the local environment of the spin-labeled fatty acids in the mycoplasma membrane is an associate lipid structure having the properties of a bilayer. Figure 1 also shows the remarkable differences in the hyperfine splitting (2Tm) of the spin-labeled

TABLE 5. *Properties and tentative identification of the polar lipids of M. hominis*

Component[a]	R_f[b]	Percentage of total polar lipids (by wt)	Percentage of total radioactivity in polar lipids[c]	Moles of fatty acid ester/g atom of P	Molybdate reaction	Periodate-Schiff reaction	Tentative identification
A	0.29	7.0	5.2	1.2	+	+	Lysophosphatidylglycerol
B	0.58	83.0	93.0	2.0	+	+	Phosphatidylglycerol
C	0.79	1.0	1.6	2.1	+	−	Phosphatidic acid
D	0.90	8.4	0	−	−	−	Unknown

[a] The ninhydrin reaction was negative for all components.
[b] Developing solvent system, chloroform-methanol-water (65:25:4, v/v).
[c] Organisms were grown with palmitic acid-*1*-^{14}C.

TABLE 6. *Fatty acid composition of M. hominis and the growth medium lipids*

Fatty acid	*M. hominis* lipids			Medium lipids[a]	
	Total lipids	Polar lipids	Neutral lipids	Total lipids	Free fatty acids
12:0[b]	0.2[c]	0.1	0.1	0.1	0.1
14:0	1.1	1.5	0.1	1.4	1.5
16:0	39.7	44.9	17.7	22.8	20.1
16:1	1.6	0.4	0.9	1.6	11.6
17:0	1.2	1.8	0.1	0.1	0.1
18:0	18.8	33.5	6.5	19.1	3.2
18:1	10.1	13.2	8.6	18.7	14.2
18:2	27.3	4.6	66.1	34.4	48.3
Sat'd/unsat'd[d]	1.56	4.49	0.33	0.82	0.35

[a] The dry ingredients of Edward medium (13) were extracted with chloroform-methanol (2:1). Free fatty acids were separated by thin-layer chromatography as described in Materials and Methods.
[b] The first number indicates chain length and the second the number of double bonds.
[c] Percentage of total peak area.
[d] Ratio of saturated to unsaturated fatty acids.

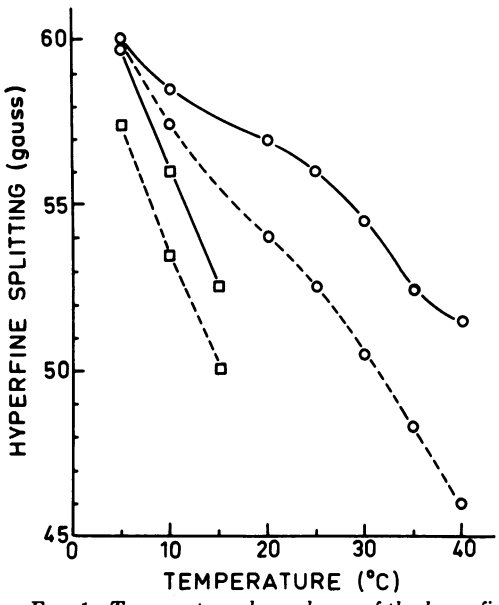

FIG. 1. *Temperature dependence of the hyperfine splitting (2Tm) of spin-labeled fatty acids incorporated into mycoplasma membranes. Symbols: O, spin label I (12,3); □, spin label I (5,10). Solid lines, M. hominis membranes; broken lines, A. laidlawii membranes.*

fatty acids incorporated into *M. hominis* and into *A. laidlawii* membranes. The higher 2Tm values observed with *M. hominis* membranes indicate that the mobility of the hydrocarbon chains of the spin-labeled fatty acids is more restricted, especially at higher temperatures. The hyperfine splitting of the spin-labeled fatty acids in *M. hominis* membranes was not affected when the cholesterol content of the membranes was reduced as much as 40% by growing the organisms with a lower concentration of PPLO serum fraction (see Table 3).

DISCUSSION

Several important differences emerge from the comparison of the lipid composition of *M. hominis* and *A. laidlawii* membranes. Whereas in *A. laidlawii* neutral lipids (excluding glycolipids) constitute a minor fraction (10%) of the membrane lipids (23), they are the major lipid fraction in the *M. hominis* membrane. This is because of the much higher cholesterol content of *M. hominis*, as is to be expected from a sterol-requiring mycoplasma (see Argaman and Razin [2]), and because of the large quantities of glycerides and free fatty acids present in the *M. hominis* membrane but not in the *A. laidlawii* membrane (25). Since the addition of radioactive oleic or palmitic acid to the growth medium failed to label the cholesterol esters and the glycerides, they do not seem to be synthesized by the organisms and are incorporated as such from the growth medium.

The finding of relatively large amounts of free fatty acids was somewhat unexpected, as no significant amount of free fatty acids was found in the membrane lipids of *A. laidlawii*, *M. gallinarum* strain J, and *M. arthritidis* strain 07 (25). Recently, however, undetermined but significant amounts of free fatty acids were found in lipid analyses of a T mycoplasma (16) and *A. axanthum* (25). The origin of the free fatty acids in the *M. hominis* membrane is not clear. They may possibly be the products of membrane phospholipid hydrolysis by one or several enzymes associated with the *M. hominis* membrane, though their undiminished presence under conditions minimizing endogenous phospholipase activity, such as extraction of the lipids directly from whole cells instead of membranes, and the very low quantities of the lyso compound found under normal conditions of membrane isolation render it more likely that most of them are incorporated as such from the growth medium. Like other sterol-requiring *Mycoplasma* species, but unlike *A. laidlawii* (19), *M. hominis* appears to be unable to synthesize long-chain fatty acids, as indicated by the inability to utilize ^{14}C-acetate for lipid synthesis and the absence of acyl carrier protein (18).

The dominant lipid in the polar lipid fraction of *M. hominis* was identified as phosphatidylglycerol, a major phospholipid of *A. laidlawii* and other mycoplasmas (25). No diphosphatidylglycerol could be detected in *M. hominis*. The minor phospholipids of the polar lipid fraction, tentatively identified as lysophosphatidylglycerol and phosphatidic acid, seem to represent breakdown products of phosphatidylglycerol.

The nonfermentative *M. hominis* also differs from the fermentative *A. laidlawii* in its lack of glycolipids. This appears to substantiate the thesis that nonfermentative mycoplasmas do not synthesize glycolipids (25), although trace amounts of glycolipids have been recently detected in a T-strain mycoplasma classified as nonfermentative (16, 24).

The radical difference in the fatty acid composition of the neutral and polar lipid fractions of *M. hominis* may perhaps be regarded as the most salient finding. Whereas in the polar lipids the saturated fatty acids were predominant, the unsaturated fatty acids predominated in the neutral lipid fraction of the cells and in the lipids of the growth medium. Hence, *M. hominis* makes a preferential use of satu-

rated fatty acids for polar lipid synthesis, which accounts for the much higher labeling of the cells when grown with ^{14}C-palmitate than with ^{14}C-oleate. *A. laidlawii*, on the other hand, incorporates considerable amounts of both types of fatty acids into its polar lipids (10, 15).

The physical state of the lipid region of the mycoplasma membrane depends largely on the chain length and the degree of unsaturation of the fatty acid residues of the membrane lipids (14, 26). How the organism controls the fluidity of the lipid region under different physiological conditions is still not clear (S. Razin. *In* A. H. Rose and D. W. Tempest, ed., Advances in microbial physiology, in press). The highly saturated nature of the hydrocarbon chains of the polar lipids of *M. hominis* might be expected to raise the phase transition temperature and reduce the fluidity of the lipid region of the membrane to a level liable to affect permeability and growth under physiological temperatures. The large quantities of unsaturated neutral lipids and cholesterol incorporated into *M. hominis* membranes during growth may, however, help to offset the condensing effect of the saturated polar lipids and to produce the lipid region fluidity essential to proper membrane function. Cholesterol has in fact been shown to prevent the crystallization of lipid membranes made of highly saturated phospholipids (3, 8). Nevertheless, the freedom of motion of spin-labeled fatty acids incorporated into *M. hominis* membranes was much lower than in *A. laidlawii* membranes, indicating the closer packing of the lipids and the higher rigidity of the lipid backbone of the *M. hominis* membrane.

The possibility that the growth requirement of parasitic mycoplasmas for cholesterol is associated with the preferential synthesis of highly saturated complex lipids warrants further investigation.

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