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# Long-Chain Fatty Acid Perturbations in *Mycoplasma pneumoniae*

OFRA LEON AND CHARLES PANOS*

*Department of Microbiology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107*

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The fatty acid content of *Mycoplasma pneumoniae* increased 2.5- to 9.6-fold when the growth medium was supplemented with a saturated, unsaturated, or beta-hydroxy fatty acid, the greatest increase occurring with palmitic acid. The amount of each supplemented fatty acid found within this organism was 2.8 to 5.5% of the total fatty acid content; the exception was palmitic acid. Up to 57% of the palmitic acid was utilized from the supplemented medium, whereas only 0.2 to 10% of the other fatty acids was utilized. Chromatographic and isotopic analyses revealed that 22% of the labeled palmitic acid incorporated from the palmitic acid-supplemented medium remained free in this organism. Also, even though complex lipid synthesis increased a minimum of 3.8-fold under these conditions, this mycoplasma continued to incorporate intact complex lipids from the growth medium. Bacteriostatic and bactericidal studies which used high concentrations of various long-chain fatty acids showed that only palmitic, myristic, and beta-hydroxydecanoic acids were not bactericidal. The addition of palmitic acid to the growth medium resulted in the formation of exceedingly long, filamentous cells in approximately 25% of the population. Osmotic fragility and electron spin resonance spectroscopy studies showed a correlation among this increased fatty acid content, decreased membrane fluidity, and the increased osmotic fragility of palmitic acid-grown cells. In addition, these cells had a lowered cholesterol content. The effect of such compositional changes on osmotic fragility is discussed in this paper. Finally, the profound increase in the total fatty acid content of palmitic acid-grown cells altered neither sensitivity to tetracycline or erythromycin nor the amount of hydrogen peroxide secreted.

While information exists on the complex lipids of *Mycoplasma pneumoniae,* none of the *Mycoplasma* species studied is able to synthesize de novo or to elongate, desaturate, or oxidize long-chain fatty acids added to the growth medium (13, 24). This metabolic deficiency is probably due to the lack of acyl carrier protein (19). However, such mycoplasmas do synthesize complex lipids (25). Sterol-requiring mycoplasmas are able to incorporate fatty acids from the growth medium, and this incorporation is not entirely indiscriminate (21). Likewise, these fatty acids, when incorporated into the phospholipids and glycolipids of the membrane, form a major portion of the hydrophobic core of the biomembrane (8). Therefore, the physical properties of this core are largely determined by fatty acid residues.

Nothing is known about the selection and incorporation of exogenous long-chain fatty acids into *M. pneumoniae* or the subsequent effects of the acids upon the lipid composition, physiology, morphology, and osmotic fragility of the organism. Also, correlations among cellular lipid modifications, resistance to antibiotics, and hydrogen peroxide secretion by this human pathogen are unknown. These studies were undertaken to fill these voids.

(This investigation was presented in part, in preliminary form, at the 3rd Conference of the International Organization for Mycoplasmology, 3 to 9 September 1980, Custer, S. Dak.)

## MATERIALS AND METHODS

**Organism, growth conditions, and assessment of growth.** A glass-adhering strain of *M. pneumoniae* (CL8P9D3), obtained from N. Somerson, was cultured in the SSR2 liquid medium described elsewhere (3). However, the source of some of the ingredients and the method of preparing this medium were different; mycoplasma broth base without crystal violet was obtained from Difco Laboratories, Detroit, Mich., and bovine serum fraction A (1×) was obtained from GIBCO Laboratories, Grand Island, N.Y. The entire medium was compounded, the pH was adjusted to 7.6, and the medium was sterilized by filtration through a pressure filter (250 $cm^3$, 60 mm) containing an asbestos

filter pad (grade D 10) capable of retaining a particle size of 0.01 μm (F. R. Hormann and Co., Milldale, Conn.). Filtration of the completed medium removed all precipitates which were in the serum fraction and 25% yeast-extract solution received from the vendors, thus preventing occlusion of lipid and protein particles with cells during centrifugation. The organism was grown at 37°C in horizontally laid milk dilution bottles (160 ml; Fisher Scientific Co., Philadelphia, Pa.), each containing 20 ml of medium. After 5 days of incubation, the medium was decanted, and the confluent mycoplasma layer was washed three times (5 ml each) with phosphate-buffered saline (0.1 M $KPO_4$; pH 7.2) before being harvested. Harvesting cells with trypsin proved unsatisfactory because a residual layer of cells remained attached to the glass. Cells were removed by being intermittently stirred with a small magnetic bar (no. 8608-581; A. H. Thomas Co., Philadelphia, Pa.) in 1 to 2 ml of phosphate-buffered saline or SSR2 medium and being scraped with a rubber policeman. The organism was viable for at least 30 months in SSR2 medium when stored in screw-capped tubes at −70°C. Growth was assessed by determining the yield of protein per bottle, which correlated well with changes in pH of the medium with time, or by determining dry weight, or by determining both. For dry-weight determinations, cells were harvested and divided into tubes to minimize the amount of buffer occluded during centrifugation. After being washed with phosphate-buffered saline, the interior of each tube was carefully dried with a soft tissue before lyophilization.

**Lipid study.** Fatty acids were dissolved in sterile 70% ethanol and were added to the sterilized medium. The final concentration of ethanol in the growth medium was 0.5%, a concentration neither toxic nor inadequate for maintaining the solubility of each fatty acid at the maximum concentrations tested. Not all of the fatty acids tested (see Table 1) showed an effect when tested at equimolar concentrations. For comparative purposes, therefore, the concentrations used were determined from curves indicating the maximal concentration of each fatty acid which was tolerated by the organism and yet was not bacteriostatic or bactericidal. Also, isotope incorporation studies utilized admixtures of carrier and labeled palmitic acid (10 μg plus 0.033 μCi/ml) or palmitoleic acid (5 μg plus 0.01 μCi/ml). Labeled cells were harvested and washed with phosphate-buffered saline to constant radioactivity before being lyophilized.

**Lipid extraction and analysis.** Lyophilized cells were extracted by being shaken with acetone at 4°C for 24 h. The supernatant was then removed, and the extraction was repeated a total of six times. The combined extracts were centrifuged, the supernatant was filtered (Whatman no. 1 paper), and its volume was reduced in vacuo under nitrogen. Acetone-extracted cells were next extracted with chloroform-methanol (2:1, vol/vol) at 45°C for 2 h and at room temperature overnight. After saponification, the combined fatty acid content of these extracts was nearly identical to that of unextracted but saponified cells. This and the total recovery of all added radioactivity proved that these extractions successfully removed all of the cellular lipids from this mycoplasma.

Acetone and chloroform-methanol extracts were examined by thin-layer chromatography and autoradiography. For thin-layer chromatography, precoated silica gel G plates (0.25 mm thick; E. M. Laboratories, Inc., Elmford, N.Y.) were prewashed with the developing solvent system (chloroform-methanol-water, 62:25:4, vol/vol/vol), dried, and activated at 105°C for 90 min before being stored in a desiccator until needed. Suitable standards included a lipid mixture from *Acholeplasma laidlawii* B kindly provided by P. F. Smith, University of South Dakota, Vermillion. Phospholipids were determined by visualization with a lipid-phosphorus-specific spray (26), and glycolipids were visualized with orcinol (23). For autoradiography, plates were dried, overlaid with X-ray film (Kodak No-Screen medical film, NS-2T; Eastman Kodak Co., Rochester, N.Y.), and exposed for 48 h at −70°C before being developed. To assess radioactivity, appropriate areas were scraped from plates, put into vials containing Brays solution (10 ml), and counted in a Packard Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

The alkaline hydrolysis-fatty acid extraction method, the methylation procedure (3.5% perchloric acid in methanol, 55°C, 15 min), and the identification of fatty acids by capillary column gas chromatography before and after selected hydrogenation have been detailed previously (10). The results in Table 1 are averages of at least six determinations, each based upon cells from 50 to 200 bottles per experiment.

The nonlabeled fatty acids were purchased from Supelco Inc., Belfonte, Pa., except for beta-hydroxydecanoic acid, which was obtained from Analabs, Inc., North Haven, Conn. [*U*-$^{14}$C]palmitic acid (specific activity, 613 μCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass., and [1-$^{14}$C]palmitic acid (specific activity, 20 μCi/mmol) was obtained from Applied Science Laboratories, State College, Pa.

**Bacteriostatic and bactericidal assessment.** Each fatty acid was tested at three concentrations (see Table 4). After 5 days of incubation, the medium was removed, its pH was determined, and the attached cells were washed three times with phosphate-buffered saline before assessment of growth. A decreased protein content per bottle, when compared with controls containing ethanol without added fatty acid, indicated the amount of growth inhibition. Continued mycoplasma viability was determined by removing the fatty acid-supplemented media from a duplicate set of bottles, washing cell layers with phosphate-buffered saline as described above, and adding fresh medium (20 ml) without the added fatty acid. The protein content per bottle was again compared with the control after 5 days of incubation. Fatty acids were considered bactericidal if growth failed to occur and bacteriostatic if growth occurred only after removal of the fatty acid being tested.

**Electron microscopy.** Cells grown with and without 10 μg of added palmitic acid per ml were washed with phosphate-buffered saline and fixed with 2% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at 4°C. After being postfixed with 1% (wt/vol) osmium tetroxide in this same buffer for 1 h at room temperature, the cells were washed three times in distilled water and stained en bloc with uranyl acetate (saturated solution in 50% ethanol) for 20 min at room

temperature before being dehydrated in ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and were examined with a Phillips 300 electron microscope.

**Cholesterol and osmotic fragility determinations.** Cholesterol was extracted from lyophilized cells with chloroform-methanol (2:1, vol/vol) at 45°C for 2 h and at room temperature overnight. The extracts were combined and evaporated to dryness under nitrogen, and the lipids were dissolved in glacial acetic acid (2 ml) with slight heating. Cholesterol was determined by the ferric chloride method (12, 27). Osmotic fragility was determined as detailed by Rottem and Panos (20).

**Electron spin resonance spectroscopy method.** *M. pneumoniae* grown with and without added palmitic acid was 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 (17) as follows. Cells suspended in 0.25 M NaCl were incubated for 30 min at 4°C with 1 ml of the above-mentioned probes (2.5 mM) dissolved in a 5% aqueous solution of bovine serum albumin fraction V. Four milliliters of 0.25 M NaCl was then added, and cells were sedimented by centrifugation (12,000 × *g*, 15 min). The pellet was washed once and suspended in 0.05 to 0.15 ml of 0.25 M NaCl. Samples were then transferred to a disposable pipette which was then sealed at one end, and electron paramagnetic resonance spectra were obtained with a Varian E-4 spectrometer equipped with a temperature control accessory (Varian Associates, Palo Alto, Calif.).

The freedom of motion of the spin-labeled fatty acids in the membrane preparation was assessed from the order parameter (6). The order parameter(s) is related to the mean angular deviation of the labeled fatty acid chain from its average orientation in the membrane. Low values of order parameter are associated with a higher freedom of motion of membrane lipids.

**Other determinations.** Protein was determined by the method of Lowry et al., with bovine serum albumin as the standard (7), and peroxide secretion was quantitated as detailed by Cohen and Somerson (2). RNA was determined by the orcinol method (4). Changes in antibiotic sensitivity were assessed by growing cells with (10 μg/ml) and without added palmitic acid in the presence of various concentrations of erythromycin (0.01 to 0.1 μg/ml) or tetracycline (0.4 to 4.0 μg/ml) for 5 days and following the change in pH of the medium over time.

## RESULTS

**Fatty acid change and cell yield.** The long-chain fatty acid content, composition, and cell yield of *M. pneumoniae* grown with various exogenous acids are given in Table 1. Palmitic, stearic, oleic, and linoleic acids comprised 82 to 92% of the total cellular fatty acid content, regardless of the exogenous fatty acid. The amount of each exogenous fatty acid found within this organism varied from 2.8 to 5.5%, with the exception of palmitic acid. Palmitic acid accounted for 53% of the total fatty acids derived from palmitic acid-grown cells, an enrichment 29 to 51% over that of control cells (Tables 1 and 2). Suprisingly, the total cellular fatty acid content increased 2.5- to 9.6-fold when the medium was supplemented with a saturated, unsaturated, or hydroxylated fatty acid (Table 1). Finally, while the saturated/unsaturated fatty acid ratio ranged from 1.2 to 2.1, that of palmitic acid-enriched cells ranged from 3.0 to 4.2. These results varied considerably from the fatty acid composition and saturated/unsaturated fatty acid ratio of the growth medium (Table 1).

The fatty acid content of the uninoculated growth medium after hydrolysis was 90 μg/ml. However, the total amount of each fatty acid present (Table 1) was probably not available to the organism. For example, *M. pneumoniae* utilized only a small fraction of the palmitic and palmitoleic acids in the unsupplemented medium (9.5 and 1.3%, respectively). However, they were used much more when added to the medium (up to 57 and 8.5% for palmitic and palmitoleic acids, respectively, Table 1). With the exception of palmitic acid, the amount of a particular fatty acid (Table 1) actually taken from the supplemented medium by this mycoplasma ranged from 0.2 to 10.0%.

Average cell yields per bottle, in terms of protein, decreased when long-chain fatty acids were added to the medium (Table 1). Subcultures in identical medium did not reduce cell yields further after 10 serial transfers. Also, since palmitic acid was incorporated most, Fig. 1 shows the change in percent protein and fatty acid content of this organism as the amount of this saturated acid was varied in the growth medium. The protein/RNA ratio of this organism, with and without added palmitic acid, remained nearly constant (9:10).

A reduced cell yield did not always result in an equivalently increased fatty acid content in this organism. For example, palmitoleic acid decreased growth to that of its saturated homolog. However, although it resulted in a much higher total fatty acid content than that of the control cells, the increase was not nearly as great as that which followed the addition of palmitic acid (Table 1). By comparison, although two positional isomers of *cis*-tetradecenoic acid (myristoleic and *cis*-5-$C_{14}$ acids) retarded growth almost equally and increased the total fatty acid content about 2.9-fold, each was taken from the medium to a different extent (myristoleic acid, 1.3%; *cis*-5-$C_{14}$, 0.18%). Conversely, beta-hydroxydecanoic acid did not inhibit growth but did increase the fatty acid content (2.5-fold), illustrating that a decreased protein content was not

TABLE 1. *Fatty acid composition of M. pneumoniae grown with various monoenoic or saturated acids*

| Fatty acid | Total fatty acids (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | None | *cis*-Vaccenic acid[a] (2 μg/ml) | Palmitoleic acid[a] (5 μg/ml) | Myristoleic acid[a] (20 μg/ml) | *cis*-$\Delta^5$-$C_{14}$[a] (20 μg/ml) | Palmitic acid[a] (10 μg/ml) | B-OH $C_{10}$[a] (5 μg/ml) | Uninoculated medium control |
| Lauric | 0.60 | 0.12 | 0.33 | 0.41 | 0.25 | 0.32 | 0.46 | 0.77 |
| Myristic | 1.70 | 1.42 | 1.30 | 1.78 | 0.84 | 0.96 | 1.09 | 1.18 |
| *cis*-$\Delta^5$-$C_{14}$ | | | | | 0.99 | | | TR[b] |
| Myristoleic | 0.37 | TR | | 5.54 | | | | 0.56 |
| Saturation unknown | 0.58 | 0.48 | 0.40 | 1.11 | 0.25 | 0.72 | 0.45 | 0.82 |
| B-OH-$C_{10}$ | | | | | | | 0.66 | |
| Saturated unknown | 1.37 | 0.69 | 0.61 | 0.87 | 0.28 | 0.70 | 0.97 | 0.89 |
| Saturated unknown | 2.22 | 0.94 | 0.88 | 1.04 | 0.62 | 0.73 | 0.34 | 0.20 |
| Saturated unknown | 1.44 | 0.31 | 0.36 | 0.57 | 0.27 | 0.86 | TR | 0.32 |
| Saturated unknown | 1.55 | 0.62 | 0.61 | 0.64 | 0.28 | 0.57 | TR | TR |
| Palmitic | 35.61 | 29.52 | 39.35 | 28.66 | 37.70 | 53.63 | 37.13 | 16.64 |
| $C_{16}$ unsaturated | 1.16 | 1.20 | | 0.71 | 0.43 | | | |
| Palmitoleic | 1.70 | 2.61 | 3.69 | 1.65 | 1.12 | 0.48 | 0.67 | 2.59 |
| Saturated unknown | 0.57 | 0.36 | 0.44 | 0.29 | 0.35 | 0.25 | 0.45 | 0.19 |
| Saturated unknown | 0.85 | 0.77 | 0.88 | 0.55 | 0.67 | 0.58 | 0.75 | 0.65 |
| Saturated unknown | 0.93 | 0.66 | 0.19 | 1.26 | 0.16 | 1.02 | 0.61 | 2.80 |
| Saturated unknown | 1.21 | 1.16 | 1.73 | 1.43 | 1.74 | 1.02 | 1.67 | 1.36 |
| Saturated unknown | 0.50 | 0.35 | 0.22 | 0.37 | 0.16 | TR | 0.17 | 0.68 |
| Stearic | 17.98 | 16.95 | 17.14 | 28.89 | 19.16 | 19.41 | 21.25 | 20.04 |
| Oleic | 17.82 | 15.46 | 14.15 | 10.71 | 20.60 | 13.70 | 22.72 | 14.45 |
| *cis*-Vaccenic | | 2.78 | | | | | | |
| Linoleic | 11.75 | 23.50 | 17.77 | 13.45 | 14.12 | 5.07 | 10.34 | 35.76 |
| Cell protein[c] | 62 | 50 | 45 | 56 | 55 | 35 | 66 | ND[d] |
| Total fatty acids[c] | 4.4 | 13.0 | 25.6 | 13.7 | 12.3 | 42.2 | 11.0 | 0.22 |
| Avg cell yield per bottle[e] (μg of protein) | 908 | 375 | 615 | 404 | 380 | 554 | 865 | |
| Saturated/unsaturated ratio | 2.05 | 1.19 | 1.81 | 2.12 | 1.68 | 4.20 | 1.89 | 0.87 |

[a] Addition to medium.
[b] TR, Trace.
[c] As a percentage of cell dry weight.
[d] ND, Not done.
[e] See text.

always merely due to an increase in cellular fatty acids.

**Lipid alteration with added palmitic acid.** The fatty acid content and composition of the saponified acetone and chloroform-methanol extracts from *M. pneumoniae* grown with and without added palmitic acid appear in Table 2. The total fatty acid content of the control cells, obtained by combining the fatty acids from these lipid extracts (5.6%), was always higher than that obtained by direct saponification of cells (4.4%, Table 1). This difference was due to a fine precipitate present in acetone extracts which was not removed by centrifugation and filtration. The dry-weight yield of this organism from medium supplemented with palmitic acid was not markedly lower (not more than 18%) than that without added palmitic acid. However, of interest was the great increase in the fatty acid content of the acetone (18-fold) and chloroform-methanol (3.8-fold) extracts from cells in this supplemented medium (Table 2). Finally, the saturated/unsaturated fatty acid ratio was highest in the chloroform-methanol extracts of control cells and was equal in both solvent systems from the palmitic acid-supplemented cells (Table 2).

The phospholipids and glycolipids of this mycoplasma have been identified previously (11, 24). However, because of the marked increase in the fatty acid content of the organism when grown with added palmitic acid, acetone and chloroform-methanol extracts were also examined for their content of free palmitic acid (i.e., palmitic acid not incorporated into complex lipids). Using thin-layer chromatography and labeled palmitic acid, we observed that the lipid content of the acetone extract was composed mostly of free palmitic acid (70% or 56,000 cpm), the remainder being distributed among three

TABLE 2. *Fatty acid composition of the acetone and chloroform-methanol extracts from M. pneumoniae grown with and without added palmitic acid*

| Fatty acid | Fatty acids (%)[a] | | | | | |
|---|---|---|---|---|---|---|
| | No addition[b] | | | Palmitic acid added[c] (10 μg/ml) | | |
| | Acetone | Chloroform-methanol | Avg | Acetone | Chloroform-methanol | Avg |
| Lauric | 0.28 | 0.11 | 0.20 | 0.37 | 0.10 | 0.24 |
| Myristic | 1.69 | 0.74 | 1.22 | 1.06 | 0.45 | 0.76 |
| Saturated unknown | 0.47 | 0.29 | 0.38 | 0.39 | 0.10 | 0.25 |
| Saturated unknown | 0.84 | 0.84 | 0.84 | 0.42 | 0.45 | 0.44 |
| Saturated unknown | 0.80 | 0.29 | 0.50 | 0.65 | 0.56 | 0.61 |
| Palmitic | 29.66 | 53.68 | 41.67 | 45.29 | 61.11 | 53.20 |
| Palmitoleic | 1.31 | 0.11 | 0.71 | 0.73 | TR[d] | 0.37 |
| Saturated unknown | 0.34 | 0.43 | 0.39 | 0.49 | 0.17 | 0.33 |
| Saturated unknown | 0.79 | 0.64 | 0.72 | 0.30 | 0.23 | 0.27 |
| Saturated unknown | 1.22 | 1.65 | 1.44 | 1.01 | 0.81 | 0.91 |
| Stearic | 25.10 | 17.45 | 21.27 | 23.90 | 11.00 | 17.45 |
| Oleic | 24.90 | 20.61 | 22.75 | 16.33 | 21.62 | 18.98 |
| Linoleic | 12.71 | 3.13 | 7.92 | 7.99 | 2.68 | 5.34 |
| Saturated/unsaturated ratio | 1.57 | 3.18 | 2.18 | 2.94 | 3.09 | 3.02 |

[a] Fatty acid content after saponification; average of two determinations.

[b] Total number of bottles used for growth of organism, 75.0; dry weight yield of cells, 112.5 mg; total protein, 64.9 mg; fatty acids from acetone extracts, 1.1 mg; fatty acids from chloroform-methanol extracts, 5.2 mg; total fatty acids, 5.6% of cellular dry weight.

[c] Total number of bottles used for growth of organism, 75.0; dry weight yield of cells, 92 mg; total protein, 32.4 mg; fatty acids from acetone extracts, 20.2 mg; fatty acids from chloroform-methanol extracts, 19.6 mg; total fatty acids, 43.3% of cellular dry weight.

[d] TR, Trace.

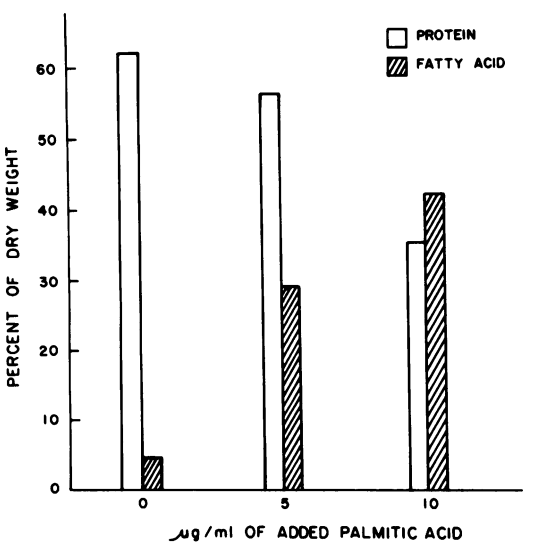


FIG. 1. *Percent fatty acid and protein content of M. pneumoniae in medium supplemented with various amounts of palmitic acid.*

phospholipids and one glycolipid. Additional two-dimensional chromatograpby (5) also separated one of the phospholipids into two labeled components, a phosphoglycolipid and a phospholipid. Distribution of radioactivity within the chloroform-methanol extract was different. While 10 labeled complex lipids were observed, radioactivity due to free palmitic acid accounted for only 4.6% (or 3,700 cpm), the remainder being distributed among the predominating phospholipids and the phosphoglycolipid and glycolipid already mentioned. Based on the radioactivity of isolated fractions after thin-layer chromatography, 22% of the labeled palmitic acid within this mycoplasma, grown with this added saturated acid, was free. Also based upon gravimetric and isotopic data, and assuming that the combined chloroform-methanol extracts from both the control and experimental cells contained only 4.6% free fatty acids (Table 2), complex lipid synthesis increased a minimum of 3.8-fold after addition of palmitic acid. The total radioactivity present in the acetone and chloroform-methanol extracts of palmitic acid-labeled cells was always 93% or more of that recovered from the intact organism when saponified directly.

Lipids were also detected in those extracts that were not isotopically labeled. One of these lipids was the most intensely phosphorus positive of all the components detected and gave a brown color when treated with orcinol. It migrated faster than the free fatty acids and had

an $R_f$ similar to that of diphosphatidylglycerol. The lipids obtained with chloroform-methanol also contained two nonlabeled phosphorus-positive but orcinol-negative components that were absent in the acetone extracts. All of these nonlabeled lipids were present in the uninoculated (control) growth medium.

Earlier, gravimetric analyses had shown that the fatty acid contents of the chloroform-methanol and acetone extracts of cells grown with added palmitic acid had increased markedly and were nearly equal (Table 2). However, determinations with labeled palmitic acid showed a distribution that differed; one-third of the radioactivity was in the acetone extracts, and the remainder was in the chloroform-methanol extracts (Table 3). This same distribution occurred when labeled palmitoleic acid served in lieu of palmitic acid (typical results, $1.5 \times 10^5$ cpm in acetone extracts and $3.4 \times 10^5$ cpm in chloroform-methanol extracts). The detection of the most prominent complex lipid in these acetone extracts being a nonradioactive but phosphorus-positive and orcinol-reactive component from the medium helped explain this discrepancy. The fatty acids from this particular lipid, plus those from lipids containing labeled palmitic acid, probably resulted in the fatty acid content of the extracts being equal in weight but unequal in isotope distribution. It is not likely that the presence of labeled and nonlabeled complex lipids in this organism is due to fatty acid-exchange reactions, because the same nonlabeled phospholipids isolated from this organism were also present in the medium, and because the mycoplasmas seem incapable of degrading polar lipids. With one exception (18), phospholipase activities in these organisms have not been detected (25).

**Fatty acid toxicity.** The ability of *M. pneumoniae* to maintain viability with higher concentrations of saturated and unsaturated acids was examined because certain long-chain fatty acids retarded growth (Table 1). All of the fatty acids tested were toxic to some degree (Table 4). Oleic and linoleic acids were bactericidal at the lowest concentration tested (20 μg/ml). Also, all but palmitoleic and myristic acids prevented recovery when tested at 32 μg/ml. Finally, palmitoleic acid was bactericidal at the highest concentration tested, whereas beta-hydroxydecanoic acid failed to inhibit growth at 64 μg/ml. Of interest was the finding that this organism did not recover but, instead, continued to disengage itself from the glass surface and died only after the removal of added linoleic and oleic acids at the two lower concentrations tested and after the removal of added myristoleic acid at 32 μg/ml. The lowest values in Table 4 (6 to 15%) reflect the inoculum size used and indicate cell attachment without continued proliferation.

TABLE 3. *Typical results of radioactivity and weight distributions in fatty acids from the acetone and chloroform-methanol extracts of M. pneumoniae grown with added palmitate*[a]

| Fatty acids | Radioactivity (cpm) | Dry wt (mg) | Fatty acid (cpm/mg) |
|---|---|---|---|
| Acetone extracts | $1.25 \times 10^6$ | 21.8 | $5.5 \times 10^4$ |
| Chloroform-methanol extracts | $3.47 \times 10^6$ | 22.6 | $15.0 \times 10^4$ |

[a] Admixture of nonlabeled (10 μg/ml) and labeled (0.033 μCi/ml) palmitate added to medium. Total cellular incorporation of isotope was 4.4%.

TABLE 4. *Bacteriostatic and bactericidal effect of various long-chain fatty acids on M. pneumoniae*

| Fatty acid added | Growth[a] with added fatty acid (μg/ml of medium) | | | Growth[a] after removal of fatty acid (μg/ml of medium) | | |
|---|---|---|---|---|---|---|
| | 20 | 32 | 64 | 20 | 32 | 64 |
| Oleic | 40 | 41 | FAP[b] | 20 | 10 | FAP |
| Linoleic | 30 | 16 | FAP | 10 | 10 | FAP |
| Linolenic | 30 | 26 | 16 | 50 | 45 | 14 |
| Palmitoleic | 44 | 39 | 7 | 90 | 95 | 6 |
| Myristoleic | 65 | 65 | 38 | 75 | 14 | 15 |
| Myristic | 33 | 28 | FAP | 85 | 80 | FAP |
| Palmitic | 50 | FAP | FAP | 90 | FAP | FAP |

[a] Values are expressed as percentages of control cells and are based upon changes in cellular protein content per bottle. Control cells (without added fatty acid) contained 908 to 950 μg of protein per bottle. Initial inoculum size per bottle was 90 to 95 μg of protein. Each figure is an average of quadruple replicates.

[b] FAP, Fatty acid precipitation from medium at these concentrations.

**Altered morphology with added palmitic acid.** The effect of the increased uptake of palmitic acid (and markedly elevated total fatty acid content) on the cellular morphology of *M. pneumoniae* was examined by electron microscopy (Fig. 2). No obvious difference was observed in the thickness of the cell membrane after it was supplemented with palmitic acid (Fig. 2A and B). However, these cultures contained an appreciable amount (approximately 25% as judged from electron photomicrographs) of very long, filamentous cells (Fig. 2B). A lower magnification (a montage) shows the extensive length of one such filament (at least 176 μm, Fig. 3) unobscured by the cross wires of the grid. These filaments were nonseptated and had an extremely variable width when viewed at a higher magnification (photo not shown), the maximum occasionally similar in size (0.45 μm) to the average diameter of the spherical components of the culture. Also, the filaments were often looped and more electron dense than the spherical cells.

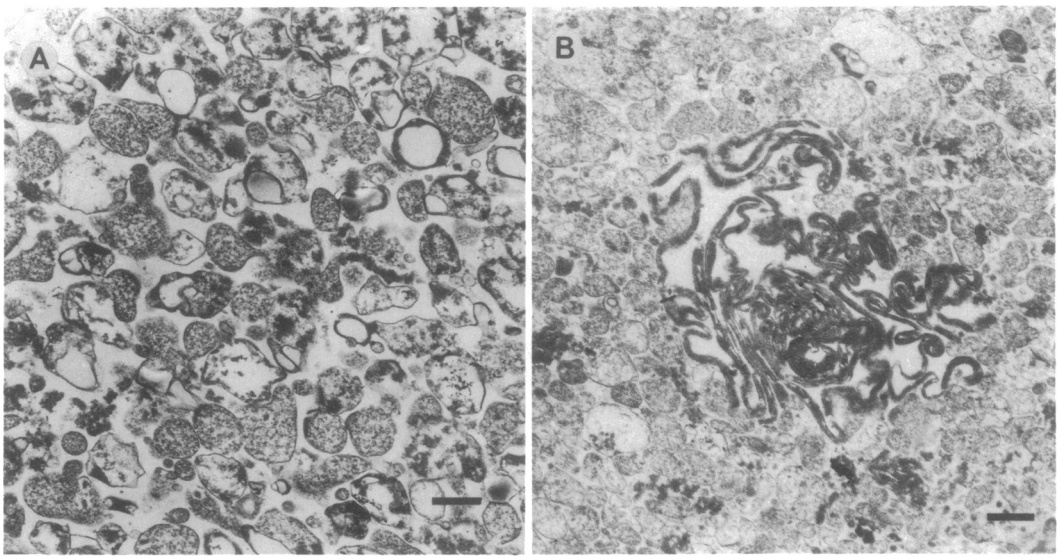


FIG. 2. *Thin sections of M. pneumoniae. (A) Control cells from medium without added palmitic acid. Bar, 0.5 μm. (B) Filamentous cells from medium supplemented with palmitic acid (10 μg/ml). Bar, 0.9 μm.*

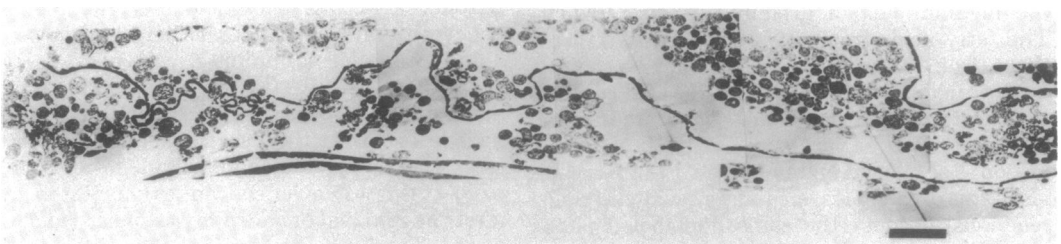

FIG. 3. *Thin sections of M. pneumoniae grown with added palmitic acid (10 μg/ml). Montage showing overall length of a typical filament. Bar, 3.57 μm.*

TABLE 5. *Effects of a homologous saturated and unsaturated fatty acid on growth, cholesterol content, and osmotic fragility of M. pneumoniae*

| Fatty acid added to growth medium (10 μg/ml) | Cellular yield (protein per bottle) | Total fatty acid content (%) | Cholesterol content (μg/mg protein) | Osmotic fragility[a] | |
|---|---|---|---|---|---|
| | | | | 0.05 M NaCl | Water |
| None | 908 | 4.4 | 116.7 | 0 | 12.5 |
| Palmitic | 554 | 42.2 | 83.3 | 15.7 | 35.1 |
| Palmitoleic | 500 | 27.0 | 110.4 | 0 | 22.9 |

[a] Percent lysis against 0.25 M NaCl as a control.

**Membrane change.** The effects of palmitic and palmitoleic acids on growth, cholesterol content, and osmotic fragility are shown in Table 5. The addition of either acid to the growth medium resulted in a greatly increased cellular fatty acid content (Table 1) but also in a decreased cholesterol content and cell yield. Also, although osmotic fragility in distilled water increased, only cells grown with added palmitic acid lysed in 0.05 M NaCl. In addition, palmitic acid-enriched cells had the greatest osmotic fragility in water, a 2.8-fold increase over that of control cells. Although the yields of cells grown with 5 and 10 μg palmitoleic acid per ml differed (Tables 1 and 5), their fatty acid contents were comparable.

Cells grown with and without added palmitic acid were examined by electron spin resonance spectroscopy to relate the observed changes in osmotic fragility with altered membrane fluidity. (Fig. 4). Fig. 4 shows representative electron spin resonance spectra of 12-nitroxystearate in the membranes of these cells. Ample evidence already exists that this and similar probes are incorporated into the membrane bilayer. The spectra show great similarity, but the large hyperfine splitting ($2T_{11}$) as well as the line height ratio ($h_0/h_{-1}$) differ. Order parameters ($S$) cal-

culated from spectra of 12- and 5-nitroxystearate taken at various temperatures were higher at each temperature for cells grown with palmitic acid than for cells grown without it (Table 6). In these experiments, the accuracy of the reported order parameter was about ±0.01. A fact to be particularly noted is that at a given temperature, the flexibility of the lipid chains in *M. pneumoniae* was considerably greater near the methyl end group of the acyl chains. Thus, $S$ values obtained with 12-nitroxystearate are lower than those obtained with 5-nitroxystearate. The restricted molecular motion of the spin-labeled fatty acid in the palmitic acid-enriched cells was also evident in the much higher height ratio ($h_0/h_{-1}$, 130 in palmitic acid-enriched cells, in contrast to 17 in control cells) observed in spectra of 12-nitroxystearate taken at 45°C. The heights of the mid- and high-field lines on a first-derivative absorption spectrum are $h_0$ and $h_{-1}$, respectively. Such a ratio may serve as a parameter for the mobility of acyl chains in the membrane (9), where higher ratios are associated with a more restricted mobility. The difference in the order parameters and height ratios indicated that in membranes of cells grown with palmitic acid, the motion of the probes was much more restricted than in membranes from control cells; i.e., the membranes had a higher viscosity.

TABLE 6. *Order parameters for 5-nitroxystearate and 12-nitroxystearate incorporated into the cell membrane of M. pneumoniae cells grown with and without added palmitic acid*[a]

| Preparation | Order parameter ($S$) | | | |
|---|---|---|---|---|
| | 5-Nitroxystearate | | 12-Nitroxystearate | |
| | 25°C | 45°C | 25°C | 45°C |
| Cells grown with palmitate | 0.76 | 0.66 | 0.65 | 0.51 |
| Cells grown without added palmitate | 0.66 | 0.57 | 0.59 | 0.45 |

[a] *M. pneumoniae* cells were grown with (10 μg/ml) or without added palmitic acid. Spin-labeling of the cells and the calculation of order parameters are described in the text. Electron spin resonance spectra were recorded at 25 and 45°C.

**Biological change with compositional and structural alteration.** Detailed studies failed to show any change in susceptibility to two antibiotics when this mycoplasma was grown with added palmitic acid; growth continued to be inhibited by 1 to 1.5 μg of tetracycline and 0.01 μg of erythromycin per ml. Likewise, catalase inhibition, a measure of peroxide secretion, was unchanged in these cultures when they were incubated for 5 to 8 days; peroxide secretion stopped after 8 days of incubation. Also, no difference in pH was observed between these and the control cultures; all decreased from 7.6 to 6.0 or lower after 5 days of incubation.

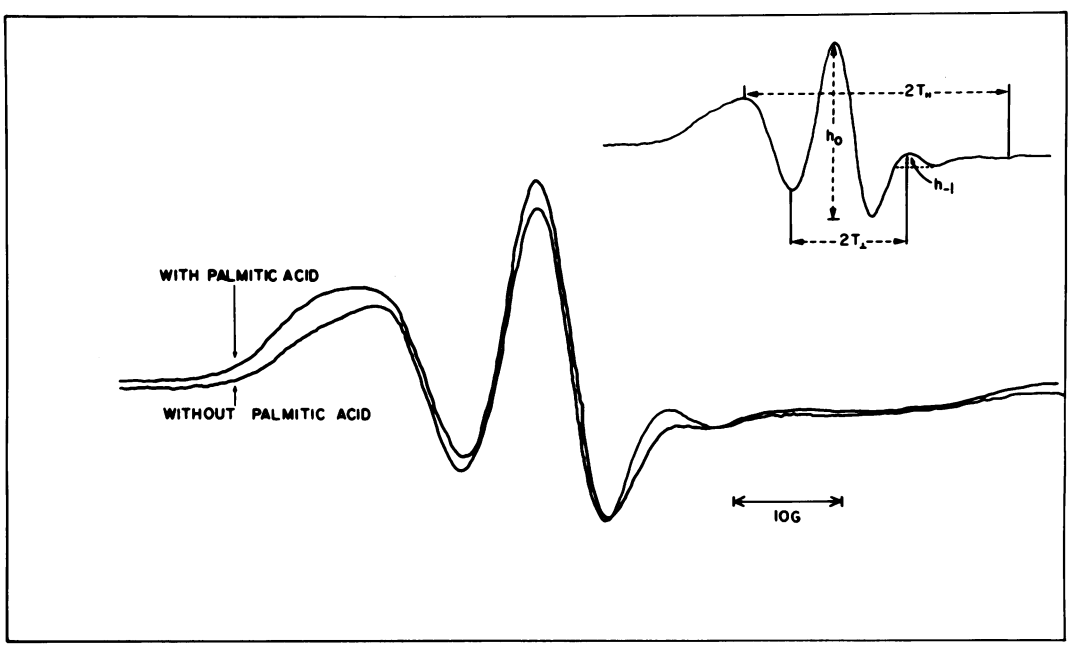


FIG. 4. *Representative electron spin resonance spectra obtained at 45°C of N-oxy-4',4'-dimethyloxazolidine derivative of 12-ketostearic acid in M. pneumoniae grown with and without added palmitic acid (10 μg/ml).*

## DISCUSSION

These studies indicate lipid perturbations and specificities previously unknown for this or any other *Mycoplasma* spp. For example, although the incorporation of a variety of exogenous fatty acids was very low (except for palmitic acid), adding each exogenous fatty acid resulted in a profound increase in the total fatty acid content of *M. pneumoniae,* which suggested possible changes in fatty acid transport, metabolic regulation of fatty acid activation and biosynthetic incorporation of activated fatty acids into complex, or both. Also, it is interesting that this increase occurred whether the exogenous acid used was saturated, unsaturated, or hydroxylated, which indicated a lack of specificity for initiating this overall accumulation. However, certain lipid characteristics, like the preferred accumulation of palmitic, stearic, and linoleic acids, remained unaffected. Likewise, the preferred retention of a particular positional isomer was apparent when oleic acid continued to be selected from an available mixture of oleic and *cis*-vaccenic acids. Finally, a comparison of the fatty acid composition of the growth medium with that of *M. pneumoniae* grown with and without fatty acid supplementation clearly revealed (i) a continued need for long-chain saturated fatty acids, as has been shown for *M. hominis* (21), (ii) that fatty acids were not indiscriminately absorbed from the growth medium, and (iii) that the low uptake of exogenously added acids is not simply due to insufficient amounts in the growth medium. It has been postulated that a proper saturated/unsaturated fatty acid ratio is needed for growth and osmotic stability of the mycoplasmas (13, 24).

It is unlikely that these results are due to cellular occlusion or nonspecific adsorption of the fatty acids added to the growth medium. This is concluded from the following: (i) complete solubilization of each added fatty acid in the growth medium for the duration of these experiments, (ii) washing of the glass-adhering mycoplasmas free from medium components before detachment and collection, and (iii) the increased concentration of all the fatty acids detected as well as the increased complex lipid content of this organism when grown in supplemented medium.

Rottem found that 30 to 40% of labeled palmitate or oleate added to the growth medium was actually free in the membrane of *M. hominis.* The percentage of free fatty acids in the neutral lipid fraction from this organism was 10.3% (21). A high concentration of free fatty acids has also been reported in a *Ureaplasma* sp. (15). Therefore, the finding that 22% of the labeled palmitic acid within *M. pneumoniae* is free was not unexpected. Other mycoplasmas (*A. laidlawii, M. gallinarium* J, and *M. arthritidis*), like the true bacteria, are known not to contain significant amounts of free fatty acids (24).

The addition of palmitic acid to the growth medium increased the complex lipid content of *M. pneumoniae* almost fourfold. Even so, finding nonlabeled complex lipids in the acetone and chloroform-methanol extracts from such cells indicated an ability of this mycoplasma to remove intact lipids (and fatty acids) from the medium even during increased complex lipid synthesis.

High concentrations of long-chain fatty acids are known to be toxic to microorganisms. Nonetheless, while high concentrations of saturated and unsaturated long-chain fatty acids were bactericidal for *M. pneumoniae,* high concentrations of palmitic and beta-hydroxydecanoic acids were not. This cannot be due solely to a low incorporation, as evident from the palmitate content of *M. pneumoniae* when grown with palmitic acid. Of the fatty acids tested, the saturated acids were less toxic than their unsaturated homologs or than the other unsaturated acids tested. These effects are similar to those observed with true bacteria. Also, the continued detachment (and death) of this mycoplasma from a glass surface after removal of only certain added unsaturated acids is not due to the position of the double bond. Removal of palmitoleic acid, whose double bond is also in the 9,10 position, permitted rapid recovery of the organism. Finally, the continued ability of this mycoplasma to attach itself to a surface in the absence of growth is also noteworthy and is suggestive of a lack of interdependence between these two activities.

Granting that some mycoplasmas normally exhibit a filamentous stage of growth, *A. laidlawii* B readily produces filaments when oleic or other enoic acids are added to the growth medium (14). These additions also decrease the osmotic fragility (14, 16) of the organism. Saturated long-chain fatty acids, including palmitic acid, inhibit growth and filament formation of this acholeplasma (14). To our knowledge, this is the first report of filament induction by a saturated fatty acid in a mycoplasma. The filamentous cells of *M. pneumoniae* differ from those of *A. laidlawii* in that they are much longer and are present as late as the stationary phase of growth. Also, although those of the oleic acid-grown acholeplasma fragment into smaller viable units, the ability of this filamentous *M. pneumoniae* to divide or fragment is unknown.

*A. laidlawii* is osmotically more stable when grown with oleic acid; however, increasing its stearic or palmitic acid content increases its cell fragility (16, 21). Thus, an increased osmotic fragility for *M. pneumoniae* grown with added palmitic acid was not unexpected. However, the increased membrane viscosity, as judged by electron spin resonance spectroscopy, was not nearly as striking as anticipated from the high amount of palmitic acid known to be incorporated. The concomitant decrease in the cholesterol content of the organism, with palmitic acid enrichment, helps explain this. Cholesterol is a regulator of mycoplasmal membrane fluidity (8, 16). The addition of cholesterol to phospholipids changes their packing mode and makes the membrane lipids more condensed above their transition temperature and more fluid below it (8, 16). Also, Rottem et al. (22) have shown that lowering the growth temperature of *M. mycoides* subsp. *capri* results in a decrease in the cholesterol content of its membrane without affecting the fatty acid composition of the membrane polar lipids. Therefore, this mycoplasma appeared to react differently from bacteria, which synthesize more unsaturated fatty acids at lower growth temperatures. Our data indicate that *M. pneumoniae* can lower its cholesterol content to compensate for an increased saturated-fatty acid content. The effect of a decreased amount of cholesterol upon membrane fluidity would be similar to minimizing a change in membrane viscosity by increasing the unsaturated fatty acid content, when the growth temperature is above the phase transition temperature of the membrane lipids. However, the decrease in the cholesterol content of palmitic acid-enriched cells of *M. pneumoniae* is probably insufficient to offset the great increase in the cells saturated fatty acid content, and this insufficiency results in increased osmotic fragility. Also, the increased but intermediate (i.e., between the control and palmitic acid-enriched cells) osmotic fragility in water of palmitoleic acid-grown cells is probably due to the fact that the increased saturated and unaltered cholesterol content is only slightly countered by the relatively small increase in incorporation of unsaturated acids.

Some information is at hand on the relationship between membrane functional properties and fatty acid structure (1). However, while palmitic acid-grown *M. pneumoniae* had altered osmotic fragility and morphology, its susceptibility to erythromycin or tetracycline or its ability to secrete hydrogen peroxide did not change. These results indicate certain biological activities within the mycoplasma that are not affected by profound changes in morphology or lipid physiology.

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