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UTILIZATION OF ACETATE-1-$^{14}$C IN THE SYNTHESIS OF LIPID BY ACHOLEPLASMAS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Patricia Kay Herring, B.S., M.T. (A.S.C.P.)

The Ohio State University

1974

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Department of Medical Microbiology
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I also wish to express my thanks to my family and friends for their support and patience during the past four years.
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INTRODUCTION

The Class Mollicutes, proposed by Edward and Freundt (14), contains those organisms which lack a cell wall, produce typical "fried egg" colonies, pass through a 450 nm membrane filter, and fail to revert to a bacterium under appropriate conditions (93). This Class has only one Order, Mycoplasmatales (13), which is further subdivided into two families. The Family Mycoplasmataceae (13) contains the genera Mycoplasma (12) and Ureaplasma (71). The second Family, Acholeplasmataceae, contains only the genus Acholeplasma (15). These genera are differentiated from each other by their cholesterol dependence and production of urease. Mycoplasmas and ureaplasmas require cholesterol for growth, but acholeplasmas do not (12,53,90). Only the ureaplasmas produce urease (69,70).

Besides serological comparisons, other criteria are useful in placing these organisms in the appropriate family or genus. Acholeplasmas can be distinguished from the mycoplasmas and ureaplasmas by their genome size. Acholeplasmas have a genome size approximately twice the size of mycoplasmas and ureaplasmas (3,4,6,7,30).

Other workers demonstrated that the localization or presence of certain enzymes can be used to differentiate between acholeplasmas and mycoplasmas. Pollack (40,42,43,45) reported that NADH oxidase activity was associated with the membranes of A. laidlawii A and B, A. granularum, A. axanthum, and A. modicum but with the soluble
fraction of eleven Mycoplasma strains. Recently, a NAD-dependent L(+)-lactic dehydrogenase (LDH) which is specifically activated by fructose-1,6-diphosphate was isolated from acholeplasmas but not from mycoplasmas (31,32). Other types of LDHs were found in all the fermentative mycoplasmas examined.

Certain compounds were found to have a greater inhibitory effect on the growth of mycoplasmas than on the growth of acholeplasmas. The reaction of these compounds with the cholesterol present in the cell membranes is probably responsible for this effect. Most mycoplasmas were sensitive to 5% sodium polyanethanol sulfonate (SPS) while all the acholeplasmas tested were resistant. M. anatis, M. iners, and M. gallinarum were resistant to 5% SPS (2,16,17,23). Therefore, if an organism is sensitive to 5% SPS it is probably a mycoplasma. Rottem (58) reported that sensitivity to Amphotericin B could be used as a test to differentiate between acholeplasmas and mycoplasmas. Amphotericin B (20 µg/ml) inhibited the growth of mycoplasmas but not the growth of acholeplasmas. Recently Mårth and Taylor-Robinson (27) demonstrated that the mycoplasmas and ureaplasmas were approximately four fold more sensitive to the inhibitory effect of lysolecithin than most acholeplasmas. However, A. modicum was found to be as sensitive to lysolecithin as most of the mycoplasmas. Inhibition of growth by digitonin has also been used to separate the acholeplasmas from mycoplasmas (52,91). Acholeplasmas are inhibited only by a high concentration of digitonin (250-500 µg/ml) while mycoplasmas are sensitive to lower concentrations (10-60 µg/ml).

It must be mentioned that two mycoplasma-like organisms have
recently been described. *Thermoplasma acidophilum* (5,11,24), which was originally isolated from a coal refuse pile, requires a pH of 1-2 and incubation at 56-60 C for optimum growth. The other mycoplasma-like organism, *Spiroplasma citri* (8,9,10,50,64), was isolated from citrus plants affected with "Stubborn disease." A definite taxonomic position in the Class Mollicutes has not been assigned to these organisms.

Previous investigations of acholeplasmas and mycoplasmas have suggested to us that these organisms might also be separated by differences in their ability to synthesize lipid.

Many workers have studied the lipids of the Mycoplasmataceae. Their research can be divided into three sections dealing with either phospholipids, glycolipids, or neutral lipids.

The predominant phospholipids isolated from the mycoplasmas, acholeplasmas, and ureaplasmas are glycerophospholipids (28,35,36,37, 38,39,55,62,76,80,84,87,88). All of the strains tested were capable of synthesis of phospholipids from labeled precursors. *M. neurolyticum*, *M. gallinarum*, *M. mycoides*, *M. species* (Y), *A. axanthum* (S743) and *U. species* (P108) synthesized phosphatidylglycerol and diphosphatidylglycerol while *M. hominis* synthesized mainly phosphatidylglycerol. *M. gallinarum* also synthesized a monoacyl glycerophosphoryl glycerophosphate. A phosphatidyl monoglyceride as well as phosphatidylglycerol was synthesized by *M. pneumoniae*. *A. laidlawii* synthesized glucose containing phospholipids besides phosphatidylglycerol (66,67, 68,83) and aminoacyl phosphatidylglycerol. *M. neurolyticum* also synthesized an aminoacyl phosphatidylglycerol.
Some experiments indicated that the glycerophospholipids were metabolically stable. It was proposed that these compounds may play a structural role in these organisms (28, 76, 80, 82, 87).

There is some indirect evidence that *M. mycoides* (35) synthesizes diphosphatidylglycerol from phosphatidylglycerol, while *M. gallinarum* (87) uses monoacyl glycerophosphoryl glycerophosphate as a precursor for diphosphatidylglycerol. Plackett and Rodwell (38) suggested that *M. species* (Y) can synthesize phosphatidylglycerol by reaction of CDP-diglyceride with sn-glycerol-3-phosphate. *A. laidlawii* B was shown to preferentially incorporate saturated fatty acids into the 1-position of phosphatidylglycerol and unsaturated fatty acids into the 2-position (29, 56).

Originally it was reported that the glucose containing phospholipid from *A. laidlawii* B had a high turnover rate (76, 80). A recent paper indicates that there is no turnover of this compound (28). This compound was found to be a glycerylphosphoryldiglucosyl diglyceride (66, 67, 68). Shaw *et al.* (67) proposed that this lipid was synthesized by the transfer of glycerophosphate from CDP-glycerol to diglucosyl diglyceride, or the transfer of phosphatidic acid from CDP-diglyceride to diglucosyl diglyceride followed by selective deacylation. A phosphatidylglycerol diglyceride was also found to be synthesized by *A. laidlawii* B (83).

Plackett *et al.* (39) suggested a possible structure for the phosphosphingolipid synthesized by *A. axanthum* (S743). The mechanism for the synthesis of this lipid has not been determined.

The mechanism of the synthesis of aminoacyl phosphatidylglycerol was described for *A. laidlawii* B by Kostra and Smith (22). In synthe-
sis of L-alanylphosphatidylglycerol, the amino acid activated via amino-
acyl-tRNA is transferred to phosphatidylglycerol. The activation of
D-alanine occurs differently. D-alanine is transferred to phosphatidyl-
glycerol via an AMP-D-alanyl enzyme complex.

Glycolipids are also synthesized by some of these organisms. M. mycoides incorporates $^{14}$C-glucose into galactosyl diglycerides which
have little turnover (35,36). $^{14}$C-glucose is also incorporated into the
monoglucosyl and diglucosyl diglycerides of A. laidlawii B without sub-
sequent loss of radioactivity from these lipids (28,65,80). M. pneu-
moniae incorporated labeled glucose, glycerol and palmitate but not
galactose into glycosyl diglycerides (37). Only trace amounts of uni-
dentified glycolipids were isolated from U. species (P108) when it was
grown in the presence of labeled oleate (55). Radioactive oleate was
also incorporated into the monoglucosyl and diglucosyl diglycerides of
M. neurolyticum (84). The triacylglucose content of M. gallinarum
remains relatively constant (89). The cholesterol glucoside isolated
from this organism will be discussed later.

Smith (79,82) has studied the mechanism for the synthesis of glyco-
lipids in A. laidlawii B. Monoglucosyl diglyceride is synthesized from
1,2-diglyceride and uridine-5'-diphosphoglucose. Diglucosyl diglyceride
is formed from monoglucosyl diglyceride and uridine-5'-diphosphoglucose.
The enzymes involved are membrane bound and the rate of synthesis of
monoglucosyl diglyceride is twice that of diglucosyl diglyceride. The
source and the biosynthesis of the precursors of these lipids has not
been studied but it is assumed that the uridine-5'-diphosphoglucose is
synthesized as in other organisms.
Pigmented carotenoids are synthesized by *A. granularum*, *A. oculi* (*A. oculusi*) (1), most *A. laidlawii* strains and several other *A.* strains (94). *A. axanthum* and *A. modicum* do not synthesize colored carotenoids, but non-pigmented carotenoids may be synthesized (96).

The *de novo* pathway for the synthesis of these polyterpenes from acetate has been determined for *A. laidlawii* B (19,20,75,78,82,85,86,98). The mycoplasmas are not capable of synthesizing neutral lipids from acetate although some of them possess parts of the pathway found in *A. laidlawii* B (19,20,77,85,98). Smith (82) summarized the data concerning the enzymes present in *A. laidlawii* B, *M. arthritidis* and *M. gallinarum*.

Cholesterol appears to competitively inhibit the biosynthesis of carotenoids at the isopentenyl pyrophosphate isomerase locus in *A. laidlawii* B (75,92). Therefore, this organism may fail to produce the characteristic pigments when grown in media containing cholesterol.

Some workers suggest that carotenoids have a function similar to that of cholesterol in mycoplasmas (57,73,74,75,86). Data from several investigations indicated that *A. laidlawii* A and B grow in media devoid of cholesterol without the production of pigmented carotenoids (51,61). Smith (82) suggested that under the conditions present in his experiments, nonpigmented carotenoids might be synthesized which function as well as the pigmented compounds.

Carotenyl esters and cholesteryl esters of fatty acids are synthesized (73,80). *A. laidlawii* B can synthesize carotenyl glucoside (80). The synthesis of cholesteryl glucoside was also studied in *M. gallinarum* (81). The transfer of glucose to membrane bound cholesterol occurs via uridine-5'-diphosphoglucose. Galactose is also transferred to
cholesterol via uridine-5'-diphosphogalactose.

Pollack and Tourtellotte (47) first suggested that the acholeplasmas, but not the mycoplasmas, could incorporate acetate into fatty acids. It was reported that A. laidlawii A and B were capable of incorporating acetate into lipids—specifically saturated fatty acids (47,60). Rottem and Razin (61) also found that acetate was incorporated into the lipids of A. laidlawii and to a lesser extent into the lipids of M. gallisepticum, M. hominis, and M. orale. They also concluded that M. fermentans, M. mycoides var. mycoides and M. strain 14 were incapable of using acetate for lipid synthesis although aceto-kinase activity was observed in these strains as well as the other strains that were tested. Tully and Razin (96) later demonstrated that A. granularum and A. axanthum also incorporated acetate into their lipids. A. laidlawii incorporated more radioactive acetate into its lipids than any of the other organisms examined (61,96). Rottem et al. (59) reported that acyl carrier protein activity, necessary for fatty acid synthesis in bacteria, was higher for A. laidlawii, A. granularum, and A. axanthum than for any mycoplasmas tested. Rottem and Panos (60) have suggested that the A. laidlawii A synthesis of long-chain saturated fatty acids occurs via the malonyl-CoA pathway. Romijn et al. (56) citing their own work and others (47) indicated that A. laidlawii B could also elongate lauric and myristic acid to palmitate.

A. laidlawii A and B, M. mycoides var. mycoides, M. fermentans, and M. species (14) are not capable of synthesizing unsaturated fatty acids from acetate (47,60). Other work has shown that A. laidlawii A and B are capable of elongating short-chain monoenoic acids (33,34).
When a β-hydroxythioester dehydrase of \textit{E. coli} was added to a cell free system obtained from \textit{A. laidlawii A}, unsaturated fatty acids were synthesized (60). The lack of this type of enzyme may be the reason \textit{A. laidlawii} does not synthesize unsaturated fatty acids from acetate.

We decided to investigate the possibility that acholeplasmas and mycoplasmas could also be differentiated by their ability to synthesize lipids from radioactive acetate. We also wanted to determine if other acholeplasmas synthesized saturated fatty acids, and if so, would this indicate any differences among the acholeplasmas.
MATERIALS AND METHODS

Organisms. The Acholeplasma strains (see Table 1) were received from J. G. Tully (National Institutes of Health).

*Mycoplasma hyorhinis* 7 was obtained from R. F. Ross (Iowa State University), and *M. pneumoniae* CL8, *M. gallisepticum* S6, *M. fermentans* PG18, *M. pulmonis* N3, and *M. neurolyticum* A were obtained from N. L. Somerson (Ohio State University).

The anaerobic *Acholeplasma bactoclasticum* JR described by Robinson and Hungate (54) was not included in our study of acholeplasmas because of a recent observation (M. Allison and I. M. Robinson, personal communication) that this strain has an absolute cholesterol requirement.

Media and Growth Conditions. All organisms were grown in SSR2 broth medium (44) and where possible in a modified Tryptose broth medium (MT) (47). The MT basal medium consisted of: tryptose (Difco)-20g; NaCl, 5g; N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES) (Calbiochem, San Diego, Calif.), 9.77g; and 938 ml of distilled water. The basal medium was adjusted to pH 8.2 with 10 N NaOH. Additions were as follows: 50% (w/v) sterile glucose solution, 20 ml; fresh yeast extract solution (Microbiological Associates, Inc., Bethesda, Md.) 50 ml; and PPLO Serum Fraction (Difco), 10 ml. The PPLO Serum Fraction used in MT or SSR2 media was from control lot 521051 or 525683.

Stock solutions of sodium acetate-1-$^{14}$C (738 μCi/mg, Amersham/
Searle Corp., Arlington Heights, Illinois) were prepared in either MT or SSR2 medium at a concentration of 125 μCi/ml. These stock solutions were filter-sterilized. Stock solutions of radioactive acetate were then added to their respective medium to give a final concentration of 0.25 μCi/ml in the complete medium. Penicillin G was also added to give a final concentration of 50 units/ml in MT medium or 100 units/ml in SSR2 medium.

For acholeplasmas, 3-6 ml of a 24-h-old MT or SSR2 culture were inoculated into 125 ml of the same medium containing radioactive acetate. Acholeplasma cells grown either in SSR2 or MT medium were harvested at 24 h and 48 h, respectively. For Mycoplasma strains, 10-15 ml of a 24-h-old culture were inoculated into 250 ml of SSR2 containing radioactive acetate and harvested at 48 h, except for M. gallisepticum S6, which was harvested at 24 h. All organisms were incubated at 36 C.

To obtain pellets of mycoplasmas, the broth cultures were centrifuged at 16,000 X g for 20 min. The pellets were washed twice by resuspension in 200 ml of cold Kappa buffer (44) followed by centrifugation. The washed organisms were resuspended in 30 ml of ammonium acetate (0.155 M, pH 7.5), immediately centrifuged at 27,000 X g for 15 min, frozen at -25 C, and freeze-dried. The freeze-dried cells were stored in vacuo at -25 C.

**Extraction and Purification.** The freeze-dried pellets (2-47 mg) were weighed to 10^-4 g and were extracted three times with 5 ml of chloroform-methanol (2:1, Lipopure Reagents, Applied Science Laboratories, Inc., State College, Pa.). The pooled extract was filtered, dried under nitrogen, and purified by Sephadex chromatography as
described by Slakotos and Rouser (63, 72). The chloroform-methanol (19:1, water-saturated) fraction was dried under nitrogen, and the residue ("purified lipid") was weighed to \(10^{-5}\) g (Cahn G-2 Electrobalance, Ventron Inst. Corp., Paramount, Calif.). Data are reported as percent lipid recovered from whole cells, designated "% lipid."

**Determination of Acetate Contamination.** The final supernatant wash fluids contained about 340 dpm/ml, presumably all acetate-\(^{14}\)C. Washed cell pellets never exceeded 1 ml in volume. Using these values, and assuming an intracellular space of 50%, we computed that harvested cells, prior to freeze-drying, were contaminated with a maximum of 170 dpm acetate-\(^{14}\)C. In other experiments, Sephadex columns were loaded only with labeled acetate and chromatographed to determine how much radioactivity would elute into the chloroform-methanol (19:1, water-saturated) fraction and, therefore, contaminate the purified lipid fraction. About 50% of the labeled acetate applied to the Sephadex columns was eluted in this fraction. With these data, we calculated that no more than 100 dpm in any of our experiments could be ascribed to acetate contamination. In experiments with *Acholeplasma* strains, the acetate contamination was computed to be 0.77% (n=127, SD+1.77%).

To determine if spots seen in thin-layer chromatography (TLC) radioautographs were contaminating acetate-\(^{14}\)C, TLC plates were spotted with only radioactive acetate and chromatographed. These experiments showed that there were no spots found on TLC of radioactive acetate that corresponded to any found in our radioautographs of purified lipid.
Distribution of Radioactivity in Lipid Classes. Samples of purified lipid, containing known amounts of radioactivity, were placed on silicic acid columns, and the neutral lipid and glycolipid fractions were eluted (63,99). These eluates were dried under nitrogen and assayed for radioactivity. The radioactivity in the non-eluted polar fraction was estimated by subtracting the sum of the recovered neutral lipid and glycolipid fraction radioactivity from the amount applied to the column.

Scintillation cocktail (45) was added to purified lipid of known weight and assayed for radioactivity in a liquid scintillation counter (Model 3310S, Packard Instrument Co.). The samples were counted for 20 min, corrected for quenching and background, and radioactivity was reported as disintegrations/min/mg lipid (45).

Fatty Acid Isolation. Fatty acid methyl esters were prepared from dry purified lipids (0.09–5.02 mg) with known amounts of radioactivity by refluxing (65–70°C) for one hr in 0.5–2.0 ml of acidified methanol (18). Two-tenths ml of saline (0.85%) was added after the tubes cooled, and the mixture was extracted four times with petroleum ether (b.p. 38.9–60.0°C). The petroleum ether extracts were dried and assayed for radioactivity as for Lipid Classes.

Thin-Layer Chromatography of Lipids. Phospholipids were isolated by the two-directional TLC technique described by Turner and Rouser (97). In this procedure we used commercially prepared Silica Gel H plates, 250 mm thick, containing 7.5% magnesium acetate (Analtech Inc., Newark, Delaware). Neutral lipids were resolved by four-directional-development TLC (4D-TLC) (41).
To visualize lipids, plates were sprayed with a sulfuric acid/dichromate spray (Applied Science Laboratories, Inc., State College, Pa.) and charred. Phospholipids were also visualized by spraying with Phospray (Supelco Inc., Bellefonte, Pa.).

Radioautography. X-ray film (No-Screen Medical X-ray Film NS-54T, Kodak, Rochester, N.Y.) was exposed to unsprayed TLC plates for seven days and developed. The final prints showed labeled lipids as dark spots on a white background.

Analysis of Fatty Acid Methyl Esters. Mercuric acetate adducts of unsaturated fatty acid methyl esters were prepared according to Jantzen and Andreas (21) and assayed for radioactivity as for the Lipid Classes.

Silica Gel G plates (Analtech, Inc., Newark, Del.) were sprayed with a 40% (w/v) aqueous solution of AgNO₃. The plates were air dried in the dark for 30 min, activated at 110 C for 45 min, cooled, and used immediately. The plates were spotted with the adducted fatty acid methyl ester samples and chromatographed with n-propyl ether:hexane (1:4). A plate of standards was also chromatographed (Fig. 3). Saturated fatty acid methyl esters migrated near the solvent front while the mercuric acetate adducts of unsaturated fatty acid methyl esters remained at the origin.

The chromatographed plates were radioautographed. Areas of the TLC plates shown to have radioactivity in the radioautographs were scraped from the glass and extracted. Saturated fatty acid methyl esters were eluted with petroleum ether:diethyl ether (2:1) and areas of interest remaining near the origin were eluted with methanol. The
samples were dried and assayed for radioactivity as for the Lipid Classes. The radioactivity was reported as the percent of recovered radioactivity isolated in the saturated fatty acid methyl esters.

Summary of Experimental Procedures. A flow sheet of the experimental procedures is summarized in Appendix A.
RESULTS

SSR2 medium supported the growth of all the strains tested. Acholeplasmas, but not mycoplasmas, grew in the MT medium (Table 1).

The percent lipid and the dpm/mg lipid were determined for the acholeplasmas grown in the SSR2 and MT media. The same data were obtained for mycoplasmas grown in SSR2 medium (Table 1). The percent lipid was not significantly different (p>0.05) between strains of the two genera grown in SSR2 medium. However, the amount of radioactivity, from acetate-1-^{14}C, incorporated into the lipids of these organisms clearly separated the two genera. *Acholeplasma* strains incorporated significantly more radioactivity into their lipids than did *Mycoplasma* strains (p<0.025).

There was no significant difference (p>0.05) in the percent lipid of *Acholeplasma* cells grown in either SSR2 or MT medium. There was significantly more (p<0.005) radioactivity incorporated into the lipids of the *Acholeplasma* cells grown in the MT medium than incorporated when grown in the SSR2 medium (Table 1).

Additional experiments demonstrated that almost all of the radioactivity was lipoidal and not contaminating acetate-1-^{14}C.

The purified lipid from five *Acholeplasma* strains grown in the MT medium was fractionated on silicic acid columns (Table 2). Only 3% of the total radioactivity was found in the neutral lipid fraction. The balance of the radioactivity was distributed without statistically
Table 1. Comparison of Acholeplasma and Mycoplasma strains: the % lipid of whole cell (dry weight) and the incorporation of acetate-1-\(^{14}\)C radioactivity into their lipids

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<td></td>
<td></td>
<td>% lipid</td>
<td>dpm/mg of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dry wt</td>
<td>lipid</td>
</tr>
<tr>
<td>Acholeplasma laidlawii A</td>
<td>PG8</td>
<td>12.1(^b)</td>
<td>41,300</td>
</tr>
<tr>
<td>A. laidlawii B</td>
<td>PG9</td>
<td>13.9(^c)</td>
<td>77,000</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>Flamm</td>
<td>16.0</td>
<td>37,200</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>Granoff</td>
<td>13.8</td>
<td>62,800</td>
</tr>
<tr>
<td>A. laidlawii (94,95)</td>
<td>Haig 179L</td>
<td>12.3</td>
<td>8,300</td>
</tr>
<tr>
<td>A. laidlawii (25)</td>
<td>3M-152</td>
<td>15.2</td>
<td>20,500</td>
</tr>
<tr>
<td>A. granularum</td>
<td>31B-1</td>
<td>19.6</td>
<td>1,000</td>
</tr>
<tr>
<td>A. granularum</td>
<td>BTS-39</td>
<td>20.7</td>
<td>32,989</td>
</tr>
<tr>
<td>A. axanthum</td>
<td>S743</td>
<td>15.1</td>
<td>9,500</td>
</tr>
<tr>
<td>A. axanthum (94)</td>
<td>H86N</td>
<td>11.0</td>
<td>14,433</td>
</tr>
<tr>
<td>A. modicum</td>
<td>Squire (PG49)</td>
<td>15.6</td>
<td>2,500</td>
</tr>
<tr>
<td>A. oculi (oculusi) (1)</td>
<td>19L</td>
<td>13.3</td>
<td>147,451</td>
</tr>
<tr>
<td>Acholeplasma sp.</td>
<td>2310-8</td>
<td>13.8</td>
<td>4,600</td>
</tr>
<tr>
<td>Acholeplasma sp.</td>
<td>B3046</td>
<td>15.1</td>
<td>97,881</td>
</tr>
<tr>
<td>Acholeplasma sp.</td>
<td>Harris</td>
<td>15.6</td>
<td>14,689</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>CL8</td>
<td>29.7(^d)</td>
<td>77</td>
</tr>
<tr>
<td>M. gallisepticum</td>
<td>S6</td>
<td>19.7(^f)</td>
<td>180</td>
</tr>
<tr>
<td>M. fermentans</td>
<td>PG18</td>
<td>14.7</td>
<td>48</td>
</tr>
<tr>
<td>M. pulmonis</td>
<td>N-3</td>
<td>13.9</td>
<td>61</td>
</tr>
<tr>
<td>M. neurolyticum</td>
<td>A</td>
<td>13.0(^g)</td>
<td>26</td>
</tr>
<tr>
<td>M. hyorhinis</td>
<td>7</td>
<td>20.1</td>
<td>75</td>
</tr>
</tbody>
</table>

\(^a\) Data are the means of three to eight different determinations
\(^b\) Rasin et al. (49) found 12.8
\(^c\) Literature values (46,49,78) have ranged from 8.3 to 12.1
\(^d\) In the same medium, Pollack et al. (42) reported a value of 26.4; Prescott et al., using another medium (48), reported a value of 11.5
\(^e\) Not determined
\(^f\) Rasin et al. (49) found 15.7
\(^g\) Rasin et al. (49) reported 20.3, and Smith (84) reported 13.7
Table 2. Radioactivity in lipid fractions of *Acholeplasma* strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Radioactivity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Neutral lipid</th>
<th>Glycolipid</th>
<th>Polar lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. laidlawii</em> A</td>
<td>PG8</td>
<td></td>
<td>3</td>
<td>37</td>
<td>61</td>
</tr>
<tr>
<td><em>A. laidlawii</em> B</td>
<td>PG9</td>
<td></td>
<td>1</td>
<td>42</td>
<td>57</td>
</tr>
<tr>
<td><em>A. granularum</em></td>
<td>31B-1</td>
<td></td>
<td>5</td>
<td>47</td>
<td>48</td>
</tr>
<tr>
<td><em>A. granularum</em></td>
<td>BTS-39</td>
<td></td>
<td>4</td>
<td>53</td>
<td>43</td>
</tr>
<tr>
<td><em>A. axanthum</em></td>
<td>S743</td>
<td></td>
<td>2</td>
<td>34</td>
<td>65</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean: 3, 43, and 55 for neutral lipid, glycolipid, and polar lipid, respectively.
significant difference (p>0.05) between the glycolipid and polar lipid fractions.

Radioactive purified lipid and non-radioactive lipid standards were chromatographed (Fig. 1) by 4D-TLC. The upper right plate of Fig. 1 is a chromatogram, visualized by charring, of lipid standards. The upper left plate of Fig. 1 shows A. laidlawii A (PG8) lipids which were also visualized by charring. In this latter plate, the spots of the A. laidlawii A (PG8) lipid near the origin (lower left corner of plate) correspond to phospholipid and glycolipid areas. There are fainter spots in the diglyceride area (upper right quadrant). The lower plate of Fig. 1 shows a reproduction of a radioautograph of a similar sample of A. laidlawii A (PG8) lipids. The radioactivity appears to be located in the same areas as the lipids in the upper left plate of Fig. 1. These localized areas as well as the entire TLC plate were assayed for radioactivity. The percent of the total plate radioactivity in the diglyceride and phospholipid+glycolipid spots was 6.3% and 93%, respectively. Only 0.7% of the radioactivity was found on the remainder of the plate. This agrees closely with the data from the silicic acid column, which show 3% of the radioactivity in the neutral lipid and 97% in the polar+glycolipid fractions.

To confirm further that the unresolved radioactivity at the origin of the TLC plates in Fig. 1 was lipid, additional TLC chromatography was conducted. Fig. 2 shows radioautographs of Acholeplasma axanthum (S743), A. granularum (BTS-39), and A. laidlawii A (PG8) chromatographed for resolution of phospholipids. These radioactive lipids have not been completely identified.
Figure 1. 4D-TLC (41) of lipid standards and purified lipids of A. laidlawii A (PG8). Upper left plate, A. laidlawii A (PG8)-purified lipids (0.50 mg) visualized by sulfuric acid-dichromate spray and charring. Lower plate, Radioautograph of A. laidlawii A (PG8) lipids (0.20 mg). Upper right plate, Lipid standards (25 to 50 μg each): tetracosane, cholesteryl palmitate, methyl palmitate, tripalmitin, 1,3-dipalmitin, 1,2-dipalmitin, palmitic acid, cholesterol, 1-monopalmitin, 2-monopalmitin, phosphatidic acid, phosphatidyl glucose, and lecithin.
Figure 2. Radioautographs of purified lipids of: A. exanthum (S743) (1.06 mg), upper left plate; A. granularum (BTS-39) (0.24 mg), upper right plate; and A. laidlawii A (PG8) (0.25 mg), lower plate. TLC plates were chromatographed by the method of Turner and Rouser (97) to separate phospholipids.
At least 42% of all the radioactivity in unfractionated purified lipids was isolated in a fatty acid methyl ester fraction from those acholeplasmas listed in Table 1.

Since earlier work showed that A. laidlawii A (PG8) and B (PG9) incorporated acetate radioactivity into saturated fatty acids, we were interested in determining if this was also true for other acholeplasmas. A TLC technique was developed which permitted the isolation of saturated long-chain fatty acid methyl esters from free saturated and unsaturated fatty acids, and mercuric acetate adducted derivatives of unsaturated fatty acid methyl esters. This technique used AgNO₃ impregnated plates and is illustrated in Fig. 3 using standards. We also found that methyl-2-OH-palmitate had a Rf similar to methyl linoleate. Fig. 4 shows a reproduction of a representative radioautograph of TLC separation of fatty acid methyl esters after adduction with mercuric acetate. All the Acholeplasma strains tested showed radioactive spots which correspond to the saturated fatty acid methyl ester area migrating with methyl palmitate. Any remaining radioactivity was located at or near the origin. These origin associated radioactive compounds have not been identified.

It appears from the data in Table 3 that there are two groups of acholeplasmas - one group which has a high percentage of the recovered radioactivity found in a saturated fatty acid methyl ester fraction migrating with methyl palmitate, and another group which has a low percentage of the recovered radioactivity found in the same fraction. The higher group contains: A. laidlawii A (PG8), A. laidlawii B (PG9), A. laidlawii (Flamm), A. laidlawii (Granoff), A. laidlawii (Haig 179L)
Figure 3. Drawing of TLC of lipid standards on Silica Gel G plates sprayed with 40% (w/v) aqueous AgNO₃. Solvent: n-propyl ether:hexane (1:4). a. methyl palmitate, b. methyl oleate, c. methyl linoleate, d. methyl oleate adduct, e. methyl linoleate adduct, f. oleic acid, g. linoleic acid, h. methyl palmitate, methyl oleate, and methyl linoleate. Spots were visualized by spraying with Rhodamine 6G reagent.
Figure 4. Radioautograph of TLC separation of saturated fatty acid methyl esters of acholplasmas on Silica Gel G plates sprayed with 40% (w/v) aqueous AgNO₃. Solvent: n-propyl ether:hexane (1:4). a. *A. laidlawii* (Flamm), b. *A. laidlawii* (Granoff), c. *A. species* (Harris), d. *A. granularum* (BTS-39), e. *A. laidlawii A* (PG8), f. methyl palmitate (PA-M), methyl oleate (OL-M), and methyl oleate adduct (OL-M-Ad).
Table 3. % recovered radioactivity in saturated fatty acid methyl esters

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>X% in SSR2 medium</th>
<th>X% in MT medium</th>
<th>X% of SSR2 &amp; MT medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acholeplasma laidlawii A</td>
<td>PG8</td>
<td>89</td>
<td>--</td>
<td>89</td>
</tr>
<tr>
<td>A. laidlawii B</td>
<td>PG9</td>
<td>92</td>
<td>--</td>
<td>92</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>Flamm</td>
<td>91</td>
<td>94</td>
<td>92</td>
</tr>
<tr>
<td>A. laidlawii</td>
<td>Granoff</td>
<td>93</td>
<td>--</td>
<td>93</td>
</tr>
<tr>
<td>A. laidlawii (94,95)</td>
<td>Haig 179L</td>
<td>84</td>
<td>96</td>
<td>90</td>
</tr>
<tr>
<td>A. granularum</td>
<td>31B-1</td>
<td>--</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>A. granularum</td>
<td>BTS-39</td>
<td>88</td>
<td>--</td>
<td>88</td>
</tr>
<tr>
<td>A. oculi (A. oculusi)(1)</td>
<td>19L</td>
<td>95</td>
<td>--</td>
<td>95</td>
</tr>
<tr>
<td>A. sp.</td>
<td>b3046</td>
<td>95</td>
<td>--</td>
<td>95</td>
</tr>
<tr>
<td>A. sp.</td>
<td>Harris</td>
<td>73</td>
<td>82</td>
<td>76</td>
</tr>
<tr>
<td>A. axanthum</td>
<td>S743</td>
<td>14</td>
<td>--</td>
<td>14</td>
</tr>
<tr>
<td>A. axanthum (94)</td>
<td>H86N</td>
<td>7</td>
<td>46</td>
<td>27</td>
</tr>
<tr>
<td>A. modicum</td>
<td>Squire (PG49)</td>
<td>40</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>A. laidlawii (25)</td>
<td>3M-152</td>
<td>8</td>
<td>43</td>
<td>26</td>
</tr>
<tr>
<td>A. sp.</td>
<td>2310-S</td>
<td>21</td>
<td>58</td>
<td>40</td>
</tr>
</tbody>
</table>

a) Data are the means of two to five experiments.

b) Data is the result of only one experiment.
A. granularum (31B-1), A. granularum (BTS-39), A. oculi (A. oculusi) (1) (191), A. species (b3046) and A. species (Harris). The lower group contains: A. axanthum (S743), A. axanthum (H86N) (94), A. modicum (Squire, PG49), A. laidlawii (3M-152) (25) and A. species (2310-S). The mean value for the high group was 89 (SD±7) when grown in SSR2 medium and 90 (SD±5) when grown in MT medium. The low group has a mean value of 18 (SD±12) when grown in SSR2 medium and a mean value of 47 (SD±7) when grown in MT medium. The two groups, high vs. low, are statistically different whether they are grown in SSR2 (p<0.0005) or MT medium (p<0.0005). The means of the values obtained from SSR2 and MT medium were averaged (col. 3, Table 3). Again, the two groups were statistically distinct (p<0.0005). The mean value for the high group was 90 (SD±5) and 29 (SD±10) for the low group.
DISCUSSION

MT medium was originally chosen for our work because of the low serum content (1%), but growth of the Mycoplasma strains in this medium was negligible or absent. Therefore, SSR2 medium (containing 3% serum), which supported growth of all of the strains studied was used as the test medium.

As shown in Fig. 1, there was an increase in the dpm/mg lipid of the Acholeplasma strains grown in MT medium when compared to SSR2 medium (p<0.005). This increase is attributed to a greater biosynthetic use of acetate as a lipid precursor in the lipid-poor MT medium compared to the SSR2 medium.

Some of the Acholeplasma strains, especially b3046, showed a wide variation in replicate experiments in dpm/mg lipid. This variation may be due in part to different lots of medium ingredients.

Rottem and Razin (61) showed that radioactivity from acetate was incorporated into the lipids of some Mycoplasma species. Their data was reported as counts per minute (cpm) in extracted total lipid. Their method of purifying isolated lipid was by washing (61) and they grew the cells in Edward medium. It is difficult to compare their work to ours since we have used a somewhat more defined medium to grow the organisms, and purified the lipids by Sephadex chromatography to remove non-lipid contaminants and retain glycolipids. Also our computations
are based on assessing radioactivity as disintegrations per minute (dpm), by adjusting for spurious quenching effects which occur during counting, and relating corrected data to a unit weight of purified lipid.

Rottem and Razin (61) found in the isolated lipids of *M. gallisepticum* (A5969) about 16% of the amount of radioactivity detected in the lipids from *A. laidlawii* A. In our work, Table 1, the dpm/mg lipid from *M. gallisepticum* S6 was less than 1% of that found in *A. laidlawii* A. When we computed the data of Rottem and Razin (61) as cpm in recovered lipid/mg whole cell protein we obtained values of 528 for *M. gallisepticum* (A5969) and 1852 for *A. laidlawii* A. In our work we reported as dpm/mg lipid (Table 1) 180 for *M. gallisepticum* S6 and 41,300 for *A. laidlawii* A.

The radioautographs of 4D-TLC separation of lipids from *A. laidlawii* A (PG8) show small amounts of radioactive components in the diglyceride area. The presence of these apparent diglycerides suggest a source for the intermediates in the synthesis of glycolipids and possibly phospholipids. Smith has shown that *A. laidlawii* B can synthesize monoglycosyl diglyceride (MGD) from 1,2-diglyceride and uridine-5'-diphosphoglucose (UDPG) and the diglucosyl diglyceride from MGD and UDPG (79). Plackett et al. (39) have found that *A. axanthum* (S743) is capable of utilizing glycerol in the synthesis of phospholipids. The relatively small amounts of the radioactive diglyceride-like compounds found in our work suggests a possible source of these compounds for more complex lipid synthesis.

Mårdh and Taylor-Robinson (27) found that the growth of strains
of 31 Mycoplasma species was inhibited by lysolecithin; strains of Acholeplasma species were less sensitive. These workers used Acholeplasma sp. (Squire, PG49), which was recently established as Acholeplasma modicum (26), and found that it was as sensitive to lysolecithin as the majority of the Mycoplasma strains. From our work, this strain is included in the genus Acholeplasma because it incorporates radioactivity into lipid.

Recently, Rottem et al. (59) detected acyl carrier protein (ACP) activity in acholeplasmas and relatively less activity in mycoplasmas. Their findings are compatible with the relative amounts of acetate radioactivity incorporated into the lipids of the acholeplasmas and mycoplasmas that we have tested.

As shown in Table 3, all the acholeplasmas tested incorporated radioactivity from acetate-1-¹⁴C into saturated fatty acids (migrating with palmitic acid). It has not been determined if the radioactivity is incorporated into these fatty acids by de novo synthesis from acetate. Rottem and Panos (60) described a cell free system isolated from A. laidlawii A (PG8) that synthesized long-chain fatty acids. They suggested that fatty acids were synthesized by the malonyl Co-A pathway. It is possible that other acholeplasmas use this pathway for the de novo synthesis of saturated fatty acids from acetate. Some of the acholeplasmas may lack enzymes necessary for the de novo synthesis of saturated fatty acids. Romijn et al. (56) citing their own work and the work of others (47) indicated that lauric and myristic acid can be elongated to palmitate. Therefore, saturated fatty acids may also be synthesized by elongation of short-chain fatty acids using
acetate as a carbon source.

Radioautographs of TLC plates chromatographed for the isolation of saturated fatty acid methyl esters also showed unidentified radioactive compounds located at or near the origin. These radioactive compounds may be mercuric acetate adducts of unsaturated fatty acid methyl esters except in the case of A. laidlawii A (PG8) and B (PG9) which are unable to incorporate acetate into unsaturated fatty acids (47,60). There are other compounds which remain near the origin in this system. For example, any free fatty acids or saturated fatty acids that were not cleaved from the glycerol backbone remain in this area. We found that hydroxy fatty acid methyl esters also remain near the origin. Plackett et al. (39) reported the possible isolation of hydroxy fatty acids from lipid extracted from A. axanthum (S743).

We were able to separate all the acholeplasmas tested into two groups according to the percentage of recovered radioactivity isolated in a saturated fatty acid methyl ester area. Recently Tully (94) compared twenty-nine acholeplasmas by their biological and serological properties. He separated these acholeplasmas into five distinct groups and one group of five ungrouped but serologically distinct acholeplasmas. In his work he classified A. species (H86N), which was originally classified as A. laidlawii (25), as A. axanthum. According to our data, we placed this strain in the low group along with A. axanthum (S743). Earlier work (25) serologically grouped A. species (34-152) with A. laidlawii A (PG8) and A. species (643-N). We found, however, that this strain fits into our low group while the remainder of the A. laidlawii
strains fit into our high group. If the serological classification of A. species (3M-152) as an A. laidlawii strain is correct, then our data may indicate a subgrouping within the A. laidlawii strains.

We propose that the ability of acholeplasmas to incorporate significant amounts of acetate-\textsuperscript{14}C into their lipid is a useful criterion for differentiating them from the mycoplasmas.
SUMMARY

Fifteen *Acholeplasma* strains incorporated radioactivity into their lipids when grown in media containing acetate-\(^{14}\)C. In most cases, a greater incorporation of acetate-\(^{14}\)C occurred when the acholeplasmas were grown in the medium with the lowest serum content. The majority of the radioactivity was found in the glycolipid (43%) and the polar lipid (53%) fractions. Radioactivity was also found in the neutral lipid fraction which contained some lipids that migrated as diglycerides when chromatographed.

The amount of acetate-\(^{14}\)C radioactivity incorporated into the lipids (disintegrations per minute per milligram) of strains of six *Mycoplasma* species was less than 1% of the amount found in the fifteen *Acholeplasma* strains.

At least 42% of the total lipid radioactivity was isolated in fatty acid methyl esters. All the acholeplasmas tested incorporated acetate-\(^{14}\)C into saturated fatty acids. This incorporation may result from de novo synthesis from acetate or elongation of pre-existing fatty acids. The acholeplasmas were grouped according to the percentage of recovered radioactivity isolated in the saturated fatty acid methyl ester fraction. The high group contains: *A. laidlawii* A (PG8), *A. laidlawii* B (PG9), *A. laidlawii* (Flamm), *A. laidlawii* (Granoff), *A. laidlawii* (Haig 179L) (94), *A. granularum* (31B-1), *A. granularum* (BTS-39), *A. oculi* (*A. oculusi*) (1) (19L), *A. species* (b3046) and
A. species (Harris). The lower group contains: A. axanthum (S743), A. axanthum (H86N) (94,95), A. modicum (Squire, PG49), A. laidlawii (3M-152) (25) and A. species (2310).

Therefore, the ability to synthesize lipids or saturated fatty acids from acetate is another useful criterion for differentiating the genus Acholeplasma from Mycoplasma.
Appendix A. Summary of experimental procedures

Growth of mycoplasmas and acholeplasmas

in the presence of acetate-1$^{14}$C

a. MT medium
b. SSR2 medium

organisms harvested by centrifugation

a. pellets washed, frozen and weighed

extraction and purification of lipid

a. % lipid of whole cell dry weight
b. dpm/mg lipid

distribution of radioactivity

Thin-layer chromatography of total purified lipids

a. 4D TLC for resolution of lipids
b. 2-D for resolution of phospholipids

Isolation of fatty acids

a. synthesis of fatty acid methyl esters
b. preparation of mercuric acetate adducts
c. TLC of adducts
d. isolation and assay of radioactive compounds


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