LIPID COMPOSITION AND SYNTHESIS IN THE PLEUROPNEUMONIA-LIKE ORGANISM *MYCOPLASMA GALLISEPTICUM*[1]

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ABSTRACT

TOURTELLOTTE, MARK E. (University of Connecticut, Storrs), ROBERT G. JENSEN, GEORGE W. GANDER, AND HAROLD J. MOROWITZ. Lipid composition and synthesis in the pleuropneumonia-like organism *Mycoplasma gallisepticum*. J. Bacteriol. **86**:370–379. 1963.—A simple method of extracting lipids from cells of *Mycoplasma gallisepticum* by use of silicic acid columns is described. Proteolipids (peptides) extracted with chloroform-methanol (2:1) by other methods were not extracted with the lipid by this method, nor were proteins and nucleic acids. Fractionation of lipids of *M. gallisepticum* demonstrated the presence of saturated hydrocarbons, free fatty acids, cholesterol, cholesterol esters, di- and triglycerides, phosphatidic acids, cephalins, inositides, phosphatidyl choline, and sphingomyelin. The fatty acid composition of the various fractions was also determined. The positive identification of cholesterol and cholesterol esters in this organism by chromatography, melting point, and infrared spectroscopy confirms reports by others that cholesterol is present in the pleuropneumonia group of microorganisms. The incorporation of P^{32} orthophosphate into four phospholipid fractions, of oleic acid-*1*-C^{14} into neutral and phospholipids, and cholesterol-*4*-C^{14} into cholesterol esters clearly demonstrated the ability of *M. gallisepticum* to synthesize these lipids from simpler compounds. Between 70 and 80% of the lipid of this organism was found in the membrane.

Pleuropneumonia-like organisms (PPLO), like the viruses, pass through very fine filters. Unlike the viruses, however, they do not require host cells in order to replicate, and they grow in bacteriological media if serum or ascitic fluid is added.

Kandler (1956) and Lynn and Smith (1960) showed that the percentage composition of nucleic acids, protein, and lipid did not differ significantly from that of bacterial cells, except for a lower nucleic acid content and the presence of cholesterol in the PPLO. Recently, Morowitz et al. (1961) reported on the chemical composition and submicroscopic morphology of the avian PPLO *Mycoplasma gallisepticum* (Edward and Kanarek, 1960). The percentages of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, and lipid were approximately 4, 8, 80, and 11%, respectively, which was also not significantly different from those found in bacteria.

DNA was double-stranded, ribosomes and soluble RNA were present, and the sedimentation pattern of the soluble protein appeared similar to that of *Escherichia coli*. Amino acid composition of the protein was also similar to that of bacterial protein, except for a somewhat lower cystine content.

The requirement for cholesterol for growth of PPLO has attracted considerable attention. Smith and Rothblat (1960) demonstrated cholesterol uptake and cholesterol esterase activity in some human strains of PPLO. Lynn and Smith (1960) partially characterized the lipids of PPLO and found, in addition to cholesterol, fatty acids and phospholipids; the composition of fatty acid fraction and phospholipids was not analyzed. A report by Edward and Fitzgerald (1951) that an acetone-insoluble lipid fraction from egg yolk, in addition to cholesterol, is necessary for growth suggests that PPLO require phospholipids. Although Smith and Lynn (1958) and Rodwell (1960) could not show a growth requirement for phospholipid, the question is still

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an open one since serum albumin and other components of the medium might contain traces of lipids.

In view of the fact that PPLO appear to require complex medium and possess limited biosynthetic powers, it would be of interest to separate and characterize the lipid fractions and determine whether they are synthesized by the cells.

This paper consists of a detailed study of the lipid composition of *M. gallisepticum* and demonstrates that this organism possesses enzymes capable of synthesizing glycerides, phospholipids, and cholesterol esters from simpler compounds.

Materials and Methods

Growth of cells. The organism used throughout this study was the A5969 strain of *M. gallisepticum*, the causative agent of a respiratory disease in poultry. Growth of cells was as described in detail by Tourtellotte and Jacobs (1960).

Tryptose Broth (Difco) supplemented with 1% glucose and 1% PPLO Serum Fraction (Difco), pH 8.2, was inoculated with *M. gallisepticum* and incubated at 37 C on magnetic stirrers for 24 hr. Cells were harvested in a Servall continuous-flow centrifuge (30,000 × *g*), and washed twice with 0.85% saline and once with distilled water. Dry weight was determined by drying a sample of cell suspension to constant weight at 105 C. In isotope-labeling experiments, the radioactive compounds were added directly to the above medium. Small quantities of cells were harvested in a Servall SS-1 centrifuge at 30,000 × *g* for 10 min and washed as above. In experiments where cells were grown with radioactive cholesterol and oleic acid as the sole lipids, Tryptose and Serum Fraction (pH 2.0) were extracted in a Soxhlet extractor with methanol and chloroform.

Extraction of lipids. Extraction of lipids was performed by two different methods.

(i) Filtration method: 1 g of cell paste (pH 2.0) was mixed with 100 ml of chloroform-methanol (2:1) at 0 C and filtered through previously extracted filter paper [ethyl ether and chloroform-methanol (2:1)] on a Büchner funnel. Additional solvent was added until 500 ml had filtered through. Solvent was removed on a rotary evaporator or under a stream of N_2 at 4 C. The residue was redissolved in chloroform-methanol (2:1), washed with water by the method of Folch et al. (1951), and dried under vacuum to constant weight.

(ii) Silicic acid method: a thick, aqueous cell suspension (pH 2.0) was triturated with silicic acid in a mortar to make a free-running powder (10 ml per 18 g of silicic acid), mixed with 200 ml of chloroform-methanol (2:1), and poured into an extraction column containing a plug of silicic acid on a sintered-glass base (Harper, Schwartz, and El-Hagarawy, 1956). Solvent was gently sucked through with the aid of an aspirator; when the solvent was nearly gone, an additional 100 ml of chloroform-methanol (2:1) was allowed to flow through, followed by 200 ml of chloroform-methanol (1:4). Removal of solvent and Folch washing was the same as in the filtration method.

Silicic acid. Silicic acid for extraction and fractionation of lipids was minus 325 mesh specially prepared for the chromatography of lipids (Bio-Rad Laboratories, Richmond, Calif.). Before use, it was washed with chloroform, chloroform-methanol (2:1), and methanol (100 ml of each per 20 g of silicic acid); dried overnight at 115 C; and stored in a vacuum desiccator. Washing with these solvents minimized elution of silicic acid in extraction and fractionation procedures.

Chemicals. All solvents including petroleum ether (bp 60 to 70 C) were reagent grade and were distilled before use. Reference lipids [β-carotene, cholesteryl acetate, triolein, oleic and palmitic acids, glycerol monooleate, L-α-cephalin (β,γ-dipalmitoyl), L-α-lecithin (β,γ-dipalmitoyl)], as well as oleic acid-*1*-C^{14}, cholesterol-*4*-C^{14}, glucose-*U*-C^{14}, galactose-*2*-C^{14}, glycine-*1*-C^{14}, thymine-*2*-C^{14}, and uracil-*2*-C^{14}, were obtained from Calbiochem. $H_3P^{32}O_4$ was obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.

Fractionation of lipids. Fractionation of lipids was carried out by silicic acid chromatography, by the stepwise elution method of Hirsch and Ahrens (1958) for neutral lipids coupled with the elution scheme for phospholipids described by Hanahan, Dittmer, and Warashina (1957). A column of the type described by Hirsch and Ahrens (1958) was packed with 18 g of silicic acid. Lipids in 10 ml of petroleum ether were applied to the column, and elution was accomplished with the solvents and volumes shown in Table 1. Changes of solvent were made slowly, just as the last of the previous solvent disappeared

TABLE 1. *Stepwise elution scheme for fractionation of lipids of Mycoplasma gallisepticum from silicic acid columns**

Tube no.†	Amt of eluant	Eluant
1–9	360	1% Ethyl ether in petroleum ether
10–17	320	4% Ethyl ether in petroleum ether
18–33	640	8% Ethyl ether in petroleum ether
34–39	240	25% Ethyl ether in petroleum ether
40–44	200	100% Ethyl ether
45–46	80	Chloroform-methanol (7:1)
47–49	120	Chloroform-methanol (4:1)
50–53	160	Chloroform-methanol (3:2)
54–56	120	Chloroform-methanol (1:4)
57–60	160	Methanol

* Columns (250 × 18 mm) contained 18 g of silicic acid.

† Tubes contained 40 ml each.

into the column; at no time was the silicic acid allowed to dry. Special caution must be taken when chloroform-methanol (7:1) is added, since rapid mixing with ethyl ether will disrupt the structure of the column.

Elution of known reference lipids, separately and as mixtures, occurred in fractions, as predicted by these authors, and thus gave a clue to the identity of the peaks eluted from PPLO lipids.

Solvent was removed in a rotary "flash" evaporator at 50 C or under a stream of N_2 at 4 C, and the resulting lipid fractions were dried in vacuo and weighed, corrections being made for elution of silicic acid.

Analytical methods. Infrared analyses were done in a Perkin-Elmer Infracord spectrophotometer (model 137). Fatty acid analyses were accomplished in a modified Aerograph model A-90C gas chromatography instrument using columns of Apiezon M (on glass beads), 10 ft long, temperature programmed 100 to 275 C and diethylene glycocyl succinate on Chromosorb W at 180 C, after conversion of fatty acids to their methyl esters by refluxing with sodium methoxide in methanol. They were identified by comparison with plots of methyl esters of known pure fatty acids and semilog plots of retention time vs. chain length. Fatty acids were quantitated by cutting out peaks and weighing the papers. Radioactivity was determined with a Nuclear-Chicago model 183B scaler with a windowless gas-flow detector. Counts were corrected for background and self-absorption.

Hydrocarbons were identified by infrared spectroscopy. Cholesterol and cholesterol esters were detected by the Liebermann-Burchard reaction. Cholesterol was further identified by melting point, digitonin precipitation, paper chromatography, and infrared spectroscopy.

Monoglycerides were determined by the method of Jensen and Morgan (1959). Di- and triglycerides were identified by infrared spectroscopy. Free fatty acids were detected by determination of titratable acidity.

Phospholipid phosphorus was determined by the method of Stewart and Hendley (1935) and by counting of radioactive P^{32}. Plasmalogens ("acetals") were detected with Schiff-Feulgen reagent (Hack and Ferrans, 1959). Choline was identified on paper chromatograms by the procedure of Levine and Chargaff (1951).

Lipids for inositol and glycerol assay were hydrolyzed with 2 N HCl at 100 C for 30 min, evaporated to dryness over KOH in vacuo, and dissolved in water. Glycerol, inositol, and other carbohydrates were detected on paper chromatograms developed in *n*-propanol-ethanol-water (5:3:2) by spraying with ammoniacal silver nitrate (Hanahan, 1958).

Purification of lipid fractions. Because free amino acids and glycerol eluted with certain phospholipid fractions (Hanahan et al., 1957; Wren and Mitchell, 1959), purification of phospholipids for subsequent analyses was necessary. These fractions were chromatographed on paper with chloroform-methanol-water (15:4:1). Phospholipids moved with the solvent front, whereas amino acids and peptides did not migrate (Hanahan et al., 1957). The front was eluted with methanol, reapplied to paper, and developed with *n*-propanol-ethanol-water (5:3:2). Phospholipids, as revealed by P^{32} counts, again migrated with the solvent front; glycerol showed an R_F of 0.67.

Cephalins, separated from free amino acids and peptides by the above procedure, were detected by spraying chromatograms with ninhydrin.

Sphingomyelin was hydrolyzed, and sphingosine was extracted and chromatographed by the method of Brady and Koval (1958) after removal of ninhydrin-positive compounds and glycerol.

For cerebroside analysis, a sample of purified total phospholipid was hydrolyzed with 3 N HCl for 2 hr at 100 C and evaporated to dryness over KOH. Paper chromatography with *n*-propanol-ethanol-water (5:3:2) and spraying with ammoniacal silver nitrate showed hexose sugars other than inositol to be absent.

Quantitative tests for cephalins, inositides, and lecithins. Crude quantitative tests to determine the relative amounts of cephalins, inositides, and lecithins were performed by spotting known amounts of L-α-cephalin, L-α-lecithin, and inositol on paper and comparing the size and intensity of the color spots with those produced by cephalins, lecithins, and inositides from PPLO.

Incorporation of glucose and galactose. Incorporation of glucose and galactose into cells was determined by growing cells in the presence of uniformly C^{14}-labeled glucose and galactose-*2-*C^{14} and counting the radioactivity present in cells.

Distribution of lipid between membrane and soluble fractions. In experiments designed to determine the relative amounts of lipid in membrane and soluble fractions, cell suspensions in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.6) were ruptured in a French pressure cell at 6,000 psi and centrifuged at 30,000 × *g* for 10 min. Examination of the pellet in an electron microscope showed predominantly membrane "ghosts." No cells were observed in the supernatant fluid.

RESULTS

Extraction of lipids. In early experiments, lipids were extracted by mixing cells with chloroform-methanol (2:1) and filtering through a Büchner funnel. However, emulsions were occasionally formed that would not allow separation of lipid from other cell components by filtration or centrifugation. In addition, chloroform-methanol (2:1) extracts contained large amounts of ninhydrin-positive material which was not removed by Folch washing. When chromatographed on paper in chloroform-methanol (2:1), the ninhydrin-positive material did not migrate from the point of application; lipids in this solvent flowed with the front (Hanahan et al., 1957). Elution of this spot and hydrolysis in 6 N HCl for 18 hr revealed a 10- to 20-fold increase in ninhydrin-positive material, suggesting that it was a peptide or "proteolipid" and not free amino acids. It was of interest that chloroform-methanol (2:1) extracts of PPLO grown in medium containing glycine-*1-*C^{14} contained over 20% of the total radioactivity of the cells (Table 2). When chromatographed on paper in chloroform-methanol (2:1), all the C^{14} remained with the peptide; no radioactivity was found in the lipid.

TABLE 2. *Extraction of lipids, proteins, peptides, and nucleic acids from Mycoplasma gallisepticum with chloroform-methanol by the filtration and silicic acid methods*

Cells grown in presence of	Per cent total radioactivity of cells extracted by	
	Filtration	Silicic acid
Oleic acid-1-C^{14}	98.9	99.3
$H_3P^{32}O_4$*	19.0	19.4
Glycine-1-C^{14}	22.0	0.4
Thymine-2-C^{14}	0.1	0.2
Uracil-2-C^{14}	0.2	0.2

* $H_3P^{32}O_4$ was also incorporated into proteins and nucleic acids.

Extraction of total lipids by the silicic acid method (Table 2), however, showed little glycine-*1-*C^{14} and no peptides in the effluent. This method of extraction thus had two advantages over the filtration method: formation of emulsions was avoided, and chloroform-methanol soluble peptides were not extracted with the lipids.

Efficiency of lipid extraction with silicic acid. As originally reported by Harper et al. (1956), neutral lipids were extracted but phospholipids were not completely eluted from silicic acid columns utilizing chloroform-methanol (2:1). It was therefore necessary to determine whether increasing solvent polarity would remove all phospholipids. In previous experiments, we had noted that lipid extraction, by the filtration method, of cells grown in the presence of oleic acid-*1-*C^{14} showed all the radioactivity to be in the chloroform-methanol extract with none in the cell residue. As will be shown later, fractionation of this lipid extract revealed radioactivity peaks which corresponded to the four phospholipid peaks, establishing the presence of oleic acid-*1-*C^{14} in all phospholipid fractions. Since oleic acid-*1-*C^{14} was completely extracted with the lipid and appeared in all phospholipid fractions, it provided a convenient index for determining efficiency of phospholipid extraction by the silicic acid method.

As shown in Table 2, elution of lipids from silicic acid columns with chloroform-methanol (2:1) followed by chloroform-methanol (1:4) gave approximately 100% recovery of oleic acid-*1*-C^{14}, showing that this method did extract all neutral and phospholipids. As a further check, cells grown in the presence of $H_3P^{32}O_4$ were extracted by both the filtration and silicic acid methods. P^{32} counts in both cases were the same.

Since it was established that all lipids were extracted by the silicic acid method, it seemed desirable to determine whether nucleic acids were eluted with the solvent. Accordingly, cells grown in the presence of thymine-*2*-C^{14} and uracil-*2*-C^{14} were washed with saline and water and extracted on silicic acid columns; the chloroform-methanol extracts contained no appreciable radioactivity. As was shown previously, proteins and peptides labeled with C^{14} glycine were also not eluted by chloroform-methanol (Table 2).

Thus, the extraction of lipids using silicic acid with subsequent Folch washing not only gave complete extraction of neutral and phospholipids but also retained protein, peptides, and nucleic acids on the column. It provided a rapid and simple method for extraction of lipids without the complications encountered with the filtration method. The extraction by this method of all oleic acid-*1*-C^{14}, which is present in neutral and phospholipids in the cells, suggests that, unlike the bacteria, this organism contains no bound lipids.

Total PPLO lipids. Extraction of 1750 mg (dry weight) of cells yielded 206.5 mg of lipid (11.8%). Small amounts (about 9 mg) of silicic acid were eluted from the columns by the solvent, and the above value is corrected for this.

Fractionation of total lipids. Fractionation on silicic acid separated *M. gallisepticum* lipids into ten peaks. The elution pattern is presented in Fig. 1.

Fraction A corresponded to saturated hydro-

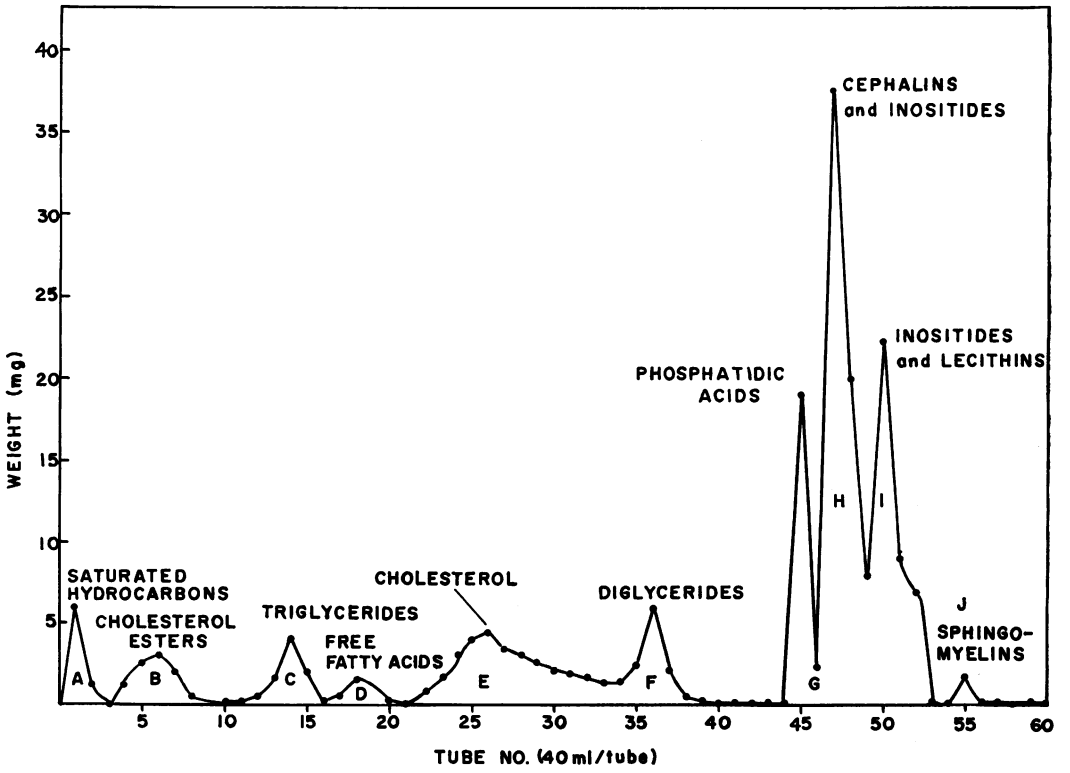

FIG. 1. *Fractionation of lipids from Mycoplasma gallisepticum by silicic acid chromatography. Abscissa: tube numbers correspond to tube numbers and solvents shown in Table 1. Ordinate represents the dry weight of lipid in each tube. Letters correspond to fractions in Table 3 also described in text.*

carbons according to the scheme of Hirsch and Ahrens (1958), B to cholesterol esters, C to triglycerides, D to free fatty acids, E to cholesterol (sterols), and F to diglycerides. There was no monoglyceride peak. Phospholipid fractionation with chloroform-methanol showed peaks G, H, I, and J, which were eluted in the corresponding glycerophosphatidic acid, cephalin, inositide-lecithin, and sphingomyelin fractions as described by Hanahan et al. (1957). Chemical characterization of these fractions is presented below.

Fraction A was a colorless material that gave a negative test for carotene and vitamin A. The infrared spectrum showed four prominent bands at 2930, 2850, 1465, and 1370 cm^{-1}.

Fraction B was Liebermann-Burchard-positive, not precipitated by digitonin. On acid hydrolysis, it contained a digitonin-precipitable compound which gave the same R_F and infrared spectrum as cholesterol, identifying it as cholesterol esters. The hydrolysate also contained fatty acids.

Fraction C's infrared spectrum was typical of that given by triglycerides. Hydrolysis revealed only glycerol and fatty acids.

Fraction D contained only fatty acids.

Fraction E gave a positive Liebermann-Burchard test, was digitonin-precipitable, gave the same R_F as known cholesterol, melted at 147 to 148.5 C, and gave an infrared spectrum after 3 × crystallization from methanol identical with that of authentic cholesterol.

Fraction F gave a spectrum typical of diglyceride, showing characteristic (OH-stretching) peaks at 3660 and 1040 cm^{-1}. It contained only glycerol and fatty acids after hydrolysis. This fraction gave a negative test for monoglycerides and was concluded to be diglyceride.

Fraction G, the first phosphorus-containing peak eluted, was ninhydrin-negative and contained no inositol or choline. Acid hydrolysis showed it to consist of fatty acids, glycerol, and glycerol phosphate, identifying this fraction as glycerophosphatidic acid.

Fraction H was ninhydrin-positive and contained inositol but no choline. The first fraction from this peak gave an infrared spectrum similar to synthetic dipalmitoyl-DL-α-cephalin. Crude quantitative tests of ninhydrin-positive material and inositol showed approximately 30% of this peak to be cephalins and 70% inositides.

Fraction I was ninhydrin-negative after purification to remove free amino acids, and gave positive tests for inositol and choline. A crude quantitative test showed approximately 85% to be choline-containing phospholipids and 15% inositol phospholipids. The latter fraction of this peak gave infrared absorbance peaks similar to those observed with dipalmitoyl-DL-α-lecithin.

Fraction J, as with fraction I, was ninhydrin-negative after removal of free amino acids. It contained no inositol but gave a positive test for choline. The infrared spectrum gave strong peaks at 1660 (CONH) and 1735 cm^{-1} (C—O ester-stretching). The latter peak suggested it contained some lecithins. The peak at 1660 cm^{-1}, which is absent or weak in lecithins, was suggestive of sphingomyelins (Nelson and Freeman, 1959). Hydrolysis of this fraction and chromatography on paper with pyridine, according to the method of Brady and Koval (1958; sphingosine R_F 0.9), revealed a ninhydrin-positive spot (R_F 0.8) which was absent in an unhydrolyzed sample. This sample was considered to be a sphingosinelike base.

Fractionation of lipids from cells grown in medium containing oleic acid-*1*-C^{14} showed radioactivity to be present in cholesterol esters, triglycerides, free fatty acids, diglycerides, and the four phospholipid fractions. $H_3P^{32}O_4$ was found only in the phospholipids (fractions G, H, I, and J). Liebermann-Burchard-positive material, as well as radioactivity from cells grown in the presence of cholesterol-*4*-C^{14}, was present only in fractions B, E, and F (Fig. 2). Traces of free glycerol were found in fractions H, I, and J. These results, in addition to others, are presented in Table 3.

Fractionation of lipids from cells grown in lipid-extracted medium supplemented with cholesterol-*1*-C^{14}, oleic acid-*1*-C^{14}, $H_3P^{32}O_4$ showed radioactivity peaks (Fig. 2) in all fractions shown in Fig. 1, except for fraction A (saturated hydrocarbons). *M. gallisepticum* did not grow in the absence of cholesterol or oleic acid.

Although glucose and galactose are metabolized by *M. gallisepticum* (Tourtellotte and Jacobs, 1960), growth of cells in the presence of C^{14} glucose and galactose showed no incorporation of radioactivity into the cells. They are presumably used solely as energy sources.

Fatty acid composition. Analysis by gas-liquid chromatography of the fatty acid content of the total cell lipid after hydrolysis revealed 65.7% of the total to be saturated acids and 34.3% un-

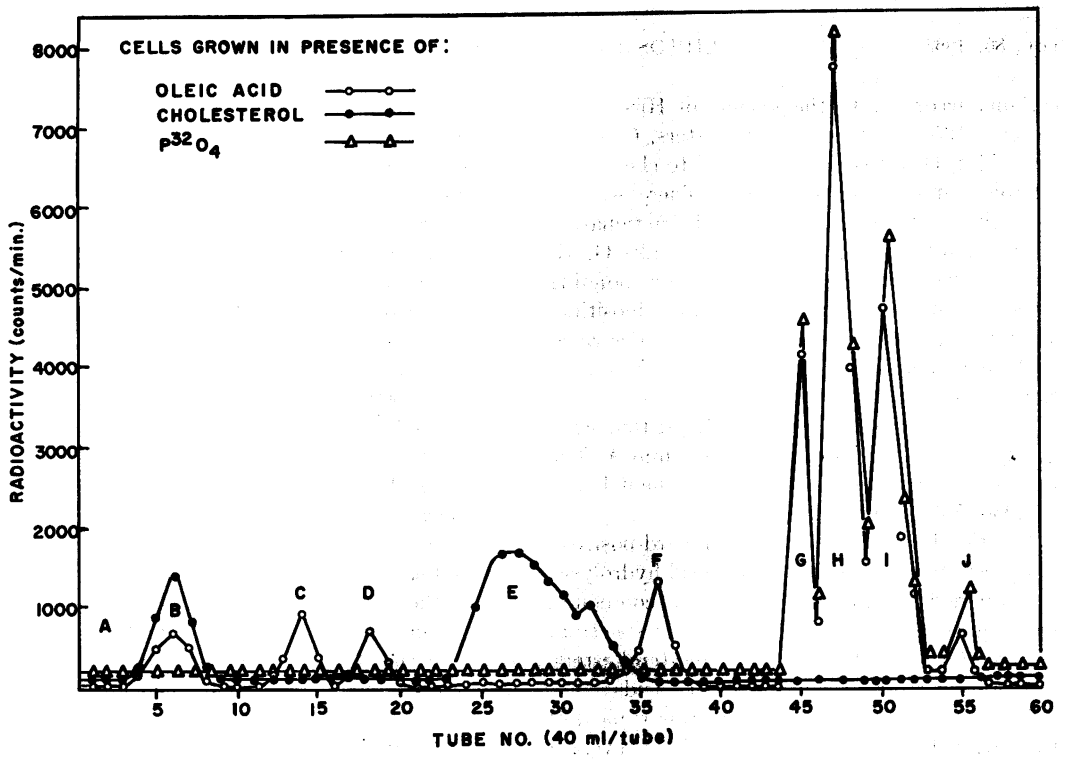

FIG. 2. *Fractionation of lipids by silicic acid chromatography from Mycoplasma gallisepticum grown separately in medium containing oleic acid-1-C^{14}, cholesterol-4-C^{14}, and P^{32} orthophosphate. Abscissa: tube numbers correspond to tube numbers and solvents shown in Table 1. Ordinate represents the radioactive counts in each tube. Letters correspond to fractions in Fig. 1.*

TABLE 3. *Composition of lipid fractions of Mycoplasma gallisepticum separated by chromatography on silicic acid columns*

Fraction	Dry weight	Total lipid*	Phosphorus	$H_3P^{32}O_4$†	Cholesterol-4-C^{14}†	Oleic acid-1-C^{14}†	Liebermann-Burchard	Titratable acidity	Glycerol (free)	Glycerol (after hydrolysis)	Inositol	Choline	Acetals	Reducing sugars	Ninhydrin	Ninhydrin (after removal of free amino acids)	Identity of compound(s)
	mg	%	*mg*	%	%	%											
A	7.9	3.8	0	0	0	0	−	−	−	−	−	−	−	−	−	−	Saturated hydrocarbons
B	11.3	5.5	0	0	31.6	6.9	+	−	−	−	−	−	−	−	−	−	Cholesterol esters
C	8.1	3.9	0	0	0	6.5	−	−	−	+	−	−	−	−	−	−	Triglycerides
D	4.0	1.9	0	0	0	4.7	−	+	−	−	−	−	−	−	−	−	Free fatty acids
E	28.1	13.3	0	0	64.2	0	+	−	−	−	−	−	−	−	−	−	Cholesterol
F	10.8	5.5	0	0	4.2	8.0	+sl	−	−	+	−	−	−	−	−	−	Diglycerides
G	21.3	10.2	0.98	16.7	0	15.6	−	ND‡	−	+	−	−	−	−	−	−	Phosphatidic acid
H	65.1	31.5	2.60	46.7	0	34.5	−	ND	+	+	+	−	−	−	+	+	Cephalins and inositides
I	37.4	18.1	1.53	28.1	0	22.6	−	ND	+	+	+	+	−	−	+	−	Inositides and lecithins
J	1.7	0.8	0.07	3.5	0	1.2	−	ND	+	+	−	+	−	−	+	−	Sphingomyelin ? (sphingosinelike base)

* Values are per cent of total lipid applied to column; 5.2% was not recovered.
† Values are per cent of total radioactivity of cell lipid.
‡ ND = not done.

TABLE 4. *Fatty acid composition of lipid fractions of Mycoplasma gallisepticum eluted from silicic acid columns**

Fatty acid	Carbon structure	Lipid fraction†				
		Free fatty acids	Cholesterol esters	Diglycerides	Triglycerides	Phospholipids
Caproic	6:0‡	—	—	—	0.6	—
Caprylic	8:0	—	—	—	0.9	—
Capric	10:0	3.8	—	0.4	6.1	—
Unidentified	10:1	—	—	—	0.6	—
Lauric	12:0	3.0	—	0.6	5.3	0.7
Myristic	14:0	1.4	1.2	3.4	14.3	3.3
Myristoleic	14:1	4.6	0.3	0.6	1.4	2.2
Pentadecanoic	15:0	1.0	0.3	0.5	1.1	—
Unidentified	15:1	1.4	—	—	—	—
Palmitic	16:0	36.2	23.4	26.3	32.2	24.8
Palmitoleic	16:1	12.6	6.3	8.8	1.1	2.9
Heptadecanoic	17:0	1.0	1.2	0.1	0.5	2.4
Unidentified	17:1	0.4	—	—	0.1	—
Stearic	18:0	21.2	13.1	32.1	10.0	36.0
Oleic	18:1	12.0	54.2	21.9	21.9	20.4
Linoleic	18:2	1.4	Trace	5.3	3.9	7.3
Total saturated		67.6	39.2	63.4	71.0	67.2
Total unsaturated		32.4	60.8	36.6	29.0	32.8

* Lipid fractions were acid-hydrolyzed and the fatty acids converted to their methyl esters by refluxing with sodium methoxide in methanol. Methyl esters were fractionated by gas chromatography.

† Results are expressed as per cent of total fatty acids in each fraction.

‡ First figure represents the number of carbons; the second figure, the number of double bonds.

saturated. The ratio of saturated to unsaturated was approximately 7:3 in the free fatty acid, di- and triglyceride, and phospholipid fractions; the cholesterol esters, however, contained more unsaturated than saturated acids (Table 4).

The predominant acids were palmitic, stearic, oleic, palmitoleic, linoleic, and myristic. We were unable to detect arachidonic acid in this organism. Fatty acid composition of the lipid fractions is also shown in Table 4.

Distribution of lipid between membrane and soluble fractions. Since electron micrographs of thin sections of *M. gallisepticum* showed the absence of a cell wall and the presence of a typical "unit" membrane approximately 75 A thick (Bullivant, *personal communication*), which is presumably lipoprotein, an experiment to determine the distribution of lipid between membrane and soluble matter was run. On the basis of dry weight, approximately 70% of the lipid was found in the membrane and 30% in the soluble fraction. Approximately 70% of the Liebermann-Burchard-positive material was also found in the membrane. When cells grown in oleic acid-*1*-C^{14} were fractionated, 80% of the radioactivity was in the membrane fraction. More detailed experiments are being conducted to determine the distribution of the various lipid components in membrane and soluble fractions.

Discussion

Although silicic acid is used routinely to separate the various components of a lipid extract, it has not been used extensively for the primary extraction of total lipids.

The extraction of total radioactivity from PPLO grown in the presence of oleic acid-*1*-C^{14} on silicic acid columns, and the extraction of the same amount of P^{32} phospholipid by this method as was extracted by filtration, established the silicic acid method as a simple and efficient means for extraction of neutral and phospholipids from biological material. Extraction of total lipid can be accomplished in 5 to 10 min, emulsions are avoided, and nucleic acids, protein, and proteolipids (proteins or peptides soluble in chloroform-methanol by the filtration method) are not eluted from the column with the lipids. As with other methods for extraction of total lipids, small water-soluble molecules were eluted and were removed by the method of Folch et al. (1951). The amount of small water-soluble molecules is not large, however, since most of these are removed by washing the cells before extraction and it is possible that Folch washing is not necessary. The possibility that cerebrosides were present but not extracted by this method was ruled out for the following reasons. (i) Wren and Mitchell (1959), using silicic acid in fractionation of Drosophila lipids, reported the elution of cerebrosides in solvent less polar than that used here (chloroform-methanol, 1:4) and, although Weiss (1956) reported the elution of strongly absorbed cerebrosides from silicic acid with methanol, this solvent failed to elute additional lipid from PPLO. (ii) Hydrolysis of total purified phospholipids extracted by both the silicic acid and filtration

methods showed that hexose sugars other than inositol were not present. (iii) Glucose-*U*-C^{14} and galactose-*1*-C^{14} are not incorporated into the cells.

In fractionation procedures, approximately 95% of the total lipid applied to the column was recovered. Since 5% of the P^{32} was retained on the column after fractionation (Table 3), it is probable that the loss is phospholipid, which is degraded owing to the length of time involved in fractionation procedures.

In agreement with Hirsch and Ahrens (1958), simple lipids were eluted in predictable fractions; phospholipids, on the other hand, were not resolved in 100% methanol, and the stepwise elution scheme of Hanahan et al. (1957), with chloroform-methanol, was employed. Although this method separated phospholipids into four fractions, we were unable to separate completely cephalins from inositides and inositides from lecithins by use of a number of different combinations of chloroform-methanol.

The water-insoluble peptide or "proteolipid" extracted from PPLO by the filtration method appears similar to that reported by Folch and Lees (1951). On removal of the solvent, part of the residue became insoluble in chloroform-methanol (2:1); it was also insoluble in water. It was of interest that the chloroform-methanol extracts of PPLO grown in medium containing glycine-*1*-C^{14} contained over 20% of the total radioactivity found in the cell protein. Characterization of this material and kinetic studies will be reported at a later date.

The large amounts of phosphatidic acid are probably due to a lecithinase C in this organism which hydrolyzes other phospholipids to phosphatidic acid (Hanahan, 1957). A recent experiment, involving the harvest of cells at 3 C followed by immediate extraction with cold chloroform-methanol, showed a smaller yield of this fraction with corresponding increase in the inositide-lecithin fraction. Since crude tests indicated that cephalins contributed only a small part of the total phospholipids, it appears that approximately 90% of the phospholipid consists of inositides and phosphatidyl choline.

The positive identification of cholesterol in this organism and the high concentration of this compound and its esters (2.2% of the dry weight of cells or 18.8% of total lipid) are of considerable interest. Cholesterol and other sterols are not found in bacteria (Asselineau and Lederer, 1960). Although sterols are found in yeast, other fungi, and plants, the presence of cholesterol has not been conclusively demonstrated (Cook, 1958) Cholesterol, however, has been reported in other PPLO. Lynn and Smith (1960) reported a sterol which was Liebermann-Burchard-positive, digitonin-precipitable, and had the same R_F as cholesterol on paper chromatograms. The further identification of cholesterol by melting point and especially by infrared spectroscopy in our experiments is conclusive evidence that one of the sterols in PPLO is indeed cholesterol. Infrared spectra of the cholesterol fraction before recrystallization suggested the presence of small amounts of other sterols which could not be resolved from cholesterol on silicic acid.

Since serum is present in the growth medium, the possibility existed that some of the lipids represented adsorbed lipids and not synthesized cellular lipids. This, however, does not appear too likely, since lipids synthesized by cells grown in lipid-extracted medium supplemented with radioactive cholesterol, oleate, and $H_3P^{32}O_4$, when fractionated, paralleled the lipid peaks from cells grown in normal medium with one exception: there was no radioactive hydrocarbon peak. Although some cholesterol is probably adsorbed (Smith and Rothblat, 1960), the fact that cholesterol esters are formed from cholesterol and cholesterol is an absolute growth requirement for this organism is strong argument that cholesterol does not solely represent adsorbed lipid.

The appearance of $H_3P^{32}O_4$ in the four phospholipid fractions, oleic acid-*1*-C^{14} in neutral and phospholipids, and cholesterol-*4*-C^{14} in cholesterol esters, when cells were grown in medium previously extracted with lipid solvents, clearly demonstrates that this organism is capable of synthesizing these lipids from simpler compounds.

The presence of a unit membrane approximately 75 A thick, the absence of a cell wall, and the high lipid content of the membrane suggest that most of the cellular lipids are structural and functional components of this membrane.

Although the high ratio of saturated to unsaturated fatty acids in this organism may reflect the fatty acid composition of the medium, the preponderance of saturated over unsaturated fatty acids, the presence of cholesterol, and a "unit" membrane with the absence of a cell wall, suggest a closer relationship of this microorganism

to animal than to plant cells. The place of this fascinating group of organisms in the biological scheme is yet to be elucidated.

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