The Glycerolipids of *Mycoplasma mycoides**

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ABSTRACT: Two strains of *Mycoplasma*, requiring exogenous glycerol and fatty acids for growth, were shown to incorporate ^{14}C-labeled precursors into glyco- and phospholipids. The main glycolipid, which contained fatty acid ester, glycerol, and galactose in the ratio 2:1:1, gave a compound identified as 1-*O*-β-D-galactofuranosylglycerol when deacylated. The deacylation products of the phospholipids were glycerylphosphorylglycerol (GPG) and 1,3-diglycerylphosphorylglycerol (GPGPG).

Lecithin and sphingomyelin were found in cells harvested from media containing serum, but were not synthesized by the *Mycoplasma* organisms. The ratio of GPGPG-lipid to GPG-lipid increased during the later stages of growth, when incorporation of glycerol into the lipid fraction had almost ceased.

The *Mycoplasma* organisms are bounded by a flexible lipoprotein membrane, unsupported by a rigid cell wall. The type species, *Mycoplasma mycoides* var. *mycoides*, requires for growth cholesterol, a saturated and an unsaturated fatty acid, and glycerol. The effects of deficiencies of these nutrients on the viability of the organisms and the characteristic morphological changes seen have been described by Rodwell and Abbot (1961). Studies of the utilization of [^{14}C]cholesterol (Rodwell, 1963) and [^{14}C]glycerol (Plackett, 1961) have been reported. This paper describes the identification of the lipid types synthesized by the organisms from glycerol and other labeled precursors.

Experimental Section

Organisms. The strains used were V5, a vaccine strain of *M. mycoides* var. *mycoides*, and Y, a goat strain (Laws, 1956) similar antigenically and biochemically to V5, but not a true *mycoides* strain.

Semidefined Media. Medium B2 (Rodwell, 1963) was modified as indicated by further studies of the nutrition of *M. mycoides* (A. W. Rodwell, personal communication). Modification SD 1 contained, per liter: disodium hydrogen phosphate, 0.05 mole; sodium DL-lactate, 0.11 mole; D-glucose, 0.04 mole; glycerol, 0.001 mole; KCl, 0.01 mole; $MgSO_4$, 0.001 mole; cholesterol, 6×10^{-5} mole; sodium oleate, 5×10^{-5} mole; sodium palmitate, 5×10^{-5} mole; bovine serum albumin fraction V (defatted with isooctane and acetic acid), 0.8 g; bovine serum fraction C (Rodwell and Abbot, 1961), 0.5 g; tryptic digest from 1.0 g of casein; acid-hydrolyzed casein (Difco, vitamin free), 10 g; L-tryptophan, 10 mg; L-cystine, 5 mg; adenine, 10 mg; guanine, 10 mg; uracil, 10 mg; thymine, 5 mg; thiamine, 2 mg; nicotinamide, 2 mg; pyridoxamine, 1 mg; calcium pantothenate, 1 mg; biotin, 0.2 mg; leucovorin, 0.2 mg; riboflavin, 2 mg; α-lipoic acid, 0.2 mg; choline chloride, 4 mg; and inositol, 4 mg.

SD 2 contained, per liter: sodium phosphate buffer (pH 8), 0.08 mole; sodium DL-lactate, 0.03 mole; glucose and glycerol as specified below; other constituents as for SD 1, except for the omission of choline chloride and inositol. SD 3 had the same composition as SD 2, with the addition of coenzyme A (2 mg/l.).

Cultures were grown at 37° without aeration. Their optical densities were measured in an Evelyn colorimeter with a red (660 mμ) filter. The organisms were harvested by centrifugation at 25,000*g* in the cold. The pellets were allowed to drain briefly and the walls of the tubes were swabbed carefully with filter paper.

Undefined Medium. The ox–serum medium (BVF-OS) of Turner *et al.* (1935) was supplemented with glucose, glycerol, and oleate (Plackett *et al.*, 1963). The organisms were grown in rotated flasks, harvested, and washed with cold 0.2 M sodium chloride and with water (Buttery and Plackett, 1960).

Extraction of Lipids. PROCEDURE A. The unwashed pellets were resuspended in a small volume (*e.g.*, 1 ml/150 ml of culture) of cold 0.1 M potassium chloride and immediately mixed with 19 volumes of chloroform–methanol (2:1, v/v) at room temperature. After 20 min, with occasional agitation, the mixture was centrifuged and the deposit was washed twice with chloroform–methanol (2:1, v/v). The combined supernatants were washed twice by partition with 0.2 volume of 0.1 M KCl. The washed lipids, obtained in this way from cells grown with [^{14}C]glycerol, accounted for about 90% of the ^{14}C incorporated by the cells. About 1% was present in the dialyzed KCl washings of the lipid fraction and 9% in the cell residue. It was thought that the extraction of unwashed cells by procedure A might allow the detection of additional labile components if such were present. Uncombined glycerol was effectively removed by washing the extracted lipids. However, no evidence for additional components was obtained, and the following procedure was used for most of the work.

PROCEDURE B. After draining and swabbing the tubes

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as above, the organisms were resuspended in cold 0.02 M sodium–potassium phosphate buffer (pH 7.1) containing 0.01 M $MgSO_4$ (30 ml/100 ml of culture) and centrifuged again. The washed pellet was drained, dispersed in a small volume of the same diluent, and mixed with 19 volumes of chloroform–methanol (2:1, v/v). Extraction at room temperature was continued for 45–60 min, after which the residue was centrifuged and washed as in procedure A. The combined supernatants were washed once by partition with 0.1 M KCl.

Lipid Separations. Intact lipids were chromatographed on silicic acid impregnated paper (Marinetti, 1962) using the solvent diisobutyl ketone–acetic acid–water (40:20:3, v/v) at 3–4°, on Whatman AE 30 paper (Mumma and Benson, 1961), in diisobutyl ketone–acetic acid–water (40:25:5, v/v) at room temperature, and on thin layers of silica gel G (Merck) with chloroform–methanol–water (65:25:4, v/v) at room temperature. Reagents used to reveal the position of the spots included Rhodamine 6G, ninhydrin (Marinetti, 1962), Dragendorff reagent (Wagner *et al.*, 1961), and (for thin layer chromatography) 10 N sulfuric acid. Adsorbents used for column chromatography were silicic acid (Mallinckrodt, 100 mesh) and DEAE-cellulose powder (Whatman, pretreated according to Rouser *et al.*, 1963).

Isolation of Glycolipid. Although the phosphatides of *M. mycoides* yielded deacylation products identifiable by comparison with known compounds, the organisms were found to synthesize a neutral lipid of unusual structure. To obtain enough of this substance for characterization, preparations were made from several batches of organisms of the V5 strain grown in the undefined BVFOS medium. A simple procedure, involving chromatography of the washed lipids on silicic acid columns, from which the glycolipid was eluted with chloroform–methanol (39:1), gave a product (G1) containing negligible amounts of other compounds. However, complete elution of the glycolipid was not obtained before phospholipid (diphosphatidylglycerol) started to emerge. A combination of silicic acid and DEAE-cellulose column chromatography was more satisfactory. In one such preparation (G2), freeze-dried organisms from 45 l. of culture were treated with chloroform–methanol by the method of Bligh and Dyer (1959). The washed lipids (1.1 g) were fractionated on a column (13 × 2.6 cm) containing 45 g of silicic acid.

Elution was started with 500 ml of chloroform–methanol (100:1, v/v) followed by 1000 ml of chloroform–methanol (39:1, v/v). Those portions of the latter eluate containing the glycolipid, with traces of two additional components detectable by thin layer chromatography, were concentrated and applied to a column of DEAE-cellulose (1.0 g) in the acetate form (Rouser *et al.*, 1963). The glycolipid was eluted with chloroform–methanol (9:1, v/v), concentrated, redissolved in chloroform–methanol (100:1, v/v), and transferred to a small (1.0 g) column of silicic acid. A small amount of impurity was eluted from this column with 4 ml of chloroform–methanol (100:1, v/v), after which the glycolipid was eluted with 13 ml of chloroform–methanol (29:1, v/v). The yield was 14.1 mg.

Another preparation (G3) was made by a procedure based on that of Rouser *et al.* (1963), in which the washed lipids were first fractionated on a column of DEAE-cellulose. After elution of a fraction, consisting mainly of cholesterol, with chloroform–methanol (100:1), the remaining nonacidic lipids were eluted with chloroform–methanol (7:3). The latter eluate was then chromatographed on silicic acid columns from which the glycolipid was eluted with chloroform–methanol (40:1) or chloroform–acetone (1:1). A similar procedure was used to obtain samples of spinach leaf galactosyl glycerides for comparison with the glycolipid of *M. mycoides.*

Deacylation of Lipids. Lipid fractions were deacylated with LiOH by the method of Brockerhoff (1963). About 5% of the glycerol compounds remained chloroform soluble after 15 min at room temperature, but if the reaction was allowed to proceed for 30 min, conversion to water-soluble products was 99% complete. The aqueous phase was passed through a column of Dowex 50 in the pyridinium form and concentrated *in vacuo* for chromatography on paper or on anion-exchange columns.

Glycolipid preparation G2 (8.05 mg) was refluxed with 0.1 M methanolic potassium hydroxide under nitrogen. After 3 hr the mixture was acidified and shaken with petroleum ether (bp 59–61°). The petroleum ether extract was retained for analysis by gas–liquid partition chromatography (glpc). The aqueous phase, deionized and dried to constant weight *in vacuo*, gave 2.85 mg of a glassy solid. Paper chromatography in solvents 2 and 6 revealed only one component. Hexose and glycerol were determined after hydrolysis in 0.05 N sulfuric acid at 100° for 90 min. Another sample of the glycolipid was refluxed with 3% (w/v) sulfuric acid in methanol for 2 hr under nitrogen. The fatty acid methyl esters were extracted into petroleum ether for estimation by the hydroxamate method. Glycerol was determined by the enzymic method after hydrolysis and deionization of the aqueous phase. Glycolipid preparation G3 was subjected to alkaline methanolysis in chloroform–0.2 M methanolic NaOH under nitrogen. After 1 hr at room temperature the mixture was neutralized with aqueous acetic acid. The chloroform phase was washed with water, dried over Na_2SO_4, and retained for analysis of the methyl esters. The water-soluble product was recovered from the aqueous phase after deionization.

Chromatography of Deacylation Products. Water-soluble compounds were chromatographed on paper in the following solvent systems: (1) isopropyl alcohol–concentrated aqueous ammonia–water (70:6:30, v/v), (2) 1-butanol–acetic acid–water (5:2:3, v/v), (3) water-saturated phenol (in the presence of ammonia and KCN), (4) isopropyl alcohol–1% aqueous ammonium sulfate (2:1, v/v) on paper impregnated with ammonium sulfate, (5) ethanol–1 M ammonium acetate (pH 3.8) (7:3), v/v), (6) 1-butanol–pyridine–water (6:4:3), and (7) 1-butanol–ethanol–water (40:11:19, v/v). Paper ionophoresis was done in an apparatus of the kind described by Bailey and Hackman (1962). Buffers used were 0.05 M ammonium formate (pH 3.5) and 0.05 M Tris-HCl

(pH 7.5). Radioactive sports were detected by autoradiography on Kodak X-ray film, phosphorus compounds with the reagent of Burrows *et al.* (1952), reducing sugars with aniline hydrogen phthalate (Cummins and Harris, 1956), or with silver nitrate (Dedonder, 1952), and nonreducing polyols with silver nitrate.

Water-soluble deacylation products were separated on columns prepared from 0.6 g of Dowex 1-X8 chloride. Before use the resin was converted to the bicarbonate form and washed with water. Samples were applied in dilute aqueous solution. Each column was connected to a linear gradient device, the mixing vessel of which contained 40 ml of water, while the reservoir contained 40 ml of 0.4 M ammonium bicarbonate (pH 8.6). The radioactivity was eluted in three separate peaks, consisting of neutral compounds, glycerylphosphorylglycerol (GPG)[1] and 1,3-diglycerylphosphorylglycerol (GPGPG), in that order. The neutral deacylation products were further fractionated on a column of Dowex 1-X2 in the hydroxyl form, using distilled water as the eluent (Austin *et al.*, 1963). A small amount of free glycerol was eluted first, followed by galactosylglycerol.

Gas–Liquid Partition Chromatography. Fatty acid methyl esters, obtained by direct alkaline methanolysis of the lipids, by transmethylation with 10% BF_3 in methanol at 68°, or by refluxing the acids with methanol containing 3% (v/v) H_2SO_4, were chromatographed on an F & M 810 instrument using the flame ionization detector. Glass columns (5 ft × 0.25 in. o.d.) were packed with 15% EGS, 2% Se30, or 10% Apiezon L on Diatoport S. Columns were calibrated with N.H.I. standard mixture D. For confirmation of peak identity samples were examined after hydrogenation with the catalyst of Vandenheuvel (1956).

Analytical Methods. For the determination of glycerol and galactose in culture media, samples were deproteinized by the method of Somogyi (1945). The supernatants were deionized by passage through columns containing a mixture of equal parts of Dowex 50 (H^+) and Dowex 3 (OH^-). After sampling for glucose determinations, the rest of the effluent was passed through a column of Dowex 1 (OH^-) to remove reducing sugars. The effluent from the second column was used for estimation of glycerol. Glucose and galactose were estimated with the Glucostat and Galactostat reagents (Worthington Biochemical Corp., Freehold, N. J.) and by the anthrone procedure of Trevelyn and Harrison (1952).

Glycerol was determined by the enzymatic method of Wieland (1963), scaled down for use with microcuvets of 1-cm light path. A reagent solution was prepared just before use by mixing stock solutions at 0°. It had pH 9.8 and contained, per milliliter: hydrazine, 1.6 mmoles; glycine, 0.32 mmole; $MgSO_4$, 3.2 μmoles; ATP, 2.7 μmoles; NAD, 1.4 μmoles; α-glycerophosphate dehydrogenase, 0.11 mg; and glycerokinase, 6 μg. This solution (0.18 ml) was added to 0.15-ml samples and to appropriate blank and standard glycerol solutions. The mixtures were incubated at room temperature until the reaction was complete. Absorbance was measured at 340 mμ. Values for glycerol, in samples prepared from culture media as described above, were about 4% lower than the values for total polyols obtained by the periodate–chromotropic acid method.

Ester groups were determined by a modification of the procedure of Antonis (1959). Samples (about 0.2 μequiv) were taken to dryness in small (75 × 10 mm) Pyrex tubes. Diethyl ether (0.15 ml) and 0.05 ml of freshly prepared hydroxylamine reagent were added and the tubes were closed immediately with Teflon stoppers. After 30 min at room temperature 0.3 ml of ferric perchlorate reagent was added. Absorbance was measured at 530 mμ. Trimyristin and methyl pertadecanoate were used as standards.

Phosphorus was estimated by the method of Bartlett (1959) as modified by Marinetti (1962).

Lipids containing 1,2-glycol groups were analyzed by the procedure of Coulon-Morelec and Giraud (1965). 1,2-Glycol groups in water-soluble compounds were estimated as described elsewhere (Plackett, 1964). Erythritol was the standard.

Periodate consumption by water-soluble compounds was measured by the decrease in absorbance at 230 mμ (Plackett and Buttery, 1964).

Degradation of Labeled Galactosylglycerol. The V5 strain was grown in medium SD2 containing [G-^{14}C]-glucose. The lipids were deacylated, and the galactosylglycerol was isolated by paper chromatography in solvent 2. It was subjected to brief (5 min at 25°) oxidation in 0.0025 M sodium metaperiodate, followed by reduction with sodium borohydride. The product was hydrolyzed in 0.1 N sulfuric acid at 100° for 90 min and chromatographed on paper in solvents 2, 3, and 6.

Degradation of Labeled Phosphatidylglycerol. Phospholipase C of *Bacillus cereus* was prepared by the method of Chu (1949). The system contained 0.5 ml of 0.1 M Tris-acetate (pH 7.2), 0.05 ml of 0.1 M calcium acetate, enzyme, and water to give a total aqueous phase of 2.0 ml, and 5.0 ml of a solution of the substrate in diethyl ether. The mixture was shaken at 28° for 5 hr. The lower phase was passed through a column of Dowex 50 (H^+), concentrated *in vacuo*, counted, and analyzed for phosphorus. Paper chromatography in solvent 1 showed a single radioactive spot migrating like glycerol 1-phosphate. The ethereal phase was taken to dryness. The residue was dissolved in chloroform–methanol (100:1, v/v) and applied to a column of silicic acid, from which the diglyceride was eluted with the same solvent mixture. Glycerol and ^{14}C were determined after deacylation with LiOH.

Radioactivity Measurements. A thin end-window Geiger counter and a Packard Tri-Carb liquid scintillation spectrometer were used. Samples for Geiger counting were pipetted onto aluminum planchets (2 cm^2) bearing disks of lens tissue which had been previously moistened with 1 drop each of 1% solutions of cetyl-

[1] Abbreviations used: GPG, glycerylphosphorylglycerol; GPGPG, 1,3-diglycerylphosphorylglycerol; GPE, glycerylphosphorylethanolamine; GPC, glycerylphosphorylcholine; Gal-G, galactofuranosylglycerol; ATP, adenosine triphosphate; NAD, nicotinamide–adenine dinucleotide.

trimethylammonium bromide and polyvinyl alcohol. Under these conditions the rapid volatilization of glycerol plated directly onto aluminum planchets, reported by deFreitas and Depocas (1964), did not occur. Heating for 30 min, with a 250-w infrared lamp placed 9 in. above the planchets, did not cause an appreciable drop in the count rate, although there was a slow decline on prolonged exposure to the atmosphere (about 6% in 10 days). The efficiency of ^{14}C counting by this method was about 7%.

Liquid scintillation counting was done in the dioxane system of Bray (1960), but with 1,4-bis-2-(5-phenyloxazolyl)benzene replaced by the dimethyl derivative. The efficiency of counting was 70–75% for ^{14}C.

Radioactive spots, located on paper chromatograms by radioautography, were cut out and immersed in the scintillator solution.

Labeled Compounds. [1-^{14}C]Palmitic acid, [G-^{14}C]-glucose, [*methyl*-^{14}C]choline, and [1-^{14}C]glycerol were obtained from the Radiochemical Centre, Amersham, England, and [1-^{14}C]oleic acid from Calbiochem. The [^{14}C]glycerol contained a number of minor radioactive constituents. Although these were probably not utilized by the organisms (they were still present in the culture medium when all the glycerol had been consumed) they were removed by paper chromatography in solvent 3 before use. The other labeled compounds were used as supplied.

Other Reagents. Glycerokinase was obtained from L. Light and Co., Colnbrook, England, and glycerophosphate dehydrogenase from Calbiochem. Glycerylphosphorylglycerol (GPG) was synthesized by the method of Fischer and Pfähler (1920), and 1,3-diglycerylphosphorylglycerol (GPGPG) as described elsewhere (Plackett, 1964).

Results

Identity of the Main Lipid Components. The lipid compositions of strains V5 and Y were qualitatively and quantitatively similar. Chromatographic mobilities of the five main components, all of which were labeled whether the isotope was supplied as [^{14}C]glycerol, [^{14}C]palmitate, or [^{14}C]oleate, are given in Table I. Glycolipid D, the characterization of which is described below, was the most heavily labeled component when the cells were grown with [^{14}C]glucose, but some ^{14}C appeared in E, which is probably also a glycolipid. Traces of three minor components migrating more slowly than A on thin layer chromatography plates (R_F 0.10, 0.16, and 0.25) were detected on radioautographs; they were not identified.

The phospholipids gave comet-shaped spots (A, B, and C) on thin layers of silica gel G, whereas D and E gave compact spots. On paper chromatograms treated with Rhodamine 6G, the phospholipids gave reddish-purple spots with a tendency to streak, and D gave compact orange-pink spots. E was detectable only by radioautography. A and D were periodate-Schiff positive. The data for silicic acid impregnated paper in Table I refer to paper prepared in this laboratory; commercially prepared (Whatman SG81) paper has since been found to give better separations of A (R_F 0.30), B (R_F 0.55), and C (R_F 0.43) in the same solvent system. A migrated in all systems like a sample of phosphatidylglycerol from spinach leaves.

TABLE I: Chromatographic Mobility of *M. mycoides* Lipids.

Component	R_F in System[a] 1	2	3	Deacylation Product
A	0.48	0.54[b]	0.41[b]	GPG
B	0.74			GPGPG
C	0.60	0.45	0.31	GPGPG
D	0.81	0.67	0.82	Gal-G
E	0.65	0.46	0.54	Not identified

[a] System 1, thin layer chromatography on silica gel G in chloroform–methanol–water (65:25:4, v/v); system 2, Whatman No. 1 paper impregnated with silicic acid (Marinetti, 1962) in diisobutyl ketone–acetic acid–water (40:20:3, v/v) at 3–4°; and system 3, Whatman AE 30 paper in diisobutyl ketone–acetic acid–water (40:25:5, v/v) at room temperature. [b] Components A and B gave a single elongated spot in systems 2 and 3.

The nature of the difference between the two GPGPG lipid components (B and C) remains to be determined. The presence of different salt forms (Rose, 1964; de Haas *et al.*, 1966) would account for the presence of two spots on thin layer chromatography plates run in neutral solvents and for the behavior observed on silicic acid columns (see below). However, the additional spot was also seen on paper chromatograms run in acidic solvents. C may be a lyso derivative with one fatty acid residue less than B.

The deacylation products of the phospholipids from cells grown with [^{14}C]glycerol were identified by chromatography in admixture with authentic samples of GPG and GPGPG. In all six systems (Table II) the labeled material coincided with the carrier spots revealed by the molybdate reagent. Further evidence of structure was obtained by estimation of 1,2-glycol groups after separation of the phosphate esters on columns of Dowex 1-bicarbonate.

Although chloroform–methanol extracts of washed cells grown in the undefined ox-serum medium contained both lecithin and sphingomyelin, there was no evidence for the synthesis of either choline or amino phosphatides by the *Mycoplasma* organisms. Glycerol was not incorporated into GPC or GPE in either the semidefined or the undefined media. There was no incorporation of [*methyl*-^{14}C]choline into the lipid fraction of cells grown in medium SD 1, nor was palmitate incorporated into sphingosine or other long-chain base. No ninhydrin-positive lipids were detected on paper chro-

TABLE II: Deacylation Products of *M. mycoides* Phosphatides.

Fraction	R_F in System[a] 1	2	3	4	5	Rel[b] Mobility at pH 7.5	1,2-Glycol:P[c]
II (GPG)	0.59	0.27	0.38	0.50	0.54	0.73	2.0
III (GPGPG)	0.55	0.09	0.15	0.33	0.35	(1.00)	1.0

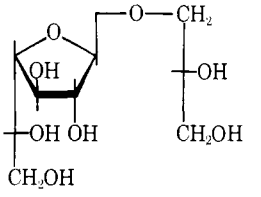
[a] Paper chromatography using the following solvent systems: (1) isopropyl alcohol–concentrated aqueous ammonia–water (70:6:30, v/v), (2) 1-butanol–acetic acid–water (5:2:3, v/v), (3) water-saturated phenol (in the presence of aqueous ammonia and KCN), (4) isopropyl alcohol–1% aqueous ammonium sulfate (2:1, v/v), and (5) ethanol–1 M ammonium acetate (pH 3.8) (7:3, v/v). [b] Ionophoretic mobility in 0.05 M Tris-HCl (pH 7.5), corrected for endosmosis. [c] Mole equivalent of 1,2-glycol per gram-atom of P.

matograms, and there was no evidence for the presence of phosphatidylglycerophosphate.

Characterization of the Glycolipid. The *M. mycoides* glycolipid was not acidic and contained no phosphorus. Alkaline methanolysis gave fatty acid methyl esters and a nonreducing water-soluble product. Hydrolysis of the latter in 0.1 N sulfuric acid at 100° for 90 min gave glycerol and galactose, identified by paper chromatography and by reaction with galactose oxidase and with the coupled glycerokinase–glycerophosphate dehydrogenase system. Although the composition of the galactolipid (Table III) corresponded to that of a monogalactosyldiglyceride, it was distinguished from the monogalactopyranosyldiglyceride of spinach leaves by its chromatographic mobility and by that of its deacylation product (Table IV).

Degradation of the galactosylglycerol, from cells grown with [^{14}C]glucose, by the periodate–borohydride method, followed by acid hydrolysis, gave a radioactive product indistinguishable from arabinose in three solvent systems. This observation, and the fact that the galactosylglycerol was not a substrate for galactose oxidase, suggested that it was a galactofuranoside.

The presence of a 1,2-glycol group in the intact galactolipid and two such groups in the deacylation product, determined by measurement of formaldehyde produced by oxidation with metaperiodate (Table III), also indicates a galactofuranose structure. When the deacylation product was treated with 0.4 mM metaperiodate (pH 4.6) at 22°, 1.9 equiv of oxidant/mole of hexose was consumed in 90 min, and 2.2 equiv/mole in 1200 min. In dilute periodate solution preferential oxidation of the 1,2-glycol groups is expected (Clancy and Whelan, 1959; Plackett and Buttery, 1964), the *trans*-hydroxyl pair (C-2,3) of the galactofuranose ring being relatively resistant to periodate (Kjølberg, 1960). The galactosylglycerol (from preparation G2) gave a large negative rotation, $[\alpha]_{5890}$ −80° (±13°) (*c* 0.157, water). The hexose content corresponded to the presence of 84% by weight of anhydrous galactosylglycerol. The observed rotation per mole of hexose ($[M]_{5890}$ − 24,200) is of the order expected for 1-*O*-β-D-galactofuranosylglycerol (I). Although the glycolipid is probably a diglyceride



derivative, the position of the fatty ester groups has not been unequivocally determined.

TABLE III: Composition of *M. mycoides* Glycolipid (mole per mole of galactose).

	Preparation No. G1	G2	G3
Ester	1.9[a]	2.1[b]	1.8[c]
Glycerol	0.91	0.97	
1,2-Glycol (intact lipid)			0.93
1,2-Glycol (deacylation product)		2.1	2.0

[a] By direct hydroxylaminolysis. [b] In petroleum ether extract after acid methanolysis. [c] In chloroform phase from alkaline methanolysis.

The fatty acid composition of *M. mycoides* lipids reflects that of the culture medium. Data for two galactolipid preparations from cells grown in BVFOS medium are shown in Table V. When grown in semidefined medium the organisms incorporate the fatty acids supplied without alteration of chain length or saturation (Rodwell, 1967). The galactolipid of strain Y grown in such media contained approximately equimolar amounts of saturated and unsaturated acids whether these were palmitic and oleic, or myristic and erucic.

Distribution of ^{14}C among Lipid Components after Growth with Labeled Glycerol. Figure 1 shows the elution profile from a silicic acid column of lipids extracted by procedure B from stationary-phase cells of strain V5. The medium (SD3) contained a low concentration (5×10^{-4} M) of [1-^{14}C]glycerol (0.3 μc/μmole). It was found (see below) that older cultures contained rela-

TABLE IV: Comparison of Galactolipids from *M. mycoides* and from Spinach Leaves.

	M. mycoides Galactolipid	Spinach Galactolipid	
		Monogalactosyl	Digalactosyl
R_F of intact lipids[a]	0.56	0.48	0.20
$R_{galactose}$ of deacylation products			
1[b]	1.61	0.89	0.20
2[c]	1.23	1.08	0.62

[a] On Whatman SG 81 paper in diisobutyl ketone–acetic acid–water at 3°. [b] On Whatman No. 1 paper in 1-butanol–pyridine–water (6:4:3) at 25°. [c] On Whatman No. 1 paper in 1-butanol–acetic acid–water (5:2:3) at 25°.

tively more of components B, C, and E. In this case the culture was harvested after 44.5 hr, when the optical density was 0.35. All the glycerol had been consumed, but the morphological changes characteristic of glycerol deficiency (Rodwell and Abbot, 1961) had not taken place. Peaks I, II, and V contained components D, E, and B, respectively. The GPG lipid A was the major component of VII, but there was some overlap with VIII, 90% of the ^{14}C in the latter being present as GPGPG lipids (B and C). Component C was not found in earlier fractions. Further small quantities of the phospholipids, together with traces of component E and at least two unidentified components, were present in the minor peaks III, IV, VI, and IX.

Another sample of the total lipids was deacylated and the water-soluble products were separated on a column of Dowex 1-bicarbonate. The sample (98%) applied to the column was recovered in three well-separated peaks

TABLE V: Fatty Acid Composition of *M. mycoides* Glycolipid.

Fatty Acids	Preparation G2	Preparation G3
14:0[a]	1[b]	2
16:0	18	25
18:0	49	40
18:1	24	25
18:2	3	3
Other[c]	5	5

[a] Number of carbon atoms to number of double bonds. [b] Mole/100 moles of fatty acids. [c] Mainly 16:1.

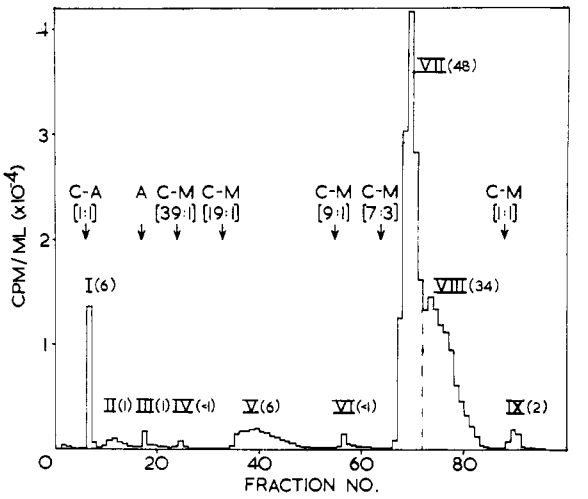


FIGURE 1: Chromatography of lipids from stationary-phase culture of *M. mycoides* grown with [^{14}C]glycerol. The column (7 × 0.6 cm) was prepared from 1.0 g of silicic acid. The sample contained 4.24 × 10^5 cpm, equivalent to 1.45 μmoles of glycerol. Eluting solvents were (abbreviations used: C, chloroform; M, methanol; and A, acetone): 8 ml of C–M (100:1), 20 ml of C–A (1:1), 20 ml of A, 15 ml of C–M (39:1), 35 ml of C–M (19:1), 20 ml of C–M (9:1), 40 ml of C–M (7:3), 30 ml of C–M (1:1), and 15 ml of M. Fractions of 128 drops were collected. Figures in brackets show percentage distribution of ^{14}C eluted in the corresponding peaks. A small peak (X), not shown, emerged after the change in methanol. It accounted for 1% of the ^{14}C.

(Figure 2). Galactofuranosylglycerol emerged in the first peak, together with a trace of free glycerol and at least five minor components separable by paper chromatography in solvents 2 and 6. The most prominent of these was the deacylation product of component E. In Table VI the distribution of ^{14}C among the major lipids is shown. The values obtained by column chromatography of the intact lipids followed by paper chromatography of the eluted fractions or their deacylation products (column 2 of Table VI) are consistent with the results of ion-exchange chromatography of the deacylation products (column 1) and of paper chromatography of the intact lipids on ion-exchange paper (column 3).

Variation of Lipid Composition with Age of Culture. Table VII shows the distribution of ^{14}C in the deacylation products of the lipids of strain V5 grown in medium SD 3 containing 0.04 M glucose and 0.002 M glycerol. Each of the two cultures (100 ml, 18 μc of [1-^{14}C]glycerol) was inoculated with the cells from 10 ml of a 4-day culture in BVFOS medium. One of them was harvested after 20.5 hr when in the early filamentous phase, and the other after 44 hr when only a few filaments remained. The lipids, extracted by procedure B, were deacylated and the water-soluble products were separated by ion-exchange chromatography on Dowex 1-bicarbonate as described above. The neutral fraction was then rechro-

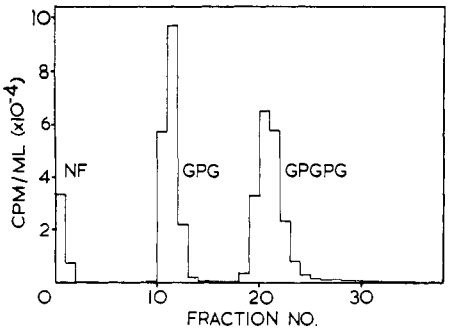


FIGURE 2: Ion-exchange chromatography of water-soluble deacylation products from another sample of the total lipid extract chromatographed in Figure 1. The column was Dowex 1-bicarbonate. The sample was 7.72×10^5 cpm (2.65 μmoles of glycerol). Elution with ammonium bicarbonate gradient (see Methods). Fractions of 1.8 ml. Abbreviations used: NF, neutral fraction; GPG, glycerylphosphorylglycerol; GPGPG, 1,3-diglycerylphosphorylglycerol.

TABLE VI: Distribution of ^{14}C in Major Lipid Components of *M. mycoides* after Growth with [1-^{14}C]-Glycerol.

Component	Deacylation Product	% of Total ^{14}C in Lipids		
		1[a]	2	3
A	GPG	42	39	79
B	GPGPG	47	38	
C	GPGPG		10	13
D	Gal-G	9	6	6
E	Not identified		<2	2

[a] (1) By ion-exchange chromatography of deacylation products, (2) by silicic acid column chromatography and paper chromatography of eluted fractions, and (3) by chromatography of intact lipids on anion-exchange paper.

matographed on Dowex 1-X2 in the hydroxyl form to separate the galactofuranosylglycerol from the minor neutral components. The latter were more numerous and more heavily labeled in the older culture.

Most of the incorporation of the labeled glycerol took place during the early phase of growth, the subsequent twofold increase in the amount of ^{14}C in GPGPG being accompanied by slight decreases in the other two fractions.

Both the GPGPG lipid components increased in activity. The proportion of the total lipid ^{14}C in component C, determined by chromatography of the intact lipids on AE 30 paper, was 9% at 20.5 hr and 15% at 44 hr. There was no decrease in the amount of GPG (as determined by analysis for P or for 1,2-glycol groups), the loss of ^{14}C being balanced by dilution with glycerol derived from unlabeled precursors.

Although no reversal of the glycerophosphate oxidase system of *M. mycoides* has been demonstrated (A. W. Rodwell, personal communication), some exchange of triose phosphate with glycerophosphate may occur. Entry of unlabeled carbon at the level of glycerophosphate would explain the observed greater dilution of the phospholipid glycerol compared with that in the medium. The low specific activity of the GPGPG from the 20.5-hr culture may be accounted for by the synthesis of part of the GPGPG-lipid from unlabeled GPG-lipid present in the large inoculum. The specific activities shown in Table VII for the glycerol residues in the phosphate esters are average values. The assumption that all the residues in a given molecule are equally labeled, may not be valid.

Studies with Strain Y. The lipid composition of strain Y closely resembled that of V5, except that a smaller proportion of label was found in the neutral fraction after growth with [^{14}C]glycerol. As with V5, the molar ratio GPGPG:GPG increased with age of culture. Observed values ranged from 0.28 to 0.58.

Strain Y, which grows faster than V5, was used to study the incorporation of [1-^{14}C]glycerol over shorter time intervals during growth in medium SD2. The lipids, extracted by procedure A, were fractionated on silicic acid columns. The specific activities of the GPG-lipid (A) and the GPGPG-lipid (B) were compared. Glycerol utilization during the phase of rapid growth was studied by growing the cells in medium containing a low concentration of glycerol of high specific activity. When about 99% of the glycerol had been consumed, one portion of the culture was harvested and a large excess of unlabeled glycerol was added to the remainder.

Ninety minutes later, at about the inflection point of the optical density curve, the rest of the culture was harvested. During the interval lipid phosphorus increased by a factor of 1.4 (Table VIII). There was little change in the relative proportion of the lipids and no loss of ^{14}C from the lipid fraction. The decrease in specific activity of the GPGPG-lipid was small compared to that of the GPG-lipid, indicating that most of the newly incorporated glycerol had entered the latter.

In other experiments cultures were grown in unlabeled medium until they were approaching the stationary phase. [^{14}C]Glycerol was then added and incubation was continued for 70–75 min. The specific activity of the GPG-lipid corresponded to the incorporation of less than 0.02 mole of glycerol/hr per g-atom of phosphorus. The specific activity of the GPGPG-lipid (B) was at least four times lower. Component C was not isolated, but fractions containing it were also of much lower specific activity than the GPG-lipid. When a sample of the GPG-lipid labeled under these conditions was degraded with phospholipase C, the specific activities of the glycerol 1-phosphate and of the glycerol obtained by deacylation of the diglyceride moiety were

TABLE VII: Incorporation of [1-^{14}C]Glycerol into Lipids of *M. mycoides* Strain V5 during Growth in Semidefined Medium.

Age at harvesting (hr)	20.5	44
Optical density	0.10	0.34
Glycerol in medium (mM)	1.88	1.05
^{14}C in lipids (cpm)	3.39×10^5 (100)[a]	4.20×10^5 (100)
Gal-G	0.36×10^5 (11)	0.23×10^5 (6)
Other neutral components	0.05×10^5 (1)	0.15×10^5 (3)
GPG	2.14×10^5 (63)	1.93×10^5 (46)
GPGPG	0.85×10^5 (25)	1.89×10^5 (45)
μmoles of GPG/100 ml of culture[b]	1.10	1.11
μmoles of GPGPG/100 ml of culture[b]	0.35	0.73
Molar ratio GPGPG/GPG	0.32	0.66
Specific activity of glycerol residues (cpm/μmole)		
Glycerol in medium	1.22×10^5	0.97×10^5
GPG in lipids[c]	0.97×10^5	0.89×10^5
GPGPG in lipids[c]	0.81×10^5	0.86×10^5

[a] Values in parentheses are percentage distribution of lipid ^{14}C in deacylation products. [b] By analysis for 1,2-glycol groups in the separated deacylation products. [c] Average value for all residues in the molecule.

in the ratio of 1.0:1.1. Since both were labeled to about the same extent, the incorportion probably represents newly synthesized lipid, and not merely turnover of part of the molecule.

Discussion

The composition of the polar lipid fraction of *M. mycoides* resembles that of the protoplast membrane of *Micrococcus lysodeikticus* (Macfarlane, 1964). It is of interest that in each of these organisms glycolipid and glycan containing similar hexose residues occur together, mannopyranose in the case of *M. lysodeikticus* and galactofuranose in *M. mycoides*. The metabolic relationship between the galactolipids and the galactan of *M. mycoides* is now being investigated in this laboratory. The galactofuranosylglycerol obtained by deacylation of the galactolipid is probably identical with the substance isolated from the lipid fraction of *Bacteriodes symbioticus* by Reeves *et al.* (1964).

The results reported here indicate that the incorporation of exogenous glycerol takes place primarily into GPG-lipid, both positions being labeled at similar but probably not identical rates, and that GPG-lipid is subsequently converted to GPGPG-lipid, the process continuing after the incorporation of exogenous glycerol has ceased. Further work is needed to determine the nature of the reactions involved in the synthesis of these phosphatides, and the physiological significance of the observed changes in relative proportions.

It may appear paradoxical that, at the stage when the breaking up of filaments to smaller elements is increasing the ratio of surface area to cell mass, there should be no new synthesis of membrane lipid. However, the process may be accompanied by a reduction in cytoplasmic volume, or the membrane may be expanded, as a result, perhaps, of the conversion of GPG-lipid to GPGPG-lipid.

TABLE VIII: Uptake of Glycerol into Phospholipids of *Mycoplasma* Strain Y during Rapid Growth.

	Sample 1[a]	Sample 2
Age of culture (min)	920	1010
Optical density	0.17	0.25
^{14}C in lipids (cpm)	2.28×10^5	2.32×10^5
Total P in lipids (μg-atom)	2.91	4.11
Specific activity of glycerol residues (cpm/μmole of glycerol)		
In GPGPG-lipid	4.7×10^4	4.3×10^4
In GPG-lipid	4.1×10^4	2.7×10^4

[a] Sample 1 was taken immediately before dilution with unlabeled glycerol. Specific activity of glycerol before inoculation was 6.9×10^4 cpm/μmole, and decreased to 5.2×10^4 cpm/μmole at 920 min. After dilution specific activity was less than 100 cpm/μmole.

References

Antonis, A. (1959), *J. Lipid Res. 1*, 485.

Austin, P. W., Hardy, F. E., Buchanan, J. G., and Baddiley, J. (1963), *J. Chem. Soc.*, 5350.

Bailey, S. W., and Hackman, R. H. (1962), *J. Chromatog. 8*, 52.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37*, 911.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Brockerhoff, H. (1963), *J. Lipid Res. 4*, 96.
Burrows, S., Grylls, F. S. M., and Harrison, J. S. (1952), *Nature 170*, 800.
Buttery, S. H., and Plackett, P. (1960), *J. Gen. Microbiol. 23*, 357.
Chu, H. P. (1949), *J. Gen. Microbiol. 3*, 255.
Clancy, M. J., and Whelan, W. J. (1959), *Chem. Ind. (London) 32*, 673.
Coulon-Morelec, M. J., and Giraud, D. (1965), *Bull. Soc. Chim. Biol. 47*, 47.
Cummins, C. S., and Harris, H. (1956), *J. Gen. Microbiol. 14*, 583.
Dedonder, R. (1952), *Bull. Soc. Chim. France*, 874.
deFreitas, A. S. W., and Depocas, F. (1964), *Can. J. Biochem. 42*, 195.
de Haas, G. H., Bonsen, P. P. M., and van Deenen, L. L. M. (1966), *Biochim. Biophys. Acta 116*, 114.
Fischer, E., and Pfähler, E. (1920), *Chem. Ber. 53*, 1606.
Kjølberg, O. (1960), *Acta Chem. Scand. 14*, 1118.
Laws, L. (1956), *Australian Vet. J. 32*, 326.
Macfarlane, M. G. (1964), *in* Metabolism and Physiological Significance of Lipids, Dawson, R. M. C., and Rhodes, D. N., Ed., New York, N. Y., Wiley, p 399.
Marinetti, G. V. (1962), *J. Lipid Res. 3*, 1.
Mumma, R. O., and Benson, A. A. (1961), *Biochem. Biophys. Res. Commun. 5*, 422.
Plackett, P. (1961), *Nature 189*, 125.
Plackett, P. (1964), *Australian J. Chem. 17*, 101.
Plackett, P., and Buttery, S. H. (1964), *Biochem. J. 90*, 201.
Plackett, P., Buttery, S. H., and Cottew, G. S. (1963), *8th Symp. Intern. Congr. Microbiol., Montreal, Can.*, 535.
Reeves, R. E., Latour, N. G., and Lousteau, R. J. (1964), *Biochemistry 3*, 1248.
Rodwell, A. W. (1963), *J. Gen. Microbiol. 32*, 91.
Rodwell, A. W. (1967), *Ann. N. Y. Acad. Sci.* (in press).
Rodwell, A. W., and Abbot, A. (1961), *J. Gen. Microbiol. 25*, 201.
Rose, H. G. (1964), *Biochim. Biophys. Acta 84*, 109.
Rouser, G., Kritchevsky, G., Heller, D., and Lieber, E. (1963), *J. Am. Oil Chemists' Soc. 40*, 425.
Somogyi, M. (1945), *J. Biol. Chem. 160*, 69.
Trevelyan, W. E., and Harrison, J. S. (1952), *Biochem. J. 50*, 298.
Turner, A. W., Campbell, A. D., and Dick, A. T. (1935), *Australian Vet. J. 11*, 63.
Vandenheuvel, F. A. (1956), *Anal. Chem. 28*, 362.
Wagner, H., Hörhammer, L., and Wolff, P. (1961), *Biochem. Z. 334*, 175.
Wieland, O. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., Weinheim, Verlag Chemie, p 211.