Glucose-containing phospholipids in *Mycoplasma laidlawii*, strain B

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SUMMARY Three major phospholipid fractions were isolated from *Mycoplasma laidlawii*, strain B, grown in lipid-free medium.

Two of these fractions were shown to contain covalently bound glucose, and had molar ratios of P:sugar:glycerol:fatty acid close to 1:1:1:2. They differed somewhat in their fatty acid compositions. Products of mild deacylation and of partial acid hydrolysis indicated both to be salt forms of phosphatidyl glucose.

Another major fraction not structurally identified yielded data suggestive of a compound resembling cardiolipin or of a phosphatidyl glycerophosphate. Small amounts of nitrogen-containing phospholipids, but no sulfolipids, were detected.

KEY WORDS *Myocoplasma laidlawii* · phosphatidyl glucose · microbial phospholipids · glucose-containing phospholipids

LIPIDS, ABOUT HALF of them unsaponifiable, comprise 10–20% of the dry weight of *Mycoplasma* (1). The unsaponifiable lipid consists of sterol and its derivatives in sterol-requiring strains (2) and of carotenoids in strains not requiring sterol (3). Little is known about the nature of the phospholipids of *Mycoplasma*. One sterol-requiring strain of avian origin, *M. gallisepticum*, contains phosphatidic acids, cephalins, inositides, phosphatidyl choline, and sphingomyelin (4). However, this composition of phospholipid mimics that of the phospholipids of the crude culture medium, from which the phospholipids could have been derived.

The enveloping membrane of *Mycoplasma* appears to be lipoprotein in nature (5). As these organisms are resistant to osmotic shock (6, 7), this membrane must be a very stable structure, in contrast to the cytoplasmic membrane surrounding bacterial protoplasts (7), and it is thought that the stability is due to the elevated lipid content (1). Hence, an examination of the nature of the phospholipids in *M. laidlawii*, strain B, was undertaken to complement what is known about the unsaponifiable lipids. Since a defined medium capable of producing large yields of *Mycoplasma* has not been devised, *M. laidlawii*, strain B, was used because of its ability to synthesize its own lipid when grown in a lipid-free medium. One major type of phospholipid, phosphatidyl glucose, was demonstrable.

MATERIALS AND METHODS

Cultivation of the Organism

M. laidlawii, strain B, was grown in a medium consisting of 2% tryptose (Difco Laboratories, Inc., Detroit, Mich.) which had been extracted in the dry state with chloroform–methanol 2:1 (v/v), 0.5% sodium acetate, and 0.5% glucose at pH 7.8, and contained thallium acetate (1:4000) to suppress possible bacterial contamination. Approximately 60 liters of 24 hr old culture, started from a 10% inoculum grown in the extracted medium, were used for each analysis. Incubation was carried out statically at 37° in 2-liter volumes contained in 3-liter flasks. The organisms were harvested and washed as previously described (2). Each batch of culture yielded 1.5–2.0 g dry wt of organisms.

Extraction and Purification of Lipids

All analytical procedures were performed and the lipids were stored in an atmosphere of nitrogen. The wet cell paste was extracted three times with 40 volumes of chloroform–methanol (C–M) 2:1 (v/v) and the pooled extracts were washed by the method of Folch et al. (8). Samples were removed for total phosphorus determinations and the washed extracts were then dried in vacuo. The dried lipid extract was resuspended in 10 ml of *n*-hexane and applied to a column of 10 g of activated

silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.). An initial separation into lipid classes was made using as eluents: benzene–hexane, 6:94, 200 ml; benzene–hexane 1:4, 250 ml; benzene–hexane 6:4, 250 ml; benzene, 200 ml; chloroform–benzene 3:1, 250 ml; chloroform, 200 ml; C–M 20:1, 200 ml; C–M 9:1, 200 ml; C–M 4:1, 200 ml; C–M 1:1, 200 ml; C–M 1:20, 200 ml. Approximately 10-ml fractions were collected. All fractions eluted with solvents up to and including chloroform were freed from solvent in vacuo and the residues weighed. These fractions contained as major components the previously identified lipids of this strain (2, 3), i.e., neurosporene, carotenols, esters of carotenols, and carotenyl glycosides, and were not analyzed further. One milliliter samples from each tube of the fraction collector were analyzed for total phosphorus. The contents of the tubes were pooled according to the results of these analyses and dried in vacuo.

The purity of each fraction then was determined by thin-layer chromatography (TLC) on Silica Gel G (E. Merck AG, Darmstadt, Germany). Layers 250 μ thick were prepared using deionized water by the technique of Skipski et al. (9). Samples were applied in 10 μl of chloroform and the plates were developed in chloroform–methanol–acetic acid–water 65:25:8:4. The solvent was allowed to flow 13 cm from the origin. Phospholipids were detected by spraying with an ammonium molybdate–perchloric acid mixture (9) and with 5 N sulfuric acid. Fractions exhibiting more than one spot were subjected to further column chromatography using various proportions of C–M, as described in Results. The additional subfractions were pooled on the basis of their phosphorus content and their purity was again checked by TLC. Usually rechromatography on a silicic acid column yielded pure fractions, which were used for further analyses.

Analytical Procedures

Infrared absorption spectra were obtained with a Beckman IR5A spectrophotometer. Samples were examined as liquid films on crystals of sodium chloride or in pellets of potassium bromide. Very small samples necessitated the use of a scale expansion apparatus.

After removal of aliquots for total phosphorus and sulfate determinations, the purified fractions were saponified by refluxing in ethanolic N potassium hydroxide for 30 min.

The fatty acids were methylated by heating with boron trifluoride in methanol (10) and the resultant esters subjected to gas–liquid chromatography in a Beckman model GC 2A instrument equipped with a Thermotrac temperature programmer, a thermistor detector, and matched 18 ft Resoflex R-446 columns under the following conditions: column temperature, 235°; gas, helium; flow rate, 85 ml/min; current, 200 ma; sensitivity, 1 or 2. Fatty acids were identified on the basis of retention times compared with standards (Applied Science Labs., State College, Pa.) and of increase in peak height when mixed with known fatty acid methyl esters. Proportions of given fatty acids were determined by measurement of the area under each peak by planimetry.

The aqueous layer from the extraction of the saponification mixtures, freed from residual ethanol and ether by a stream of nitrogen, were made 2 N in hydrochloric acid and refluxed for 48 hr (11). Samples were then removed for glycerol determinations. The remainder was dried in vacuo and the residues were extracted three times with 1.5 ml of pyridine to separate the sugars from residual salts (12). Pyridine was removed in vacuo and the residues were dissolved in deionized water. These solutions were employed for identification and determination of sugars and amino compounds. Samples of unhydrolyzed fractions served as controls in all procedures for analysis of hydrolytic products.

To ensure complete degradation of any inositol phosphate present to free inositol, a sample (1 mg) of the unfractionated phospholipid was heated for 15 hr in 6 N hydrochloric acid at 120° in a sealed tube (13). Excess acid was removed in vacuo and the residue used for paper chromatographic detection of inositol.

Samples (5–10 mg) of each major purified fraction were subjected to the mild deacylation procedure of Benson and Maruo (14) for detection of water-soluble phosphate diesters and the products were chromatographed on paper by the method of Benson et al. (15). The phosphate diesters were detected with a modified Hanes-Isherwood reagent under ultraviolet irradiation (16). Quantities of hydrolysis products sufficient for analysis were obtained by preparative chromatography on Whatman No. 1 paper (15). Spots were eluted with water and the eluates acidified with hydrochloric acid (2 N) and refluxed for 48 hr. The hydrolysates were analyzed for glucose, glycerol, and total phosphorus.

Samples (2 mg) of one purified fraction (J, see Results) were subjected to mild acid and alkaline hydrolyses (11). The water-soluble fractions were examined qualitatively for α-glycerol phosphate (17) and glucose-6-phosphate (18) by measuring reduction of NADP by the respective dehydrogenases and for glucose-1-phosphate by isomerization to glucose-6-phosphate with phosphoglucomutase followed by detection of glucose-6-phosphate (19).

Phosphorus was determined by the method of King (20); reducing sugar by the method of Park and Johnson (21); inositol by the microbiological assay procedure of Atkin et al. (22) as described by Snell (23); glucose by the glucose oxidase method (Worthington Biochemicals Corporation, Freehold, N. J.); glycerol by measurement

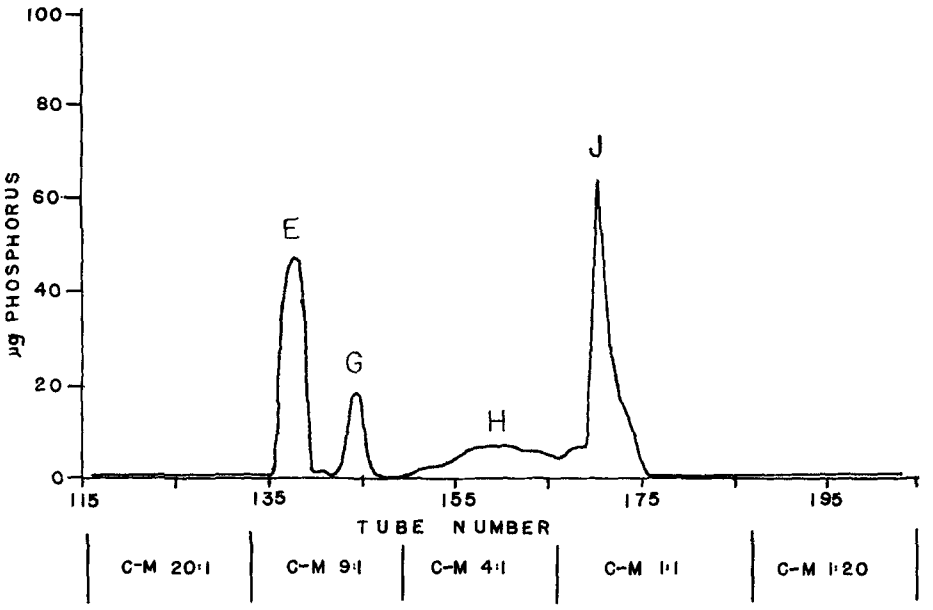

FIG. 1. Elution pattern from silicic acid column of phospholipids from *M. laidlawii*, strain B, grown in lipid-free culture medium.

of reduction of NAD by α-glycerophosphate dehydrogenase following glycerol phosphorylation by glycerokinase and adenosine triphosphate (24); and sulfate by the method of Rosenberg (25). All enzymatic reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

Paper chromatography of sugars was performed by the descending method using *n*-butanol–acetic acid–water 4:1:5 (26) and ammoniacal silver nitrate as the color reagent (27). Inositol was detected by the Fleury reagent followed by alcoholic resorcinol (16). Paper chromatography of amino compounds was carried out as previously described (28). Analyses for plasmalogens were made using the Feulgen reagent (29). Sodium and potassium were measured with a Beckman flame photometer (Model 4100).

TABLE 1 TLC OF PHOSPHOLIPID FRACTIONS FROM *M. laidlawii*, STRAIN B, OBTAINED BY COLUMN CHROMATOGRAPHY ON SILICIC ACID

Fraction	R_F Values	
	First Column	After Rechromatography
E	0.69	0.70
G	0.69 (Major)	
	0.58 (Minor)	
H	0.58 (Major)	0.61
	0.31 (Minor)	
J	0.58 (Minor)	
	0.31 (Major)	0.33

TLC solvent: Chloroform–methanol–acetic acid–water 65:25:8:4. R_F of lecithin, sphingomyelin in this system: 0.89, 0.29 respectively.

RESULTS

An initial experiment was performed to determine the pattern of phospholipids contained in *M. laidlawii*, strain B, when grown in the unextracted tryptose medium. Silicic acid chromatography of C–M extracts of the medium showed it to contain at least three phospholipids, whose infrared spectra resembled those of phosphatidyl choline and sphingomyelin. *Mycoplasma* grown on this medium contained minor amounts of sphingomyelin, presumably derived by adsorption or incorporation from the medium, since sphingomyelins have never been detected in bacteria (30). Further experiments were therefore conducted with cells grown on media made after extraction of their dry ingredients, a procedure which did not affect growth of *M. laidlawii*, strain B.

Figure 1 presents the typical elution pattern of the bacterial phospholipids from a silicic acid column. Thin-layer chromatography of fractions E, G, H, and J showed them to contain three components of R_F 0.69, 0.58, and 0.31 (Table 1). Another phosphorus-containing lipid appeared in the fraction eluted with C–M 20:1 together with the carotenyl glucoside (3); it moved with the solvent front on thin-layer chromatograms and probably represents phosphatidic acid. Rechromatography of fractions E plus G; H; and J on silicic acid yielded purified E, eluted with C–M 14:1; purified H, eluted with C–M 4:1; and purified J, eluted with C–M 5:2. These fractions moved as single spots on TLC (Table 1, second column).

The infrared spectra of the three purified fractions were very similar (Fig. 2). None was identical with the

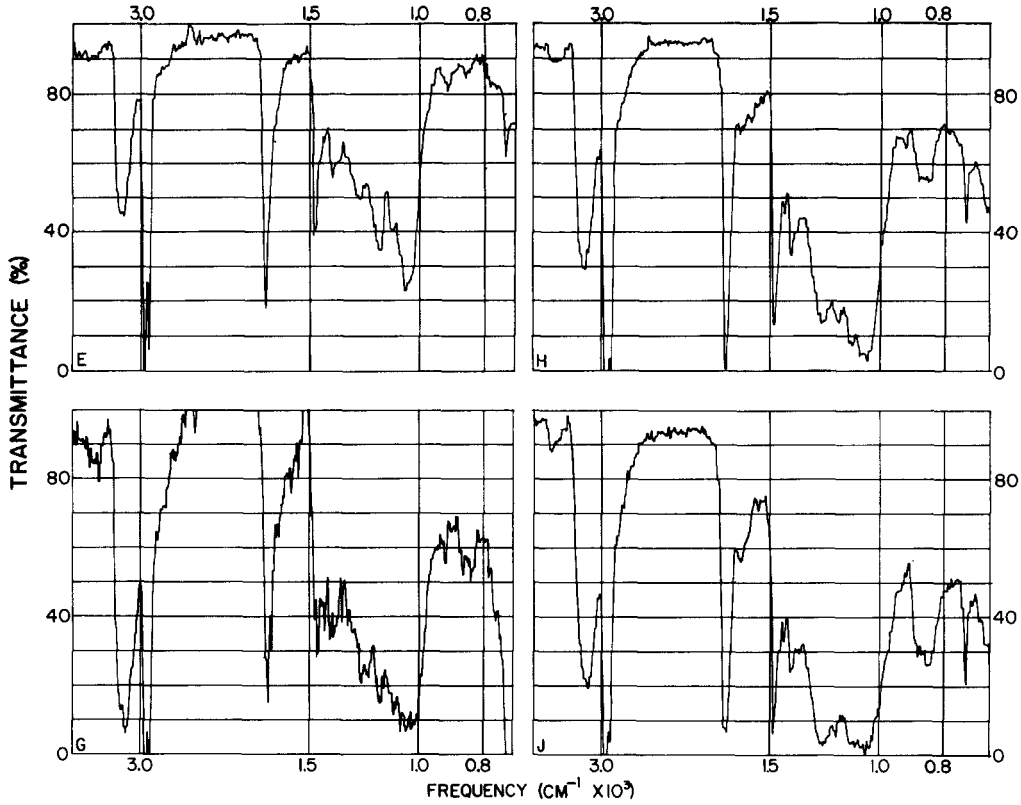

FIG. 2. Infrared spectra of major phospholipids isolated from *M. laidlawii*, strain B.

spectra of known phospholipids but they possessed some resemblance to those of phosphatidyl inositol. Notable were the presence of strong absorption at 3400 cm^{-1}, indicating hydroxyl groups, and the absence of absorption between 1500 and 1700 cm^{-1}, indicating the absence of nitrogen. None of the purified fractions gave a positive ninhydrin reaction.

Following purification and infrared spectrophotometry, 8–10 mg of fractions E and H and 20–25 mg of fraction J remained from each 60 liter batch of culture for analysis (Table 2). In fractions E and J, phosphorus, glycerol, and reducing sugar were found in equimolar proportions and fatty acid in titrable equivalents twice that of the other components. Fraction H, on the other hand, contained only a small amount of reducing sugar, perhaps because of contamination by fraction J.

All three major fractions were tested for plasmalogens using the Feulgen reaction. Fractions E and J gave no reaction; a 2 mg sample of fraction H was calculated to contain 0.01–0.02 μmole aldehyde. Fraction H thus contains only small amounts of plasmalogen, and additional analyses are necessary to determine its structure.

No inositol was detectable on paper chromatograms, nor was any growth response to *Saccharomyces carlsbergensis*, ATCC No. 9080, evident, in contrast to the control. Paper chromatograms indicated that all the reducing sugar existed as glucose. Chromatography of the unknown reducing sugar with authentic glucose resulted in one spot. Confirmation of the identity of the reducing sugar as glucose was obtained by its specific determination with glucose oxidase. No free reducing sugar was detectable in unhydrolyzed fractions when the dried lipid was suspended in water and subjected to chemical and enzymatic assays for reducing sugar.

Incomplete acid hydrolysis of fractions E and J resulted in the production of compounds migrating as α-glycerophosphate (R_F 0.10) and glucose-6-phosphate

TABLE 2 ANALYSIS OF MAJOR PHOSPHOLIPID FRACTIONS FROM *M. laidlawii*, STRAIN B

Fraction	Phosphorus		Fatty Acid		Reducing Sugar		Glycerol	
	μmoles	*Ratio*	*μeq*	*Ratio*	*μmoles*	*Ratio*	*μmoles*	*Ratio*
E	2.3 ± 0.2	1.00	5.2 ± 1.0	2.26	2.8 ± 0.3	1.22	2.39 ± 0.16	1.04
H	7.3 ± 0.7	1.00	9.3 ± 1.0	1.27	1.1 ± 0.3	0.16	12.44 ± 0.32	1.70
J	17.4 ± 0.8	1.00	38.3 ± 1.3	2.20	17.8 ± 0.5	1.02	17.70 ± 0.16	1.02

Mean values ± SE. Number of determinations, 4.

TABLE 3 ANALYSIS OF WATER-SOLUBLE PHOSPHATE DIESTERS FROM PHOSPHOLIPID FRACTIONS E AND J

Fraction	Phosphorus		Reducing Sugar		Glycerol	
	μmole	*Ratio*	*μmole*	*Ratio*	*μmole*	*Ratio*
E	0.067	1.00	0.062	0.93	0.054	0.81
J	0.16	1.00	0.14	0.88	0.12	0.75

(R_F 0.31 relative to glucose) on paper chromatograms. Confirmation of the presence of α-glycerol phosphate and glucose-6-phosphate in the partial acid hydrolysate of fraction J was made by demonstrating NADP reduction with the respective dehydrogenases. Chromatography of phosphate diesters resulting from mild deacylation of each of the three major purified phospholipids resulted in the appearance of a single phosphate-containing spot in each, with the following R_F values in the butanol–propionic acid solvent: Fraction E, 0.04; H, 0.07; J, 0.03. None of the known phosphorylated compounds examined (glycerol phosphate, glucose-1-phosphate, choline phosphate, serine phosphate) and none of the phosphate diesters resulting from mild deacylation of known phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol) gave spots with R_F values approximating those of the unknowns. The acid hydrolysates of eluted phosphate diesters of the unknown fractions E and J contained glycerol, reducing sugar, and phosphate in equimolar amounts (Table 3). Erratic results were obtained with fraction H.

Paper chromatograms of hydrolysates of each fraction exhibited very faint traces of ninhydrin-positive material with the R_F value of glutamic acid. Analyses of the total lipid extracts and hydrolysates for sulfate proved negative, indicating the absence of sulfolipids in these organisms.

The only detectable differences between fractions E and J were in polarity, as revealed by TLC, and in composition of fatty acids (Table 4). Fraction E differs qualitatively from fraction J by the presence of 13:0 and 15:0 fatty acids and the absence of a 10:0 acid.

TABLE 4 FATTY ACID COMPOSITION OF PHOSPHOLIPID FRACTIONS E AND J

Fatty Acid	Fraction E	Fraction J
	% total fatty acid	
10:0	none	Tr.
12:0	3.2	7.7
13:0	1.7	none
14:0	33.2	29.0
15:0	Tr.	none
16:0	53.8	49.0
18:1	8.1	14.2
18:2	Tr.	Tr.

The predominant fatty acids in both fractions were 18:1, 16:0, and 14:0, and the differences are too slight to account for the difference in polarity between E and J. Potassium and sodium analyses showed that fraction J contained 0.24 μeq K and 1.4 μeq Na per mole of phosphatide (assuming a molecular weight of 860) while fraction E contained only 0.07 μeq K and 1.2 μeq Na per mg. These cation values are in excess of the maximum expected value of 1.0 and probably indicate contamination with inorganic salts.

DISCUSSION

The analytical data are compatible with a structure for two of the major phospholipid fractions in *M. laidlawii*, strain B, of a phosphatidyl glucose. The fatty acids are attached, presumably to glycerol, by alkali-labile ester linkages. The glucose probably is attached to glycerol through a phosphodiester linkage between C_6 of glucose and C_3 of glycerol. Such an attachment is substantiated by the presence of α-glycerophosphate and glucose-6-phosphate in partially hydrolyzed preparations. The appearance of only one phosphorus-containing component on paper chromatograms after mild deacylation and the equimolar proportions of phosphate, reducing sugar, and glycerol as its hydrolysis products further confirms the structure of phosphatidyl glucose.

LeFevre et al. (31) have shown recently that monosaccharides form weakly associated complexes with phospholipids. The phospholipid of *M. laidlawii*, strain B, does not appear to be such a phospholipid–sugar complex. No free sugar was detected, either by chemical or enzymatic methods, in aqueous suspensions of the unhydrolyzed dried lipid. All glucose-containing phospholipids moved on thin-layer chromatograms more slowly than choline-containing phospholipids, without dissociation of glucose from the lipid. The appearance of glucose-6-phosphate and a phosphate diester that contained reducing sugar in partially hydrolyzed preparations are also indicative of covalently bound glucose.

Hexose-containing phospholipids have been demonstrated in other bacteria. Vilkas and Lederer (32) have described a phosphatidyl inosito-dimannoside isolated from *Mycobacterium tuberculosis*. Other phosphatidyl inosito-mannosides as well as glucose-containing phosphoinositides have been reported (32–34). Ballou et al. (35) recently identified positively the four inositides in mycobacteria to be glycerol myo-inositol phosphate, and its mono-, di-, and pentamannosides, the glycerol phosphate in each being attached to the L-1 position of the myo-inositol ring. The glucose reported in phospholipid fractions of mycobacteria was shown not to be glucose but trehalose octaacetate. It is not an integral part of phospholipids but a contaminant derived from

cord factor. In addition to the mycobacteria, some Gram-positive bacteria (*Lactobacillus*, *Staphylococcus*) and some Gram-negative bacteria (*Salmonella*) have yielded hexoses in the hydrolysis products of phospholipid fractions (30). However, in most of these cases, the purity of the phospholipids is questionable.

The structure of the second major phospholipid, fraction H, remains to be elucidated. The analytical data showing equimolar proportions of phosphorus and fatty acid but almost twice that amount of glycerol and the presence of some aldehyde might be indicative of a cardiolipin type compound, a phosphatidal glycerol, or phosphatidyl glycerophosphate (36).

M. laidlawii, strain B, appears similar to the mycobacteria and Gram-positive bacteria in its lack of nitrogen-containing phospholipids (37, 38). It is dissimilar in that no inositides are present. The complete absence of nitrogen-containing phospholipids in *M. laidlawii*, strain B, cannot be assumed from our data since traces of a compound similar to glutamic acid were detected. Although there were no detectable amounts of choline- or ethanolamine-containing phospholipids and no sphingomyelins, small amounts of phospholipids containing amino acids probably occur.

The possibility exists that the phospholipids of the organism are a mixture of phosphatidic acids which might serve in membrane transport of glucose and amino acids.

The helpful suggestions of Dr. David Kritchevsky in the preparation of this manuscript are kindly acknowledged.

This work was supported in part by a research grant (AI-04410-03) and training grant 1T1 AI 232-01 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

Manuscript received July 10, 1964*; accepted September* 10, 1964.

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