The Lipid Composition of *Mycoplasma laidlawii* Strain B

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1. Total lipid was extracted from *Mycoplasma laidlawii* strain B with chloroform-methanol mixtures and fractionated into neutral lipid, glycolipid and phospholipid components by chromatography on silicic acid. 2. Saponification of the glycolipid fraction, which represented nearly half of the total lipid, yielded two glycosides for which the structures *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-D-glycerol and *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-D-glycerol were established. 3. The ratio of monoglucosyl diglyceride to diglucosyl diglyceride increased with the age of the culture, though the total glycolipid concentration remained virtually constant. The glycolipid concentration was unaffected by the addition of cholesterol to the culture medium. 4. The phospholipid fraction consisted of two components, phosphatidylglucose and phosphatidylglycerol. Organisms harvested at acidic pH also contained *O*-amino acyl esters of phosphatidylglycerol. No lipids containing inositol could be detected.

The *Mycoplasma* species are a group of organisms devoid of the normal rigid bacterial cell wall and are surrounded by a thin membrane similar to the bacterial plasma membrane. This membrane, which is lipoprotein in nature (Van Iterson & Ruys, 1960), is more resistant to osmotic shock than is the bacterial protoplast membrane (Smith & Sasaki, 1958; Razin & Argaman, 1963). This is thought to be due to the high content of lipids, which constitute as much as 35% of the dry weight of the membrane (Razin, Argaman & Avigan, 1963). A knowledge of the chemical composition of lipids from the *Mycoplasma* species is therefore of interest. Smith & Koostra (1967) have described the lipid composition of two sterol-requiring (parasitic) *Mycoplasma* species, the fermentative *Mycoplasma* sp. avian strain J and the non-fermentative *M. hominis* type 2 strain 07, when grown under carefully defined conditions. The non-sterol-requiring (saprophytic) *M. laidlawii* strain B is able to synthesize its own lipid when grown in a lipid-free medium. The non-saponifiable lipids of this organism have been shown to consist of neurosporene, a hydroxylated carotene and a carotenyl glucoside (Smith, 1963). The present paper describes the chemical composition of the saponifiable lipids.

EXPERIMENTAL AND RESULTS

Materials. Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, 'Mallinckrodt 100 mesh' silicic acid from Kodak Ltd., Kirkby, Lancs., and Merck 'Kieselgel G nach Stahl' for thin-layer chromatography from Anderman and Co. Ltd., London, S.E. 1. Almond emulsin β -glucosidase was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and Glucostat reagent from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Glycerol kinase (EC 2.7.1.30) and α -glycerol phosphate dehydrogenase (EC 1.1.1.8) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. A sample of diglycerol phosphate was synthesized by the method of Brundish, Shaw & Baddiley (1967a) and phosphatidylglycerol was isolated from *Streptococcus faecalis* (Vorbeck & Marinetti, 1965). A sample of diglucosylglycerol was obtained by deacylation of the diglucosyl diglyceride from *S. faecalis* and a sample of glucosylglycerol by partial acid hydrolysis of the diglucosylglycerol (Brundish, Shaw & Baddiley, 1966). Solvents for chromatography were purified and dried before use.

Analytical methods. Phosphorus was determined by the method of Chen, Toribara & Warner (1956), fatty acid esters were determined by the method of Rapport & Alonzo (1955), reducing sugar was determined by the method of Park & Johnson (1949) and the anthrone procedure (Scott & Melvin, 1953), and D-glucose was determined with the Glucostat reagent. Glycerol was determined by phosphorylation with glycerol kinase and ATP followed by

measurement of the reduction of NAD by α -glycerol phosphate dehydrogenase (Wieland, 1963).

Paper chromatography. Paper chromatography of sugars, polyols and glycosides was carried out on Whatman no. 1 paper by the descending method in the solvent system butan-1-ol-pyridine-water (6:4:3, by vol.; solvent A) (Jeanes, Wise & Dimler, 1951). Compounds were detected by the periodate-Schiff reagents for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956) and the alkaline AgNO_3 reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950). Paper chromatography of phosphate esters was carried out on Whatman no. 4 paper by the ascending method in the solvent system propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.; solvent B) (Hanes & Isherwood, 1949) and by the descending method in the system propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (7:2:1, by vol.; solvent C) (LeCocq & Ballou, 1964). Compounds were detected by the reagents described above where appropriate and by the acid molybdate spray for phosphoric esters (Hanes & Isherwood, 1949).

Thin-layer chromatography. Lipids were examined in the solvent systems chloroform-methanol-water (65:25:4, by vol.; solvent D) (Wagner, Hörhammer & Wolff, 1961) and chloroform-methanol-acetic acid-water (65:25:8:4, by vol.; solvent E) (Skipiski, Peterson & Barclay, 1962). Components were located by exposure to iodine vapour and with reagents specific for phosphorus (Dittmer & Lester, 1964), α -glycols (Baddiley *et al.* 1956) and amino compounds (Marinetti, 1962). Preparative thin-layer chromatography was carried out on 20 cm. $\times$ 20 cm. plates coated with silicic acid 1 mm. thick. Control experiments showed that under the conditions employed both glycolipids and phospholipids were eluted with an efficiency $\geq 95\%$.

Deacylation. Lipids were deacylated with sodium methoxide by the method of Marinetti (1962).

Acid hydrolysis. Total acid hydrolysis was carried out in 2N-HCl for 3 hr. at 100°. The hydrolysate was evaporated to dryness *in vacuo* over KOH, and the residue dissolved in the minimum quantity of water and examined by paper chromatography.

Growth of organism. *M. laidlawii* strain B was grown on a lipid-free medium as described by Smith & Henrickson (1965). Organisms used for the isolation of *O*-amino acyl esters of phosphatidylglycerol were grown in a medium containing 2% (w/v) Bactotryptose [previously extracted in the dry state with chloroform-methanol (2:1, v/v)], 0.5% glucose and 0.5% NaCl at pH 8. The inoculum consisted of a 10% 16 hr. culture in the same medium. Incubation was carried out statically at 37° in 3 l. flasks each containing 2 l. of medium. After 22 hr. the pH was lowered to 5.0 by the addition of sterile HCl and incubation continued for a further 2 hr. The organisms were harvested and washed as described by Rothblat & Smith (1961).

Extraction, purification and fractionation of the lipids. The cell paste was extracted three times with 40 vol. of chloroform-methanol (2:1, v/v) and the extract purified by the method of Wells & Dittmer (1963). A sample (5 mg.) of the lipid was hydrolysed with 2N-HCl for 3 hr. at 100° and the hydrolysate examined in solvent A. Glucose and glycerol were observed; inositol was absent. The remaining lipid (288 mg.) was dissolved in chloroform (5 ml.) to give a clear yellow solution, which was introduced on to a column of silicic acid (2 cm. $\times$ 10 cm.) made up in chloroform. The column was eluted successively with chloroform (200 ml.,

fraction A), acetone (200 ml., fraction B) and chloroform-methanol (1:1, v/v; 100 ml., fraction C). The yields obtained were: fraction A, 28 mg. (10%); fraction B, 119 mg. (43%); fraction C, 131 mg. (47%). Examination by thin-layer chromatography in solvent D showed that fraction A contained neutral components only. It was not examined further. Fraction B contained two glycolipids, B1 (R_F 0.75) and B2 (R_F 0.65), both of which gave a slow blue colour with the periodate-Schiff reagents. Fraction C contained two phospholipids, C1 (R_F 0.5) and C2 (R_F 0.3).

Examination of the glycolipids. The remainder of fraction B was dissolved in the minimum quantity of chloroform and streaked across the origin of four thin-layer chromatography plates coated with silicic acid (1 mm. thick). After development in the system chloroform-methanol (9:1, v/v) the bands were located by exposure to iodine vapour and scraped off the plates. The lipids were eluted with chloroform-methanol (2:1, v/v). Total acid hydrolysis of both components and examination by paper chromatography in solvent A showed the presence of glucose and glycerol. Analysis gave fatty acid:D-glucose:glycerol proportions 2:1:1:0:0:9 for compound B1 and 1:8:2:0:1:1 for compound B2, consistent with a monoglucosyl diglyceride structure for compound B1 and a diglucosyl diglyceride structure for compound B2.

(a) Component B1, 1-(*O*- α -D-glucopyranosyl)-2,3-diacyl-D-glycerol (I). Deacylation yielded a single water-soluble glycoside which on paper chromatography corresponded to glucosylglycerol (R_{G10} 0.95 in solvent A) and gave a rapid purple colour with the periodate-Schiff reagents indicative of a 1-substituted glycerol (Baddiley, Buchanan & Roberts, 1963). The intact lipid did not give the rapid purple colour, indicating that the 2- and 3-positions of the glycerol were acylated. Instead it gave a much slower blue colour characteristic of hexoses. The glycoside was unaffected by incubation with β -glucosidase and had $[\alpha]_D + 69^\circ$ (c 1.5 in water).

(b) Component B2, 1-[*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl]-2,3-diacyl-D-glycerol (II). The lipid gave a slow blue colour with the periodate-Schiff reagents and on deacylation gave a single water-soluble glycoside that on paper chromatography corresponded to diglucosylglycerol (R_{G10} 0.53 in solvent A) and now gave the rapid purple colour characteristic of a 1-substituted glycerol. The glycoside was unaffected by incubation with β -glucosidase and had $[\alpha]_D + 104^\circ$ (c 0.87 in water). A sample of the glycoside (3 mg. in 1 ml. of water) was oxidized with 100 mm- NaIO_4 solution (2 ml.) for 16 hr. NaBH_4 (5 mg.) was added and the solution left for a further 16 hr. Excess of NaBH_4 was destroyed by the addition of Dowex 50 (H^+ form) resin and the supernatant evaporated to dryness. The boric acid was removed by evaporation with methanol (3 $\times$ 5 ml.) on a steam bath and the residue hydrolysed with 2N-HCl. Paper chromatography of the hydrolysate showed only glycerol and ethylene glycol. The absence of either erythritol or glucose, which would be produced from a diglucosylglycerol containing a (1 $\rightarrow$ 4)- or a (1 $\rightarrow$ 3)-linkage respectively between the two sugars, suggested that the linkage must be either (1 $\rightarrow$ 6) or (1 $\rightarrow$ 2). The latter was confirmed by methylation studies. A sample of the glycoside (10 mg.) was methylated and the methyl ethers produced by hydrolysis with methanolic HCl were examined by gas-liquid chromatography as described by Brundish, Shaw & Baddiley (1967b). Comparison with authentic standards

showed the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-glucose were produced, establishing the linkage between the two sugars as (1→2). The configuration of the glycerol in the glycosides was established as *D* by the method of Brundish, Shaw & Baddiley (1965).

Glycolipid composition as a function of age of culture. Duplicate experiments were carried out with 301. of culture. The cells were harvested at 6 hr. (81.), 10 hr. (61.), 14 hr. (41.), 16 hr. (41.), 24 hr. (41.) and 32 hr. (41.). The lipids were extracted and purified, and the glycolipid fraction was isolated as described above. The ratio of monoglucosyl diglyceride to diglucosyl diglyceride was determined by separation of the two components by thin-layer chromatography as described above and determination of glucose by the anthrone procedure. The results given in Table 1 are the mean of two determinations.

Effect on glycolipid composition of the presence of cholesterol in the growth medium. *M. laidlawii* was grown for 16 hr. in a medium supplemented with various concentrations of cholesterol. The glycolipid concentration and ratio of the two components in the harvested cells was determined as above. The results are given in Table 2.

Examination of the phospholipids. Fraction C, containing the phospholipids, was examined, as described for fraction B, by thin-layer chromatography in solvent *D*. The bands were located by exposure to iodine vapour and scraped off the plates. The lipids were eluted with chloroform-methanol (2:1, v/v).

(a) Component C1, phosphatidylglycerol. Compound C1 co-chromatographed on thin-layer chromatography with authentic phosphatidylglycerol, both compounds giving a rapid purple colour with the periodate-Schiff reagents. Deacylation gave a single water-soluble phosphate ester that co-chromatographed with diglycerol phosphate in solvents *B* and *C* and that, on treatment with sodium periodate followed by dimethylhydrazine, yielded P₁ (Letters, 1966).

(b) Component C2, phosphatidylglucose. Compound C2

Table 1. *Glycolipid composition as a function of age of culture*

Age of culture (hr.)	Total glycolipid (μmoles/g. dry wt. of organism)	Monoglucosyl diglyceride/diglucosyl diglyceride ratio
6	46	1.3
10	36	0.7
14	28	1.4
16	46	2.0
24	39	2.1
32	33	2.6

Table 2. *Effect on glycolipid composition of cholesterol in the growth medium*

Concn. of cholesterol in medium (mg./l.)	Total glycolipid (μmoles/g. dry wt. of organism)	Monoglucosyl diglyceride/diglucosyl diglyceride ratio
0	13	1.2
0.5	14	1.4
5	12	1.4
10	11	1.4

gave a blue colour with the periodate-Schiff reagents and on total acid hydrolysis liberated glucose. Deacylation gave a single water-soluble phosphate ester that on total acid hydrolysis yielded glycerophosphates and glucose. The detailed structure of this lipid is under investigation.

Isolation of *O*-amino acyl esters of phosphatidylglycerol. Extraction of the lipids and separation of the *O*-amino acyl esters were accomplished by the method of Houtsmuller & van Deenen (1965), acidic conditions being maintained throughout. The *O*-amino acyl esters were eluted from a silicic acid column with chloroform-methanol (3:1, v/v). They moved with *R_F* 0.53 on thin-layer chromatography in solvent *E*, whereas phosphatidylglycerol had *R_F* 0.62. When the lipid was emulsified sonically and incubated in borate buffer at pH 9 at room temperature for 30 min., water-soluble amino acids were liberated. The other product from this reaction was extractable into lipid solvents and was identified as phosphatidylglycerol by the methods described above. The amino acids were also liberated on total acid hydrolysis and identified by paper chromatography with known standards in the systems propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.), butan-1-ol-acetic acid-water (4:1:5, by vol.), phenol-water (5:2, v/v), butan-1-ol-benzyl alcohol (1:1, v/v, saturated with water) and pyridine-3-methylbutan-1-ol-water-diethylamine (10:10:7:3, by vol.). Alanine, glutamic acid, glycine, leucine/isoleucine, lysine and tyrosine were detected. The configuration of the amino acids was not determined.

Examination of the cell residue for polysaccharide. The cell residues after lipid removal were extracted with phenol by the procedure of Westphal as described by Plackett & Buttery (1964). Duplicate determinations of carbohydrate by the anthrone reagent gave a glucose concentration of 0.32 mg./g. of cell residue.

DISCUSSION

The non-saponifiable lipids of *M. laidlawii* strain B, grown in a lipid-free medium, have previously been shown to consist of neurosporene, a hydroxylated carotene and a carotenoid glucoside (Smith, 1963). The saponifiable lipids have now been shown to consist of approximately equal amounts of glycolipids and phospholipids.

Previous studies suggested that the phospholipids consisted of two components, bisphosphatidylglycerol (Smith, Koostra & Henrickson, 1965) and a new phospholipid, phosphatidylglucose. The present study has confirmed the presence of the latter compound and its detailed structure is under investigation. The other lipid component has been characterized as phosphatidylglycerol and not bisphosphatidylglycerol as previously reported. At acidic pH *O*-amino acyl esters of phosphatidylglycerol have been isolated from *M. laidlawii*, but it is doubtful if these occur naturally since the organism will not grow at pH values below 7.0. These esters have also been isolated from *M. hominis* under similar growth conditions (Smith & Koostra, 1967). It was suggested that they were synthesized by the organisms in an attempt to adjust their environment to a more favourable pH. No phospholipids containing inositol could be detected in *M. laidlawii*.

The glycolipid fraction yielded two glycosyl diglycerides identified as 1-(*O*- α -D-glucopyranosyl)-2,3-diacyl-D-glycerol and 1-[*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl]-2,3-diacyl-D-glycerol. Plackett & Shaw (1967) showed that a partially characterized diglucosyl diglyceride isolated from both *M. laidlawii* and *Streptococcus* MG gave similar end-points in complement-fixation tests with antisera to either *M. laidlawii* or *Streptococcus* MG and suggested that the glycolipids had similar structures. Glycolipids of structure identical with those from *M. laidlawii* described here have been found in streptococci (Vorbeck, 1966; Brundish *et al.* 1966, and unpublished work) thereby confirming the observations of Plackett & Shaw (1967). Glycosyl diglycerides, though widely distributed in Gram-positive bacteria (Brundish *et al.* 1966), usually represent only a small fraction of the total lipid; in *M. laidlawii* they represent almost half. The presence of large concentrations of the monoglucosyl diglyceride is also unusual; Brundish *et al.* (1965, 1966) were unable to detect the presence of any monoglucosyl diglycerides in most of the organisms they investigated, and in the *Pneumococcus* type I it only represented 1% of the total glycolipid. In *M. laidlawii* the total glycolipid concentration remained approximately constant throughout the culture period, though the ratio of monoglucosyl diglyceride to diglucosyl diglyceride

steadily increased (Table 1). Labelling experiments showed that the terminal glucose of the diglucosyl diglyceride did not undergo any turnover during growth of the organism, as might have been expected if the glycolipid were an intermediate in some glycosylation reaction (Smith, 1967). Moreover, *M. laidlawii* did not contain either polysaccharides or other polymers of the type normally found in bacterial cell walls whose biosynthesis might involve the utilization of glycolipid intermediates.

The presence of relatively large amounts of cholesterol in the parasitic mycoplasma may be partly responsible for maintaining structural integrity of the cell (see the introduction). It is possible that when the saprophytic *M. laidlawii* is grown in the absence of cholesterol large amounts of glycolipids are synthesized as a substitute for cholesterol. It is noteworthy that the derived L-form of *Streptococcus pyogenes* showed a 100% increase in glycolipid concentration over the parent bacterium (Cohen & Panos, 1966). However, when grown in the presence of cholesterol, *M. laidlawii* still synthesized glycolipids (Table 2) and certain fermentative sterol-requiring *Mycoplasma* species also contain glycolipids, although apparently not in such high concentrations. *Mycoplasma* sp. strain J contains an acylated glucose derivative (Smith & Koostra, 1967) and *M. mycoides* contains a galactofuranosyl diglyceride (Plackett, 1967). The additional function of providing substrate permeability has been attributed to cholesterol in the mycoplasma. This function could also be carried out by the glycolipids; an examination of the molecular configuration of glycosyl diglycerides led Brundish *et al.* (1967b) to suggest such a function for the glycolipids found in Gram-positive bacteria.

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REFERENCES

- Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.
- Baddiley, J., Buchanan, J. G. & Roberts, W. K. (1963). *Biochem. J.* **88**, 1.
- Brundish, D. E., Shaw, N. & Baddiley, J. (1965). *Biochem. J.* **97**, 158.
- Brundish, D. E., Shaw, N. & Baddiley, J. (1966). *Biochem. J.* **99**, 546.
- Brundish, D. E., Shaw, N. & Baddiley, J. (1967a). *Biochem. J.* **104**, 205.
- Brundish, D. E., Shaw, N. & Baddiley, J. (1967b). *Biochem. J.* **105**, 885.
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- Cohen, M. & Panos, C. (1966). *Biochemistry*, **5**, 2385.

- Dittmer, J. C. & Lester, R. L. (1964). *J. Lipid Res.* **5**, 126.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Houtsmuller, U. M. T. & van Deenen, L. L. M. (1965). *Biochim. biophys. Acta*, **106**, 564.
- Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
- LeCocq, J. & Ballou, C. E. (1964). *Biochemistry*, **3**, 976.
- Letters, R. (1966). *Biochim. biophys. Acta*, **116**, 489.
- Marinetti, G. V. (1962). *J. Lipid Res.* **3**, 1.
- Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
- Plackett, P. (1967). *Biochemistry*, **6**, 2746.
- Plackett, P. & Buttery, S. H. (1964). *Biochem. J.* **90**, 201.
- Plackett, P. & Shaw, E. J. (1967). *Biochem. J.* **104**, 616.
- Rapport, M. M. & Alonzo, N. (1955). *J. biol. Chem.* **217**, 193.
- Razin, S. & Argaman, M. (1963). *J. gen. Microbiol.* **30**, 155.
- Razin, S., Argaman, M. & Avigan, J. (1963). *J. gen. Microbiol.* **33**, 477.
- Rothblat, G. H. & Smith, P. F. (1961). *J. Bact.* **82**, 479.
- Scott, T. A. & Melvin, E. H. (1953). *Analyt. Chem.* **25**, 1650.
- Skipiski, V. P., Peterson, R. F. & Barclay, M. (1962). *J. Lipid Res.* **3**, 467.
- Smith, P. F. (1963). *J. gen. Microbiol.* **32**, 307.
- Smith, P. F. (1967). *J. Amer. Oil Chem. Ass.* **44**, 361A.
- Smith, P. F. & Henrickson, C. V. (1965). *J. Lipid Res.* **6**, 106.
- Smith, P. F. & Koostra, W. L. (1967). *J. Bact.* **93**, 1853.
- Smith, P. F., Koostra, W. L. & Henrickson, C. V. (1965). *J. Bact.* **90**, 282.
- Smith, P. F. & Sasaki, S. (1958). *Appl. Microbiol.* **6**, 184.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Van Iterson, W. & Ruys, A. C. (1960). *J. Ultrastruct. Res.* **3**, 282.
- Vorbeck, M. L. (1966). *Abstr. Pap. Amer. chem. Soc.*, 152nd Meet., p. C-296.
- Vorbeck, M. L. & Marinetti, G. V. (1965). *Biochemistry*, **4**, 296.
- Wagner, H., Hörhammer, L. & Wolff, P. (1961). *Biochem. Z.* **334**, 175.
- Wells, M. A. & Dittmer, J. C. (1963). *Biochemistry*, **2**, 1259.
- Wieland, O. (1963). In *Methods of Enzymatic Analysis*, p. 211. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.