The Structure of a Glycerylphosphoryldiglucoyl Diglyceride from the Lipids of *Acholeplasma laidlawii* Strain B

By N. SHAW

Microbiological Chemistry Research Laboratory, Department of Organic Chemistry,
University of Newcastle upon Tyne NE1 7RU, U.K.

and P. F. SMITH

Department of Microbiology, University of South Dakota, Vermillion, S. Dak. 57069, U.S.A.

and H. M. VERHEIJ

Biochemisch Laboratorium der Rijksuniversiteit, Utrecht, The Netherlands

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1. The phosphatidylglucose structure proposed previously (Smith & Henrikson, 1965) for the glucose-containing phospholipid from *Acholeplasma laidlawii* is incorrect. 2. The structure now proposed is 3-(*sn*-glycerol-3-phosphoryl-6'-[*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl])-*sn*-1,2-diglyceride, a new type of bacterial lipid. 3. Deacylation of the lipid gave a single water-soluble phosphate ester which could be distinguished on chromatography from synthetic samples of glucosylphosphorylglycerols. 4. Hydrolysis of the lipid with alkali gave a mixture of fatty acids, glycerol 2-phosphate, *sn*-glycerol 3-phosphate and *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-D-glycerol. 5. The lipid was unaffected on incubation with phospholipases A, C and D. 6. Diglucoyl diglyceride was isolated after treatment of the lipid with 60% HF, establishing the location of the fatty acid residues. 7. Periodate oxidation studies showed that the *sn*-glycerol 3-phosphate was esterified to the 6-hydroxyl group of one of the glucose residues in diglucoyl diglyceride.

In a previous publication (Shaw *et al.*, 1970) we suggested a revised structure for the glucose-containing phospholipid isolated from *Acholeplasma laidlawii* B (formerly denoted *Mycoplasma laidlawii* B) and originally called 'phosphatidylglucose' (Smith & Henrikson, 1965). This was the first example of a new type of bacterial lipid, a phosphoglycolipid (for a discussion on nomenclature see Shaw & Stead, 1972), which has subsequently been found in several other bacteria (Shaw & Stead, 1972). In the present paper we describe the structural elucidation of the phosphoglycolipid from *A. laidlawii* as 3-(*sn*-glycerol-3-phosphoryl-6'-[*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl])-*sn*-1,2-diglyceride.

Experimental and Results

Materials

D-Glucose 6-phosphate and α -D-glucose 1-phosphate were purchased from Sigma (London) Chemical Company, London S.W.6, U.K. and β -D-glucose 1-phosphate was prepared by the method of MacDonald (1966). α -Glucosidase, glycerol kinase and α -glycerol phosphate dehydrogenase were purchased from Sigma. The Glucostat reagents were purchased from Worthington Biochemical Corp.,

Freehold, N.J., U.S.A. Phospholipase A was a gift from Dr. G. de Haas, Rijksuniversiteit, Utrecht, The Netherlands, phospholipase C was isolated from *Bacillus cereus* and *Clostridium welchii* and phospholipase D was isolated from cabbage (Davidson & Long, 1958). Mallinckrodt 100-mesh silicic acid from Kodak Ltd., Kirkby, Lancs., U.K., was used for column chromatography and Merck Kieselgel G 'nach Stahl' for thin layer chromatography was from Anderman and Co., London S.E.1, U.K.

Analytical methods

Phosphorus was determined by the method of Chen *et al.* (1956), fatty acids 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 by the Glucostat reagent. Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954); glycerol was determined enzymically by phosphorylation with glycerol kinase and ATP followed by measurement of the reduction of NAD⁺ by α -glycerol phosphate dehydrogenase (Wieland, 1963). Formaldehyde was determined by the procedure of Hanahan & Olley (1958).

Paper chromatography

Paper chromatography of sugars, polyols and glycosides was done 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 *et al.*, 1951). Compounds were detected by the periodate-Schiff reagents for α -glycols (Baddiley *et al.*, 1956) and the alkaline AgNO_3 reagent for sugars and polyols (Trevelyan *et al.*, 1950). Paper chromatography of phosphate esters was done 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 on Whatman no. 1 paper 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 system chloroform-methanol-water (65:25:4, by vol.; solvent D) (Wagner *et al.*, 1961). Compounds were located by reagents specific for phosphorus (Dittmer & Lester, 1964) and α -glycols (Shaw, 1968).

Isolation of the phosphoglycolipid

The growth of *A. laidlawii*, the extraction of the lipids, and the purification of 'phosphatidylglucose' have been described previously (Shaw *et al.*, 1968). The phosphoglycolipid was homogeneous on t.l.c., R_F 0.3 in solvent D.

Analysis. Fatty acid, glycerol, phosphate and D-glucose analyses were done after hydrolysis in 2M-HCl at 100°C for 48 h on a sample repurified several times by preparative t.l.c. The molar proportion of fatty acid:glycerol:phosphate:D-glucose determined was 2:2:1:2 which is at variance with the 2:1:1:1 reported by Smith & Henrikson (1965). It seems probable that the original determinations were done on less-pure material contaminated with more highly acylated derivatives which may occur in some lipid preparations from this organism (P. F. Smith, unpublished work).

Synthesis of glycerylphosphorylglucoses

This method is based upon the procedure of Brown *et al.* (1959) for the preparation of glycerol-1-phosphoryl-2'-myoinositol. Glucose 6-phosphate (NH_4^+ salt, 30mg) was dissolved in water (5ml) containing 2,3-epoxypropanol (glycidol, 0.3ml) and the mixture was heated at 50°C for 6h. After being cooled the

solution was introduced on to a column of DEAE-cellulose (HCO_3^- form) (15 cm $\times$ 2 cm) and eluted with water (250 ml) to remove neutral products. The phosphates were then eluted from the column with 500 ml of a linear gradient (0-0.1 M) of $(\text{NH}_4)_2\text{CO}_3$. Fractions were collected (5 ml) and samples (0.1 ml) analysed for phosphorus. One major product was observed and was recovered by combining appropriate fractions and removing water by rotary evaporation. No attempt was made to crystallize the glucose-6-phosphoryl-1'-glycerol which was a colourless glassy solid. It was homogeneous on paper chromatography and gave a rapid purple colour indicative of a 1-substituted glycerol with the periodate-Schiff reagents (Roberts *et al.*, 1963). Analysis gave a proportion of glycerol:phosphorus:glucose of 0.95:1:1.02 and the products of acid hydrolysis (1M-HCl at 100°C for 1 h) were α , β -glycerol phosphates and glucose. Samples of α - and β -glucose-1-phosphoryl-1'-glycerol were prepared by using α -glucose 1-phosphate and β -glucose 1-phosphate in the above procedure. The chromatographic properties of the synthetic compounds and the deacylation product of the phosphoglycolipid are shown in Table 1.

A sample of glucose-6-phosphoryl-1'-glycerol (2mg) was dissolved in water (0.5 ml) and sodium borohydride (5mg) was added. After 4 h at room temperature, the excess of sodium borohydride was destroyed by the addition of a drop of acetic acid and the solution was passed through a small column of Dowex 50 (H^+ form) resin and evaporated to dryness. The boric acid was removed by evaporation with three successive portions (1 ml) of methanol on a steam bath. The residue was hydrolysed in 1M-HCl at 100°C for 1 h and the hydrolysate examined by chromatography in solvent A for sugars and polyols: glycerol

Table 1. *Paper chromatographic properties of the synthetic glucosylphosphorylglycerols compared with those of the deacylated phosphoglycolipid*

For further details see text.

	$R_{\alpha\text{-glycerol-P}}$	
	Solvent B	Solvent C
α -Glycerol phosphate	1.0	1.0
Glucose-1-phosphoryl-1'-glycerol*	1.6	2.7
Glucose-6-phosphoryl-1'-glycerol	1.5	2.5
Deacylation product of phosphoglycolipid	1.3	1.9

* Both α and β isomers had identical chromatographic properties.

and glucitol were detected but not glucose. This procedure was repeated on a sample of the deacylation product of the phospholipid: glucose and glycerol were observed, but glucitol was absent.

Alkaline hydrolysis of the phosphoglycolipid

The lipid (1 mg) was heated for 2 h at 100°C in 1 M-NaOH (0.5 ml). After being cooled Na⁺ ions were removed by passage through a small bed of Dowex 50 (NH₄⁺ form) resin and the eluate was evaporated to dryness. The products were examined by paper chromatography in solvents A and B. α,β -Glycerol phosphates were the only products containing phosphorus; no glucose phosphates were produced. The sole neutral product was a non-reducing glycoside which rapidly gave a purple colour characteristic of a 1-substituted glycerol with the periodate-Schiff reagents, and which had the chromatographic properties of a diglucosylglycerol.

Identification of the glycoside. The lipid (75 mg) was hydrolysed in 1 M-NaOH as above and the de-ionized hydrolysate was introduced on to a column of DEAE-cellulose (HCO₃⁻ form) (15 cm × 2 cm) and eluted consecutively with water (250 ml) and 0.1 M-(NH₄)₂CO₃ (250 ml). The glycoside was recovered from the aqueous fraction and its structure determined from studies of acid hydrolysis, periodate oxidation and methylation as previously described for the diglucosylglycerol obtained from the glycolipid of this organism (Shaw *et al.*, 1968). The structure, *O*- α -D-glucopyranosyl-(1→2)-*O*- α -D-glucopyranosyl-(1→1)-D-glycerol, was identical with that of the glycoside from the glycolipid.

Stereochemistry of the glycerol phosphate. Alkaline hydrolysis of a *sn*-glycerol-3-phosphodiester derivative proceeds through a cyclic phosphate intermediate to yield *sn*-glycerol 3-phosphate (42% of total P) and glycerol 2-phosphate (58% of total P) (Brown *et al.*, 1958; Olsen & Ballou, 1971). Samples of the mixture of glycerol phosphates recovered from the (NH₄)₂CO₃ fraction by evaporation were assayed enzymically for *sn*-glycerol 3-phosphate (Wieland, 1963). Of the total phosphate 39% was present as *sn*-glycerol 3-phosphate. Thus at least 93% of the glycerol 3-phosphate present in the phosphoglycolipid is of the *sn*-glycerol 3-phosphate configuration.

Oxidation with sodium periodate

Oxidation of the phosphoglycolipid. The lipid (containing 4 μ mol of P) was dried under a stream of N₂ and then dissolved in 0.1 M-NaHCO₃ (2 ml) and a solution of 0.01 M-NaIO₄ (3 ml) was added. [A sample of α -glycerol phosphate (10 μ mol) was oxidized under identical conditions as a control.] Samples were removed (1 ml) after 0, 5, 19 and 26 h and analysed for formaldehyde and periodate uptake. After 26 h

4.0 mol.prop. of periodate were consumed with the liberation of 1.03 mol of formaldehyde/mol of P.

Oxidation of the deacylation product. A sample of the water-soluble deacylation product (4 μ mol of P) was dissolved in 2 ml of 0.1 M-NaHCO₃ and a solution of NaIO₄ (0.01 M, 3 ml) was added. Consumption of periodate and liberation of formaldehyde were determined after 0, 1, 2, 4 and 24 h. After 24 h 5.0 mol of periodate/mol of P were consumed with the liberation of 2.0 mol of formaldehyde.

Controlled periodate oxidation for the detection of furanose residues. The deacylated lipid (72 μ mol of P) dissolved in water was allowed to react with NaIO₄ (144 μ mol) at 0°C. After 20 min the reaction was stopped by the addition of glycerol (150 μ mol). Sodium borohydride (40 mg) was added and the solution left overnight at room temperature. The excess of sodium borohydride was removed as described above and the residue was hydrolysed in 1 M-H₂SO₄ for 15 min at 100°C. After de-ionization the hydrolysate was examined by paper chromatography for sugars. Glucose was the only carbohydrate detected; xylose, the product expected from degradation of a glucofuranose residue, was absent. The same procedure done on a sample of α -methyl glucofuranose gave xylose. A further sample of lipid was oxidized as above and the product was hydrolysed in 1 M-HCl at 100°C for 2 h. Chromatography showed the presence of ethylene glycol phosphate.

Hydrolysis with hydrogen fluoride

The phosphoglycolipid (2 mg) was treated with 60% HF (0.5 ml) at 0°C for 8 h in a polythene tube fitted with a cap. An ice-cold mixture of chloroform-water (1:1, v/v, 5 ml) was added, and the reaction mixture was shaken rapidly. The chloroform layer was separated off and washed well with ice-cold saturated NaHCO₃ solution and then with water (5 ml of each). The chloroform layer was dried over anhydrous Na₂SO₄, evaporated to dryness and examined by t.l.c. One major product was observed which co-chromatographed with a sample of the diglucosyl diglyceride from *A. laidlawii*. The reaction was repeated on a larger sample of lipid (10 mg) and the product introduced on to a small column of silicic acid. The column was eluted successively with 100 ml portions of chloroform, acetone and chloroform-methanol (1:1, v/v). The product behaved as expected of a glycolipid, being recovered in the acetone fraction, and upon deacylation yielded a diglucosylglycerol identical with that obtained from the glycolipid.

Enzymic studies

Phospholipase A. The lipid (2 μ mol of P) in 0.1 M-tris-HCl buffer at pH 7 (0.5 ml) containing CaCl₂ (0.03 M) and ether (1 ml) was treated with hog

pancreatic phospholipase A (0.2ml). The reaction mixture was shaken at room temperature and samples were removed at frequent intervals and examined by t.l.c. After 24h there was no detectable loss of starting material, and no free fatty acids had been produced. The enzyme quantitatively degraded phosphatidylcholine in 20min.

Phospholipase C. The lipid (4 μ mol of P) in 0.5ml of 0.1M-tris-HCl buffer at pH7 containing ZnCl₂ (0.01M) and ether (1ml) was treated with enzyme (0.2ml) and was shaken at room temperature for 72h. Samples were removed at frequent intervals and examined by t.l.c. There was no detectable loss of starting material and examination of the aqueous layer by paper chromatography did not reveal any phosphate esters. The enzyme rapidly degraded phosphatidylglycerol and more slowly bis-phosphatidylglycerol.

Phospholipase D. The lipid (4 μ mol of P) in 0.5ml of 0.1M-sodium acetate buffer at pH5.6 containing CaCl₂ (0.03M) and ether (1ml) was treated with enzyme (0.2ml) and shaken at room temperature. No detectable degradation had occurred after 72h; the enzyme completely degraded phosphatidylcholine.

α -Glucosidase. The water-soluble deacylation product from the phosphoglycolipid (2mg) was incubated with an aqueous solution of α -glucosidase (0.2ml of a 1% solution) at 37°C overnight. Examination of the products by paper chromatography did not reveal any glucose, even after prolonged incubation. A sample of diglucosylglycerol from the glycolipid was degraded slowly by the enzyme to glucose and glycerol.

Discussion

The structure originally suggested for the glucose-containing phospholipid from *A. laidlawii* was a 'phosphatidylglucose' in which the phosphatidyl residue was esterified to the 6-hydroxyl of glucose (I). This proposal was based upon analytical results and identification of the products from acid hydrolysis (Smith & Henrikson, 1965). Studies intended to confirm this proposal by chemical synthesis now show that it is untenable.

Brown *et al.* (1958) described a general method for the formation of phosphodiester derivatives of glycerol 1-phosphate by the reaction of 2,3-epoxypropanol (glycidol) with phosphomonoesters. This route has been used for the synthesis of glycerol-1-phosphoryl-2'-myoinositol, the deacylation product of phosphatidylmyoinositol (Brown *et al.*, 1959) and subsequently for diglycerol phosphate and bis(glycerolphosphoryl)glycerol, the deacylation products of phosphatidylglycerol and bis-phosphatidylglycerol respectively (Brundish *et al.*, 1967). Reaction of glucose 6-phosphate with glycidol gave a product with the properties of glucose 6-phosphoryl-1'-

glycerol, the deacylation product of phosphatidylglucose (I): chromatographic comparison of the synthetic product with the natural product showed that they were not identical. Reduction of the glucose-6-phosphoryl-1'-glycerol with sodium borohydride followed by acid hydrolysis gave the expected glucitol, whereas the natural product still gave glucose, showing that in the latter the glucose was bound through its reducing group. Accordingly α - and β -glucose-1-phosphoryl-1'-glycerol, the deacylation products of the isomeric phosphatidylglucose (II) were prepared by a similar method: neither co-chromatographed with the natural product. The synthesis of phosphatidylglucose (II) itself (Verheij *et al.*, 1970) confirmed that this structure was incorrect. The observation that formaldehyde was liberated from the lipid on oxidation with sodium periodate initially suggested that another isomeric 'phosphatidylglucose' structure was possible containing a glucofuranose residue. Controlled periodate oxidation followed by reduction and acid hydrolysis did not liberate xylose, which should be produced from a glucofuranose residue under these conditions. Thus all possible isomers of the 'phosphatidylglucose' structure were eliminated.

The alternative structure, glycerylphosphoryldiglycosyl diglyceride (III), is supported by a series of degradative studies summarized in Scheme 1. Treatment with 1M-NaOH at 100°C for 1h completely hydrolysed the lipid to a mixture of fatty acids, α,β -glycerophosphates and diglucosylglycerol. The structure of the diglucosylglycerol was established as *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-D-glycerol by the methods used previously for the glycoside from the glycolipid present in this organism (Shaw *et al.*, 1968). Enzymic determination of the α -glycerophosphate produced in the hydrolysis showed that the original lipid is a derivative of *sn*-glycerol 3-phosphate.

The lipid consumed 4mol.prop. of sodium periodate with the liberation of 1mol of formaldehyde. The deacylation product consumed a further mol of sodium periodate and liberated a further 1mol of formaldehyde. Controlled oxidation of the lipid with sodium periodate followed by reduction with sodium borohydride and hydrolysis with acid yielded ethylene glycol phosphate; no xylose indicative of a glucofuranose residue was produced. The isolation of ethylene glycol phosphate precludes the presence of a 'phosphatidyl' residue in the lipid, a finding confirmed by the inability of phospholipases A, C and D to cause hydrolysis. The location of the fatty acids was established by hydrolytic studies with HF. This reagent has been used to cleave polymers containing phosphodiester linkages without hydrolysis of glycosidic linkages (Glaser & Burger, 1964; Archibald *et al.*, 1968) and the reaction with the phospholipid gave a good yield of diglucosyl

diglyceride. This reaction conclusively established that the lipid was not a phospholipid containing a 'phosphatidyl' residue but was a new type of bacterial lipid derived from a glycolipid, diglucosyl diglyceride.

The structural feature which remains to be resolved is the precise location of the glycerol phosphate residue. The results obtained from periodate oxidation show that it is linked to either of the 6-hydroxyl groups of the glucose residues. Predictably from stereochemical considerations, alkaline hydrolysis of the phosphodiester proceeds through preferential formation of a cyclic glycerol phosphate ester, subsequently yielding a mixture of α,β -

glycerol phosphates, and no products containing a glucose phosphate residue have been observed.

Previous studies have shown that the other major lipid components of *A. laidlawii* are phosphatidylglycerol, glucosyl diglyceride and diglucosyl diglyceride (Shaw *et al.*, 1968). The structural similarity between the latter and the phosphoglycolipid implies a biosynthetic relationship which could involve transfer of glycerophosphate from CDP-glycerol to diglucosyl diglyceride, or transfer of phosphatidic acid from CDP-diglyceride to diglucosyl diglyceride followed by selective deacylation. The stereochemistry of the glycerophosphate residue is in accord with both these routes.

Scheme 1. Degradative sequence establishing the structure of the phosphoglycolipid

Since our previous publication (Shaw *et al.*, 1970), reports have appeared on the occurrence of phosphoglycolipids in other bacteria. *Streptococcus faecalis* (Ambron & Pieringer, 1971) and *Streptococcus lactis* (Fischer, 1970) also contain glycerylphosphoryldiglycosyl diglycerides but in both cases the glycerophosphate residue has been located on the internal glucose, as treatment with α -glucosidase liberates glucose. We have been unable to repeat this experiment on the phosphoglycolipid from *A. laidlawii*, which suggests it may have the isomeric structure with the glycerophosphate on the terminal glucose, but confirmatory evidence is still required. Ambron & Pieringer (1971) have been able to demonstrate the utilization of diglycosyl diglyceride as a substrate in the biosynthesis of the phosphoglycolipid but the donor of the glycerophosphate residue has yet to be determined. Phosphoglycolipids structurally related to other glycosyl diglycerides have been isolated from bacteria, suggesting their probable widespread occurrence, and a possible function has been described (Shaw & Stead, 1972; Toon *et al.*, 1972).

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