Isomers of Glucosaminylphosphatidylglycerol in *Bacillus megaterium*

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1. The lipids of *Bacillus megaterium* were extracted and three lipids containing glucosamine were identified. One of these is not a phospholipid, but the other two, which differ in their chromatographic behaviour, contain phosphorus, glycerol, fatty acid and D-glucosamine in the molar proportions 1:2:2:1. 2. In both phosphoglycerolipids, the fatty acids are bound in ester linkage, and both yield 2,5-anhydromannose and 3-*sn*-phosphatidyl-1'-*sn*-glycerol on treatment with sodium nitrite. 3. Both phosphoglycerolipids were *N*-acetylated and, after removal of fatty acids by mild alkaline hydrolysis, in both cases *N*-acetylglucosamine was quantitatively released by β -*N*-acetylhexosaminidase. 4. The glucosaminylglycerols derived from the two phosphoglycerolipids by partial acid hydrolysis differ in their behaviour towards periodate. In one case 1 mole of periodate is rapidly consumed/mole of glucosaminylglycerol, but in the other case under identical conditions the consumption of periodate is negligible. 5. The phosphoglycerolipids were identified as 1'-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-3'-*O*- β -(2-amino-2-deoxy-D-glucopyranosyl)-*sn*-glycerol and as 1'-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-2'-*O*- β -(2-amino-2-deoxy-D-glucopyranosyl)-*sn*-glycerol. 6. Both phosphoglycerolipids are good substrates for phospholipase A: neither is a substrate for phospholipase C from *Clostridium perfringens*, and only the 3'-glucosaminide is a substrate for phospholipase D.

The purpose of this paper is to present evidence for the occurrence in *Bacillus megaterium* of two isomers of glucosaminylphosphatidylglycerol. A preliminary account of some of this work has been presented to the Biochemical Society (Phizackerley & MacDougall, 1968).

The lipid nomenclature used is that suggested by the IUPAC-IUB Commission on Biochemical Nomenclature (1967).

METHODS AND MATERIALS

Growth of organisms. *B. megaterium* was grown in liquid medium containing 50 mM-KH₂PO₄ adjusted to pH 7.2 with 2 M-NaOH, 50 mM-NH₄Cl, 0.1 M-glucose and essential salts (40 mg. of CaCl₂·6H₂O, 80 mg. of MgSO₄·7H₂O, 4.0 mg. of MnSO₄·4H₂O and 4.0 mg. of FeSO₄·7H₂O/l. of growth medium). The glucose was autoclaved separately (115° for 20 min.) as a 1 M solution and added to the sterilized salt solution. Conical flasks (2 l.) containing 1 l. of the above medium were inoculated from a freshly grown culture and the flasks shaken for 48 hr. on a Gyrotary Shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The cells were harvested by centrifugation at 30 000g at 2° in a Servall automatic superspeed centrifuge. The yield was about 6 g. wet wt. of hard-packed cells/l. of medium.

Extraction of lipid. The procedure was designed to

minimize the extraction of poly- β -hydroxybutyrate. Cells (100 g. wet wt.) suspended in 50 ml. of water were added to 1.5 l. of ethanol and shaken overnight at room temperature. The suspension was filtered and the filtrate evaporated to dryness under N₂ under reduced pressure at room temperature. The residue was extracted with chloroform-methanol (2:1, v/v) and purified by the procedure of Folch, Lees & Sloane-Stanley (1957). The final extract was dissolved in 20 ml. of chloroform and stored at 0°. It contained 11.3 mg. of phospholipid P.

Purification of glucosaminylphosphatidylglycerols. Step 1. Column chromatography on silicic acid. This step yielded an extract in which both glucosaminylphosphatidylglycerols were concentrated. Silicic acid (100 mesh; Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.) (100 g.), prepared as described by Phizackerley & Francis (1966), was used as a 20 cm. × 12 cm.² column. The lipid extract in chloroform (50 mg. of phospholipid P) was applied to the column, which was eluted under gravity with 500 ml. of chloroform and then with a discontinuous chloroform-methanol gradient. The chloroform-methanol volume ratio was varied from 120:1 to 10:1 in 12 steps, each of 200 ml., and from 10:1 to 3:2 in eight steps, also of 200 ml. Fractions (50 ml.) were collected and each fraction was monitored by measurement of P and t.l.c. The recognition of lipids containing glucosamine was facilitated by the fact that they formed a pink derivative when the plates were developed by spraying with ninhydrin, which contrasts with the purple colour formed

by other amino lipids. A lipid containing glucosamine but not P was eluted as the gradient extended from chloroform-methanol volume ratios 20:1 to 10:1; the properties of this lipid are not reported here. Both glucosaminylphosphatidylglycerols were eluted as the gradient extended from chloroform-methanol volume ratios 6:1 to 4:1, and these fractions, which contained other lipids, were pooled and evaporated to dryness under N_2 , and the residue was extracted with chloroform. This extract (17.2 mg. of phospholipid P) was applied to a second silicic acid column, which was eluted with a discontinuous gradient in which the chloroform-methanol volume ratio was varied from 10:1 to 3:2 in ten steps, each of 200 ml. The fractions rich in glucosaminylphosphatidylglycerols were pooled and treated as described above. The final extract contained 12.2 mg. of phospholipid P; the main contaminant was phosphatidylethanolamine.

Step 2. Preparative t.l.c. It was found in preliminary experiments that the use of a mixture of silica gel HF₂₅₄ and silica gel G (1:1, w/w) allowed the recovery of about 80% of phospholipid P by using the elution procedure described below, whereas if either form of silica gel were used alone the recovery was very much less. Plates (20 cm. $\times$ 20 cm., 0.5 mm. thick) were activated at 120° for 75 min. and the load was 0.5 mg. of phospholipid P/plate. The plates were developed in solvent 1 (see below), and three main bands were seen. The upper band consisted mainly of phosphatidylethanolamine, which largely concealed 2'-(O- β -glucosaminyl)phosphatidylglycerol, and the middle band consisted mainly of 3'-(O- β -glucosaminyl)phosphatidylglycerol. These bands were scraped separately from the plates and extracted with 200 ml. of solvent 1, followed by 200 ml. of chloroform-methanol (1:4, v/v) and finally 50 ml. of methanol. The extracts were evaporated to dryness at reduced pressure under N_2 , and the residues extracted with chloroform. The final purification of 3'-(O- β -glucosaminyl)phosphatidylglycerol was achieved by repeating the preparative t.l.c. as described above. The pure lipid was dissolved in chloroform-methanol (2:1, v/v) as it is only slightly soluble in pure chloroform. The yield was 0.78 mg. of phospholipid P. The final purification of 2'-(O- β -glucosaminyl)phosphatidylglycerol was achieved by developing the plates in solvent 3. The yield was 3.1 mg. of phospholipid P.

Acid hydrolysis of glucosaminylphosphatidylglycerols. This was carried out in evacuated sealed tubes under the conditions described in the text.

Treatment of glucosaminylphosphatidylglycerols with sodium nitrite. A portion (3 μ moles) of lipid was incubated with 2 ml. of methanol and 0.5 ml. of $NaNO_2$ (100 mg./ml. in 10 mM-sodium acetate buffer, pH 5.6) in a sealed tube at 60° for 72 hr. Then 2.0 ml. of chloroform and 1.4 ml. of water were added, the mixture was agitated on a Whirlimixer for 2 min. and centrifuged on a bench centrifuge, and the upper and lower phases were separated. 2,5-Anhydromannose was recovered from the upper phase as described by Ellwood, Kelemen & Baddiley (1963). The lower phase was evaporated to dryness and the residue extracted with chloroform. Phosphatidylglycerol was purified from this extract by preparative t.l.c. with solvent 2.

Preparation of glycerophosphorylglyceryl N-acetylglucosaminides. These derivatives were prepared by incubating 0.5 μ mole of lipid dissolved in 5 ml. of chloroform-methanol (2:1, v/v) in a stoppered tube with 0.3 ml. of 0.1 M- $NaHCO_3$ and 0.25 ml. of freshly prepared 1% (v/v) acetic anhydride

in water. The N-acetylated lipid was recovered by extracting the reaction mixture by the procedure of Folch *et al.* (1957); on t.l.c. in solvent 1 a single ninhydrin-negative compound was seen. Fatty acids were removed and the water-soluble product was extracted by the procedure of Dawson, Hemington & Davenport (1962) except that alkali was neutralized with 2 M-HCl.

Hydrolysis with β -N-acetylhexosaminidase. The conditions of hydrolysis and the method of determination of N-acetylglucosamine were as described by Frohwein & Gatt (1967).

Hydrolysis of lipids with phospholipases. (1) Phospholipase A. The reaction mixture contained 0.3 μ mole of lipid, 1 ml. of ether and 50 μ g. of Russell-viper venom in 50 μ l. of 0.1 M-tris-HCl buffer, pH 7.2, and was shaken for 4 hr. at room temperature. (2) Phospholipase C. The reaction mixture contained 0.3 μ mole of lipid, 1 ml. of ether, 50 μ l. of 0.1 M- $CaCl_2$ and 50 μ g. of phospholipase C from either *Clostridium perfringens* or *Bacillus cereus*. (3) Phospholipase D. The enzyme was partially purified and freed from glycerol by passage of 10 mg. of the commercial preparation dissolved in 0.2 ml. of 15 mM- $CaCl_2$ and 5 mM-sodium acetate buffer, pH 5.4, down a column (10 cm. $\times$ 1 cm.²) of Sephadex G-50. Fractions (2 ml.) were collected, and those fractions containing the first peak to emerge after the void volume that contained material absorbing at 280 nm. were used as enzyme. The reaction mixture contained 0.3 μ mole of lipid, 5 ml. of ether and 1 ml. of enzyme (0.3 mg. of protein), and was shaken for 18 hr. at 25°.

Thin-layer chromatography. The t.l.c. for purposes of identification was carried out on plates of silica gel G 0.25 mm. thick. The following solvents were used and are designated by their numbers in the text: solvent 1, chloroform-methanol-water (65:25:4, by vol.); solvent 2, chloroform-methanol-acetic acid (35:10:1, by vol.); solvent 3, chloroform-methanol-aq. NH_3 (sp. gr. 0.880) (35:10:1, by vol.); solvent 4, butan-1-ol-acetic acid-water (3:1:1, by vol.); solvent 5, chloroform-methanol-aq. 30% (v/v) methyl amine (65:25:8, by vol.); solvent 6, hexane-ether-formic acid (60:50:1, by vol.). Lipids were located with I_2 vapour; ninhydrin-positive compounds were located by spraying the plate with 0.2% (w/v) ninhydrin in butan-1-ol followed by heating at 105° for 15 min.; P-containing compounds were located with the reagent of Dittmer & Lester (1964).

Paper chromatography. This was carried out on Whatman no. 1 paper. The following solvents were used: solvent 7, pyridine-ethyl acetate-water-acetic acid (5:5:3:1, by vol.); solvent 8, butan-1-ol-ethanol-water (4:1:1, by vol.). Reducing sugars were located with alkaline $AgNO_3$ (Smith, 1961) and α -diols were located with the benzidine-periodate reagent of Cifonelli & Smith (1954).

Paper electrophoresis. This was carried out with a Locarte high-voltage electrophoresis apparatus (Locarte Co. Ltd., London E.C.3) for 30 min. at 100 v/cm. in pyridine-acetic acid-water, pH 3.6 (1:10:89, by vol.), with Whatman no. 1 paper. When used to purify glucosaminylglycerol, the paper was washed by elution for 24 hr. with 2 M-acetic acid and then for 72 hr. with water. Untreated paper contained material that on elution with water strongly inhibited the Elson-Morgan reaction for amino-2-deoxy sugars, and in addition interfered with the measurement of periodate consumption.

Gas-liquid chromatography. The g.l.c. of fatty acid

methyl esters, prepared as described by Schlenk & Gellerman (1960), was carried out on an F & M 402 high-efficiency gas chromatograph with a column of 20% (w/w) Apiezon L on Supersorb AW-HMDS.

Column chromatography. Column chromatography by the method of Moore & Stein (1951) was carried out on a Technicon amino acid analyser.

Optical rotation. This was measured on a Bendix 66 polarimeter.

Analytical methods. Orthophosphate was determined by the method of Bartlett (1959); total nitrogen was determined by the method of Sloane-Stanley (1967); fatty acids were determined by the method of Duncombe (1963) with palmitic acid as a standard; glycerol was determined by the method of Wieland (1963) and *sn*-glycero-3-phosphate by the method of Hohorst (1963). The Elson-Morgan reaction for the determination of amino-2-deoxy sugars was carried out by the method of Boas (1953) and the determination of *N*-acetylglucosamine by the method of Frohwein & Gatt (1967). Degradation of amino sugars with ninhydrin was carried out by the method of Stoffyn & Jeanloz (1954).

Materials. 3-*sn*-Phosphatidyl-1'-glycerol was synthesized from egg-yolk lecithin and glycerol by the method of Dawson (1967) and its lyso derivative was prepared by treatment of the product with phospholipase A. Phosphatidic acid was prepared from egg-yolk lecithin by treatment with phospholipase D and lysophosphatidic acid by treating the product with phospholipase A. Phospholipase C (EC 3.1.4.3) from *B. cereus* strain 8035 was prepared by the method of Ottolenghi (1965). Phenyl *O*- α -*N*-acetylglucosaminide was synthesized by a combination of the methods of Leabach & Weissman (1963) and Roseman & Dorfman (1951). Russell-viper venom containing phospholipase A (EC 3.1.1.4), phospholipase C from *Cl. perfringens* and phospholipase D (EC 3.1.4.4) from cabbage were all obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Glycerol kinase (EC 2.7.1.30) and *sn*-glycero-3-phosphate dehydrogenase (EC 1.1.1.8) were obtained from Boehringer Corp. (London) Ltd., London W.5. *p*-Nitrophenyl *O*- β -*N*-acetylglucosaminide was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6. Silica gel G and silica gel HF₂₅₄ were obtained from E. Merck A.-G., Darmstadt, Germany. All other chemicals were of the highest quality commercially available, and all solvents were redistilled before use.

RESULTS

3'-(*O*- β -Glucosaminyl)phosphatidylglycerol. Table 1 shows that within the limits of experimental error this lipid contained phosphorus, glycerol, fatty acids and total nitrogen in the molar proportions 1:2:2:1. Its behaviour on t.l.c. on silica gel G is

summarized in Table 2, which shows that it can be distinguished from 2'-(*O*- β -glucosaminyl)phosphatidylglycerol in solvents 1, 2 and 4.

Products of acid hydrolysis. On strong acid hydrolysis (5.8M-hydrochloric acid at 105° for 18hr.) the lipid yielded, in addition to P_i, glycerol and fatty acids, a single ninhydrin-positive compound. This compound was recovered by column chromatography on Dowex 50 (H⁺ form) as described by Boas (1953) and was identified as D-glucosamine. It behaved as a reducing sugar when tested by the method of Park & Johnson (1949); it gave the Elson-Morgan reaction for amino-2-deoxy sugars by the method of Boas (1953); it behaved identically with glucosamine and differently from galactosamine and mannosamine both on paper chromatography in solvent 7 (Fischer & Nebel, 1955) and on column chromatography by the method of Moore & Stein (1951); deamination with nitrite yielded a product chromatographically identical with 2,5-anhydromannose (Ellwood *et al.* 1963); and degradation with ninhydrin yielded a product that co-chromatographed with arabinose in solvent 8 (Stoffyn & Jeanloz, 1954). The molecular optical rotation was measured and was indistinguishable from that of D-glucosamine.

On mild acid hydrolysis (2M-hydrochloric acid at 105° for 8hr.), in addition to P_i, about 70% of the phosphate was released as a mixture in approximately equimolar proportions of *sn*-glycero-3-phosphate and *sn*-glycero-1-phosphate. In contrast with the results obtained on complete acid hydrolysis, two ninhydrin-positive compounds were obtained, which were well separated from each other and from the phosphates by high-voltage paper electrophoresis at pH 3.6. The compound migrating more rapidly towards the cathode is glucosamine; the other on strong acid hydrolysis yielded glucosamine and glycerol in the molar proportions 1.00:0.91 and for this and other reasons given below was identified as 3'-(*O*- β -glucosaminyl)-*sn*-glycerol.

Products of mild alkaline hydrolysis. Mild alkaline hydrolysis by the method of Dawson *et al.* (1962) yielded 1.82 moles of fatty acid/mole of P (which indicates that the fatty acids are bound by ester linkages) and a ninhydrin-positive water-soluble derivative containing P, glycerol and glucosamine in the molar proportions 1.00:2.02:1.06. This

Table 1. Composition of glucosaminylphosphatidylglycerols from *B. megaterium*

Compound	Molar ratios of constituents			
	P	Glycerol	Fatty acid	N
3'-(<i>O</i> - β -Glucosaminyl)phosphatidylglycerol	1.00	1.76	1.93	1.09
2'-(<i>O</i> - β -Glucosaminyl)phosphatidylglycerol	1.00	1.82	1.90	1.06

Table 2. *Thin-layer chromatography on silica gel G of glucosaminylphosphatidylglycerols and related compounds*

The preparation of plates and the nature of the developing solvents is described in the Methods and Materials section. About 1 μ g. of phospholipid P was spotted on the plate and for each solvent R_f values were determined on the same plate.

Compound	Solvent ...	R_f			
		1	2	3	4
3'-(<i>O</i> - β -Glucosaminyl)phosphatidylglycerol		0.37	0.12	0.03	0.69
Lyso-3'-(<i>O</i> - β -glucosaminyl)phosphatidylglycerol		0.17	—	—	—
3'-Glycerophosphorylglyceryl <i>O</i> - β -glucosaminide		—	—	—	0.56
2'-(<i>O</i> - β -Glucosaminyl)phosphatidylglycerol		0.49	0.31	0.06	0.73
Lyso-2'-(<i>O</i> - β -glucosaminyl)phosphatidylglycerol		0.25	—	—	—
2'-Glycerophosphorylglyceryl <i>O</i> - β -glucosaminide		—	—	—	0.63
Phosphatidylethanolamine		0.50	0.30	0.27	—

product, in contrast with the parent lipid, reacted rapidly with periodate and was identified as 3'-glycerophosphorylglyceryl *O*- β -glucosaminide. As shown in Table 2, this compound can be distinguished from 2'-glycerophosphorylglyceryl *O*- β -glucosaminide by t.l.c. in solvent 4.

Products of degradation by nitrite. Treatment of 3'-(*O*- β -glucosaminyl)phosphatidylglycerol with nitrite cleaved the glycosidic bond and yielded a water-soluble product that behaved identically on paper chromatography with 2,5-anhydromannose, which is the product expected after treatment of glucosamine with nitrite (Ellwood *et al.* 1963). The lipid product was identified as 3-*sn*-phosphatidyl-1'-*sn*-glycerol (phosphatidylglycerol). It gave a strongly positive reaction with periodate and co-chromatographed with authentic 3-*sn*-phosphatidyl-1'-glycerol (and differently from the lyso derivative of this compound) on t.l.c. in solvent 1. Degradation with phospholipase D yielded 0.95 mole of glycerol/mole of P and a lipid that behaved identically with authentic phosphatidic acid (and differently from lysophosphatidic acid) on t.l.c. in solvent 5. Alkaline hydrolysis of the phosphatidic acid obtained as described above by the method of Dawson *et al.* (1962) yielded 2.00 moles of fatty acid and 0.97 mole of *sn*-glycero-3-phosphate. Finally, degradation with phospholipase C from *B. cereus* of the phosphatidylglycerol obtained by degradation with nitrite of the parent lipid yielded a glyceromonophosphate, which was not a substrate for *sn*-glycero-3-phosphate dehydrogenase, and a diglyceride. The diglyceride behaved similarly to 1,2-dipalmitin and differently from 1,3-dipalmitin on t.l.c. in solvent 6.

Nature of the linkage between glucosamine and phosphatidylglycerol. The fact that the glycerophosphorylglyceryl glucosaminide obtained from the parent lipid by mild alkaline hydrolysis did not give the reaction for a reducing sugar indicated that

Fig. 1. Time-course of consumption of periodate by 0.02 μ mole of glycerol (○), 3'-(*O*- β -glucosaminyl)-*sn*-glycerol (●), 2'-(*O*- β -glucosaminyl)-*sn*-glycerol (○) and glucosamine (▲). The test cuvette also contained 5 μ moles of sodium acetate buffer, pH 4.5, 0.1 μ mole of freshly prepared NaIO₄ and water to 1.0 ml.; oxidizable substrate was omitted from the control cuvette. The change in E_{223} was measured at the times shown in a Cary model 14 recording spectrophotometer at 20°.

glucosamine was bound as a glycoside; and the identification of glucosaminylglycerol as a product of both mild acid hydrolysis and of degradation of the parent lipid with phospholipase D (see below) showed that glucosamine was bound to the glycerol moiety of phosphatidylglycerol.

Further examination of the glucosaminylglycerol obtained as described above showed that glucosamine was bound to the terminal carbon atom of glycerol. The time-course of the consumption of periodate by this derivative is shown in Fig. 1. Approx. 1 mole of periodate/mole of glucosaminylglycerol was rapidly consumed, whereas under the same experimental conditions glycerol rapidly consumed 2 moles of periodate/mole of glycerol and

Fig. 2. Time-course of hydrolysis of 0.5 μ mole of *p*-nitrophenyl *O*- β -*N*-acetylglucosaminide ($\blacktriangle$), phenyl *O*- α -*N*-acetylglucosaminide ($\circ$), and either 2'- or 3'-(glycerophosphorylglyceryl) *O*- β -*N*-acetylglucosaminide ($\bullet$) by β -*N*-acetylhexosaminidase. The hydrolyses were carried out at 37°, and each tube contained, in addition to substrate, 15 μ moles of sodium phosphate buffer, pH 4.2, 15 μ moles of sodium citrate buffer, pH 4.2, enzyme (0.5 mg. of protein) and water to 1 ml. Samples were removed at the times shown and *N*-acetylglucosamine was determined by the method of Frohwein & Gatt (1967).

Fig. 3. Structures of glucosaminylphosphatidylglycerols from *B. megaterium*.

glucosamine did not show a rapid phase of consumption of periodate.

The glycosidic bond was shown enzymically to have the β -configuration. The parent lipid was converted into its *N*-acetyl derivative and fatty acids were then removed by mild alkaline hydrolysis. The product is glycerophosphorylglyceryl *N*-acetylglucosaminide and, as shown in Fig. 2, *N*-acetylglucosamine was quantitatively released by β -*N*-acetylhexosaminidase prepared from calf brain (Frohwein & Gatt, 1967). Fig. 2 also shows that, although this enzyme preparation rapidly hydrolysed *p*-nitrophenyl *O*- β -*N*-acetylglucosaminide, it had no effect on phenyl *O*- α -*N*-acetylglucosaminide.

2'-(*O*- β -Glucosaminyl)phosphatidylglycerol. The composition of this lipid is given in Table 1 and its chromatographic behaviour and that of some of its derivatives is summarized in Table 2. The evidence identifying the carbohydrate moiety as D-glucosamine and the lipid moiety as 3-*sn*-phosphatidyl-1'-*sn*-glycerol is identical with that described above.

Similarly, it was shown that glucosamine was bound as a β -glycoside to the glycerol moiety of phosphatidylglycerol (Fig. 3). However, the glucosaminylglycerol derived from this lipid by partial acid hydrolysis consumed periodate even more slowly than did glucosamine (Fig. 2). This result indicates that glucosamine is attached to C-2 of glycerol and that the parent lipid is 1'-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-2'-*O*- β -(2-amino-2-deoxy-D-glucopyranosyl)-*sn*-glycerol.

Fatty acids of 3'-(O- β -glucosaminyl)phosphatidylglycerol and 2'-(O- β -glucosaminyl)phosphatidylglycerol. The fatty acids obtained from these lipids by alkaline hydrolysis under nitrogen were converted into their methyl esters and examined by g.l.c. No striking difference in fatty acid composition was observed.

Degradation of 3'-(O- β -glucosaminyl)phosphatidylglycerol and 2'-(O- β -glucosaminyl)phosphatidylglycerol with phospholipases. Both lipids were good substrates for the phospholipase A of Russell-viper venom, yielding fatty acid and the corresponding lyso compounds; the latter can readily be distinguished from each other, from their parent lipids and from the corresponding glycerophosphorylglycosaminylglycerols by t.l.c. in solvents 1 and 4 (Table 2). Neither lipid was a substrate for phospholipase C from *Cl. perfringens* under the conditions described in the Methods and Materials section. 2'-(*O*- β -Glucosaminyl)phosphatidylglycerol appeared to be resistant to the action of phospholipase D from cabbage, but 3'-(*O*- β -glucosaminyl)phosphatidylglycerol was rapidly hydrolysed, yielding phosphatidic acid and glucosaminylglycerol.

DISCUSSION

The occurrence of phosphatidylglycerol has been demonstrated in a wide variety of micro-organisms, in plants and in animal tissues (for review see Goldfine, 1968) after its discovery in *Scenedesmus* by Benson & Maruo (1958). The stereochemical configuration of the phosphatidylglycerol from spinach leaves was established by Haverkate & van Deenen (1964), who showed it to be 3-*sn*-phosphatidyl-1'-*sn*-glycerol. Debuch & Rotsch (1966) have, however, obtained evidence suggesting that spinach leaves also contain 3-*sn*-phosphatidyl-2'-*sn*-glycerol.

It has also been found that derivatives of phosphatidylglycerol occur in bacteria. The first to be described were the *O*-amino acid esters (Macfarlane, 1962), and the L-alanyl, L-ornithyl and L-lysyl derivatives have been isolated (Houtsmuller & van Deenen, 1963, 1965), and it is possible that other derivatives of this kind exist (Vorbeck & Marinetti, 1964). In many cases structural information is incomplete, but the work of Houtsmuller & van Deenen (1965), Lennarz, Bensen & van Deenen

(1967) and Molotkovsky & Bergelson (1968) has shown that the lysyl derivative is 1'-(3-*sn*-phosphatidyl)-3'-*O*-L-lysyl-*sn*-glycerol.

More recently, the occurrence of a diglucosyl derivative of phosphatidylglycerol in *Streptococcus haemolyticus* strain D58 has been reported and the lipid has been characterized as a di-*O*-acyl derivative of α -kojibiosyl-(1 $\rightarrow$ 1)-glycerylphosphoryl-1'-glycerol (Ishizuka & Yamakawa, 1968).

The presence of a glucosamine derivative of phosphatidylglycerol in *Pseudomonas ovalis* Chester was reported by Phizackerley, MacDougall & Francis (1966) and in *B. megaterium* by Op den Kamp Houtsmuller & van Deenen (1965). Op den Kamp & van Deenen (1966), van Deenen, Op den Kamp & Bonsen (1967) and Op den Kamp, Bonsen & van Deenen (1969) identified the phosphoglycolipid from *B. megaterium* as 1'-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-2'-*O*-(2-amino-2-deoxy-D-glucopyranosyl)-*sn*-glycerol and this structure has been confirmed by synthesis (Gurr, Bonsen, Op den Kamp & van Deenen, 1968), although the configuration of the glycosidic bond in the natural and the synthetic compound was not established.

The present work shows that at least three glucosamine-containing lipids are present in *B. megaterium*. One of these is not a phospholipid, but the other two are both β -glucosaminides of 3-*sn*-phosphatidyl-1'-*sn*-glycerol. They differ in that in one case the glucosamine residue is attached to the 3'-position of the glycerol moiety of phosphatidylglycerol and in the other case it is attached to the 2'-position (Fig. 3). These two lipids can be distinguished from each other in a number of ways. First, they can be separated by t.l.c., and it is noteworthy that in lipid extracts from whole cells the 2'-glucosaminide is in some solvents largely concealed by phosphatidylethanolamine (Table 2). Secondly, the glycerophosphorylglyceryl glucosaminides prepared from the parent lipids by mild alkaline hydrolysis can also be distinguished from each other chromatographically (Table 2). Thirdly, the 3'-glucosaminide is rapidly hydrolysed by phospholipase D whereas under identical conditions the 2'-glucosaminide is inert. Finally, it has been shown that the glucosaminylglycerols obtained by partial acid hydrolysis differ in that in one case, but not in the other, there is a rapid consumption of 1 mole of periodate/mole of glucosaminylglycerol (Fig. 2).

Gurr *et al.* (1968) and Op den Kamp *et al.* (1969) have measured the consumption of periodate by the chemically synthesized isomers of glucosaminylglycerol. Under their experimental conditions oxidation of the glucosamine moiety occurred. Thus glucosaminyl-1-glycerol consumed 2 mol.prop. of periodate (i.e. 1 mol.prop. was consumed by the *trans* vicinal hydroxyl groups at C-3 and C-4 of the

glucosamine residue and 1 mol.prop. was consumed by the vicinal hydroxyl groups at C-2 and C-3 of the glycerol residue), and glucosaminyl-2-glycerol consumed 1 mol.prop. of periodate (due to the glucosamine residue only). Oxidations by periodate are very sensitive to experimental conditions (Jeanloz & Forchielli, 1951) and it is also known that this reagent attacks *cis*-1,2-diols more rapidly than *trans*-1,2-diols (Bobbitt, 1956). It was found that, when using 0.1 mM-periodate at pH 4.2 and 20°, although glycerol rapidly consumed 2 mol.prop. of periodate, there was very little oxidation of glucosamine (Fig. 1). It is therefore to be expected that under these conditions 3'-(*O*- β -glucosaminyl)-*sn*-glycerol will rapidly consume 1 mol.prop. of periodate and that there will be little oxidation of 2'-(*O*- β -glucosaminyl)-*sn*-glycerol (Fig. 1).

The close similarity in structure between the two glucosaminylphosphatidylglycerols isolated from *B. megaterium* raises the question as to whether one or other of these lipids is formed by migration of the glucosamine residue on the glycerol moiety. Although it is possible that a metabolic relationship of this kind exists, it is improbable that such an isomerization occurs non-enzymically during the purification procedure. First, the two lipids are chromatographically distinguishable in the original lipid extract and no interconversion has been observed during prolonged storage at 0°. Secondly, in addition to displaying the expected stability to alkali, the glycosidic bond is unusually resistant to acid hydrolysis. As shown above, alkaline hydrolysis deacylates both lipids and yields the corresponding glycerophosphorylglyceryl glucosaminide (Table 2), i.e. the glycosidic bond remains intact. Moggridge & Neuberger (1938) showed that glucosaminides are only slowly hydrolysed by strong acid, probably because of the proximity of the amino group to the glycosidic bond, and in the present experiments it has been found that treatment with 5.8 M-hydrochloric acid at 105° for 18 hr. is required to release glucosamine.

The glucosaminylphosphatidylglycerol from *Ps. ovalis* (Phizackerley, MacDougall & Francis, 1966) is a 2'-glucosaminide of 3-*sn*-phosphatidyl-1'-*sn*-glycerol. However, it does not co-chromatograph with 2'-(*O*- β -glucosaminyl)phosphatidylglycerol prepared from *B. megaterium* and the deacylated derivatives of these two lipids can also be distinguished in this way. These results suggest that the difference between the 2'-glucosaminylphosphatidylglycerol prepared from *B. megaterium* and that prepared from *Ps. ovalis* must lie in the configuration of the glycosidic bond. In confirmation of this view it has been found that the deacylated *N*-acetyl derivative of the 2'-glucosaminylphosphatidylglycerol from *Ps. ovalis* is not a substrate for β -*N*-acetylhexosaminidase, but that instead *N*-acetyl-

glucosamine is quantitatively released by a preparation from *Patella vulgata* that contains α -*N*-acetylhexosaminidase (Conchie & Levvy, 1957). It has been concluded that the phosphoglycolipid from *Ps. ovalis* is 1'-(1,2-diacyl-*sn*-glycero-3-phosphoryl)-2'-*O*- α -(2-amino-2-deoxy-D-glucopyranosyl)-*sn*-glycerol (Phizackerley & MacDougall, 1968).

Op den Kamp, van Iterson & van Deenen (1967) showed that the amount of glucosaminylphosphatidylglycerol in *B. megaterium* strain MK10D was dependent on the pH of the growth medium. In cells grown at pH 7 the amount of glucosaminylphosphatidylglycerol was too small to measure, but in cells grown at pH 5 it constitutes 32% of the total phospholipid. However, in our experiments we have not found any obvious effects on the phospholipid composition of *B. megaterium* of varying the pH of the growth medium, and in particular the yield of glucosamine-containing lipids appears to be unaffected between pH 5 and pH 7.

Since in most of the naturally occurring compounds containing glucosamine it is present as its *N*-acetyl derivative, we incubated the deacylated derivatives of the total phospholipid extract obtained from *B. megaterium* with β -*N*-acetylhexosaminidase for 72 hr. The release of *N*-acetylglucosamine was not detected, which suggests that *N*-acetyl derivatives of the glucosaminylphosphatidylglycerols are not present in *B. megaterium*.

It is apparent from the above results that at least three isomers of glucosaminylphosphatidylglycerol occur in micro-organisms. The functional significance of these compounds remains to be established.

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