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STRUCTURAL INVESTIGATIONS ON GLUCOSAMINYL PHOSPHATIDYLGLYCEROL FROM *BACILLUS MEGATERIUM*

J. A. F. OP DEN KAMP, P. P. M. BONSEN AND L. L. M. VAN DEENEN
Laboratory of Biochemistry, The State University, Utrecht (The Netherlands)
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SUMMARY

1. Glucosaminyl phosphatidylglycerol from *Bacillus megaterium* was converted into phosphatidylglycerol and 2,5-anhydromannose. The stereochemical configuration of phosphatidylglycerol was investigated with phospholipase A, phospholipase C, and glycero-3-phosphate dehydrogenase, and was found to be 1,2-diacyl-*sn*-glycero-3-phosphoryl-1′-*sn*-glycerol.

2. Partial acid hydrolysis of the phospholipid yielded glucosaminylglycerol, which was shown to be identical with 2′-*O*-(2-amino-2-deoxy-β-glucopyranosyl)-glycerol.

3. 1′-*O*-(2-Amino-2-deoxy-β-D-glucopyranosyl)glycerol, 2′-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)glycerol and 2′-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-1′-phosphorylglycerol were synthesized and compared with glucosaminylglycerol and glucosaminyl glycerophosphate derived from the phospholipid.

4. The structure of the phospholipid was established as 1,2-diacyl-*sn*-glycero-3-phosphoryl-1′[2′-*O*-(2″-amino-2″-deoxy-D-glucopyranosyl)]-*sn*-glycerol.

INTRODUCTION

Bacillus megaterium has been shown to contain a glucosamine derivative of phosphatidylglycerol[1]. Previous studies on the structure of this compound have indicated that the glucosamine is bound to the terminal glycerol moiety of phosphatidylglycerol[1–3]. In the present work, the stereochemical configuration of phosphatidylglycerol which was isolated after removal of the amino sugar has been studied in detail. PHIZACKERLEY *et al.*[4] showed the presence of a similar phosphoglyceride in *Pseudomonas ovalis*. Applying their method of partial acid hydrolysis, we were able to isolate glucosaminylglycerol and to determine the mode of attachment of the glucosamine moiety to the phospholipid. A number of chemically well-defined glucosaminylglycerol compounds were synthesized in order to make comparisons with glucosaminylglycerol obtained from the phospholipid.

MATERIALS AND METHODS

Cultivation of *B. megaterium* MK 10D, extraction of lipids and purification of glucosaminyl phosphatidylglycerol have been carried out as described previously[1].

Phospholipase A from porcine pancreas was a gift from Dr. G. H. DE HAAS and Dr. N. M. POSTEMA from this laboratory. Phospholipase C was prepared by $(NH_4)_2SO_4$ precipitation of the culture filtrate of *B. cereus* (NCTC 6349) grown in an akaline broth. Glycero-3-phosphate dehydrogenase was obtained from Boehringer, Mannheim, Germany. Wheat germ phosphomonoesterase was purchased from Sigma, St. Louis, Mo., U.S.A. *β-N*-Acetylglucosaminidase was donated by Dr. J. H. Veerkamp, Laboratory of Biochemistry, University of Nijmegen, The Netherlands.

Synthetic compounds (Figs. 1 and 2)

2-Phthalimido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl bromide (I). This compound was prepared according to the method outlined by BERGMAN AND ZERVAS[12], ZERVAS AND KONSTAS[13] and AKIYA AND OSAWA[14,15].

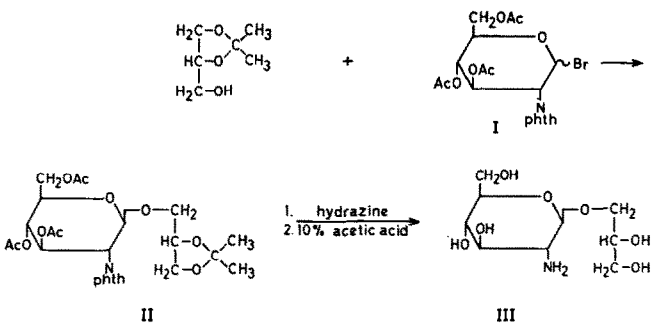

Fig. 1. Synthesis of *rac*-1′-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)glycerol. Ac, acetyl; phth, phthaloyl.

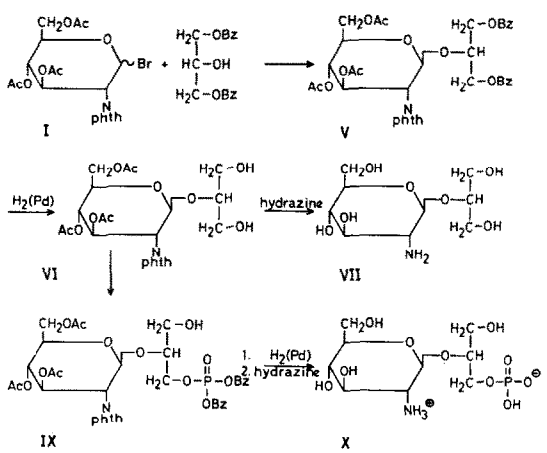

Fig. 2. Synthesis of 2′-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)glycerol and *rac*-2′-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-1′-phosphorylglycerol. Ac, acetyl; phth, phthaloyl; Bz, benzyl.

rac-1'-O-(2-Phthalimido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-2',3'-isopropylidene glycerol (II). 13.5 g of $Hg(CN)_2$, 14.0 g of the bromo sugar derivative (I) and 10 g of *rac*-isopropylidene glycerol in 200 ml of dry benzene were refluxed for 1 h. After cooling, the reaction mixture was washed with 1% NaCl solution twice and then with water. After drying over $MgSO_4$, the benzene solution was evaporated *in vacuo*. The residue was crystallized from ethanol, giving (II) as a white crystalline product in a yield of 84%, m.p. 82–92°; $[\alpha]_D^{20}+24.2°$ (*c* 3.1 in chloroform). (Found: C, 56.4; H, 5.6; N, 3.0. $C_{26}H_{31}NO_{12}$ requires: C, 56.82; H, 5.69; N, 2.55.)

rac-1'-O-(2-Amino-2-deoxy-β-D-glucopyranosyl)glycerol as hydrochloric acid salt (III). 2.2 g of the condensation product (II) were dissolved in 10 ml of dioxan. 1 ml of hydrazine hydrate (98%) was added and the mixture was shaken for 24 h at room temperature. After dilution with 20 ml of water, acetic acid was added until a pH of 4 was reached. Much of the phthaloylhydrazide precipitated after some time, and was removed by filtration. The filtrate was taken to dryness, and the residue dissolved in 50 ml of 10% acetic acid in water and heated on a water bath for 1 h to remove the isopropylidene group. The solvent was removed by evaporation, and the residual syrup dissolved in water and treated with Dowex 2 (carbonate form) in order to remove anions. The mixture was chromatographed on a column with Amberlite IRC-50 (H^+). Elution was performed with water and 0.001 M acetic acid. The combined pure fractions were again treated with Dowex 2 (carbonate form) and evaporated *in vacuo*. The solid residue was extracted with hot ethanol. Some insoluble material was removed by filtration. 1 ml of 5% HCl in abs. ethanol was added to the hot ethanolic solution. Crystallization of the hydrochloric acid salt was effected by the addition of some ether, giving III as a very hygroscopic white solid in a yield of about 50%. This purification was mainly done according to the procedure of HARDY[16]. There was no real m.p.: the compound softened at 25°–30°; $[\alpha]_D^{20}$-20.5° (*c* 1.1 in H_2O). (Found: C, 34.9; H, 7.3. $C_9H_{20}ClNO_7 \cdot H_2O$ requires: C, 35.12; H, 7.21.)

rac-1'-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)glycerol (IV). 130 mg of III were dissolved in water, 260 mg of acetic anhydride were added, after which the solution was adjusted to pH 9 with triethylamine. After 4 h at room temperature, the solution was acidified with acetic acid and evaporated *in vacuo*. This material was dissolved in water and percolated over a column with Amberlite IR-120 (H^+) to remove all cations. The eluent was taken to dryness, and the residue was crystallized from ethanol–ether, giving a white, glassy material in a yield of 70%. M.p.: the compound softened at 30°; $[\alpha]_D^{20}-24.5°$ (*c* 1 in H_2O). (Found: C, 44.2; H, 6.8. $C_{11}H_{21}NO_8$ requires: C, 44.74; H, 7.16.)

2'-O-(2-Phthalimido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-1',3'-di-O-benzyl glycerol (V). 8.5 g of the bromo sugar (I) were mixed with 8.5 g of $Hg(CN)_2$ and 5 g of 1,3-di-*O*-benzylglycerol[18] in 50 ml of benzene and refluxed for 30 min. The working up procedure was carried out as for II. The residue was chromatographed over silica with benzene–ether mixtures as eluents, giving V as a colourless oil in a yield of 82%. $[\alpha]_D^{20}+25.8$ (*c* 2 in chloroform). (Found: C, 64.4; H, 5.8; N, 2.2. $C_{37}H_{39}NO_{12}$ requires: C, 64.43; H, 5.69; N, 2.03.)

2'-O-(2-Phthalimido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)glycerol (VI). Hydrogenolysis of V was effected in ethanol with 5% palladium on charcoal as a catalyst. Some impurities were removed by chromatography on silica with chloroform–methanol (98:2, by vol.) as eluent, giving VI as a glassy material after drying

in high vacuum over P_2O_5 in a yield of 90%. M.p., 64–67°; $[\alpha]_D^{20}+25.4$ (*c* 3 in chloroform). (Found: C, 53.5; H, 5.4; N, 2.9. $C_{23}H_{27}NO_{12}$ requires: C, 54.22; H, 5.34; N, 2.75.)

2'-O-(2-Amino-2-deoxy-β-D-glucopyranosyl)glycerol as hydrochloric acid salt (VII). The blocking groups of VI were removed by a treatment with hydrazine hydrate (5 moles) in dioxan. Purification was carried out as described for III. M.p., 212–214° (decomp.); $[\alpha]_D^{20}-19.7°$ (*c* 1.1 in H_2O); reported[16] m.p., 214°; $[\alpha]_D^{20}-20.0°$ (*c* 1.4 in H_2O). (Found: C, 37.4; H, 7.10; N, 4.7; Cl 12.5. $C_9H_{20}ClNO_7$ requires: C, 37.31; H, 6.95; N, 4.83; Cl, 12.24.)

2'-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)glycerol (VIII). This material was prepared as described previously for the isomer (IV). Yield, 90%; glassy material which softens at 25°; $[\alpha]_D^{20}-14.4°$ (*c* 2.1 in H_2O). (Found: C, 44.3; H, 6.9; N, 4.2. $C_{11}H_{21}NO_8$ requires: C, 44.74; H, 7.16; N, 4.74.)

rac-2'-O-(2-Phthalimido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucopyranosyl)-1'-(dibenzyl)phosphorylglycerol (IX). 2.28 g of VI were phosphorylated with 2.5 g of dibenzylphosphoric acid as described by THEODOROPOULOS AND SOUCHLERIS[17]. The precipitate of the dicyclohexylurea was removed by filtration and the filtrate evaporated *in vacuo*. The residue was dissolved in benzene and washed with 0.25 M H_2SO_4, with a 5% $NaHCO_3$ solution and water. After drying over Na_2SO_4, the benzene solution was taken to dryness. The residual oil was purified by chromatography on silica using 30% ether in benzene as eluent. Traces of dicyclohexylurea were still present. The yield was 38%. The product was obtained as a colourless syrup. $[\alpha]_D^{20}+6.4°$ (*c* 6 in chloroform). (Found: C, 58.0; H, 5.1. $C_{37}H_{40}NO_{15}P$ requires: C, 57.73; H, 5.24.)

rac-2'-O-(2-Amino-2-deoxy-β-D-glucopyranosyl)-1'-phosphorylglycerol (X). 470 mg of IX were treated with 0.4 ml of hydrazine hydrate in 10 ml of dioxan and shaken for 2 days at room temperature. After adding water and acidifying, the solvent was removed by evaporation *in vacuo*. The residue was subjected to catalytic hydrogenolysis in acetic acid with 5% palladium on charcoal as a catalyst. The catalyst was removed by filtration and the filtrate evaporated *in vacuo*. X was crystallized once from water–acetone. Purification on a small scale was done by means of high voltage electrophoresis. (Found: P/N = 1.1. $C_9H_{20}NO_{10}P$ requires: P/N = 1.0.)

RESULTS AND DISCUSSION

The stereochemical configuration of the phosphatidylglycerol moiety

In an earlier report, it was described how alkaline hydrolysis of the glucosaminyl phosphatidylglycerol removed not only the fatty acids, but also the glucosamine moiety giving rise to the formation of glyceryl phosphorylglycerol[21]. However, refining of the chromatographic procedures which were used to investigate the water-soluble products obtained after alkaline hydrolysis of phosphoglycerides, prompted us to reinvestigate the hydrolysis of glucosaminyl phosphatidylglycerol. ^{32}P-labelled glucosaminyl phosphatidylglycerol was isolated from *B. megaterium* as described previously[1] and hydrolysed by following the procedure of BENSON AND MARUO[5].

The water-soluble products were investigated by means of a two-dimensional combination of high-voltage electrophoresis and paper chromatography. High-voltage electrophoresis on paper was carried out at 50 V/cm for 30 min in pyridine–acetic acid–water (1:10:89, by vol.; pH 3.6). After drying at room temperature, the papers were developed in the second direction in propanol–conc. ammonia–water (6:3:1, by

vol.). As a result of this procedure, the deacylated ^{32}P-labelled breakdown product of the glucosamine-containing phospholipid was clearly separated from glyceryl phosphorylglycerol, whereas in the two-dimensional paper-chromatographic system applied formerly[2], both compounds appeared to have the same mobility. The staining properties of the compound under investigation indicated that the glucosamine was not liberated by the alkaline hydrolysis. For this reason, glucosaminyl phosphatidylglycerol was converted into phosphatidylglycerol by hydrolysis with nitrous acid—a reagent which is known to hydrolyse glycosidic bonds[6]. 7.5 mg of phospholipid were solubilized with 1 ml of methanol to which 1 ml of water, containing 50 mg $NaNO_2$, was added. The pH was adjusted to 4.0 with the aid of HCl, and the mixture was heated to 50° for 3 h. The water-soluble product was investigated by paper chromatography in butane-1-ol–pyridine–water (6:4:3, by vol.) and appeared to be identical to 2,5-anhydromannose prepared from D-glucosamine by deamination as described above. The remaining lipid was extracted with chloroform and identified as phosphatidylglycerol by chromatographic comparison with known phospholipids on silica-gel-impregnated paper in diisobutyl ketone–acetic acid–water (40:25:5, by vol.). Its identity as phosphatidylglycerol was further confirmed by the results of the following experiments which were carried out in order to determine the stereochemical configuration of both glycerol moieties of the phosphatidylglycerol (Fig. 3).

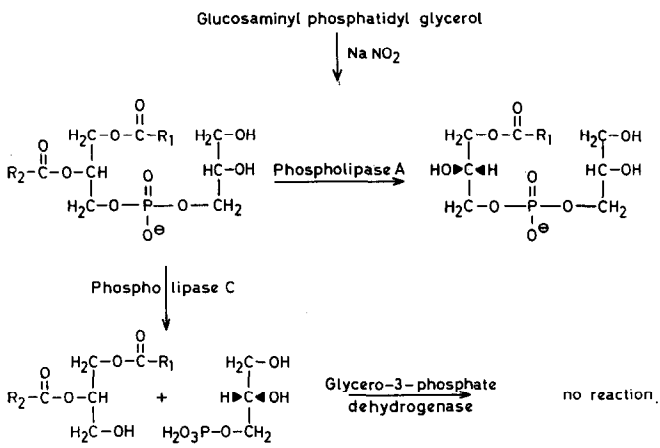

Fig. 3. Determination of the stereochemical configuration of phosphatidylglycerol.

The configuration of the fatty-acyl-containing glycerol moiety of phosphatidylglycerol, isolated after deamination of its glucosamine derivative, was determined by incubation with porcine pancreatic phospholipase A (EC 3.1.1.4), which is known to be stereospecific in such a way that hydrolysis occurs only in the case of a 3-*sn*-phosphoglyceride[7]. The phosphatidylglycerol was investigated in this way, and as shown by a complete conversion into the monoacyl phosphatidylglycerol derivative and free fatty acids, appeared to be a 3-*sn*-phosphoglyceride. On the other hand, the terminal glycerol moiety appeared to be esterified to the phosphate in the opposite stereochemical configuration. Hydrolysis of phosphatidylglycerol with phospholipase C from *B. cereus* (EC 3.1.4.3) resulted in the formation of diglyceride and glycerophosphate. The latter compound was found to be resistant to dehydrogenation with glycero-3-phosphate dehydrogenase, whereas in a control experiment, glycero-3-phos-

phate was completely converted. Furthermore, the presence of a glycero-2-phosphate has to be excluded, since the isolated glycerophosphate as well as the phosphatidylglycerol exhibited a strong colour development with the periodate Schiff reagent. Thus, the phosphatidylglycerol isolated after nitrous acid degradation of glucosaminyl phosphatidylglycerol appeared to have the following structure: 1,2-diacyl-*sn*-glycerol-3-phosphoryl-1'-*sn*-glycerol.

Attachment of the glucosamine moiety

The intact glucosaminyl phosphatidylglycerol showed only a faint colour development with the periodate Schiff reagent, an observation which indicated that the vicinal hydroxyl groups of the phosphatidylglycerol were not accessible for periodate oxidation. Therefore, the glucosamine was thought to be linked to one of these hydroxyl groups of the phosphatidylglycerol. Conclusive evidence for the glucosamine–glycerol linkage was obtained by isolation of glucosaminylglycerol after partial acid hydrolysis of the phospholipid according to PHIZACKERLEY *et al.*[4].

Hydrolysis of glucosaminyl phosphatidylglycerol in 1 M HCl at 100° for 4 h was followed by preparative high-voltage electrophoresis at 50 V/cm for 30 min in pyridine–acetic acid–water (1:10:89, by vol.; pH 3.6). The only aminonitrogen-containing compound was eluted from the paper and hydrolysed in 6 M HCl at 100° for 4 h. The products formed by the latter hydrolysis procedure appeared to be chromatographically indistinguishable from glucosamine and glycerol (Fig. 4). Quantitative determination of the amounts of glucosamine[8] and glycerol[9] revealed a ratio of 1.0:1.1, which was in accordance with the observations of PHIZACKERLEY *et al.*[4]. Further study of the glucosaminylglycerol obtained by partial acid hydrolysis revealed that this compound showed only a faint colour development with periodate-Schiff reagent and appeared to be devoid of reducing activity, as could be shown by the

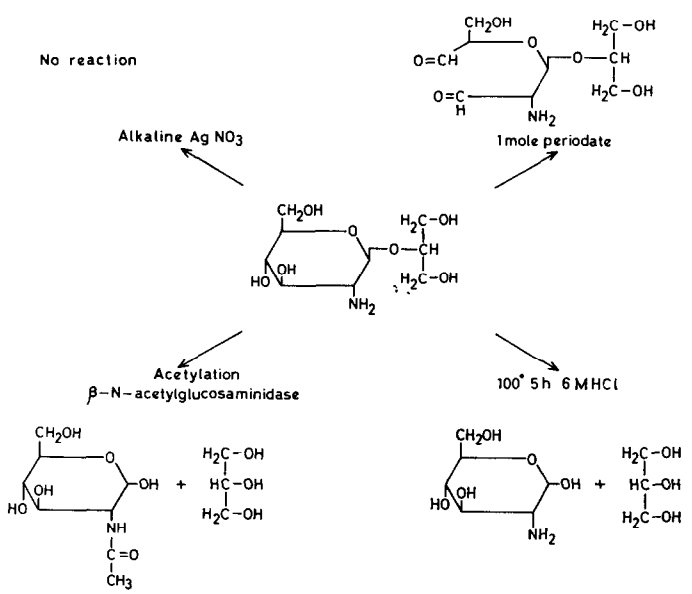

Fig. 4. Elucidation of the structure of glucosaminylglycerol.

alkaline $AgNO_3$ reaction[10]. Both observations suggest that a glycosidic linkage connects the C-1 atom of the glucosamine with the 2-OH group of the glycerol. In order to confirm the latter structure of the glucosaminylglycerol obtained from the bacterial phospholipid, a comparison was made with synthetic glucosaminyl-1-glycerol and glucosaminyl-2-glycerol. Paper chromatography in butane-1-ol–ethanol–water–ammonia (40:10:49:1, by vol.) and the different staining reactions mentioned above indicated that the natural compound was identical to the synthetic glucosaminyl-2-glycerol (Fig. 4). Measurement of the periodate consumption of the natural and synthetic compounds confirmed these preliminary observations. Per mole of glucosaminyl-2-glycerol and per mole of the natural glucosaminyl glycerol, one mole of sodium periodate was consumed, whereas the glucosaminyl-1-glycerol consumed twice as much periodate. Since the glucosamine is glycosidically linked to the glycerol and the aminonitrogen is not attacked under the conditions used, the trans vicinal hydroxyl groups at the 3 and 4 positions of the glucosamine have to be free and responsible for the periodate consumption. The results rule out the possibility that the glucosamine is linked to the 1 or 3 positions of the glycerol, for in the latter case a second set of vicinal hydroxyl groups would be available for the periodate consumption. We have to conclude, therefore, that the glucosaminylglycerol has the following structure: 2′-*O*-(2-amino-2-deoxy-D-glucopyranosyl)glycerol.

The presence of the latter compound in the intact phospholipid can be shown by hydrolysis of the phospholipid with phospholipase C followed by phosphomonoesterase degradation (Fig. 5). Glucosaminyl phosphatidylglycerol was incubated with

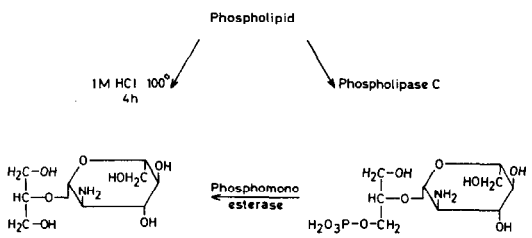

Fig. 5. Formation of glucosaminylglycerol.

phospholipase C (EC 3.1.4.3) from *B. cereus* in a mixture of equal volumes of ether and buffer at pH 6.6. The 1,2-diacylglyceride was identified by silica-gel thin-layer chromatography (ether–hexane, 1:1, by vol.). Furthermore, a water-soluble phosphorus and aminonitrogen-containing compound was formed which appeared to have identical chromatographic properties to those of the glucosaminyl phosphorylglycerol synthesized chemically. Incubation of the natural and synthetic products with phosphomonoesterase from wheat germ (EC 3.1.3.1) resulted in the formation of inorganic phosphate and glucosaminylglycerol. When 2-glucosaminyl-1-phosphorylglycerol was investigated in this way, the resulting glucosaminyl-2-glycerol appeared to be indistinguishable from the natural glucosaminylglycerol.

Finally, the nature of the glycosidic bond was investigated. In view of this, both the natural and the synthetic glucosaminyl-2-glycerol were converted into the *N*-acetyl derivatives with a fivefold excess of acetic acid anhydride at pH 9.0. After purification, the *N*-acetylglucosaminylglycerol was incubated with a specific β-*N*-acetylglucosaminidase (EC 3.2.1.30) in 0.06 M phosphate buffer, pH 7.2 at 37° (Fig. 4). Both the natural and synthetic *N*-acetylglucosaminyl-2-glycerol showed a maximal

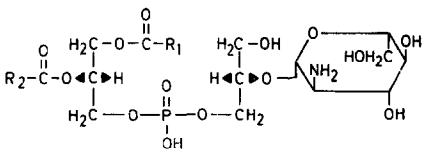

Fig. 6. Structure of glucosaminyl phosphatidylglycerol.

breakdown of about 50%. The entire degradation of the synthetic compound could be expected, however, because synthesized glucosaminyl-2-glycerol is supposed to have the β-configuration. Therefore, it cannot be concluded that in the natural glucosaminyl-2-glycerol, the α-configuration is also present. Since, furthermore, a specific α-glucosaminidase was not available, we were not able to determine the nature of the glycosidic bond conclusively. With the exception of the nature of the glycosidic linkage, the structure of this phospholipid appears to be fully established and to be identical to 1,2-diacyl-*sn*-glycero-3-phosphoryl-1′[2′-*O*-(2″-amino-2″-deoxy-D-glucopyranosyl)]-*sn*-glycerol (Fig. 6). Confirmation of this was recently obtained by chemical synthesis of this phospholipid[11]. The synthetic compound was subjected to the same degradation procedure as described in the present paper, giving identical results. However, one of us (P.P.M.B.), in collaboration with Dr. A. Kornberg (Stanford University Medical School, Stanford, Calif., U.S.A.), detected a glucosamine-containing phospholipid with a structure different from that described in the present paper. The new compound, isolated from *Bacillus megaterium*, strain QM B1551, was established to be an isomer having D-glucosamine in a glycosidic linkage at the 3′-position of phosphatidylglycerol (to be published).

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