BIOORGANIC CHEMISTRY 1, 174–187 (1971)

Two Isomers of Glucosaminylphosphatidylglycerol. Their Occurrence in *Bacillus megaterium*, Structural Analysis, and Chemical Synthesis

J. A. F. OP DEN KAMP, H. M. VERHEIJ, AND L. L. M. VAN DEENEN

Laboratory of Biochemistry, State University of Utrecht, Vondellaan 26, Utrecht, The Netherlands

Received April 2, 1971

When *Bacillus megaterium* is harvested in the stationary phase of growth from a medium in which the pH was lowered gradually during growth from 7.2 to 5.0, two isomers of glucosaminylphosphatidylglycerol are present. Both isomers were isolated and characterized using chemical and enzymatic degradation procedures. Selective removal of the glucosamine moieties yielded phosphatidylglycerol which was shown to have the stereochemical configuration 3-*sn*-phosphatidyl-1′-*sn*-glycerol. Partial acid hydrolysis of the 2′ and 3′ isomers of glucosaminylphosphatidylglycerol gave glucosaminyl-2-glycerol and glucosaminyl-3-glycerol, respectively. Identification of these breakdown products was facilitated by comparison with the two synthetic isomers.

Chemical synthesis of the two glucosaminylphosphatidylglycerol isomers was carried out and the properties of the synthetic and the natural phospholipids were compared. In all the chemical and enzymatic degradation studies, the corresponding natural and synthetic compounds appeared to be fully identical and the structures of the two phospholipids were established as 3-*sn*-phosphatidyl-1′-[3′-(2-amino-2-deoxy-β-D-glucopyranosyl)]-*sn*-glycerol and 3-*sn*-phosphatidyl-1′-[2′-(2-amino-2-deoxy-β-D-glucopyranosyl)]-*sn*-glycerol.

Whereas both glucosaminylphosphatidylglycerol isomers are good substrates for phospholipase A, phospholipases C and D are hydrolyzing the 3′ isomer slowly, and the 2′ isomer is hardly degraded by the enzymes. This difference between the two isomers in susceptibility towards phospholipases C and D is correlated with differences in area per molecule as measured in monolayer experiments. Glucosaminyl-2′-phosphatidylglycerol occupied 82 Å^2 per molecule at pH 9 and a pressure of 20 dynes/cm. The 3′ isomer occupied 77 Å^2 per molecule under the same conditions. These data indicate the presence of a large polar headgroup which might interfere with the enzyme–substrate interaction.

Glucosaminyl-3′-phosphatidylglycerol accounts for about 12% of the total phospholipids extracted, whereas the amount of the corresponding 2′ isomer is 2 to 3 times higher. The accumulation of the latter compound is favored by a low environmental pH. After cultivation of the cells at neutral pH, this phospholipid could not be detected, whereas the amount of glucosaminyl-3′-phosphatidylglycerol appears to be rather constant.

INTRODUCTION

The aminoacyl derivatives of phosphatidylglycerol have been detected as important constituents of the phospholipids of several gram-positive bacteria. The structures of these compounds as well as their biosynthesis has been elucidated, whereas their occurrence and metabolism in a number of organisms has been the subject of many studies (for a review, see Ref. (*1*)).

Another type of phosphatidylglycerol derivative, containing glucosamine, has been discovered in *Bacillus megaterium* (*2*). Initial studies on the structure of this compound demonstrated that glucosamine was linked glycosidically to the 2′ hydroxyl group of the nonacylated glycerol moiety (*3*, *4*). Phizackerley et al. (*5*) detected a similar glucosaminylphosphatidylglycerol in *Pseudomonas ovalis*, whereas Bertsch et al. (*6*) found the 3′ isomer of this phospholipid in another strain of *B. megaterium*.

Both isomers appeared to be present in a *B. megaterium* strain investigated by MacDougall and Phizackerley (*7*), and in the strains of this organism which were used by Bertsch et al. (*6*) and in this laboratory (*2*).

This contribution, which is based on some of our earlier results and on more recently obtained data, gives a complete structural analysis of both glucosaminylphosphatidylglycerol isomers, isolated from *B. megaterium* MK 10 D. Emphasis is laid thereby on the importance of synthetic reference compounds (*8*, *9*) in the elucidation of the structure of these lipids, and on variations in ratios of both isomeric compounds in dependency on growth conditions.

EXPERIMENTAL

Cultivation of Organisms

Bacillus megaterium MK 10 D was cultivated at 37°C under continuous aeration in one of the following media: medium A contained 1% of pepton (Difco), 1% of yeast extract (Difco), 0.5% of NaCl, and 0.04% of Na_2HPO_4 (pH 7.2); medium B contained in addition to these components 2% of glucose and 0.2% of $(NH_4)_2SO_4$. Phospholipids were labelled by addition of 300 μCi of ^{32}P-orthophosphate/liter of medium. Cells were harvested in the stationary phase of growth by centrifugation and washed with distilled water which was acidified with HCl to pH 3. The procedure of Bligh and Dyer (*10*) was used to extract the lipids. During these treatments the pH was maintained at pH 3.0.

Chromatography

Phospholipids were separated by one of the following techniques:

(i) Silica gel-impregnated paper chromatography by use of the solvent system of Marinetti et al. (*11*): diisobutylketone–acetic acid–water (40:25:5, v/v) which will be referred to as solvent 1.

(ii) One-dimensional thin-layer chromatography on silica gel covered plates in chloroform–methanol–water (65:25:4, v/v), solvent 2, and chloroform–methanol–concentrated ammonia (35:10:1, v/v), solvent 3.

(iii) Two-dimensional thin-layer chromatography on silica gel with solvent 4, chloroform–methanol–concentrated ammonia–water (65:35:4:1, v/v) in the first direction and solvent 5, chloroform–methanol–acetic acid–water (125:37:10:1, v/v) in the second direction.

Column chromatography of the lipids was performed on Malinkrodt silicic acid (60–140 mesh) activated at 110°C overnight. Phospholipids were eluted with increasing amounts of methanol in chloroform.

Water-soluble degradation products were investigated by paper chromatography in propanol–concentrated ammonia–water (6:3:1, v/v) solvent 6 and the upper phase of *n*-butanol–ethanol–water–concentrated ammonia (40:10:49:1, v/v), solvent 7 and by paper electrophoresis at 50 V/cm for 30 min in pyridine–acetic acid–water (1:10:89, v/v, pH 3.6), solvent 8.

Identification methods were the ninhydrin reagent for free amino groups; molybdate reagent for phosphate (*12*); periodate–Schiff reagent for vicinal hydroxyl groups; alkaline silver nitrate reagent for reducing sugars; the tricomplex staining method (*13*) for paper chromatograms of the lipids and charring with 30% (v/v) sulfuric acid on thin-layer plates.

Nonenzymatic Degradation

Acid hydrolysis was carried out in 2 *N* HCl at 100°C for 4 hr or in 6 *N* HCl at 100°C for 6 hr. Alkaline hydrolysis was according to Benson and Maruo (*14*). $NaNO_2$ degradation of the glucosamine containing phospholipids was performed by heating the lipid in 1 ml of methanol–water (1:1, v/v) containing 50 mg of $NaNO_2$ (pH 4.0) at 50°C for 3 hr.

Enzymatic Hydrolysis

Degradation of the phospholipids with pure porcine pancreatic phospholipase A which was a gift from Dr. G. H. de Haas from this laboratory (*15*), was performed in 0.1 *M* Tris buffer pH 7.1 containing $5 \cdot 10^{-3}$ *M* $CaCl_2$. Hydrolysis with pure phospholipase C from *B. cereus* (*16*) (a gift from Dr. R. F. A. Zwaal and Dr. B. Roelofsen from this laboratory) was carried out in 0.2 *M* Tris buffer pH 7.5. Phospholipase D was prepared from savoy cabbage and incubated with the lipid in 0.1 *M* acetate buffer, pH 5.6 containing 10^{-2} *M* calcium acetate. β-*N*-acetylhexosaminidase was isolated from beef liver according to Weismann (*17*). The glucosaminylphosphatidylglycerol isomers were *N*-acetylated (*7*) and deacylated (*14*) prior to the incubation with β-*N*-acetylglucosaminidase. Glycero-3-phosphatedehydrogenase was obtained from Boehringer.

Chemical Synthesis

Glucosaminyl-2-glycerol and glucoaminyl-3-glycerol were synthesized as described earlier (*4*). A detailed description of the chemical synthesis of the two glucosaminylphosphatidylglycerol isomers has been recently published (*9*).

RESULTS AND DISCUSSION

The Occurrence of the 2′ and 3′ Isomers of Glucosaminylphosphatidylglycerol

Growth conditions have been shown to influence the phospholipid composition of several bacteria considerably. In a previous report (*3*) we described that glucosaminyl-2′-phosphatidylglycerol could be detected in *B. megaterium* MK 10 D only when this organism was harvested from a medium of pH 5.0. This low pH could be obtained either by growing the cells in a nonbuffered medium plus glucose or by titrating the growing cells with lactic acid or HCl in such a way that a similar drop in pH during growth was obtained as that observed during glucose fermentation (Fig. 1).

A similar pH-dependent accumulation of glucosaminyl-2′-phosphatidylglycerol was observed in *B. megaterium* QM B1551 grown in medium B and grown in the medium used by Bertsch et al. (*6*) supplemented with 2% glucose. Because these investigations used 0.1% glucose without noticing any accumulation of the phospholipid, we investigated the relationship between the added amount of glucose and the drop in pH during growth. Accumulation of glucosaminyl-2′-phosphatidylglycerol was observed only at a pH value lower than 6.0 which could be obtained by the addition of at least 0.2% of glucose. Addition of more glucose resulted in a lower pH at harvesting and an increase in the total amount of glucosaminyl-2′-phosphatidylglycerol present.

Contradictory to these results are the observations of MacDougall and Phizackerley (*7*). The *B. megaterium* strain investigated by these authors appeared to contain both glucosaminylphosphatidylglycerol isomers independent of the pH of the growth medium.

The other phospholipids present in *B. megaterium* MK 10 D were characterized as cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, and lysylphosphatidylglycerol. From these phospholipids only the amount of phosphatidylglycerol appeared

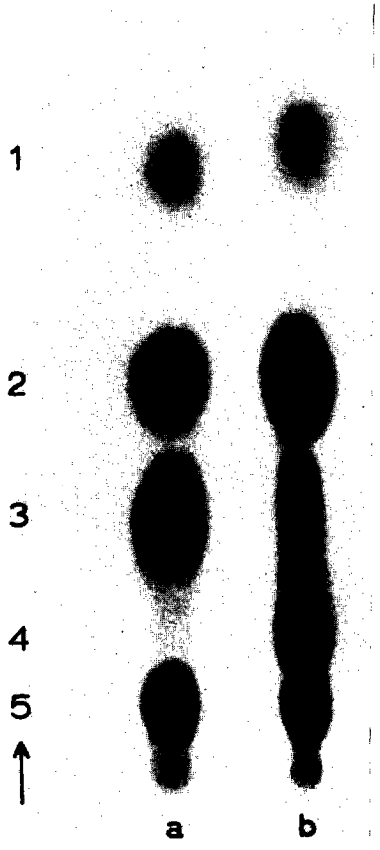

FIG. 1. Autoradiograms of the phospholipids of *B. megaterium* MK 10 D. Paper chromatograms were developed on silica-impregnated paper in solvent 1. Phospholipids of cells grown in medium A at pH 7.2 for 18 hr, a. Phospholipids of cells grown in medium B at pH 5.0 for 18 hr, b. The compounds are: cardiolipin, 1; phosphatidylethanolamine, 2; phosphatidylglycerol, 3; glucosaminyl-2′-phosphatidylglycerol, 4; a mixture of lysyl phosphatidylglycerol and glucosaminyl-3′-phosphatidylglycerol, 5.

to be influenced by the environmental pH, but the opposite way from that observed for the amount of glucosaminyl-2′-phosphatidylglycerol. A decrease in the pH of the medium induced an accumulation of glucosaminyl-2′-phosphatidylglycerol, but a decrease in the absolute amount of phosphatidylglycerol (*3*). The amount of the other phospholipids remained constant under the growth conditions which were tested. Even the amount of lysylphosphatidylglycerol, which in other gram-positive bacteria depended strongly on the environmental pH, did not increase or decrease under these growth conditions tested.

It must be emphasized, however, that contradictory to our earlier report (*3*), lysylphosphatidylglycerol is only a minor component of the phospholipids of *B. megaterium*. Stimulated by the observations of Bertsch et al. (*6*) and of MacDougall and Phizackerley (*7*) which showed the presence of glucosaminyl-3′-phosphatidylglycerol in *B. megaterium*, we reinvestigated the "lysylphosphatidylglycerol" (component 5, Fig. 1) more carefully. A two-dimensional chromatographic system on silica gel thin-layers was developed and it could be demonstrated that this spot having the lowest R_f value on the paper chromatogram (Fig. 1) consisted of two phospholipids (Fig. 2). We were able to detect only a small amount of a phospholipid containing lysine as shown after

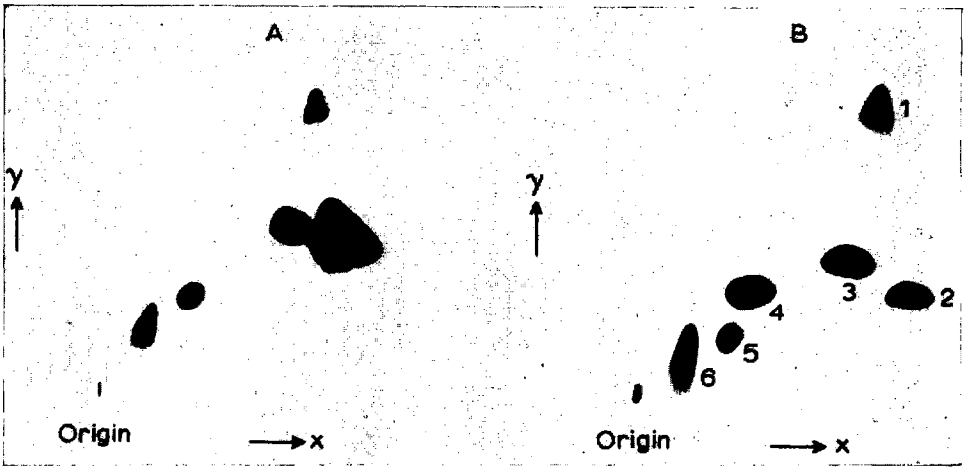

FIG. 2. Autoradiograms of the phospholipids of *B. megaterium* MK 10 D. Silica gel covered plates were developed first in the *x*-direction in solvent 4 and, after drying overnight at room temperature, in the *y*-direction in solvent 5. A shows the phospholipids from cells grown in medium A at pH 7.2 for 18 hr and B shows the phospholipids from cells grown in medium B at pH 5.2 for 18 hr. Compounds are: cardiolipin, 1; phosphatidylglycerol, 2; phosphatidylethanolamine, 3; glucosaminyl-2′-phosphatidylglycerol, 4; glucosaminyl-3′-phosphatidylglycerol, 5; and lysylphosphatidylglycerol, 6.

acid hydrolysis and coinciding in this chromatographic system with lysylphosphatidylglycerol from *Staphylococcus aureus*. The main component appeared to be glucosaminyl-3′-phosphatidylglycerol, evidence of which will be given in the following section. Preliminary observations on the occurrence of glucosaminyl-3′-phosphatidylglycerol indicate that this compound is present in *B. megaterium* in a rather constant amount regardless of the environmental pH; this is in contrast to the presence of the 2′ isomer of glucosaminylphosphatidylglycerol (Fig. 2).

Structure of Glucosaminyl-2′-phosphatidylglycerol

Compound 4 (Fig. 2) was isolated and purified by column chromatography of the total lipid extract of *B. megaterium* followed by preparative thin-layer chromatography as described earlier in detail (*2*, *3*). The phospholipid contains 0.9 moles of glucosamine and 2.0 moles of glycerol per mole of phosphorus, whereas $NaNO_2$ degradation yields 2,5 anhydromannose and phosphatidylglycerol. These data and the observations that the phospholipid does not contain a reducing group and gives only a faint color development with the periodate–Schiff reagent indicate that the glucosamine is linked

glycosidically to phosphatidylglycerol. The elucidation of the stereochemical configuration of the latter phospholipid is shown in Fig. 3. The hydrolysis of phosphatidylglycerol, which was obtained after removal of the glucosamine with $NaNO_2$, with porcine pancreatic phospholipase A demonstrates the presence of a 3-*sn*-phosphoglyceride structure. Phospholipase C degradation followed by treatment of the formed glycerophosphate with glycero-3-phosphate dehydrogenase, which did not degrade the glycerophosphate, demonstrated that the nonacylated glycerol is bound to the

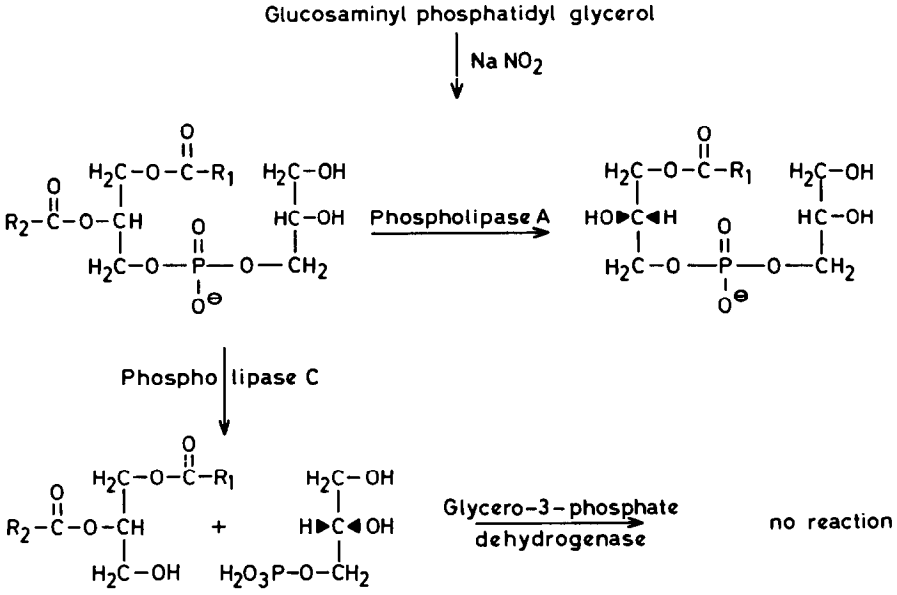

FIG. 3. Determination of the stereochemical configuration of phosphatidylglycerol.

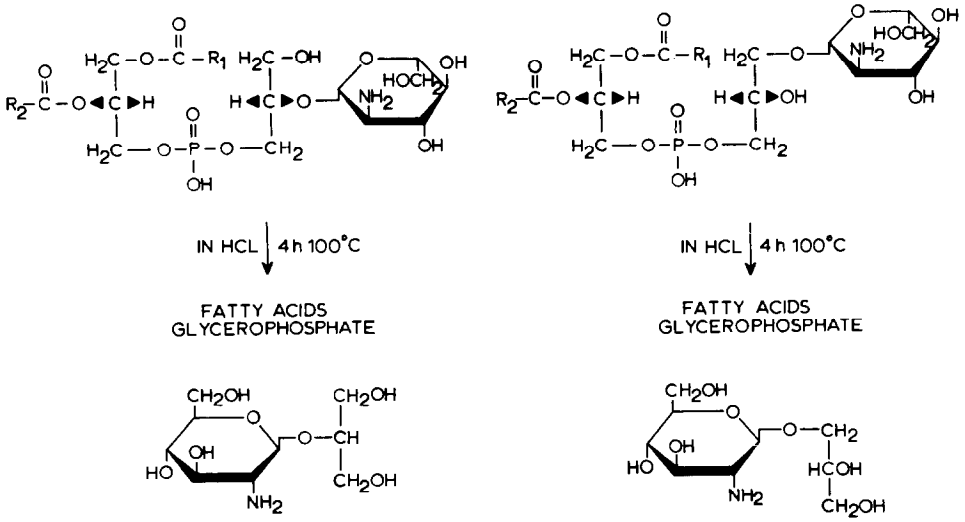

FIG. 4. Structure of the glucosaminylphosphatidylglycerol and glucosaminylglycerol isomers.

phosphate at the 1- or 2-position. The presence of glycero-2-phosphate seems unlikely because the isolated glycerophosphate as well as the phosphatidylglycerol exhibit a strong color development with the periodate–Schiff reagent.

The nature of the linkage between glucosamine and the nonacylated glycerol was studied after isolation of glucosaminylglyercol by acid hydrolysis of the phospholipid in 2 *N* HCl (Fig. 4). The absence of reducing activity and the consumption of 1 mole of periodate per mole of glucosaminylglycerol demonstrate that the isolated product

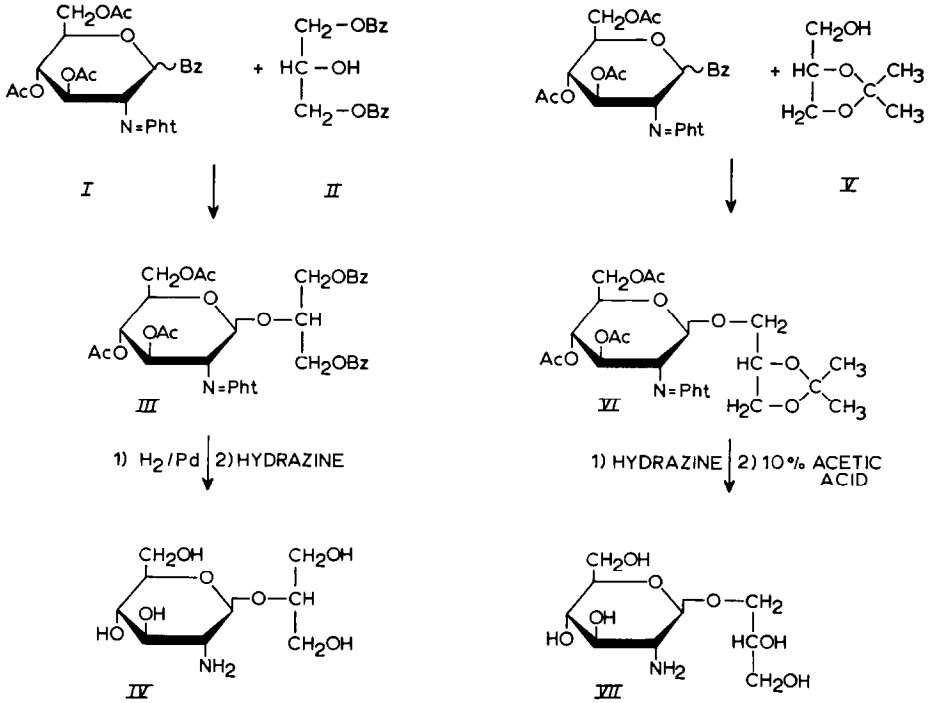

FIG. 5. Synthesis of glucosaminyl-2-glycerol (IV) and glucosaminyl-3-glycerol (VII). The protecting groups on the glucosamine and glycerol moieties are abbreviated as: Bz = benzyl; Pht = phtaloyl; and Ac = acetyl.

has the following structure: 2′-*O*-(2-amino-2-deoxy-D-glucopyranosyl)glycerol. At this stage it appeared to be necessary to have well defined synthetic compounds in order to compare these with the natural products. Glucosaminyl-2-glycerol and glucosaminyl-3-glycerol were synthesized, therefore, as shown in Fig. 5. Details of the synthesis are described elsewhere (*4*). Comparison of the chromatographic behavior, staining characteristics, and periodate consumption of the natural glucosaminylglycerol and the synthetic compounds confirmed that the natural product was identical to glucosaminyl-2-glycerol.

Based on these results, the structure of the phospholipid is identical to 3-*sn*-phosphatidyl-1′-[2′-(2-amino-2-deoxy-D-glucopyranosyl)]-*sn*-glycerol.

Structure of Glucosaminyl-3′-phosphatidylglycerol

Bacillus megaterium QM B1551, investigated by Bertsch et al. (*6*) and a strain of this organism investigated by MacDougall and Phizackerley (*7*) appeared to contain another glucosamine containing derivative of phosphatidylglycerol. Similar experiments

as described here for the elucidation of the structure of glucosaminyl-2′-phosphatidylglycerol were carried out by these investigators and the structure of the phospholipid was established as: 3-*sn*-phosphatidyl-1′-[-3′-(2-amino-2-deoxy-D-glucopyranosyl)]-*sn*-glycerol.

As described in the first section, this phospholipid was isolated also from our strain of *B. megaterium*. It could be purified by silicic acid column chromatography followed by preparative thin-layer chromatography. Glucosaminyl-3′-phosphatidylglycerol was eluted from the silicic acid column with 14% methanol in chloroform together with the remaining phosphatidylethanolamine and glucosaminyl-2′-phosphatidylglycerol. Separation of the three phospholipids was obtained by preparative thin-layer chromatography in solvent 2 which yielded pure glucosaminyl-3′-phosphatidylglycerol and a mixture of the 2′ isomer with phosphatidylethanolamine; the latter phospholipids were purified by preparative thin-layer chromatography in solvent 3. Glucosaminyl-3′-phosphatidylglycerol was compared with glucosaminyl-3′-phosphatidylglycerol isolated from *B. megaterium* QM B1551 by chromatography in solvents 1, 2, 4, and 5. Both phospholipids appeared to be chromatographically identical. In addition the acidic degradation (Fig. 4) confirmed this conclusion. A further demonstration of this identity was obtained by comparison of this phospholipid with the synthetic 2′ and 3′ isomers of glucosaminylphosphatidylglycerol which is presented below.

Synthesis of the Two Isomers of Glucosaminylphosphatidylglycerol and Comparison of Synthetic and Natural Compounds

Glucosaminyl-2′-phosphatidylglycerol was first synthesized in this laboratory by Gurr et al. (*8*). It appeared to be necessary, however, to synthesize this phospholipid and the corresponding 3′ isomer via a different route in order to introduce an unsaturated fatty acid. This was carried out by Verheij et al. (*9*) and the method of synthesizing glucosaminyl-3′-phosphatidylglycerol is illustrated in Fig. 6. Details of the synthesis are given in a separate paper (*9*).

Radioactive glucosaminyl-2′-phosphatidylglycerol and the corresponding 3′ isomer were isolated from *B. megaterium* MK 10 D grown in the presence of $[^{32}P]H_3PO_4$ in medium B (pH at harvesting 5.2) and were purified as described above. The natural, radioactive 2′ isomer had the same R_f value as the synthetic glucosaminyl-2′-phosphatidylglycerol when tested on paper- (solvent 1) and on thin-layer chromatography (solvents 2, 4, and 5) (Fig. 7). Naturally occurring glucosaminyl-3′-phosphatidylglycerol appeared to be identical to the synthetic 3′ isomer in these experiments. Hydrolysis in 6 *N* HCl degraded all the phospholipids in the same way giving free glucosamine, glycerol, and glycerolphosphates.

Partial acid hydrolysis was carried out in 2 *N* HCl (Fig. 4) giving a mixture of free glucosamine and glucosaminylglycerol in the ratio of about 1:2. A chromatographic (solvents 6 and 7) and electrophoretic comparison of the glucosaminylglycerols obtained from the 4 phospholipids showed that glucosaminylglycerol from the natural 3′ isomer was identical to the one obtained from the synthetic glucosaminyl-3′-phosphatidylglycerol but differed in R_f value and staining characteristics with the periodate–Schiff reagent from the hydrolysis products of the natural and synthetic glucosaminyl-2′-phosphatidylglycerol (Table 1).

The absence of any reducing activity in the natural and synthetic glucosaminylglycerol isomers demonstrated that the sugar is bound glucosidically to the glycerol. The nature of the glucosidic bond was indicated already by the chromatographic investigation of the glucosaminylglycerol isomers. 2′-β-Glucosaminylglycerol and the 2′-α-isomer which were obtained by chemical synthesis exhibited a different R_f value

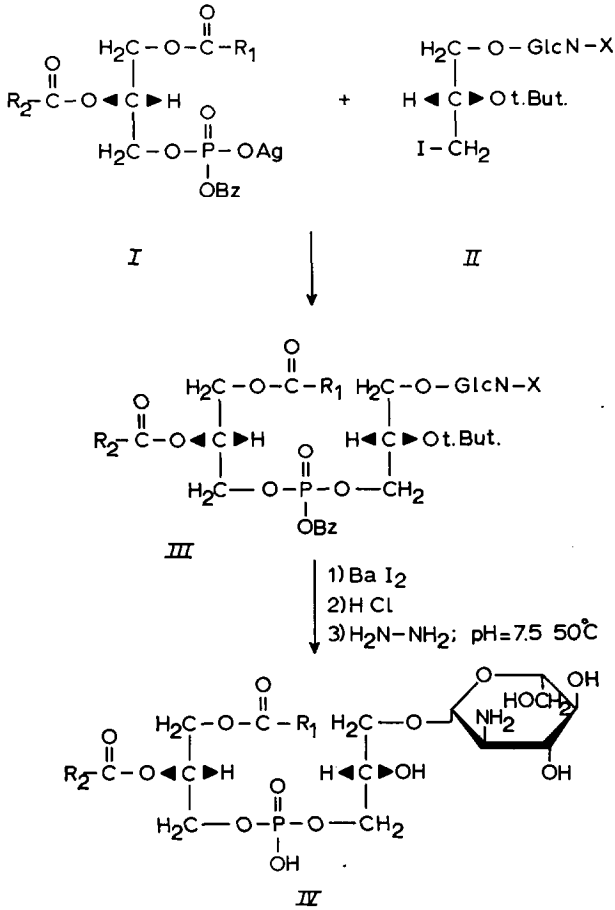

FIG. 6. Synthesis of glucosaminyl-3′-phosphatidylglycerol (IV). Bz = benzyl; t. But = tertiary butyl; GlcNX = 3,4,6-tri-*O*-acetyl-2-deoxy-2-phtalimidoglucopyranosyl; R1 = myristic acid; R2 = oleic acid.

TABLE 1

Compound	R GlcN in solvent 7[a]	Periodate–Schiff staining[b]	Periodate consumption[c] pH 7 10^{-2} *M*	pH 7 10^{-4} *M*	pH 4 10^{-2} *M*	pH 4 10^{-4} *M*
2-β-*O*-(2-amino-2-deoxy-D-gluco-pyranosyl)glycerol	1.04	–	2	—	1	—
2-α-*O*-(2-amino-2-deoxy-D-gluco-pyranosyl)glycerol	0.97	–	—	—	—	—
1-β-*O*-(2-amino-2-deoxy-D-gluco-pyranosyl)glycerol	0.91	+	3	1	2	1

[a] Values on paper relative to glucosamine.
[b] Staining of the compounds on paper chromatograms in solvent 7.
[c] Quantitative measurements of the periodate consumption as described in the text.

FIG. 7. Paper chromatogram of the synthetic and natural isomers of glucosaminylphosphatidylglycerol. Chromatograms were developed in solvent 1 and stained with the tricomplex staining (*13*). Compounds are: glucosaminyl-2′-phosphatidylglycerol from *B. megaterium*, 1; synthetic glucosaminyl-2′phosphatidylglycerol, 2; glucosaminyl-3′-phosphatidylglycerol from *B. megaterium*, 3; and synthetic glucosaminyl-3′-phosphatidylglycerol, 4.

in solvent 7 and it was shown that the isomer obtained from the natural glucosaminyl-2′-phosphatidylglycerol has the β-configuration (Table 1). In order to confirm this observation and to investigate the nature of the glucosidic bond in glucosaminyl-3-glycerol, an enzymatic procedure was followed. Both the synthetic and the natural isomers of glucosaminylphosphatidylglycerol were N-acetylated according to MacDougall and Phizackerley (*7*). The fatty acids were then removed by alkaline hydrolysis (*14*) and the resulting water soluble *N*-acetylglucosaminylglycerylphosphorylglycerols were incubated with β-*N*-acetylhexosaminidase; the reaction sequence for the 3′ isomer is as follows:

$$\text{PG-3′-GLcN}^{1} \rightarrow \text{PG-3′-GLcNAcetyl}$$

[1] PG-3′-GLcN stands for glucosaminyl-3′-phosphatidylglycerol, PG-3′-GLcNAcetyl for *N*-acetylglucosaminyl-3′-phosphatidylglycerol and GPG-3′-GLcNAcetyl for the corresponding *deacylate* D compound. Glycerylphosphorylglycerol and *N*-acetylglucosamine are abbreviated as GPG and GLcNAcetyl.

PG-3′-GLcNAcetyl → GPG-3′-GLcNAcetyl

GPG-3′-GLcNAcetyl → GPG + GLcNAcetyl

All the compounds were completely hydrolyzed by the enzyme as measured by the release of *N*-acetylglucosamine (*18*).

As mentioned before, glucosaminyl-3′-glycerol obtained from both the naturally occurring and the synthetic phospholipid was stained immediately with the periodate–Schiff reagent, whereas the 2′ isomers reacted much more slowly. This behavior was investigated quantitatively by measuring the periodate consumption of the isomeric glucosaminylglycerols. In an earlier report (*4*) we demonstrated that glucosaminyl-2-glycerol consumed 1 mol. prop. of periodate, whereas the synthetic glucosaminyl-3-glycerol consumed 2 mol. prop. of periodate. However, the experimental conditions in these quantitative studies appeared to be very important. MacDougall and Phizackerley (*7*) found in their assay that glucosaminyl-3-glycerol consumed only 1 mol. prop. of periodate while the 2 isomer was hardly oxidized at all. We investigated, therefore, the periodate consumption more carefully under a variety of conditions. As shown in Table 1, the periodate consumption appeared to depend on both the periodate concentration and the pH. At a periodate concentration as low as 10^{-4} *M*, the glucosamine ring is not oxidized at all either at pH 4 or pH 7.5. The oxidation of the glucosamine moiety is complete only in the case of a high periodate concentration in combination with a pH of 7.5, whereas an acidic pH protects the NH_2-group containing carbon atoms against oxidation (*19*, *20*).

The experiments described in this and earlier reports demonstrate clearly that chemically synthesized, well-defined compounds are useful tools in elucidating the complete structure of natural compounds.

Enzymatic Degradation of the Glucosaminylphosphatidylglycerols

The four glucosaminylphosphatidylglycerols were incubated with the phospholipases A, C, and D (Fig. 8). Marked differences were found in susceptibility of the 2′ and the 3′ isomers towards phospholipase C and D. The synthetic and the natural

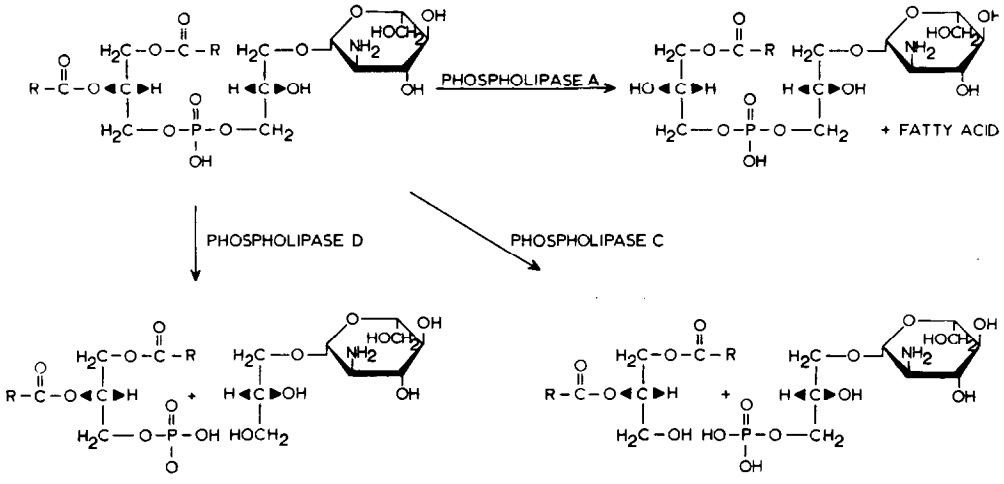

FIG. 8. Enzymatic hydrolysis of glucosaminyl-3′-phosphatidylglycerol.

compound of each isomer, however, reacted identically to the phospholipase, demonstrating again the identity of the synthetic and the naturally occurring phospholipids.

When glucosaminylphosphatidylglycerol is converted into phosphatidylglycerol by $NaNO_2$ treatment, this phospholipid appeared to be degraded completely by phospholipase A from porcine pancreas. As could be expected, therefore, also the intact glucosaminylphosphatidylglycerol isomers are hydrolyzed by this enzyme, yielding free fatty acids and the lysocompounds (Fig. 8).

Phospholipase C from *Bacillus cereus*, which was purified recently in this laboratory (*16*), hydrolyzed the 3′ isomer of glucosaminylphosphatidylglycerol completely (Fig. 9). Compared with egg-yolk lecithin, however, glucosaminyl-3′-phosphatidylglycerol is a

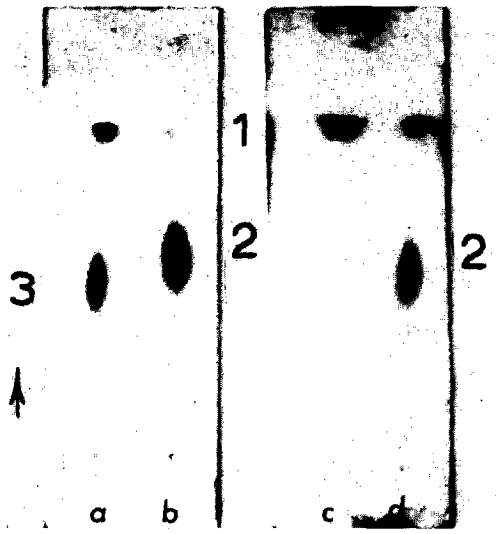

FIG. 9. Hydrolysis of two isomers of glucosaminylphosphatidylglycerol with phospholipase C. Incubations were carried out for 30 min (a and b) and 135 min (c and d). The reaction was followed by thin-layer chromatography (76 × 26 mm plates) in solvent 2. Compounds are: 1,2-diglyceride, 1; glucosaminyl-2′-phosphatidylglycerol, 2; and glucosaminyl-3′-phosphatidylglycerol, 3.

poor substrate for this enzyme because the lecithin was degraded about 250 times faster than the glucosamine containing phospholipid. The 2′ isomer is hydrolyzed even more slowly by phospholipase C and could not be degraded completely (Fig. 9). With phospholipase D from savoy cabbage similar results were obtained: the 3′ isomer is hydrolyzed completely, whereas the hydrolysis of the 2′ isomer proceeds very slowly.

Monomolecular Layers of Glucosaminylphosphatidylglycerol

It is apparent from the data presented above that the position of the glucosamine moiety in the glucosaminylphosphatidylglycerol isomers determines a number of properties by which the two isomers can be distinguished from each other. The 2′ isomer is less susceptible to hydrolysis by phospholipase C and D. The mobility of the 2′ isomer during chromatography is greater than that of the 3′ isomer, especially in acidic solvent systems. The explanation of these phenomena has to be sought in the properties of the polar headgroup of the phospholipids because the fatty acid compositions are, at least in the synthetic compounds, identical. We, therefore, studied the

behavior of the two synthetic glucosaminylphosphatidylglycerols on the air–water interface (Fig. 10).

Measurements of the area per molecule occupied by both isomers show that they contain, as could be expected, a large polar headgroup when compared with other phospholipids. Phosphatidylcholine with a comparable fatty acid composition (dimyristoyl lecithin) occupies at a pressure of 20 dynes/cm an area of only 64 Å^2 (*21*), whereas the areas per molecule occupied by glucosaminyl-2′-phosphatidylglycerol and the 3′ isomer are 82 and 77 Å^2, respectively (when measured at pH 9). The low susceptibility of the two isomers towards phospholipase C, when compared with other

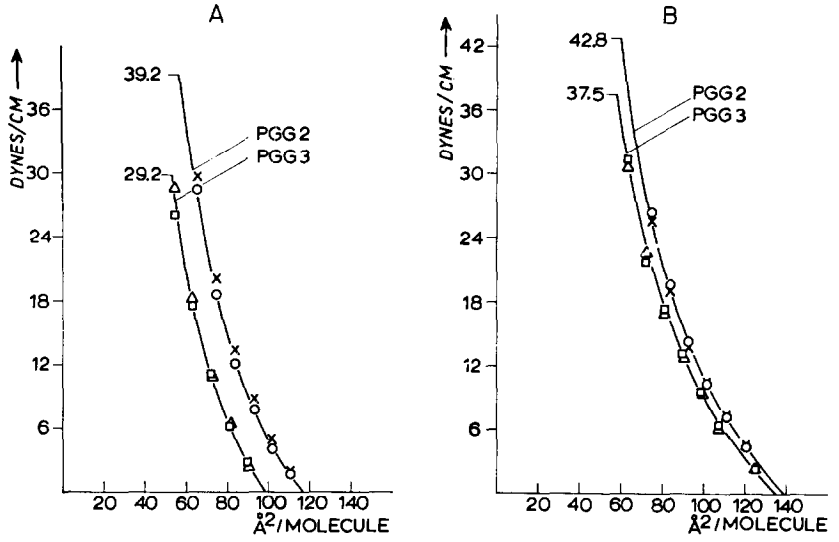

FIG. 10. Force-area characteristics of glucosaminyl-3′-phosphatidylglycerol at pH 9 (B, PGG 3) and pH 4 (A, PGG 3) and of glucosaminyl-2′-phosphatidylglycerol at pH 9 (B, PGG 2) and pH 4 (A, PGG 2). Force-area measurements were performed at the air–water interface in a paraffin-coated glass trough. The trough was filled with 0.01 *M* borate buffer pH 9 or 0.01 *M* acetate buffer pH 4. Surface pressures were determined with a conventional Langmuir–Adam surface balance.

phospholipids, might be due, therefore, to steric hindrance of this bulky polar headgroup in the enzyme–substrate interaction. The 3′ isomer occupies a smaller area per molecule than the 2′ isomer which makes it a better substrate for the enzyme.

At pH 9 the monolayers obtained from both isomers have a more expanded character than at pH 4 (Fig. 10). Since the fatty acid composition of both compounds is identical, this difference in behavior has to be attributed entirely to the polar headgroup of the phospholipids. It seems likely that at low pH value, when the molecules have a zwitterionic structure, an intramolecular interaction of the phosphate and amino group occurs, thus leading to a larger area per molecule occupied by the phospholipids. That this effect is more pronounced in the case of the 2′ isomer is supported by the observation that the area per molecule occupied by this isomer is larger than that of the 3′ isomer especially at low pH. For this reason the glucosaminyl-2′-phosphatidylglycerol might have a higher R_f value in acidic solvent systems than the corresponding 3′ isomer. It is not clear whether this difference in dimensions of the polar headgroup of both isomeric glucosaminephosphatidylglycerols has a significance for membrane properties.

ACKNOWLEDGMENTS

The present investigations were partly carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organisation for the advancement of Pure Research (Z.W.O.).

The authors thank Dr. R. A. Demel for carrying out the monolayer experiments and Miss M. Th. Kauerz for her expert technical assistance.

REFERENCES

1. J. A. F. Op den Kamp, V. Tomasi, and L. L. M. van Deenen, "Structural and Functional Aspects of Lipoproteins in Living Systems" (E. Tria and A. Scanu, Eds.). Academic Press, London, 1969.
2. J. A. F. Op den Kamp, U. M. T. Houtsmuller, and L. L. M. van Deenen, *Biochim. Biophys. Acta* **106**, 438 (1965).
3. J. A. F. Op den Kamp, W. van Iterson, and L. L. M. van Deenen, *Biochim. Biophys. Acta* **135**, 862 (1967).
4. J. A. F. Op den Kamp, P. P. M. Bonsen, and L. L. M. van Deenen, *Biochim. Biophys. Acta* **176**, 298 (1969).
5. P. J. R. Phizackerley, J. C. MacDougall, and M. J. O. Francis, *Biochem. J.* **99**, 21C (1966).
6. L. L. Bertsch, P. P. M. Bonsen, and A. Kornberg, *J. Bacteriol.* **98**, 75 (1969).
7. J. C. MacDougall and P. J. R. Phizackerley, *Biochem. J.* **114**, 361 (1969).
8. M. I. Gurr, P. P. M. Bonsen, J. A. F. Op den Kamp, and L. L. M. van Deenen, *Biochem. J.* **108**, 211 (1968).
9. H. M. Verheij, P. P. M. Bonsen, and L. L. M. van Deenen, *Chem. Phys. Lipids* **6**, 46 (1971).
10. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
11. G. V. Marinetti, J. Erbland, and J. Kochen, *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **16**, 837 (1957).
12. J. C. Dittmer and R. L. Lester, *J. Lipid Res.* **5**, 126 (1964).
13. H. G. Bungenberg de Jong, *Proc. Kon. Ned. Akad. Wetensch. Ser. B* **64**, 458 (1961).
14. A. A. Benson and B. Maruo, *Biochim. Biophys. Acta* **27**, 189 (1958).
15. G. H. de Haas, N. M. Postema, W. Nieuwenhuizen, and L. L. M. van Deenen, *Biochim. Biophys. Acta* **159**, 103 (1968).
16. R. F. A. Zwaal, B. Roelofsen, P. Comfurius, and L. L. M. van Deenen, *Biochim. Biophys. Acta* **233**, 474 (1971).
17. B. Weissmann, S. Hadjiioannou, and J. Tornheim, *J. Biol. Chem.* **239**, 59 (1964).
18. Y. Z. Frohwein and S. Gatt, *Biochemistry* **6**, 2775 (1967).
19. R. W. Jeanloz and E. Forchielli, *J. Biol. Chem.* **188**, 361 (1951).
20. P. F. Fleury, J. E. Courtois, and M. Grandchamp, *Bull. Soc. Chim. Fr.*, 88 (1949).
21. P. Joos and R. A. Demel, *Biochim. Biophys. Acta* **183**, 447 (1969).