1-(*O*- β -Glucosaminyl)-2,3-diglyceride in *Bacillus megaterium*

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1. A lipid that contains glucosamine but not phosphorus has been isolated from *Bacillus megaterium*. It constitutes about 5% of the total lipid glucosaminide in this organism and can be distinguished chromatographically from 2'-(*O*- β -glucosaminyl)phosphatidylglycerol and 3'-(*O*- β -glucosaminyl)phosphatidylglycerol, which are also present. 2. The lipid contains glycerol, fatty acids and glucosamine in the molar proportion 1:2:1. The fatty acids are bound by an ester linkage and are similar to those found in other lipids of this organism. Partial acid hydrolysis or alkaline hydrolysis of the lipid yields 1-(*O*- β -glucosaminyl)glycerol and degradation with nitrite yields 2,5-anhydromannose and diglyceride. 3. The lipid has been identified as 1-(*O*- β -glucosaminyl)-2,3-diglyceride.

It has been shown that several strains of *Bacillus megaterium* contain both 2'-(*O*- β -glucosaminyl)-phosphatidylglycerol and 3'-(*O*- β -glucosaminyl)-phosphatidylglycerol (Phizackerley & MacDougall, 1968; MacDougall & Phizackerley, 1969; Bertsch *et al.*, 1969), and both isomers have been synthesized chemically (Verheij *et al.*, 1971). Phizackerley & MacDougall (1968) also reported the presence in *B. megaterium* of a lipid that contains glucosamine but not phosphorus, and since it is possible that the lipid glucosaminides in this organism are metabolically and functionally related, this lipid has been purified and its structure determined. The evidence reported below shows that it is 1-(*O*- β -glucosaminyl)-2,3-diglyceride.

Materials and Methods

Maintenance and growth of organisms

Cultures of *B. megaterium* were maintained on agar slopes and grown in liquid culture as described by MacDougall & Phizackerley (1969); at the end of the 48 h growth period the pH of the medium was 5.5. After being harvested by centrifugation at 1500g at 2°C, the cells were washed with 0.1M-KCl, then centrifuged and the washing repeated. The washing minimized contamination of the lipid extract with amino acids and peptides. The cells were stored at -15°C until required.

Extraction of lipid

Lipid was extracted from cells (50g dry wt.) and purified as described by MacDougall & Phizackerley (1969). The final extract was dissolved in 100ml of chloroform, and contained 410 μ g-atoms of phospholipid P, 320 μ g-atoms of N and 144 μ mol of lipid glucosaminide.

Purification of 1-(*O*- β -glucosaminyl)-2,3-diglyceride

Step 1, hexane extraction. The lipid extract obtained as described above was evaporated to dryness under N₂ at reduced pressure at room temperature, and extracted with 100ml of hexane, used in several portions. The brown residue was discarded. The pale-yellow solution of hexane-soluble lipid contained 400 μ g-atoms of phospholipid P, 226 μ g-atoms of N and 144 μ mol of lipid glucosaminide.

Step 2, first acetone precipitation. The hexane extract obtained from step 1 was evaporated to dryness and dissolved in 10ml of hexane. Acetone (50ml) was added with stirring and the mixture left for 2 h at -15°C. It was then centrifuged at 20000g at -15°C for 30 min. The supernatant was discarded and the white precipitate was dissolved in 10ml of chloroform. This extract contained 374 μ g-atoms of phospholipid P, 223 μ g-atoms of N and 120 μ mol of lipid glucosaminide. In this step, glucosaminyl-diglyceride was precipitated together with phospholipid, but most of the yellow pigment remained in solution.

Step 3, column chromatography on silica gel-calcium sulphate. Silicic acid (100 mesh; Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.) (30g) was mixed with anhydrous CaSO₄ (3g), washed as described by Phizackerley & Francis (1966), activated by heating overnight at 120°C, then suspended in chloroform and used as a 5 cm $\times$ 12 cm² column. The lipid extract from step 2 was applied to the column, which was eluted under gravity with 200ml of chloroform. This eluate was discarded. The column was then eluted with chloroform-methanol (20:1, v/v) and 50ml fractions were collected. The fractions were monitored by t.l.c. and fractions 4–10, which contained most of the glucosaminyl diglyceride, were pooled and evaporated to dryness. The residue was extracted with hexane and a small yellow deposit was removed by centrifugation. The clear colourless

supernatant contained 19.5 μg -atoms of phospholipid P, 12.7 μg -atoms of N and 8.0 μmol of lipid glucosaminide. This step yielded an extract in which glucosaminyl diglyceride had been separated from most of the phospholipid.

Step 4, second acetone precipitation. The hexane extract from step 3 was evaporated to dryness and the residue dissolved in 1 ml of hexane. Acetone (5 ml) was added with stirring and the mixture left for 2 h at -15°C . It was then centrifuged at 20000 g at -15°C for 30 min. The supernatant was decanted and stored; the precipitate was dissolved in 0.5 ml of hexane, 5 ml of acetone was added, and the mixture was left for 2 h at -15°C , then centrifuged as described above. The supernatant was added to that obtained previously and the procedure repeated twice. The combined extracts were evaporated to dryness and dissolved in 2 ml of chloroform. This extract contained 12.0 μg -atoms of N and only a trace of phospholipid P was detected by t.l.c. At this stage of the purification, in contrast with step 2, most of the glucosaminyl diglyceride remained soluble in acetone-hexane (5:1, v/v) at -15°C , whereas most of the remaining phospholipid was precipitated.

Step 5, column chromatography on silicic acid. Silicic acid (2 g) was washed and activated as described above, then suspended in chloroform and used as a 10 cm $\times$ 0.35 cm² column. The extract from step 4 was applied to the column which was then eluted under gravity with 10 ml of chloroform and then with 10 ml of chloroform-methanol (100:1, v/v) followed with 10 ml of chloroform-methanol (50:1, v/v) and so on, until the chloroform-methanol ratio was 10:1 (v/v). Fractions (2 ml) were collected and were monitored by t.l.c. Pure glucosaminyl diglyceride was eluted as the chloroform/methanol volume ratio was varied from 100:4 to 100:6 (i.e. in tubes 24-33 inclusive). Eluates collected in tubes 17-22 contained, in addition to glucosaminyl diglyceride, a ninhydrin-positive lipid, which on t.l.c. in solvent 1 (see below) had a slightly greater R_F value and which yielded a mixture of amino acids on strong acid hydrolysis. The final yield of glucosaminyl diglyceride was 6.6 μmol . The purpose of this step was to separate glucosaminyl diglyceride from peptides and from traces of phospholipid.

Analysis of lipids

Acid hydrolysis of glucosaminyl diglyceride. This was done in evacuated sealed tubes under conditions described in the text.

Treatment of glucosaminyl diglyceride with sodium nitrite. A portion (0.3 μmol) of lipid was incubated with 0.2 ml of methanol and 0.05 ml of NaNO_2 (100 mg/ml in 10 mM-sodium acetate buffer, pH 5.6) in a sealed tube at 60°C for 72 h. Then 0.5 ml of chloroform, 0.3 ml of methanol and 0.4 ml of water

were added, the mixture was agitated on a Whirlimixer for 2 min and centrifuged on a bench centrifuge to separate the upper and lower phases.

Preparation of *N*-acetylglucosaminylglycerol. Lipid (0.5 μmol) dissolved in 5 ml of chloroform-methanol (2:1, v/v) was incubated in a stoppered tube with 0.3 ml of 0.1 M- NaHCO_3 and 0.25 ml of freshly prepared aq. 1% (v/v) acetic anhydride. The *N*-acetylated lipid was recovered by extracting the reaction mixture by the procedure of Folch *et al.* (1957). Fatty acids were removed and the water-soluble product was extracted by the procedure of Dawson *et al.* (1962) except that alkali was neutralized with 2 M-HCl.

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

Analytical methods

Thin-layer chromatography. This was done on plates of silica gel G that were 0.25 mm thick. The following solvents were used: 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. 0.88) (35:10:1, by vol.); solvent 4, butan-1-ol-acetic acid-water (3:1:1, by vol.); solvent 5, hexane-ether-formic acid (60:50:1, by vol.).

Paper electrophoresis. This was done with a Locarte high-voltage electrophoresis apparatus (Locarte Co. Ltd., London E.C.3, U.K.) for 30 min at 100 V/cm in pyridine-acetic acid-water, pH 3.6 (1:10:89, by vol.), on Whatman no. 1 paper.

Column chromatography. Column chromatography was done in a Technicon amino acid analyser by the method of Moore & Stein (1951).

Gas-liquid chromatography. The g.l.c. of fatty acid methyl esters, prepared as described by Schlenk & Gellerman (1960), was done on an F & M 402 high-efficiency gas chromatograph with columns of 20% (w/w) Apiezon L and 5% (w/w) polyethylene glycol succinate, both on Supersorb AW-HMDS (80-100 mesh). Catalytic hydrogenation of fatty acid methyl esters was done in hexane in the presence of a small amount of platinum dioxide for 24 h at room temperature.

Other analyses. These were done by the following methods: P., Bartlett (1959); total N, Sloane-Stanley (1967); fatty acids, by the method of Duncombe (1963) with palmitic acid as a standard; esters, by the method of Rapport & Alonzo (1955) with tripalmitin as a standard; glycerol, Wieland (1963); amino-2-deoxy sugars by the Elson-Morgan reaction, Boas (1953); reducing sugar, Park & Johnson (1949); formaldehyde, Hanahan & Olley (1958).

Materials

β -*N*-Acetylhexosaminidase was prepared from calf brain by the method of Frohwein & Gatt (1967). Glycerol kinase (EC 2.7.1.30) and *sn*-glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Silica gel G was 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 and Discussion

The main difficulty in purifying glucosaminyl-diglyceride was caused by several peptides in the lipid extract, some of them ninhydrin-negative, which co-chromatographed with it on both silicic acid column chromatography and on t.l.c. until the final stage of purification. Glucosaminyl diglyceride contained N, glycerol and fatty acids in the molar proportions 1.00:0.98:2.03. P₁ was not detected; on t.l.c. on silica gel G it migrated as a single spot in solvent 1 (*R_F* 0.73), solvent 2 (*R_F* 0.66), and solvent 3 (*R_F* 0.66). It is therefore readily distinguished from the lipid glucosaminides that have been described by MacDougall & Phizackerley (1969).

Products of acid hydrolysis

On strong acid hydrolysis (5.8M-HCl at 105°C for 18h) glucosaminyl diglyceride yields, in addition to glycerol and fatty acids, a single ninhydrin-positive compound, which has been identified as glucosamine. Thus it behaves identically with glucosamine and differently from galactosamine and mannosamine on paper chromatography (Fischer & Nebel, 1955), and on column chromatography (Moore & Stein, 1951); degradation with ninhydrin yielded a product that co-chromatographed with arabinose (Stoffyn & Jeanloz, 1954). No evidence was obtained by these

methods for the presence of any other ninhydrin-positive compound and this conclusion was confirmed by the finding that strong acid hydrolysis yielded 1.1 μ mol of glucosamine/ μ mol of lipid N. It was also shown that in the hydrolysate, the glucosamine content measured by the Elson-Morgan reaction for the determination of amino-2-deoxy sugars (Boas, 1953) was identical with that of reducing sugar, measured by the method of Park & Johnson (1949). These results indicated that glucosamine was the only sugar present.

On mild acid hydrolysis (2M-HCl at 105°C for 8h) two ninhydrin-positive compounds were obtained, and were separated from each other by high-voltage paper electrophoresis at pH 3.6, and by t.l.c. on silica gel G in solvent 4. One of these compounds is glucosamine and the other, for reasons given below, was identified as 1-(*O*- β -glucosaminyl)glycerol.

Products of mild alkaline hydrolysis

Hydrolysis [80mm-NaOH in ethanol-water (17:3, v/v) at 40°C for 30min] yielded 1-(*O*- β -glucosaminyl)glycerol and fatty acid in the molar ratio 1:2.08. This result implied that fatty acids were bound to the parent lipid solely by an ester linkage; this conclusion was confirmed by the finding that in glucosaminyl diglyceride the ester/fatty acid molar ratio was 1:1.04.

Products of degradation by nitrite

Treatment of the parent lipid with nitrite (Ellwood *et al.*, 1963) yielded a mixture of lipid products, and a product that behaved on paper chromatography identically with 2,5-anhydromannose similarly derived from glucosamine. The major constituents of the lipid products were identified chromatographically as 1,2-diglyceride and 1,3-diglyceride and on visual inspection they appeared to be present in

Table 1. Analysis by *g.l.c.* of fatty acid methyl esters

The esters were derived from whole lipid, glucosaminyl diglyceride (GDG), 3'-(*O*- β -glucosaminyl)phosphatidylglycerol (3'GPG) and 2'-(*O*- β -glucosaminyl)phosphatidylglycerol (2'GPG). The equivalent chain lengths (Miwa *et al.*, 1960) of the fatty acids were determined from the retention times of saturated straight chain fatty acid methyl esters of chain length 12–18 inclusive, on Apiezon L and polyethylene glycol succinate (PEGS) as described in the Materials and Methods section.

Equivalent chain length of fatty acid		% of individual fatty acids in each lipid			
Apiezon L	PEGS	GDG	3'GPG	2'GPG	Whole lipid
13.62	13.80	13	13	13	11
14.72	14.80	45	42	44	46
15.60	15.65	16	17	17	16
16.00	16.05	12	16	13	13
16.72	16.75	14	12	12	14

approximately equal amounts. Traces of free fatty acid and monoglyceride were also found, together with an unidentified derivative. These results indicated first that in the parent lipid, glucosamine is bound in glycosidic linkage to the glycerol moiety, and secondly (since diglyceride was the major lipid product) that fatty acids are esterified to glycerol and not to glucosamine. The fact that both 1,2-diglyceride and 1,3-diglyceride were formed does not indicate that the parent lipid is a mixture of isomeric glucosaminyl diglycerides, because 1,2-diolein when incubated with nitrite under these conditions yielded an identical pattern of lipid products, and in particular extensive isomerization occurred, leading to the formation of 1,3-diolein.

Identification of 1-(O- β -glucosaminyl)glycerol

This derivative can be obtained from the parent lipid either by partial acid hydrolysis or by alkaline hydrolysis. It contained glycerol and glucosamine in the molar ratio 1:1.04. Unlike the parent lipid it gave a rapid reaction when tested with the benzidine-periodate reagent of Cifonelli & Smith (1954), which suggested that glucosamine is bound to the primary hydroxyl group of the glycerol moiety. This conclusion was confirmed quantitatively by showing that in the presence of 0.1 mM-periodate at pH 4.2 at 20°C, 0.98 mol.prop. of periodate was rapidly consumed and 1.07 mol.prop. of formaldehyde was formed. Under these conditions 2-(O- β -glucosaminyl)glycerol consumes very little periodate (MacDougall & Phizackerley, 1969).

The glycosidic bond was shown enzymically to have the β configuration. The parent lipid was converted into its *N*-acetyl derivative and the fatty acids were removed by mild alkaline hydrolysis. The product is 1-(O- β -*N*-acetylglucosaminyl)glycerol (glycerol/nitrogen ratio 1:1.02) and it migrated as a single ninhydrin-negative spot on t.l.c. in solvent 1. When incubated with β -*N*-acetylhexosaminidase from calf brain (Frohwein & Gatt, 1967; MacDougall & Phizackerley, 1969), 0.94 mol.prop. of *N*-acetylglucosamine was liberated.

Fatty acids of glucosaminyl diglyceride

Fatty acids, obtained by alkaline hydrolysis under N_2 were converted into their methyl esters and examined by g.l.c. (Table 1). Five main peaks were observed, one of which was identified as palmitate, both by its retention time and by co-chromatography with an authentic sample of palmitic acid methyl ester. The other fatty acids were not identified. Their retention times, which were unaffected by catalytic hydrogenation, differed significantly from those of straight-chain saturated fatty acids of chain length 12–16 inclusive. Although small differences in proportions were observed, the fatty acid composition

of glucosaminyl diglyceride closely resembled that of the isomers of glucosaminyl phosphatidyl glycerol found in this organism, and of the fatty acids obtained from the lipid extract prepared from whole cells.

Considered together, these results show that the structure of the lipid is 1-(O- β -glucosaminyl)-2,3-diglyceride. Under the growth conditions employed, it constitutes about 5% of the total lipid extract from whole cells. Only very small quantities of sugars other than glucosamine were found in this extract. Thus, on strong acid hydrolysis (5.8 M-HCl at 105°C for 18 h) the reducing sugar content of the extract was equal to the glucosamine content. Under milder conditions (1 M-HCl at 100°C for 3 h), although glucose and other reducing sugars were released, the amounts in each case were less than 1% of the glucosamine content of the extract.

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