The Glycolipids of *Lactobacillus casei* A.T.C.C. 7469

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1. The lipids were extracted from *Lactobacillus casei* A.T.C.C. 7469 with chloroform-methanol mixtures. The glycolipids were obtained by chromatography on silicic acid and DEAE-cellulose (acetate form). 2. Hydrolysis of the glycolipids with alkali gave two glycerol glycosides and a mixture of fatty acids. 3. The glycosides were separated and their structures elucidated. The major component was *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol and the minor component *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol. 4. Analysis of the fatty acids by gas-liquid chromatography showed that they were predominantly palmitic acid, octadecenoic acid and lactobacillic acid.

The lipids of many Gram-positive bacteria are now known to contain glycolipids of the diglycosyl diglyceride type. Preliminary studies have shown that *Lactobacillus casei* A.T.C.C. 7469 contains a galactosylglucosyl diglyceride and smaller amounts of a larger compound, probably a triglycosyl diglyceride (Brundish, Shaw & Baddiley, 1966). The detailed structures of these lipids have now been elucidated.

EXPERIMENTAL AND RESULTS

Materials and methods

Materials. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden), Mallinckrodt 100-mesh silicic acid from Kodak Ltd. (Kirkby, Lancs.), Merck Kiesegel G (nach Stahl) for thin-layer chromatography from Alderman and Co. Ltd. (London, S.W. 1) and Whatman DEAE-cellulose (DE-11) from H. Reeve Angel and Co. Ltd. (London, E.C. 4). Glucostat and Galactostat reagents were from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) and almond-emulsin β -glucosidase was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). A sample of α -glucosidase was a gift from Koch-Light Laboratories Ltd. Solvents for chromatography were purified and dried before use.

Analytical methods. Hexoses were determined by the phenol-H₂SO₄ method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) and with the Glucostat and Galactostat reagents. Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954); glycerol and formaldehyde were determined by the procedure of Hanahan & Olley (1958).

Paper chromatography. Paper chromatography of sugars, polyols and glycosides was carried out on Whatman no. 1 paper by the descending method in the solvent system butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951). Compounds were detected by the periodate-Schiff reagents for α -glycols (Baddiley,

Buchanan, Handschumacher & Prescott, 1956) and the alkaline AgNO₃ reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950).

Thin-layer chromatography. Lipids were examined by thin-layer chromatography in the solvent system chloroform-methanol-water (65:25:4, by vol.) (Wagner, Hörhammer & Wolff, 1961). Components were located by exposure to iodine vapour and with reagents specific for phosphorus (Dittmer & Lester, 1964), α -glycols (Baddiley *et al.* 1956) and amino compounds (Marinetti, 1962).

Acid hydrolysis. This was carried out in 2N-HCl for 3 hr. at 100°. The hydrolysate was evaporated to dryness *in vacuo* over KOH, and the residue was dissolved in the minimum quantity of water and examined by paper chromatography.

Enzymic hydrolysis. Incubations of glycosides (0.5 mg.) with α - and β -glucosidases (0.2 ml. of an aqueous 1% solution) were carried out at 37° overnight.

Growth of organisms. *L. casei* was grown for 16 hr. at 30° in a medium of the following composition: sodium acetate (1%, w/v), tryptone (2%, w/v), glucose (2%, w/v), yeast extract (0.5%), KH₂PO₄ (0.45%) and Barton-Wright (1946) salts (0.05%). The cells were harvested at the stationary phase in a Sharples centrifuge and washed twice with M-potassium phosphate buffer, pH 7.

Isolation of the glycolipids

The washed moist cells (300 g.) were stirred first with methanol (500 ml.) and then with chloroform-methanol (2:1, v/v) (3 $\times$ 300 ml.). The combined extracts were evaporated to dryness *in vacuo*. The residue was dissolved in chloroform-methanol-water (40:20:3, by vol.) and passed through a column of Sephadex G-25 (Wells & Dittmer, 1963). The purified lipid (1.75 g.) was fractionated on a column of DEAE-cellulose (acetate form) as described by Brundish, Shaw & Baddiley (1965) and the fractions were analysed by thin-layer chromatography. The fraction eluted with 10% (v/v) methanol in chloroform (305 mg.)

contained the glycolipids together with traces of phospholipids. One major glycolipid was observed (R_F 0.65); it was probably a mixture of two components as judged by the elongated shape of the spot. Traces of other components with slightly higher R_F values were also observed. They all gave with the periodate-Schiff reagents a slow-developing blue colour, which is characteristic of glycosides; they gave negative reactions with the phosphorus and ninhydrin reagents. Attempts at further purification by chromatography on silicic acid according to the method of Vorbeck & Marinetti (1965) were unsuccessful. Acid hydrolysis showed the presence of galactose, glucose and glycerol.

Examination of the glycolipids

The glycolipids (280 mg.) were heated at 100° for 1 hr. with 0.1 N-NaOH (30 ml.) in an atmosphere of nitrogen, when a homogeneous solution was obtained. The solution was cooled, adjusted to pH 1 with 4 N-HCl and extracted with light petroleum (b.p. 60–80°) (3 × 25 ml.). The extracts were combined, washed with water and dried over Na_2SO_4 . The drying agent was removed and the solvent evaporated *in vacuo*, giving a residue of fatty acids (157 mg.). The aqueous solution was deionized by passage through columns (10 ml.) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form) resins and the eluate was evaporated to dryness. Paper chromatography showed the presence of two non-reducing glycosides, R_F 0.42 (glycoside A) and R_F 0.23 (glycoside B). The two glycosides were separated by preparative paper chromatography on Whatman 3MM paper and were eluted from the paper with water. The yield of glycoside A was 60 mg. and of glycoside B was 20 mg. Samples (1 mg.) of the glycosides were hydrolysed with 2 N-HCl; paper chromatography of the hydrolysates showed the presence in each of glucose, galactose and glycerol. Both glycosides gave a rapidly-developing purple colour with the periodate-Schiff reagents, indicative of formaldehyde produced from a 1-substituted glycerol (Roberts, Buchanan & Baddiley, 1963).

Analysis of glycoside A

A solution of the glycoside (8.6 mg./ml. in water) had $[\alpha]_D^{25} +150^\circ$. Portions (0.2 ml.) of the solution were hydrolysed with 2 N-HCl for 1 hr. at 100°, neutralized with N-KOH and made to 10 ml. with water. D-Glucose and D-galactose were determined enzymically and glycerol was determined by the chromatotropic acid method; the glycoside contained D-glucose, D-galactose and glycerol in the molar proportions 0.88:0.92:1.0.

Partial acid hydrolysis. A sample of the glycoside (1 mg.) was hydrolysed with 0.025 N-HCl at 100° for 5 hr. and the hydrolysate was examined by paper chromatography. Small amounts of glucose and glycerol were observed together with larger amounts of galactose and glucosylglycerol. A further sample of the glycoside (10 mg.) was hydrolysed and the glucosylglycerol isolated by preparative paper chromatography. It rapidly gave a purple colour with the periodate-Schiff reagents and was unchanged on incubation with β -glucosidase. Acid hydrolysis yielded glucose and glycerol. Incubation with α -glucosidase also gave glucose and glycerol. These results suggested the structure 1-O- α -D-glucosylglycerol, which was confirmed by gas-liquid-chromatographic studies (see below).

Oxidation with periodate. The glycoside solution (0.6 ml.), water (1.9 ml.) and 20 mM- NaIO_4 (2.5 ml.) were mixed and

kept at about 21° in the dark. Samples (0.1 ml.) were withdrawn periodically and diluted to 10 ml. with water for analysis. Another portion of the solution (0.1 ml.) was withdrawn and diluted to 10 ml. with water for the determination of hexose. After about 0.5 hr., 0.47 mole of periodate had been reduced/mole of hexose; this quantity rose gradually during 48 hr. to a final value of 1.94 moles/mole. A sample of the oxidation mixture was analysed for formaldehyde; 0.52 mole was produced/mole of hexose. The molar proportions hexose:formaldehyde produced: periodate reduced rapidly:total periodate reduced were 2.0:1.04:0.94:3.88.

Oxidation with periodate and reduction with borohydride. The glycoside solution (0.6 ml.) was oxidized with 10 mM- NaIO_4 as described above. Samples (1 ml.) were withdrawn after 0.5 hr., 24 hr. and 48 hr. and NaBH_4 (10 mg.) was added to each. After 24 hr. at 4°, a drop of acetic acid was added to each to destroy excess of borohydride. The solutions were passed through columns of Dowex 50 (H^+ form) resin (3 ml.) and evaporated to dryness *in vacuo*. The boric acid was removed by evaporation with three successive portions (1 ml.) of methanol on a steam bath. The residues were dissolved in 2 N-HCl and heated at 100° for 3 hr. After neutralization with Dowex 2 (HCO_3^- form) resin, water was removed by evaporation and the products were examined by paper chromatography. After 0.5 hr. glucose, galactose and ethylene glycol were detected, whereas after 24 hr. glucose, glycerol and ethylene glycol were present; after 48 hr. only glycerol and ethylene glycol were detected.

Studies with almond emulsin. Samples of the glycoside solution (0.2 ml.) were incubated with almond emulsin (which in addition to the β -glucosidase also contains an α -galactosidase). One sample was treated with the enzyme alone and another with the enzyme in the presence of Ag_2O to inhibit the α -galactosidase activity (Hestrin, Feingold & Schramm, 1955). Examination of the hydrolysates showed that treatment with the enzyme alone gave mainly galactose and glucosylglycerol whereas in the presence of Ag_2O negligible hydrolysis was observed.

Gas-liquid chromatography of glycosides (with D. E. Brundish). The glucosylglycerol (0.5 mg.) obtained from partial acid hydrolysis of the galactosylglucosylglycerol was dissolved in dry pyridine (0.25 ml.) and treated with hexamethyldisilazine (0.025 ml.) and trimethylsilyl chloride (0.015 ml.). The mixture (1 μ l.) was analysed by gas-liquid chromatography on a column (150 cm.) of 3% SE-52 silicone on Celite (100 mesh) maintained at 185° in a Pye Panchromatograph. Nitrogen was used as carrier gas at a pressure of 2.15 kg./cm.². Samples of synthetic 1-O- α -D-glucopyranosyl-D-glycerol and 1-O- β -D-glucopyranosyl-D-glycerol were silylated and used as reference compounds. The natural glycoside had a retention time of 17.25 min., identical with that of 1-O- α -D-glucopyranosyl-D-glycerol. The β -isomer had a retention time of 24.5 min.

The galactosylglucosylglycerol was also silylated and analysed on the same column at 255°. It had a retention time of 13.25 min., identical with that of synthetic O- α -D-galactopyranosyl-(1→2)-O- α -D-glucopyranosyl-(1→1)-D-glycerol and the glycoside from *Pneumococcus* type I with the same structure (Brundish *et al.* 1965).

Analysis of glycoside B

Acid hydrolysis. Acid hydrolysis of the glycoside (0.5 mg.) and examination of the hydrolysate by paper chromato-

graphy showed the presence of glucose, galactose and glycerol, and analysis gave a glucose:galactose molar ratio about 2:1.

Partial acid hydrolysis. The glycoside (1 mg.) was heated with 0.025 N-HCl at 100° for 5 hr. and the hydrolysate was examined by paper chromatography. Small amounts of glucose and galactose were observed together with larger amounts of glucosylglycerol and a strongly reducing component, R_{Glc} 0.38, with the characteristics of a disaccharide. A larger quantity of the glycoside (15 mg.) was hydrolysed and the glucosylglycerol and the disaccharide were isolated by preparative paper chromatography. Gas-liquid chromatography of the glucosylglycerol by the method described above established its structure as 1-*O*- α -D-glucosylglycerol. The disaccharide (0.5 mg.) yielded glucose and galactose on acid hydrolysis. A further sample (0.5 mg.) in water (1 ml.) was treated with NaBH₄ (5 mg.) at 4° for 16 hr. and, after removal of excess of borohydride and boric acid (see above), the residue was hydrolysed with 2 N-HCl at 100° for 1 hr. The products were glucose and galactitol, which establishes the structure of the original disaccharide as a glucosylgalactose. A further sample (1 mg.) was oxidized with 10 mM-NaIO₄ for 48 hr. and then reduced with NaBH₄ as described above for glycoside A. The reduced product was hydrolysed with 2 N-HCl at 100° for 1 hr. and yielded only glycerol; only a disaccharide possessing a (1→2)- or a (1→6)-link would give glycerol under these conditions. The glucosylgalactose was unaffected by β -glucosidase. It was readily distinguished from a synthetic sample (kindly provided by Dr D. E. Brundish) of *O*- α -D-glucopyranosyl-(1→2)-D-galactopyranose on paper chromatography. The synthetic compound had R_{Glc} 0.56 and gave a yellow colour with the periodate-Schiff reagents; this behaviour has been observed with 2-*O*-methyl sugars and disaccharides possessing a (1→2)-linkage (Roberts *et al.* 1963; Hardy & Buchanan, 1963), whereas the natural disaccharide gave a blue colour. The probable structure is therefore *O*- α -D-glucopyranosyl-(1→6)-D-galactose.

Enzymic hydrolysis. A sample of the glycoside (2 mg.) was incubated with α -glucosidase and the products were examined by paper chromatography. Small amounts of glucose were produced together with a component indistinguishable from glycoside A. This was confirmed by gas-liquid-chromatographic analysis.

Analysis of the fatty acids

The fatty acids obtained from hydrolysis of the glycolipids with alkali were converted into their methyl esters

and analysed by gas-liquid chromatography by the method described by Brundish *et al.* (1965). The results are given in Table 1.

DISCUSSION

The lipids of *L. casei* A.T.C.C. 7469 were extracted from whole cells with chloroform-methanol mixtures and the glycolipid fraction was isolated by column chromatography. Hydrolysis with alkali yielded two glycosides of glycerol and a mixture of fatty acids. Analysis of the fatty acids (Table 1) showed that palmitic acid, octadecenoic acid and lactobacillic acid accounted for almost 90% of the total, and the composition was therefore similar to that of the whole lipid (Thorne & Kodicek, 1962). The water-soluble glycosides were separated by paper chromatography into two components, glycosides A and B, in the ratio 3:1; both gave galactose, glucose and glycerol on acid hydrolysis. Degradative studies have established the structure *O*- α -D-galactopyranosyl-(1→2)-*O*- α -D-glucopyranosyl-(1→1)-glycerol (I) for glycoside A, which is therefore identical with the glycoside previously isolated from *Pneumococcus* type I (Brundish *et al.* 1965). Further confirmation of the identity of the two glycosides was obtained by gas-liquid chromatography. Both glycosides as their trimethylsilyl derivatives had the same retention time as synthetic *O*- α -D-galactopyranosyl-(1→2)-*O*- α -D-glucopyranosyl-(1→1)-D-glycerol. This method for the analysis of both monoglycosyl- and diglycosyl-glycerols has been developed in this Laboratory by Dr D. E. Brundish (unpublished work).

Glycoside B, which contained glucose and galactose in the ratio 2:1, had a much lower mobility on paper chromatography than had glycoside A, suggesting that it was a larger molecule. Like glycoside A, it gave a purple colour rapidly with the periodate-Schiff reagents, indicative of a 1-substituted glycerol. Treatment with α -glucosidase yielded glucose and glycoside A, which suggested that it was a glucosylgalactosylglucosylglycerol. Partial acid hydrolysis gave small amounts of glucose and galactose, together with 1-*O*- α -D-glucopyranosylglycerol and a glucosylgalactose. Oxidation of the glucosylgalactose with 10 mM-sodium periodate, followed by reduction with sodium borohydride and acid hydrolysis, gave glycerol and no other polyols, indicating a (1→2)- or a (1→6)-linkage between the two sugars. The disaccharide could be readily distinguished from a synthetic sample of *O*- α -D-glucopyranosyl-(1→2)-D-galactopyranose by its mobility on paper chromatography and by its colour reaction with the periodate-Schiff reagents, thereby suggesting the alternative structure *O*- α -D-glucopyranosyl-(1→6)-D-galactopyranose. The evidence outlined above is

Table 1. *Analysis as their methyl esters of the fatty acids produced by hydrolysis of the glycolipids with alkali*

Fatty acid	Composition (%)
C ₁₄ :0 acid	2
C ₁₆ :0 acid	37
C ₁₆ :1 acid	3
C ₁₈ :0 acid	3
C ₁₈ :1 acid	34
C ₁₉ cyclopropane acid	17

summarized in Scheme 1 and indicates that the structure of glycoside B is *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol (II).

The configuration of the glycerol residue in both glycosides has not been established with certainty, although the synthetic reference compounds used in the gas-liquid-chromatographic analysis all had the D-configuration. The glycerol residue in the galactosylglucosylglycerol from *Pneumococcus* type I was shown to have the D-configuration with glycosyl substituents at position 1 (Brundish *et al.* 1965), and it is reasonable to suppose that the glycosides from *L. casei* will also have this configuration.

The biosynthesis of diglycosyl diglycerides involves the stepwise transfer of sugar units from the appropriate nucleotide precursor first to an $\alpha\beta$ -diglyceride and then to the monoglycosyl diglyceride (Lennarz & Talamo, 1966; Kaufman, Kundig, Distler & Roseman, 1965). The nature of the sugar linkages in glycosides A and B suggest that the glucosylgalactosylglucosyl diglyceride is synthesized by a further transfer of glucose from UDP-glucose to the galactosylglucosyl diglyceride. The occurrence of triglycosyl diglycerides is uncommon, as most Gram-positive bacteria usually contain only

diglycosyl diglycerides (Brundish *et al.* 1966). This unusual phenomenon is apparently characteristic of the lactobacilli, since *L. plantarum* 17-5 has been shown to contain a glucosylgalactosylglucosyl diglyceride (Brundish *et al.* 1966) and other work in progress in this Laboratory has established the presence of these larger compounds in both *L. plantarum* N.C.I.B. 7220 and *L. helveticus* N.C.I.B. 8025.

No evidence has yet been obtained to support earlier suggestions that glycosyl diglycerides might be intermediates in the transfer of sugar residues during the biosynthesis of polysaccharides, although the occurrence of these larger compounds would be consistent with such a function. However, the recent discovery of a polyisoprenoid pyrophosphate intermediate in the biosynthesis of both peptidoglycans (muropeptides) (Higashi, Strominger & Sweeley, 1967) and lipopolysaccharides (Wright, Dankert, Fennessey & Robbins, 1967) makes this hypothesis even more unlikely. Preliminary experiments designed to see whether extraction of cells with more polar solvents would remove more hydrophilic glycolipids with more extended oligosaccharide chains have so far failed. The rate of accumulation of these compounds during cell growth also remains to be established. On the

Scheme 1. Degradative sequence establishing the structure of the triglycosylglycerol.

other hand, Brundish, Shaw & Baddiley (1967) have suggested that the hydrophilic part of the glycolipids form regions (pores) in the membrane through which small molecules, e.g. water, may

pass. The presence of variable amounts of trisaccharide chains in these regions may represent a mechanism whereby, in the lactobacilli at least, the size of these hypothetical regions may be regulated.

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