The Glycolipids from the Non-Capsulated Strain of *Pneumococcus* I-192R, A.T.C.C. 12213

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1. The total lipid was extracted from the non-capsulated strain of *Pneumococcus* I-192R, A.T.C.C. 12213, with chloroform-methanol mixtures. Two glycolipids were isolated by chromatography on silicic acid and DEAE-cellulose (acetate form). 2. The major glycolipid was obtained pure in a yield of 640 mg./34 g. dry wt. of cells and represents about 34% of the total lipid. It contained galactose, glucose, glycerol and fatty acid ester residues in the proportions 1:1:1:2, and yielded on saponification a crystalline non-reducing glycoside. 3. The structure of the glycoside was shown to be *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-D-glycerol. The fatty acids obtained on saponification were identified by gas-liquid partition chromatography of their methyl esters. 4. The minor glycolipid was obtained as a 1:1 (w/w) mixture with the major component, but after saponification the two glycosides were separated by paper chromatography. Evidence was obtained for the structure of the glycoside derived from the minor glycolipid as 1-*O*- α -D-glucosylglycerol. 5. A general method is described for determining the stereochemistry of the glycerol moiety in 1-linked glycerol glycosides.

It has been shown that glycolipids of the dihexosyl diglyceride type are widely distributed in Gram-positive bacteria (Brundish, Shaw & Baddiley, 1965a). We have investigated the structure of the dihexosyl diglyceride in the non-capsulated strain of *Pneumococcus* I-192R, A.T.C.C. 12213, and established it as 2,3-diacyl-1- [*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl]-D-glycerol. A glucosyl diglyceride present in small amounts in this organism gave on saponification a glycoside characterized as 1-*O*- α -D-glucosylglycerol. A preliminary account of this work has been published (Brundish, Shaw & Baddiley, 1965b).

Kaufman, Kundig, Distler & Roseman (1965) have independently proposed identical structures for the glycolipids of *Pneumococcus* type XIV. These authors demonstrated the utilization of the glucosyl diglyceride in the enzymic synthesis of the galactosylglucosyl diglyceride. A possible role for these compounds in the biosynthesis of polysaccharides has been suggested (Distler & Roseman, 1964).

EXPERIMENTAL AND RESULTS

Materials

Pneumococcus A.T.C.C. 12213 was obtained from Dr C. M. MacLeod and grown here by Dr A. L. Davison. Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden, DEAE-cellulose from H. Reeve Angel and Co. Ltd., London,

silicic acid 'for chromatography' from L. Light and Co. Ltd., Colnbrook, Bucks., and 'Mallinckrodt 100 mesh' silicic acid from Kodak Ltd., Kirkby, Lancs.; Glucostat and Galactostat reagents were from Worthington Biochemical Corp., Freehold, N.J., U.S.A., NADH was from Sigma Chemical Co., St Louis, Mo., U.S.A., almond-emulsin β -glucosidase was from L. Light and Co. Ltd., and fatty acid methyl esters were from British Drug Houses Ltd., Poole, Dorset, Sigma Chemical Co. and California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Crude yeast hexokinase was a gift from Sigma Chemical Co. Solvents for chromatography were purified and dried before use.

Analytical methods

Phosphorus was determined by the method of Chen, Toribara & Warner (1956), fatty acid esters by the method of Rapport & Alonzo (1955), hexoses (before and after acid hydrolysis) by the phenol-H₂SO₄ method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) and (after hydrolysis) by the Glucostat and Galactostat reagents. Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954), glycerol, glyceric acid and formaldehyde were determined by the procedure of Hanahan & Olley (1958) and formic acid was measured by a modification of the method of Goldstein & Whelan (1962) according to information kindly supplied by Professor W. J. Whelan.

Paper chromatography

Whatman no. 1 and no. 4 papers, previously washed with 2*N*-acetic acid and then water, were used with the following solvent systems: *A*, propan-1-ol-aq. NH₃ (sp.gr. 0.88)-

water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); *C*, butan-1-ol-pyridine-water-benzene (5:3:3:1, by vol.) (Albon & Gross, 1950); *D*, butan-1-ol-ethanol-water-aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol.; organic phase) (Hirst, Hough & Jones, 1949); *E*, pyridine-ethyl acetate-water (2:5:5, by vol.; organic phase) (Sastri & Kates, 1964).

Compounds were detected by the periodate-Schiff reagents for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956) and the alkaline AgNO_3 reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950).

Growth of organisms

Bacteria were grown on a medium containing 3.7% (w/v) of Difco brain-heart infusion, 0.1% of Difco Neopeptone and 1% of glucose in water. Batches of medium (6l., without glucose) were inoculated with portions (10 ml.) of a young culture, and after incubation for 12 hr. at 37° sterile 50% (w/v) glucose solution was added to give a final concentration of 1%, together with 0.5% phenol red indicator (6 ml.). 3*N*-NaOH was added to the culture at half-hour intervals to restore neutrality, and when 36 ml. of 3*N*-NaOH/l. of culture had been consumed the cells were harvested in a Sharples refrigerated centrifuge. From 1 l. of culture, 3.5 g. of wet cells was obtained. They were washed by suspending in 0.9% NaCl, resuspended in water and freeze-dried. From 100 g. of wet cells 23 g. of dry cells was obtained.

Isolation and purification of glycolipids

Freeze-dried cells (34 g.) were stirred at room temperature for three consecutive 2 hr. periods with portions (500 ml.) of chloroform-methanol (2:1, v/v), the cells being recovered by filtration. The combined extracts were dried by rotary evaporation at room temperature, dissolved in the minimum volume of chloroform-methanol-water (40:20:3, by vol.) and non-lipid contaminants removed by passing the solution through a column (30 cm. $\times$ 1.5 cm.) of Sephadex G-25 (fine grade) that was then washed with chloroform-methanol (2:1, v/v) (Wells & Dittmer, 1963). The solution containing lipids was evaporated to dryness and dried thoroughly over silica gel *in vacuo* at 37°. The weight of purified lipid was 1.96 g. This was applied to a column (25 cm. $\times$ 2 cm.) of silicic acid [Light's silicic acid-Mallinckrodt silicic acid (1:1, w/w), packed under a N_2 pressure of 0.14 kg./cm.²] in chloroform containing 2% (v/v) of methanol, and material was eluted under N_2 at 0.14 kg./cm.² with successively 300 ml. of 2% (v/v), 150 ml. of 3% (v/v), 200 ml. of 4% (v/v), 100 ml. of 5% (v/v), 200 ml. of 10% (v/v), 200 ml. of 20% (v/v) and 200 ml. of 15% (v/v) methanol in chloroform and finally pure methanol (200 ml.). Fractions of volume 15 ml. were collected. Samples (0.1 ml.) were analysed for phosphorus. Appropriate fractions were combined and evaporated to dryness at 20° in a rotary evaporator. The residues were dissolved in chloroform-methanol mixtures to give solutions containing 50 mg./ml., which were kept under N_2 at 2° in the dark in glass-stoppered tubes. Hydrolysis of samples (0.05 ml.) by acid and alkali, followed by paper chromatography in solvent *B*, showed the mono- and di-hexosyl diglycerides to be mainly in the 5% and 10% methanol eluates respectively. These fractions were combined,

evaporated to dryness and redissolved in 5% (v/v) methanol in chloroform. The solution was applied to a column (20 cm. $\times$ 2 cm.) of DEAE-cellulose (acetate form) prepared in 5% (v/v) methanol in chloroform as described by Rouser, Bauman, Kritchevsky, Heller & O'Brien (1961). Elution was effected with portions (600 ml.) of the following increasing concentrations (v/v) of methanol in chloroform: 5%, 10%, 20%, 33% and finally with 33% methanol in chloroform (600 ml.) containing ammonium acetate (2.4 g.). Fractions (40 ml.) were collected at a flow rate of 4 ml./min. and portions (0.2 ml.) were removed for determination of fatty acid ester and total phosphorus. A small amount of fatty acid ester was observed in the 5% methanol eluate and a large amount in the 10% methanol eluate. Phosphorus was present only in the fractions collected after the application of ammonium acetate solution to the column. The fractions containing the two glycolipids were combined appropriately and evaporated to dryness. Samples (1 mg.) of the residues were saponified and the water-soluble products were examined on paper; the major component gave only galactosylglucosylglycerol whereas the minor component gave a mixture of this with glucosylglycerol. A yield of 640 mg. of galactosylglucosyl diglyceride was obtained. The lipid was colourless, soluble in chloroform or methanol with difficulty, but dissolved readily in mixtures of these two solvents. It was insoluble in cold but soluble in hot acetone.

Examination of glycolipids

Analysis. Galactosylglucosyl diglyceride (about 5 mg.) was dissolved in chloroform (10 ml.) containing 10% (v/v) of methanol. Samples (1 ml.) were analysed for fatty acid ester, with methyl palmitate as a reference compound. Samples (0.2 ml.) were analysed for hexose after the following treatment: 0.4*N*-sodium methoxide in methanol (0.05 ml.) was added to the sample in a boiling tube and deacylation was allowed to proceed for 10 min. at room temperature (Marinetti, 1962); the tubes were placed in a vacuum desiccator and solvents removed by a water pump; the residue was dissolved in water (1 ml.) and hexoses were determined in the normal manner. The fatty acid ester:hexose ratio was 0.82:1.00. The minor glycolipid was not obtained sufficiently pure for similar analysis.

Alkali-hydrolysis products. Galactosylglucosyl diglyceride (640 mg.) was heated in 0.1*N*-NaOH (60 ml.) at 100° for 1 hr. under a stream of N_2 ; at the end of this time all material had dissolved. The solution was cooled and adjusted to pH 1 with 4*N*-HCl, and fatty acids were removed by extraction with portions (3 $\times$ 50 ml.) of light petroleum (b.p. 60–80°). The extracts were combined, washed with water and dried over anhydrous Na_2SO_4 . The drying agent was removed by filtration and washed with a little dry light petroleum. The combined filtrate and washings were evaporated under reduced pressure, giving 332 mg. of fatty acids, which were kept *in vacuo* at room temperature. The aqueous solution was passed through columns of Dowex 1 (OH^- form) resin (20 ml.) and Dowex 50 (H^+ form) resin (20 ml.), and the eluate was evaporated to dryness. The clear colourless gum (262 mg.) crystallized on trituration with diethyl ether. A sample was recrystallized from hot moist ethanol for analysis.

The galactosylglucosylglycerol had m.p. 165° (with softening) and $[\alpha]_D^{25} + 176 \pm 5^\circ$ (c 4.4 in water) (Found: C, 43.1;

Table 1. *Paper chromatography of water-soluble saponification products of glycolipids*

Solvents A, B, C and E are described in the text.

	R_{glycerol}	R_{glucose}		
	Solvent A	Solvent B	Solvent C	Solvent E
Glycerol	1.00	—	—	—
Glucose	—	1.00	1.00	1.00
1-O-Glucosyl-glycerol	0.86	1.02	0.95	0.92
2-O-Glucosyl-glycerol	—	1.14	—	—
Galactosylglucosylglycerol	0.71	0.43	0.40	0.47

H, 6.9. $\text{C}_{15}\text{H}_{28}\text{O}_{13}$ requires C, 43.2; H, 6.8%). The glycoside behaved as a single compound on paper chromatography in solvents A, B, C and E (Table 1) and rapidly gave the purple colour characteristic of formaldehyde produced from a 1-substituted glycerol with the periodate-Schiff reagents (Roberts, Buchanan, & Baddiley, 1963); the purple changed to blue during 48 hr. The glycoside was also detected by a modification of the alkaline AgNO_3 procedure of Trevelyan *et al.* (1950). After the paper had been dipped in the acetone containing AgNO_3 and then in ethanolic NaOH solution, it was allowed to hang until all surplus ethanol had evaporated (about 5 min.). The glycoside was just visible as a darkish spot at this stage. The paper was then passed through steam, when the colour intensified to give a normal reaction.

The minor glycolipid from the DEAE-cellulose (acetate form) column was hydrolysed in alkali and products were isolated as described above. A colourless gum (8 mg.) was obtained and shown by chromatography in solvent B to be a mixture of glucosylglycerol and the galactosyl glucoside. The material was applied as a band (12 cm.) to the origin of a Whatman no. 1 paper (previously washed with solvent B) and developed (descending) for 16 hr. in solvent B. The glycosides were eluted from appropriate areas of the paper with water. From the faster-running band glucosylglycerol (2.5 mg.) was obtained as a gum. It was chromatographically indistinguishable in solvents B and C from synthetic 1-O- α -D-glucopyranosylglycerol (prepared by Dr F. E. Hardy); both samples rapidly gave a purple colour with the periodate-Schiff reagents. By contrast, 2-O- α -glucopyranosylglycerol reacted slowly, giving a blue colour.

Fatty acid composition of the major glycolipid. The fatty acids obtained by extraction of the saponification mixture (332 mg.) with light petroleum were dissolved in anhydrous light petroleum (b.p. 60–80°) (150 ml.) and converted into their methyl esters by the method of Hornstein, Alford, Elliott & Crowe (1960). The esters were analysed by gas-liquid chromatography both before and after catalytic hydrogenation. Analyses were performed on a column (150 cm.) of polyethylene glycol adipate maintained at 185° with a Pye Panchromatograph. The esters were applied in light petroleum (b.p. 60–80°) (100 mg./ml.), and N_2 was used as carrier at a rate of 60 ml./min. at 0.7 kg./cm.². Samples (1 μ l.) were applied to the column with a Hamilton micro-syringe. Authentic methyl esters and their mixtures

Table 2. *Retention times on gas-liquid chromatograms of standard fatty acid methyl esters and those derived from the glycolipid*

Conditions of chromatography are given in the text. The fatty acid methyl esters are designated $x:y$, where x is the no. of carbon atoms/fatty acid mol. and y is the no. of double bonds/fatty acid mol. Times are given in minutes.

Methyl esters	Standard	Glycolipid	Glycolipid (hydrogenated)
12:0	4.60	4.65	4.50
14:0	7.75	7.85	7.50
14:1	—	8.65	—
16:0	13.50	13.90	13.50
16:1	—	15.65	—
18:0	25.15	26.40	26.00
18:1	27.65	28.60	—

Table 3. *Percentage composition of fatty acid methyl ester mixture from glycolipid*

The designation of the fatty acid methyl esters is as indicated in Table 2.

Methyl ester	Before hydrogenation	After hydrogenation
12:0	11.4	17.1
14:0	12.1	16.7
14:1	5.0	—
16:0	33.1	55.1
16:1	26.1	—
18:0	4.6	11.1
18:1	7.7	—
	100.0	100.0

were also analysed for reference purposes. Retention times of the standards and the components of the mixtures under investigation are given in Table 2. Percentage composition of the mixtures was determined by cutting out the peaks of the trace and weighing the paper pieces, with correction for the molecular weights of the separate esters. The results are given in Table 3.

Analysis and degradation of glycosides

Acid hydrolysis. Samples (200 μ g.) of the glycosides were hydrolysed (2N-HCl for 1 hr. at 100°), and paper chromatography of the hydrolysates in solvents B and C showed the presence of galactose, glucose and glycerol from the slower-running glycoside and glucose and glycerol from the faster-running glycoside.

Solutions of galactosylglucosylglycerol (22 mg./ml.) and the glucosylglycerol (8 mg./ml.) were prepared. Portions (0.1 ml.) were hydrolysed with 2N-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 total hexose was determined by the phenol- H_2SO_4 procedure; glycerol was determined by the chromotropic acid method. Galactosylglucosylglycerol gave the D-glucose: D-galactose:glycerol:total hexose proportions as 1.00:1.04:

1:27:1:93, and glucosylglycerol gave the D-glucose:glycerol ratio as 1:00:1:15.

Partial acid hydrolysis. A sample (200 μ g.) of galactosylglucosylglycerol was hydrolysed in 0.015N-HCl for 5 hr. at 100° and the hydrolysate was examined by paper chromatography in solvent B. Detection was by the modified AgNO₃ procedure. Before the paper was steamed, galactose was the only visible product; after the steaming, unchanged material and glucosylglycerol were observed. The hydrolysis was repeated with 3 mg. of material and the reaction mixture was applied as a band to a Whatman no. 1 paper; descending chromatography was carried out for 60 hr. in solvent B and the glucosylglycerol was recovered by elution. It behaved identically with both synthetic 1-O- α -D-glucosylglycerol and the glycoside isolated from the minor glycolipid on paper chromatography in solvent B. Examination of an acid hydrolysate by paper chromatography showed only glucose and glycerol.

Oxidation of galactosylglucosylglycerol with periodate. A portion of glycoside solution (1 ml., containing about 2.5 mg. of material) was added to 20 mM-NaIO₄ solution (2.5 ml.) in a standard flask and the mixture was diluted to 5 ml. with water. Another portion of the solution of glycoside (1 ml.) was diluted to 50 ml. and samples (1 ml.) were withdrawn for the determination of hexose. The oxidation mixture was kept at room temperature in the dark and samples (0.1 ml.) were withdrawn periodically and diluted to 10 ml. with water for analysis. Oxidation was complete after 72 hr., 3.96 mol. prop. of periodate being reduced. A sample (0.5 ml.) of the oxidation mixture was adjusted to 1 ml. with water and formaldehyde was determined: 0.94 mol. prop. of formaldehyde was formed after 72 hr.

Crystalline glycoside (9.3 mg.) was oxidized with 50 mM-NaIO₄ solution (5 ml.) overnight. Ethylene glycol (0.5 ml.) was added and, after 30 min., 10% (w/v) KI solution (2 ml.) was added. The I₂ liberated was determined by titration with 5 mM-Na₂S₂O₃ solution: 0.84 mol. prop. of formic acid was produced. All solutions in this experiment were made with water freed from CO₂.

Oxidation with 0.1 mM-sodium periodate. A portion of the glycoside solution (1 ml.) and 20 mM-NaIO₄ solution (2.5 ml.) were diluted to 500 ml. and periodate was determined at intervals. No obvious discontinuity in the rate curve was observed, but the rate of reaction decreased significantly after 1 mol. prop. of periodate had been reduced.

Oxidation with periodate and reduction with borohydride. A sample of glycoside (2.5 mg.) was oxidized with 10 mM-NaIO₄ as described above. Samples were withdrawn after 0.5, 24 and 72 hr. and NaBH₄ (10 mg.) was added to each. After 16 hr. at 2° a few drops of acetic acid were 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 2N-HCl and heated at 100° for 3 hr. After neutralization with Dowex 2 (HCO₃⁻ form) resin water was removed by cautious evaporation and the products were examined by paper chromatography in solvent B. After 0.5 hr., glucose, galactose and ethylene glycol were detected, whereas after 24 hr. glucose, glycerol and ethylene glycol were present; after 72 hr., only glycerol and ethylene glycol were detected.

Studies with almond emulsin. A sample of the galactosyl-

glucosylglycerol (0.5 mg.) was incubated at 37° for 16 hr. with a 1% (w/v) solution of almond-emulsin β -glucosidase (0.2 ml.). The hydrolysate was examined by paper chromatography in solvent B. A trace of unchanged material and higher transglycosylation products were detected but the major products were galactose and glucosylglycerol. The experiment was repeated with the addition of a saturated Ag₂O solution (0.1 ml.) to inhibit α -galactosidase activity (Hestrin, Feingold & Schramm, 1955). Negligible hydrolysis was observed.

The glucosylglycerol obtained from the minor glycolipid was unaffected by the almond emulsin.

Glyceric acid from the glycerol moiety. A sample of galactosylglucosylglycerol (10 mg.) was treated with a 2% (w/v) solution of almond emulsin (1 ml.) at 37° for 16 hr. The hydrolysate was passed through a small column (1 ml.) of Dowex 1 (OH⁻ form) resin to remove free galactose and the eluate was evaporated *in vacuo* to a small volume. The solution was transferred to a tube (6 cm. x 1 cm.) bearing a B10 ground-glass socket, freeze-dried and oxidized by NO₂ (130 mg.) in an apparatus similar to that described by Nevell (1963).

Oxidation was allowed to proceed at room temperature for 24 hr. and then the system was evacuated for 15 min. with an oil pump to remove excess of NO₂. The oxidized material was dissolved in a few drops of water and the solution adjusted to pH 7 with N-NH₃. The solution was transferred to a graduated tube, the volume adjusted to 1 ml. and 100 mM-NaIO₄ (1 ml.) was added. The mixture was kept at room temperature in the dark for 24 hr., 0.1 M-ethylene glycol (1 ml.) was added, and after 30 min. a freshly prepared solution of 1,1-dimethylhydrazine (1%) in dilute acetic acid (1 ml.) at pH 6 was added (modified procedure of Barry, 1943). The mixture was kept at 37° for 16 hr. and extracted with several portions (1 ml. each) of chloroform. The solution was passed through a small column (1 ml.) of Dowex 50 (NH₄⁺ form) resin and the eluate evaporated to a small volume. The material was applied as a band to a Whatman no. 4 paper (previously washed in solvent D) and the chromatogram was developed (descending) for 16 hr. in solvent D. The glyceric acid was eluted from the paper with water, and a drop of N-NH₃ was added to the eluate, which was evaporated carefully to dryness. The residue (368 μ g., 14.3% yield) was immediately dissolved in water (1 ml.) and the solution was stored at -40°.

Glyceric acid in the solution was determined by the chromatotropic acid procedure (0.1 ml. sample) and the configuration was determined (0.3 ml. sample) by the procedure of Archibald, Buchanan & Baddiley (1961), which involves phosphorylation and enzymic assay of glyceric acid phosphate with NADH and a preparation of enzymes from rabbit muscle. Authentic D-glyceric acid oxidized 1.96 mol. prop. of NADH and that prepared by the oxidation sequence described above applied to synthetic D-glucosyl-D-glycerol oxidized 0.93 mol. prop. of NADH. The glyceric acid obtained from the galactosylglucosylglycerol oxidized 0.006 mol. prop. of NADH.

DISCUSSION

Glycolipids are important components of the cells of the rough strain of *Pneumococcus* type I (1.97% of the dry weight), and account for 34.3% of the weight of the total extractable lipid of the organism.

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Scheme 1. Course of oxidation of the galactosyl glucoside with periodate, demonstrated by paper chromatography.

Two glycolipids have been separated from the neutral fats and phospholipids and have been shown to be present in a ratio of about 100:1 (w/w). The major glycolipid was obtained pure; it contained D-glucose, D-galactose, glycerol and fatty acid ester residues in the approximate proportions 1:1:1:2. Saponification yielded a non-reducing glycoside as the only water-soluble product and this was composed of equimolar quantities of D-glucose, D-galactose and glycerol. The rapidly developing purple

colour given by this compound with the periodate-Schiff reagents and the destruction of the glycerol residue after 30 min. by 10mM-periodate indicated that glycerol was substituted at its 1-position. Partial acid hydrolysis of the glycoside gave galactose and glucosylglycerol, thus establishing the sequence galactosylglucosylglycerol.

Almond emulsin hydrolysed the glycoside, giving galactose and glucosylglycerol. As almond emulsin contains galactosidases but no α -glucosidase, this

indicated that the glucosidic linkage to glycerol had the α -configuration. The high positive rotation of the compound suggested that the galactosyl residue also had the α -configuration; this was confirmed by inhibiting the α -galactosidase activity of the preparation by silver oxide, when the glycoside was not attacked.

Quantitative studies with periodate showed that nearly 4 mol.prop. of periodate was reduced by the glycoside, with the formation of approx. 1 mol.prop. of formaldehyde and of formic acid. For a 1-substituted glycerol derivative these results indicate a structure in which both sugars are in the pyranose form and the linkage between them is (1 $\rightarrow$ 2) or (1 $\rightarrow$ 4). Reduction of the fully oxidized glycoside followed by acid hydrolysis gave no erythritol, which would be expected from a 4-substituted glucose moiety, and so the structure of the glycoside must be *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 2)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 1)-glycerol. Paper-chromatographic studies of the course of oxidation with periodate showed a definite sequence of oxidation that was not evident from the rate curve (Scheme 1). A structurally analogous situation occurs in certain glycerol teichoic acids, where a terminal 1-substituted glycerol residue occurs together with hexose residues elsewhere in the polymer chain. Thus, with the intracellular teichoic acid from *Lactobacillus arabinosus* 17-5, after removal of alanine ester residues 0.1 mm-periodate oxidized the terminal glycerol rapidly at first and this was followed by a steady rate of oxidation of the less sterically accessible *trans*-diol groups in the glucose rings. The difference in rates for the two stages of oxidation was large enough for the method to be developed for the quantitative determination of terminal glycerol residues (Critchley, Archibald & Baddiley, 1962). With the glycoside from the glycolipid, the galactopyranosyl unit possesses a *cis*-diol grouping that was oxidized at a rate intermediate between that for the glycerol end group and the glucose *trans*-diol grouping, and the overall effect was to produce a smooth rate curve from which no extrapolation was possible; the rapid oxidation of the glycerol group corresponded to an initial reduction of about 1 mol.prop. of periodate, after which the rate decreased steadily. Clearly the sequence in Scheme 1 does not represent the actual course of oxidation, as some merging of the separate steps would be expected to occur, but the sequence can be demonstrated, nevertheless, by examination of the oxidation mixture at intervals. The products were identified by reduction with sodium borohydride followed by hydrolysis in acid and paper chromatography of resulting polyols.

Less detailed evidence was obtained for the structure of the glycoside obtained from the minor glycolipid as 1-*O*- α -D-glucosylglycerol. With the

periodate-Schiff reagents it rapidly gave a purple colour, which slowly turned blue. It was resistant to hydrolysis by almond emulsin.

Glycolipids identical with those described above have been isolated independently and simultaneously by Kaufman *et al.* (1965) from a capsulated strain of *Pneumococcus* type XIV. They proposed identical structures for their glycosides, except that the configuration of the glycerol residues was not stated. Confirmation of the 2-linkage to glucose and the demonstration of substitution at the 2- and 3-positions by fatty acid ester residues was obtained from methylation studies. The presence of two fatty acid ester residues suggests, on biosynthetic grounds, that these glycolipids belong to the class of natural diglycerides that are widely distributed in the plant kingdom and were first investigated by Carter, McCluer & Slifer (1956). They established the structures 2,3-diacyl-1-[*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactopyranosyl]-D-glycerol and the corresponding monogalactosyl diglyceride for two lipids from wheat flour (Carter, Hendry & Stanacev, 1961). In each case the configuration of the glycerol unit was determined by comparison of the infrared spectrum in the region 730-960 cm.⁻¹ with those of synthetic stereoisomers prepared by Wickberg (1958). However, the infrared spectrum of the *Pneumococcus* glycoside was unhelpful.

Benson & Miyano (1961) used nitrogen dioxide to prepare L-glyceric acid from the glycerol unit of digalactosylglycerol. In our case, however, the free 6-position of the glucose unit would also be oxidized by nitrogen dioxide and the glycosidic linkage in the resulting uronic acid derivative would be stable towards hydrolysis with acid. This difficulty was overcome by oxidation of the presumed glucuronylglyceric acid derived from the glycoside with periodate at pH 7 and subsequent elimination of the glyceric acid with 1,1-dimethylhydrazine at pH 6. At pH 7 uronic acids show a maximum rate of oxidation with periodate (Edington, Hirst & Percival, 1955) whereas α -hydroxy acids show a minimum rate of oxidation at pH 6.5 (Fleury & Courtois, 1948). To use periodate and dimethylhydrazine to eliminate the glyceric acid, the 2-position of the glucose unit must be unsubstituted, so the first step in the reaction sequence was the removal of the galactose residue by hydrolysis with almond emulsin (Scheme 2). Of the glyceric acid obtained from galactosylglucosylglycerol 99.7% was in the L-form, and 46.5% of that from synthetic D-glucopyranosyl-DL-glycerol was D-glyceric acid. This combined chemical and enzymic method for determining the configuration of the 1-substituted glycerol unit in galactosylglucosylglycerol is conveniently carried out on 10 μ moles of glycoside and could be applied directly to the determination of the configuration of glycerol residues in 1-substi-

Scheme 2. Formation of L-glyceric acid from galactosyl-glucosyl-(1→1)-D-glycerol.

tuted aldopyranosyl- and aldofuranosyl-glycerols, their 4-, 5- or 6-substituted derivatives, and, as in the present case, to 2- or 3-substituted derivatives from which the simpler glycerol monoglycosides could be obtained by partial acid or enzymic

hydrolysis. The reaction sequence, but not the enzymic assay, has been successfully carried out on as little as $2\mu\text{moles}$ of material.

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Note added in proof. Subsequent work has shown that preliminary removal of galactose from the glycoside is not essential for the formation of glyceric acid by the reaction sequence described. Moreover, phenylhydrazine may be used instead of dimethylhydrazine; in this case the mechanism presumably involves elimination of C-4 to C-6 of the original glucose residue followed by hydrolysis of the resulting extremely labile hemiacetal. Thus the method should be applicable to all glycosides of glycerol in which the sugar attached to glycerol contains a glycol grouping.

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