Biochem. J. (1972) **127**, 399–409
Printed in Great Britain

The Lipid–Teichoic Acid Complex in the Cytoplasmic Membrane of *Streptococcus faecalis* N.C.I.B. 8191

By P. TOON, P. E. BROWN and J. BADDILEY
Microbiological Chemistry Research Laboratory, School of Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(*Received* 30 *November* 1971)

1. A lipid–teichoic acid complex was isolated from *Streptococcus faecalis* N.C.I.B. 8191. The covalent nature of the linkage between teichoic acid and lipid was established. 2. The complex exhibits macromolecular properties in solution, and ultracentrifugation studies show that these are due to micelle formation. 3. From chemical studies it is concluded that the teichoic acid is a poly(glycerol phosphate) in which some of the glycerol hydroxyl groups possess kojibiosyl [2-*O*-α-D-glucopyranosyl-(1→2)-α-D-glucopyranosyl] substituents, together with D-alanine ester residues. 4. The lipid is 1-kojibiosyl diglyceride, already known as a membrane component of this organism, with probably a phosphatidyl substituent. The phosphatidyl kojibiosyl diglyceride is attached to the teichoic acid through a phosphodiester linkage, and the chain of the teichoic acid contains 28–35 units. 5. Although the complex represents the whole of the membrane teichoic acid in this organism, only about 12% of the membrane glycolipid is associated with teichoic acid. 6. Two phosphatidyl glycolipids, closely resembling that bearing the teichoic acid, were isolated from the lipids of the organism and were partly characterized.

Glycerol teichoic acids of the type poly(glycerol phosphate) are believed to occur in association with the cytoplasmic membrane of all Gram-positive bacteria and these compounds, formerly called 'intracellular teichoic acids', have more recently been given the name 'membrane teichoic acids' (Archibald & Baddiley, 1966); their main function is related to their ability to bind bivalent cations required for maintaining the correct physical and enzymic activities of the membrane (Heptinstall *et al.*, 1970; Hughes *et al.*, 1971). In strains of *Streptococcus faecalis* the serological-group-D-specific substance is the membrane teichoic acid (Wicken *et al.*, 1963), and its structure is that of a poly(glycerol phosphate) to which are attached 2-*O*-α-D-glucopyranosyl-α-D-glucopyranosyl (kojibiosyl) or kojitriosyl residues and D-alanine ester residues (Wicken & Baddiley, 1963). Although it was shown that this polymer is located in, or on the outer surface of, the cytoplasmic membrane (Hay *et al.*, 1963; Shattock & Smith, 1963; Shockman & Slade, 1964; Smith & Shattock, 1964), the nature of the association was unknown.

Much of the work on the structure of membrane teichoic acids has been done on material extracted from disrupted cells by methods employing acidic conditions, and separation from other cell components is likely to have involved the hydrolysis of acid-labile linkages. When disrupted cells are treated with phenol–water, the membrane teichoic acid and nucleic acids are recovered from the aqueous phase, thereby affording a convenient fractionation (Burger & Glaser, 1964). The suggestion (Archibald *et al.*, 1968) that in such preparations the linkage to other membrane components should remain intact has been followed up by a study of teichoic acid that had been extracted by this method from lactobacilli (Wicken & Knox, 1970; Knox & Wicken, 1971). Such preparations contained both teichoic acid and membrane lipids, and have been called 'lipoteichoic acids'. Although the lipid components could not be removed by organic solvents, and the preparations behaved as homogeneous entities in agar-gel diffusion studies with specific antisera, the nature of the association between lipids and teichoic acid was not established.

In the present paper the isolation of a lipoteichoic acid from *S. faecalis* N.C.I.B. 8191 is described. All of the membrane teichoic acid is believed to occur in this form, and it is shown that the glycerol phosphate chain is covalently attached, presumably through its terminal phosphate group, to a diglucosyl diglyceride identical with the main diglucosyl diglyceride of the membrane; it is likely that a phosphatidyl residue is also attached to the glycolipid.

Experimental

Materials

Calf intestinal phosphomonoesterase, deoxyribonuclease and ribonuclease were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Sepharose 6B and Sephadex G-200 gels and Blue Dextran were obtained from Pharmacia, Uppsala, Sweden, and DE-32 cellulose from British Drug Houses, Poole, Dorset, U.K. Mallinckrodt '100 mesh' silica was obtained from Kodak, Liverpool, U.K., Celite 560 from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and Merck silica gel PF_{254} from Anderman and Co., London S.E.1, U.K. Concanavalin A was obtained from Calbiochem, Los Angeles, Calif., U.S.A., and *Streptococcus* group D grouping serum from Wellcome Laboratories, Beckenham, Kent, U.K. Glucostat was obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. A sample of synthetic *O*-α-D-glucopyranosyl-(1→2)-D-glycerol was kindly provided by Professor J. G. Buchanan.

Methods

Analytical methods. Phosphate was determined by the method of Chen *et al.* (1956), glucose by Glucostat, hexose by the phenol–H_2SO_4 method of Dubois *et al.* (1956) and glycerol by the chromotropic acid procedure of Hanahan & Olley (1958). RNA was determined by the method of Ogur & Rosen (1950), DNA by the method of Burton (1956), and protein by the method of Lowry *et al.* (1951). Fatty acids were determined by a micromodification of the method of Snyder & Stephens (1959) after acid hydrolysis, extraction with diethyl ether and conversion into methyl esters with diazomethane. This procedure prevents the simultaneous analysis of ester-linked amino acids. Amino acids were determined automatically (Technicon and Jeol instruments).

Paper chromatography and t.l.c. Whatman no. 1 paper was used in the following solvent systems: *A*, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.; Hanes & Isherwood, 1949), descending; *B*, butan-1-ol–pyridine–water (6:4:3, by vol.; Jeanes *et al.*, 1951) descending. Compounds were detected by the following reagents wherever appropriate; periodate–Schiff for polyols and glycosides (Baddiley *et al.*, 1956); molybdate for phosphates (Hanes & Isherwood, 1949); alkaline $AgNO_3$ for reducing compounds (Trevelyan *et al.*, 1950).

T.l.c. plates (0.4mm thick) for qualitative analysis were prepared from a slurry of Merck silica gel PF_{254} (40g) in aq. 0.2% sodium acetate solution (100ml) and dried at 60°C overnight (Minnikin & Abdolrahimzadeh, 1971). They were developed in the following solvent systems: *C*, chloroform–methanol–water (65:25:4, by vol.); *D*, light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (70:30:3, by vol.) and sprayed with the periodate–Schiff reagents or aq. 20% (w/v) $(NH_4)_2SO_4$ followed by heating to 200°C for 30min (Walker, 1971).

Preparative t.l.c. plates (0.8mm thick) were similarly prepared from silica (60g) and 0.2% sodium acetate solution (150ml) and developed with solvent *C*. Lipids were detected by spraying with water; marked areas of silica were removed from the dried plates and the lipids were extracted with chloroform–methanol (1:1, v/v).

Gas–liquid chromatography. Fatty acids were analysed as methyl esters on a Pye–Argon chromatograph by using a column (2.1m×3.5mm) of 15% polyethylene glycol succinate on Celite (100–120 mesh) with argon as carrier gas. Glycosides were analysed as trimethylsilyl ethers on a Perkin–Elmer F11 chromatograph, with argon as carrier gas, through a column (2m×2mm) of 2.5% Silicone OV-1 on Chromosorb AW-DMCS (80–100 mesh).

Electrophoresis. High-voltage paper electrophoresis was done on Whatman no. 1 paper in a buffer (pyridine–acetic acid–water, 5:2:43, by vol.), pH5.3, at 25V/cm for 3h.

Enzymic dephosphorylation. Monophosphates were hydrolysed with a 0.01% solution of alkaline phosphomonoesterase in 0.05M-$(NH_4)_2CO_3$, pH8.9, at 37°C for 16h. The $(NH_4)_2CO_3$ was removed by repeated evaporation to dryness *in vacuo*.

Acid hydrolysis. Samples of polymer (2mg/ml) were hydrolysed in 2M-HCl for 3h at 100°C in sealed tubes. Those for the determination of the proportions glucose:hexose:phosphorus were neutralized with NaOH; for others acid was removed *in vacuo* over KOH pellets. Fatty acids were extracted with ether, converted into methyl esters with ethereal diazomethane and analysed quantitatively for individual fatty acids by g.l.c. or for total fatty acids. Glycerol/phosphorus ratios were determined after enzymic dephosphorylation of monophosphates.

Alkali hydrolysis. Samples of polymer (5mg/ml) were hydrolysed in 1M-NaOH for 3h at 100°C in sealed tubes *in vacuo*. Na^+ ions were removed by passage of solutions through short columns of Dowex 50 (NH_4^+ form) resin. Products of acid and alkali hydrolysis were examined by chromatography and electrophoresis before and after enzymic dephosphorylation. Neutral products were examined after removal of phosphates by passage of solutions through a short column of Dowex-2 (HCO_3^- form) resin.

Preparation of trimethylsilyl ethers for g.l.c. Samples of glycosides were treated with dry pyridine (six drops), hexamethyldisilazane (three drops) and trimethylchlorosilane (one drop) in a stoppered flask. After 15min, the solutions were evaporated to dryness and the residues were extracted with light petroleum (b.p. 60–80°C). The resulting solutions were filtered rapidly, then concentrated before examination by g.l.c.

Analytical ultracentrifugation. Sedimentation studies were done with a Beckman–Spinco model E

analytical ultracentrifuge in an AnD rotor operated at 20°C and 59780rev./min.

Growth of cells. Streptococcus faecalis N.C.I.B. 8191 was obtained from a culture that has been maintained in this laboratory for some years. Batches (18 litres) were grown at 37°C for 17h in a liquid medium of the following composition: Oxoid tryptone, 1%; Difco yeast extract, 0.5%; glucose, 1%; K_2HPO_4, 1%. Bacteria were harvested in a refrigerated Sharples centrifuge and washed with 0.85% NaCl at 0°C; the average yield of wet cells was 3g/l.

Preparation of crude teichoic acid. Batches of cells (120g wet wt.) were disrupted in a 50% (w/v) suspension in water by shaking for 2.5min with no. 11 Ballotini beads in a MSK–Braun cell disintegrator. The beads were removed by filtration on a no. 1 sintered-glass funnel and walls were removed by centrifugation at 16300*g* for 20min. The supernatant solution containing fragmented membrane was stirred with an equal volume of aq. 80% (w/v) phenol at 4°C for 1h. The resulting emulsion was centrifuged, then the upper aqueous layer was removed and the lower phenol layer was washed with an equal volume of water. Insoluble material at the interface of the two layers was discarded. The combined aqueous layers were stirred with an equal volume of chloroform at 4°C for 3h, then they were evaporated to a small volume at 30°C *in vacuo* and dialysed overnight against water.

The volume of non-diffusible material was adjusted to 25ml and this solution was mixed with an equal volume of 0.1M-tris–HCl buffer, pH8.0, containing 0.02M-$MgCl_2$, and was incubated with ribonuclease (2mg) and deoxyribonuclease (1mg) under toluene at 37°C. The incubation was continued for 3 days to remove ester-linked alanine from the teichoic acid. After being re-extracted with phenol followed by chloroform, the digest was dialysed against water and then freeze-dried.

Purification. The freeze-dried extract was dissolved in water (2ml) and the solution was passed through a column (68cm×2.4cm) of Sepharose 6B by using 0.2M-ammonium acetate, pH6.9, containing 0.02% of NaN_3; material was eluted with the same buffer. Fractions (5ml) were collected automatically and analysed for phosphate. Approximate values of nucleic acid phosphorus were estimated from measurements of the E_{260} (degraded nucleic acid containing 1μg of phosphorus/ml has E_{260} 0.33). The column void volume was determined from the elution volume of Blue Dextran. Typically three peaks were observed (Fig. 1). Material corresponding to peak 1 was eluted at the void volume and this contained 1% of the total phosphorus; it was probably nucleic acid. Material from peak 2 represented 10% of the total phosphorus and gave the typical degradation products of a glycerol teichoic acid on acid hydrolysis;

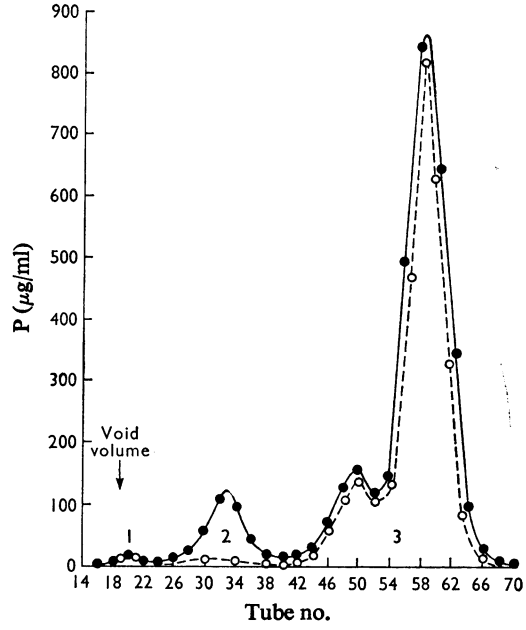


Fig. 1. *Fractionation of the components of the phenol extract on a column* (68cm×2.4cm) *of Sepharose 6B*

Nucleic acids were degraded by treatment with ribonuclease and deoxyribonuclease. ●, μg of P/ml by analysis; ○, μg of P/ml, calculated from the extinction of nucleic acid at 260nm.

it contained a small amount of nucleic acid, identified as RNA by chemical analysis, which accounted for about 4% of the phosphorus in the peak. Material corresponding to peak 3 contained 89% of the total phosphorus and was characterized as nucleic acid; it was shown by chemical analysis to contain RNA and DNA.

Fractions representing peak 2 were combined, evaporated at 30°C and dialysed overnight at 4°C against water. The solution of non-diffusible material was evaporated *in vacuo* to 2ml, applied to a column (52cm×2.4cm) of Sephadex G-200 in 0.2M-ammonium acetate containing 0.02% of NaN_3 and the products were eluted with the same buffer; fractions (5ml) were collected and analysed as described above. The teichoic acid was excluded from Sephadex G-200; on a few occasions, however, a small variable amount of an unidentified phosphate-containing substance penetrated the column material, and acid hydrolysis showed that it contained glycerol and glucose.

Fractions containing the teichoic acid were combined, evaporated to small volume and dialysed at 4°C during 24h against two changes of water. The

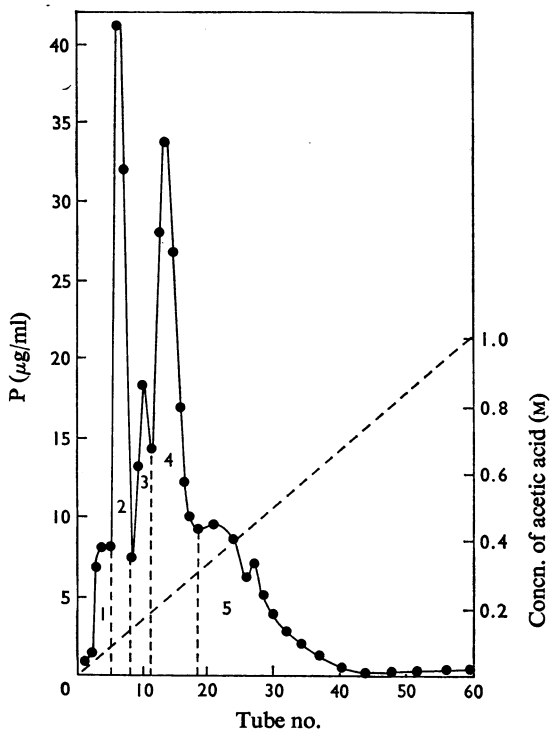


Fig. 2. *Chromatography of products of alkali hydrolysis of the lipoteichoic acid*

A column (10cm×1.5cm) of DE-32 cellulose was used; phosphates were eluted with a linear gradient (0–1.0M in acetic acid) of pyridine–acetic acid, pH5.3. ●, μg of P/ml. Peak 1, inorganic phosphate; peak 2, glycerol monophosphates; peak 3, unidentified phosphate; peak 4, glycerol diphosphates; peak 5, teichoic acid fragments.

non-diffusible material was freeze-dried to give the lipoteichoic acid (60mg) as a white solid containing 5.6% of phosphorus (molar proportions of P: glycerol:glucose, 1.00:1.05:1.20).

Determination of amino acid ester residues. Lipoteichoic acid (3mg) was treated with 0.1M-NaOH (0.5ml) for 3h at room temperature. The solution was neutralized with 1M-HCl and amino acids were determined quantitatively by autoanalysis.

Hydrolysis of the lipoteichoic acid with alkali. The lipoteichoic acid (85mg) was hydrolysed in 1M-NaOH (10ml) for 3h at 100°C in an evacuated sealed tube. The products were passed through a small column of Dowex 50 (NH_4^+ form) resin to remove Na^+ ions and then applied to a column (10cm× 1.5cm) of DE-32 cellulose (acetate form). The neutral products were eluted with water (200ml) and the phosphates with a linear gradient (0–1.0M in acetic acid) of pyridine–acetic acid, pH5.3. Fractions (5ml) were collected and analysed for phosphorus. The fractionation is illustrated in Fig. 2. The major phosphate represented by each peak was identified by paper chromatography and electrophoresis of samples from appropriate fractions. The solution of neutral products was evaporated to dryness and the constituents were examined by paper chromatography and by g.l.c.

Partial acid hydrolysis of the above neutral products. Samples of the purified deacylated glycolipid of this organism, and of the mixture of neutral products from the alkali hydrolysis of the lipoteichoic acid, were treated separately with 0.2M-HCl at 100°C for 2h. The solutions were neutralized with 1M-$(NH_4)_2CO_3$, then were evaporated and dried *in vacuo* over P_2O_5. After conversion into trimethylsilyl ethers, the products were examined by g.l.c. on a temperature programme consisting of 25min at 220°C followed by heating at 10°C/min to a final temperature of 280°C. A sample of the trimethylsilyl ether of *O*-α-D-glucopyranosyl-(1→2)-glycerol was examined for comparison.

Studies on the linkage between lipid and teichoic acid

The lipoteichoic acid was treated with various reagents to clarify the nature of the linkage between the lipid and teichoic acid. Where appropriate, products were separated by ion-exchange chromatography on a column of DE-32 cellulose (acetate form). Neutral products were eluted with water (100ml) and phosphates with a linear gradient (0–1.0M in acetic acid) of pyridine–acetic acid, pH5.3 (total vol., 300ml). Fractions (5ml) were collected and analysed for phosphorus. Appropriate fractions were combined, evaporated at 30°C to a small volume, then were dialysed overnight against water and finally freeze-dried. Fatty acids and glycosides were detected by chromatography after alkali hydrolysis.

Treatment with chloroform–methanol. The lipoteichoic acid (5mg) was stirred with chloroform–methanol (2:1, v/v) for 24h at room temperature. The insoluble material was filtered and analysed.

Treatment with 6M-urea and chloroform. The lipoteichoic acid (20mg) was dissolved in 6M-urea (4ml) and stirred with chloroform (10ml) for 24h at room temperature. The aqueous layer was separated, then was applied to a column (40cm×1.8cm) of Sephadex G-200 in 6M-urea that was eluted with 6M-urea. Fractions (5ml) were collected and analysed for phosphorus. All of the material containing phosphorus was eluted at the void volume as a single peak. Fractions corresponding to this peak were combined and dialysed for 48h against two changes of water. The solution of non-diffusible material was evapor-

ated to a small volume *in vacuo* and traces of urea were removed on a small column of Sephadex G-10. Material that was eluted by water at the void volume was freeze-dried before analysis.

Ion-exchange chromatography. The lipoteichoic acid (20mg) was chromatographed on DE-32 cellulose as described above. Material containing phosphate was eluted as a single peak at 0.7M-acetic acid. Appropriate fractions were combined for analysis.

Treatment with phenol at 70°C. The lipoteichoic acid (35mg) in water (2ml) was mixed with aq. 80% (w/v) phenol (2ml) and heated at 70°C for 30min; on being cooled the single phase separated into two layers. The upper aqueous layer was separated, extracted with chloroform, applied to a column of DE-32 cellulose and developed as described above. Material containing phosphate appeared as a single peak at 0.7M-acetic acid. Appropriate fractions were combined for analysis.

Partial hydrolysis with trichloroacetic acid at 4°C. The lipoteichoic acid (80mg) was stirred in aq. 10% (w/v) trichloroacetic acid (10ml) at 4°C. The treatment was continued for 7 days after a preliminary experiment had indicated that approx. 30% of the phosphodiester linkages had been hydrolysed during 2 days. Trichloroacetic acid and lipids were removed by stirring with diethyl ether until the pH of the aqueous solution had risen to 4; the solution was then extracted with chloroform and the products were separated on DE-32 cellulose. The fraction containing neutral products was evaporated to dryness at 30°C, the residue was dried *in vacuo* over P_2O_5, converted into trimethylsilyl ethers and examined by g.l.c. Phosphates were eluted from the column as a large broad fraction between 0.35 and 0.75M-acetic acid. The solution was evaporated *in vacuo* to a small volume before being dialysed overnight. The non-diffusible material was applied to a column (46cm × 1.8cm) of Sephadex G-200 that had been equilibrated with 0.2M-ammonium acetate containing 0.02% NaN_3. Material was eluted with the same buffer and fractions (5ml) were analysed for phosphorus; the elution diagram (Fig. 3) showed two peaks. Appropriate fractions were combined, solvent was removed at 30°C *in vacuo* and the material was dialysed overnight and freeze-dried. Product I (8mg) corresponded to peak I and product II (54mg) to peak II (molar proportions of P:glycerol:glucose in product I, 1.00:1.30:1.40; in product II, 1.00:1.01:1.20).

The combined ether and chloroform washings were evaporated to small volume and were washed repeatedly with water to remove trichloroacetic acid. The solution was dried over Na_2SO_4 and was analysed by t.l.c. in solvents *C* and *D*. A portion of this material was esterified with diazomethane and the fatty acid methyl esters were examined by g.l.c.

Deacylation with hydroxylamine. Hydroxylamine in methanol was prepared by the method of Beinert

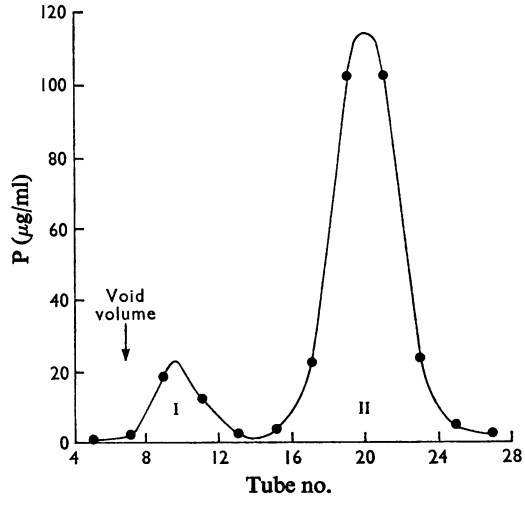


Fig. 3. *Fractionation of the products obtained by treatment of the lipoteichoic acid with trichloroacetic acid*

The column (46cm × 1.8cm) of Sephadex G-200 was loaded with material eluted as a broad peak between 0.35 and 0.75M-acetic acid from a DE-32 cellulose column by pyridine–acetic acid buffer, pH5.3. ●, μg of P/ml.

et al. (1953) and it was diluted to 5M with water by the standardization procedure of Frear & Burrell (1955). Portions (2 × 5ml) of this solution were added to the lipoteichoic acid at consecutive intervals of 24h. After 48h the mixture was extracted with ethyl acetate (4 × 10ml) before being dialysed overnight. The water-soluble materials were separated by DE-32 ion-exchange chromatography followed by gel-filtration through Sephadex G-200 as described above. Most of the phosphate-containing material was eluted from the DE-32 column as a broad peak (0.5–0.9M in acetic acid). This fraction was separated into two components (peak I, 5mg; peak II, 24mg) on the Sephadex G-200 column (Fig. 4) (molar proportions of P:glycerol:glucose in peak II, 1.00:1.15:1.20).

The ethyl acetate was evaporated and the residue was heated under reflux with methanol (4.2ml) and conc. HCl (0.8ml) for 6h. The solution was cooled and neutralized to pH5 with 1M-$(NH_4)_2CO_3$. Methyl esters were extracted with diethyl ether and analysed by g.l.c.

Dephosphorylation with 60% (w/v) HF. The lipoteichoic acid (45mg) was dissolved in 60% (w/v) HF (1ml) at 0°C in a polythene tube fitted with a cap. After 24h at 0°C, the solution was poured into a stirred, saturated solution of LiOH at 4°C; the

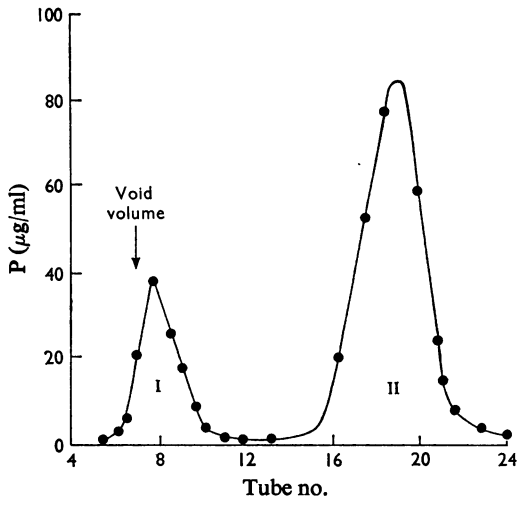


Fig. 4. *Fractionation of products obtained by treatment of the lipoteichoic acid with* 5M-*hydroxylamine*

The column (46cm×1.8cm) of Sephadex G-200 was loaded with material eluted as a broad peak between 0.5 and 0.9M-acetic acid from a DE-32 cellulose column by pyridine–acetic acid, pH5.3. ●, μg of P/ml.

appropriate amount of the latter was determined by titrating a sample (1ml) of the HF against saturated LiOH and then using only 95% of this amount. The pH of the final mixture was adjusted rapidly to 7.0 by dropwise addition of 1M-LiOH.

The mixture was freeze-dried and the residue was extracted with 10ml of chloroform–methanol (2:1, v/v). The residue was treated with water (5ml) and the solution filtered to remove LiF. Remaining Li^+ ions were removed by passing the solution through a column of Dowex 50 (NH_4^+ form) resin. The eluate was evaporated and the residue was incubated overnight with phosphomonoesterase. This solution was evaporated and dried *in vacuo* over P_2O_5. The residue was converted into trimethylsilyl ethers and was then examined by g.l.c. at 270°C and also by the temperature programme described for the identification of the products of partial acid hydrolysis of the glycosides produced by alkali hydrolysis of the lipoteichoic acid. The organic extract was examined by t.l.c. in solvents *C* and *D*.

Examination of cell lipids

Extraction and fractionation of lipids from whole cells. Wet cells (260g) were freeze-dried (yield, 50g) and extracted with chloroform–methanol (2:1, v/v; 2×200ml) during two consecutive periods each of 24h. The combined extracts were filtered and evaporated to dryness at 30°C. The residual oil (2.0g) in chloroform (25ml) was run on to a column of a mixture of silicic acid (20g) and Celite (20g) made up in chloroform. Lipids were eluted stepwise with chloroform (500ml), acetone (500ml) and 200ml quantities of increasing amounts of methanol in chloroform (3, 5, 10, 20, 33 and 50%, v/v) respectively (Vorbeck & Marinetti, 1965). Each fraction was collected separately and evaporated to dryness. Selected lipids were purified further as follows. (*a*) Glycolipid. A sample (100mg) of the acetone fraction (total weight 260mg) was chromatographed on five preparative t.l.c. plates (20cm×20cm each) to give glycolipid (60mg), the properties of which were the same as those of the material previously identified from this organism (Brundish *et al.*, 1966; Shaw & Baddiley, 1968). (*b*) Phosphatidylglycolipid I. The 33% methanol-in-chloroform fraction (0.10g) was applied to a column of Celite (5g) and silicic acid (5g) and the lipids were eluted with a linear gradient of methanol (15–33%, v/v) in chloroform (total vol., 500ml). Fractions (10ml) were collected automatically and analysed by t.l.c. in solvent *C*; fractions 30–40 were combined and evaporated to give pure phosphatidylglycolipid (10mg). (*c*) Phosphatidylglycolipid II. The 5% methanol-in-chloroform fraction (0.18g) was applied to seven t.l.c. plates (20cm×20cm each). After chromatography with solvent *C*, the lipid was still contaminated with other phospholipids, especially cardiolipin. After further preparative t.l.c. phosphatidylglycolipid II still contained small amounts of cardiolipin.

Deacylation of lipids. Lipids were deacylated by the method of Marinetti (1962). Lipid (10mg) in chloroform–methanol (1:1, v/v; 1ml) was treated with 0.2M-sodium methoxide in methanol (0.3ml); after 0.5h at room temperature, water (2ml) was added and Na^+ ions were removed by passing the mixture fairly rapidly through a column of Dowex 50 (H^+ form) resin. The deacylated product was eluted with water and was identified by paper chromatography in solvents *A* or *B*.

Results and Discussion

The membrane teichoic acid was extracted from disrupted cells of *Streptococcus faecalis* N.C.I.B. 8191 by a modification of the procedure developed by Burger & Glaser (1964) and used by Wicken & Knox (1970) for the extraction of lipoteichoic acids. The method uses aq. 80% (w/v) phenol at 4°C; lipids and some proteins are found in the phenol-rich layer, denatured protein at the interface and nucleic acids and membrane teichoic acid in the aqueous layer. Further purification by digestion with ribonuclease

Table 1. *Comparison of the fatty acid composition of the purified lipoteichoic acid and the total lipids extracted by chloroform–methanol from whole cells*

Proportions are relative to $C_{16:0}$ acid = 1.00.

Fatty acid	Total lipids	Lipoteichoic acid
$C_{14:0}$	0.09	0.15
$C_{16:0}$	1.00	1.00
$C_{16:1}$	0.20	0.23
$C_{18:1}$	0.95	1.06
C_{19}-cyclopropane	0.41	0.21

and deoxyribonuclease followed by chromatography on Sepharose and Sephadex gave the pure lipoteichoic acid. Alanine ester residues were partly lost by hydrolysis during the incubation with the nucleases (molar ratio P:alanine after 24h at pH8 and 37°C was 1:0.06, and after 72h 1:0.01); consequently the incubation was extended to 72h to give a homogeneous product lacking alanine ester residues. The stability towards alkali of these ester residues is higher than usual for a teichoic acid, and this is in agreement with the conclusion (Wicken & Baddiley, 1963) that in this case the ester linkages involve hydroxyl groups on sugar residues rather than on glycerol.

Studies of the membrane teichoic acid extracted from this organism by using trichloroacetic acid (Wicken & Baddiley, 1963) indicated the presence of 2-kojibiosyl- and 2-kojitriosyl-glycerol residues in the polymer chain. However, the glycolipid from this organism has been shown to be 3[*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diglyceride (Brundish *et al.*, 1966), which contains a 1-kojibiosylglycerol residue. Thus if the lipoteichoic acid contained a glycolipid residue, then its hydrolysis should yield both 1- and 2-kojibiosylglycerol. In fact, hydrolysis in 2M-HCl gave products typical of a glucosylated poly(glycerol phosphate), namely glycerol, its mono- and di-phosphates, P_i and glucose, together with fatty acids (5.2%, calc. as C_{16} acid) and a small amount of an unidentified phosphate. The composition of the fatty acids differed only quantitatively from that of the fatty acids present in lipids extracted from whole cells by chloroform–methanol (Table 1). Hydrolysis in alkali gave a similar range of products, but, whereas glucose was absent and glycerol was present only in traces, two isomeric glycosides of glycerol were formed.

One of these glycosides was identified as 1-kojibiosylglycerol by chromatographic comparison with the deacylation product of the glycolipid from the organism. With the periodate–Schiff spray reagents on paper it rapidly gave a purple colour that slowly changed to deep blue; this is characteristic of a glycoside that would yield formaldehyde in this test and is thus consistent with the presence of a 1-substituted glycerol (Roberts *et al.*, 1963).

The second glycoside ($R_{glucose}$ 0.54 in solvent *B*) slowly gave a blue-grey colour with the periodate–Schiff spray reagents, indicating a 2-substituted glycosylglycerol; the retention time of its trimethylsilyl ether on g.l.c. at 270°C was 0.89 relative to that of 1-kojibiosylglycerol. It was characterized by partial acid hydrolysis (0.2M-HCl at 100°C for 2h) of the neutral fraction obtained from the alkali hydrolysis of the lipoteichoic acid. This fraction, which contained two isomers of diglucosylglycerol, gave glucose together with *O*-α-D-glucopyranosyl-(1→1)-D-glycerol and *O*-α-D-glucopyranosyl-(1→2)-glycerol, which were indistinguishable by paper chromatography and g.l.c. from authentic synthetic materials. The ratio of the two monoglucosides was the same as that of the two isomers in the neutral fraction (ratio of 1-isomer:2-isomer, 0.61:1). This indicates that the glucose-to-glucose linkage is the same in both glycosides. The second glycoside is therefore *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-glycerol or 2-kojibiosylglycerol.

Although the earlier work (Wicken & Baddiley, 1963) on the structure of the membrane teichoic acid from *S. faecalis* N.C.I.B. 8191 suggested that kojitriosylglycerol residues were present, and few or no unsubstituted glycerol residues were observed, the present study is not in agreement with this. No evidence for kojitriosylglycerol residues was obtained, whereas there were undoubtedly unsubstituted glycerol residues. Presumably the organism has undergone a change during the years of laboratory culture. It is not known whether this is a genetic change or whether the original culture conditions differed in some minor but critical manner from those used in the present work.

The formation of the two isomers of kojibiosylglycerol suggests that the lipoteichoic acid might comprise a poly(glycerol phosphate), with some substitution at the 2-positions on glycerol by glucosyl-glucosyl residues, and a glycolipid identical with that already known to occur in the membrane. It is noteworthy that Wicken & Knox (1970) reported the presence of glycolipid among the lipid components of the lipoteichoic acids from the lactobacilli. However, the precise nature of the lipid component, and the association between lipid and teichoic acid, remained obscure. Experiments were therefore done to establish the nature of the association between these components. The lipid could not be separated from the teichoic acid by treatment with chloroform–methanol, 6M-urea solution in the presence of chloroform or by ion-exchange chromatography. Thus it is unlikely that the linkage is through hydrophobic interaction, hydrogen bonding or electrovalent interaction.

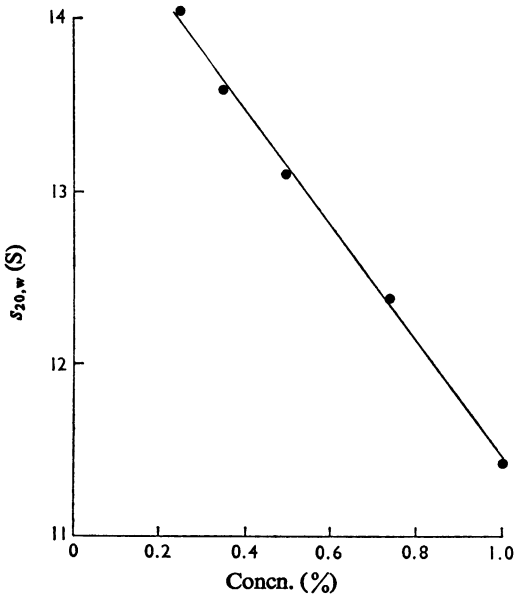


Fig. 5. *Variation of the sedimentation coefficient, $s_{20,w}$, of the lipoteichoic acid with concentration*

Solutions of the teichoic acid in 0.15M-sodium chloride–0.015M-sodium citrate buffer, pH7.1, were centrifuged at 59780rev./min at 20°C in a Beckman Spinco model E analytical ultracentrifuge in an AnD rotor.

On ultracentrifugation the lipoteichoic acid behaved like a large molecule. Thus, in 0.15M-NaCl–0.015M-sodium citrate buffer, pH7.1, it gave sedimentation coefficients ($s_{20,w}$) between 11.4 and 14.1S depending on the concentration (Fig. 5). These high values were a consequence of micelle formation, as at a concentration of 0.5% in 6M-urea containing 0.15M-NaCl, the sedimentation coefficient was decreased to 5.7S. Only one peak, of 2.0S, was observed at a concentration of 0.5% in the presence of 0.2M-NaCl containing 1.5% sodium dodecyl sulphate. This peak is attributed either to the sodium dodecyl sulphate micelle or to a mixed micelle of detergent and lipoteichoic acid. These properties suggest that the lipoteichoic acid shows a marked tendency to form aggregates in solution, with the lipid component buried within a cluster of teichoic acid chains; this aggregate is destroyed by high concentrations of urea and by detergents. If the aggregate were composed of lipid and hydrophilic teichoic acid molecules held together by either hydrophobic forces or hydrogen bonds, then a single-phase mixture of equal volumes of phenol and water at 70°C should disrupt the components, and as the two phases separated on cooling the lipid should separate with the organic-rich layer and the teichoic acid with the aqueous layer. In fact, no separation occurred, the lipoteichoic acid being recovered unchanged with $s_{20,w}$ 13.2S. This supports the conclusion from the other experiments, that the linkage between lipid and teichoic acid is covalent.

A solution of hydroxylamine at pH8 removed the acyl substituents from the molecule. The acids were extracted with ethyl acetate then were converted into methyl esters by treatment with methanolic HCl and examined by g.l.c. The major water-soluble product still contained both 1- and 2-kojibiosylglycerol, but its fatty acid content was decreased to 0.23%. At a concentration of 0.5% in NaCl–sodium citrate buffer its sedimentation coefficient was 0.7S, indicating that it was no longer capable of micelle formation. The macromolecular behaviour shown by the lipoteichoic acid is therefore a consequence of the presence of the long-chain fatty acid ester residues; the other component of the glycolipid in the molecule, 1-kojibiosylglycerol, must be covalently joined to the teichoic acid.

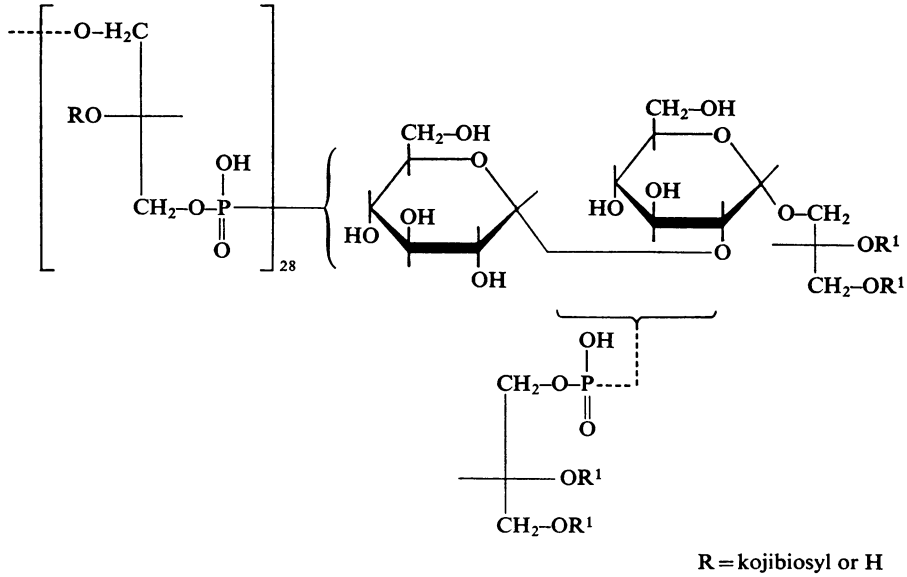
Gradual hydrolysis of the phosphodiester linkages occurred with 10% (w/v) trichloroacetic acid at 4°C during 7 days. The products included fatty acids, which could be extracted from the aqueous solution with chloroform, 1- and 2-kojibiosylglycerol in the molar ratio 1.72:1 and two phosphate fractions, products I and II. Product I, containing 8.2% of fatty acid, gave both 1- and 2-kojibiosylglycerol on hydrolysis in alkali; a 0.5% solution in NaCl–sodium citrate buffer had a sedimentation coefficient of 11.1S. It is probably a relatively undegraded form of lipoteichoic acid, in which part of the teichoic acid chain has been lost by phosphodiester cleavage. Product II, containing 1.51% of fatty acid, gave 2-kojibiosylglycerol and a trace only of the 1-isomer on hydrolysis; a 0.5% solution had a sedimentation coefficient of 1.3S. This degraded product probably corresponds approximately to the teichoic acid preparations usually obtained from cells by extraction procedures that use trichloroacetic acid. Apparently the linkage between the lipid component and the main chain of the teichoic acid is more readily hydrolysed by acid than are the phosphodiester linkages in the chain itself; this is evident from the ratio of 1- to 2-isomers formed by the hydrolysis, compared with the ratio of these components found in the polymer obtained by degradation with HF (see below).

Evidence that the linkage between lipid and teichoic acid involves a phosphodiester was obtained by studying the action of 60% (w/v) HF at 0°C; this procedure is commonly used for the conversion in high yield of phosphomonoesters and phosphodiesters into alcohols and P_i without concurrent hydrolysis of glycosidic or carboxylic ester linkages. The products were glycerol, 2-kojibiosylglycerol, a mixture of diglycerides, a 1-kojibiosyl diglyceride identical with that

present in the membrane lipids, and a small amount of monoglyceride and fatty acids. This confirms that the polymer comprises a teichoic acid attached through a phosphodiester linkage to kojibiosyl diglyceride, with probably a phosphatidyl residue attached to either the glycolipid or independently to another teichoic acid molecule. The occurrence of monoglyceride among the products of dephosphorylation might be a consequence of partial deacylation of diglyceride, although no corresponding kojibiosyl monoglyceride was detected; only traces of 1-kojibiosylglycerol were found among the reaction products.

An examination of the lipids of the organism showed that, in addition to kojibiosyl diglyceride, at least two related phospholipids were present in small amounts. One of these (R_F 0.2 in solvent *C*) was eluted from the silicic acid column by 33% (v/v) methanol in chloroform and was further purified by repetition of the chromatography. The other (R_F 0.6 in solvent *C*) was eluted with 5% (v/v) methanol in chloroform, and was further purified by t.l.c.; it contained a small amount of cardiolipin. Both phospholipids gave 1-kojibiosylglycerol and glycerol monophosphates on hydrolysis in 1M-NaOH at 100°C for 3h, and with sodium methoxide in methanol both were deacylated to a compound ($R_{glycerol}$ 0.4 in solvent *A*) that rapidly gave a purple colour with the periodate–Schiff spray reagents; this property indicates the presence of an unsubstituted glycol yielding formaldehyde on oxidation (Roberts *et al.*, 1963). The phospholipid with the lower R_F value in solvent *C* rapidly gave a purple colour with the periodate–Schiff reagents, whereas the other only gave the slowly developing blue colour typical of a glycoside. This suggested that an unsubstituted glycerol phosphate residue was present in the slower-running compound, whereas the corresponding residue in the other compound must have been acylated. These properties are in agreement with structures in which 1-kojibiosyl diglyceride bears in one case a glycerol phosphate residue on one glucose and in the other a phosphatidyl residue on a glucose. Similar phosphatidyl glycolipids and related incompletely acylated derivatives have been isolated from mycoplasma and streptococci (Shaw *et al.*, 1970; Dos Santos Mota *et al.*, 1970; Ishizuka & Yamakawa, 1968; Fischer, 1970; Ambron & Pieringer, 1971). The presence of phospholipids of this type in the membrane lipids of *S. faecalis* N.C.I.B. 8191 supports the conclusion that the lipid component of the lipoteichoic acid is of this type.

Biosynthetic considerations indicate that there should be a glycolipid or phosphatidyl glycolipid at only one end of the teichoic acid chain. Moreover, the degradative evidence indicates that the linkage between the two components involves the terminal phosphate of the teichoic acid and a hydroxyl of one of the two sugar residues. Thus there should be either two or four fatty acid residues in the lipoteichoic acid molecule. From the amount of fatty acid in the preparation the molar ratio of phosphorus:



R = kojibiosyl or H
R^1 = fatty acid

(I)

fatty acid was 1:0.115, thus giving a chain length of 35 on the basis of there being four fatty acid residues in the molecule. This value is a little higher than that calculated by the method described below, and so it is possible that rather less than four fatty acid residues may be present.

An alternative method for the determination of the chain-length of the polymer involves the measurement of the ratio 2-kojibiosylglycerol:1-kojibiosylglycerol after the complete hydrolysis of all phosphate linkages. This was achieved by hydrolysis with alkali followed by treatment of the products with HF and determination of the ratio of the two glycosides by g.l.c. of their trimethylsilyl ethers. Because of the relatively small difference in retention times of these derivatives and the small amount of the 1-isomer, the method is not especially accurate. The ratio of 2-kojibiosylglycerol:1-kojibiosylglycerol was 16.8 (±1): 1; since about 95% of the glycosyl residues in the lipoteichoic acid are kojibiosyl and the ratio phosphorus:glucose is 1:1.2, then about 60% of the glycerol residues in the molecule must bear kojibiosyl substituents. Again on the basis of one glycolipid residue in the polymer, the chain-length is 28 (±2), in good agreement with the value determined by the other method. A partial structure of the molecule is given in formula (I); the uncertainty lies in the position of substitution of the teichoic acid and the phosphatidyl residues on the hydroxyl groups of the two glucosyl residues, and the precise location of alanine ester on the glucosyl residues in the teichoic acid.

Material that had been extensively purified still contained about 2.8% of nucleic acid and 3.3% of protein. Most of the nucleic acid and about half of the protein was still present in material that had been treated with trichloroacetic acid or hydroxylamine, but it is not known whether these represent minor contaminants or whether they are covalently attached to the lipoteichoic acid. The serological properties of the material were examined by agar-gel double-diffusion, by which it was shown that, like the material that had been extracted with trichloroacetic acid (Wicken *et al.*, 1963), it reacted with the specific antiserum to group D streptococci. It gave a single line at concentrations between 0.5 and 5mg/ml. At similar concentrations the material that had been deacylated by treatment with hydroxylamine also gave a line close to the well of antiserum, whereas the material that had been partly degraded by hydrolysis with trichloroacetic acid gave no line. Previously it was found that the teichoic acid gave only a poor precipitin reaction at 2mg/ml, and we conclude that trichloroacetic acid causes hydrolysis of the main teichoic acid chain to give smaller fragments that do not react with the antiserum. Similar results were obtained by using concanavalin A (cf. Archibald & Coapes, 1971).

The structure of the lipoteichoic acid supports the previous conclusion that this is a membrane component. The glycolipid part of the molecule would intercalate with the other lipids and phospholipids in the membrane, being held by hydrophobic forces and possibly through salt linkages involving Mg^{2+} ions. Our studies have failed to reveal the presence in the cell extracts of any molecular species of teichoic acid other than lipoteichoic acid, and we conclude that all of the 'intracellular' teichoic acid in the cell is of this type. In preliminary experiments with a number of other bacteria (J. S. Gatley, J. Coley, M. Duckworth & J. Baddiley, unpublished work) it has been found that the membrane teichoic acid is lipoteichoic acid, and it is likely that this will be true for all Gram-positive bacteria. However, not all of the glycolipid or its phosphatidyl derivatives are present in the membrane in this form. From the amounts of 1- and 2-kojibiosylglycerol present in alkali hydrolysates of whole cells and purified lipoteichoic acid (molar ratios, 4.87:1 and 0.61:1 respectively) we conclude that about 12% of the membrane glycolipid is in the form of lipoteichoic acid.

We thank the Science Research Council for a Studentship (P. T.) and Dr. D. E. Minnikin for assistance with g.l.c.

References

Ambron, R. T. & Pieringer, R. A. (1971) *J. Biol. Chem.* **246**, 4216–4225

Archibald, A. R. & Baddiley, J. (1966) *Advan. Carbohyd. Chem.* **21**, 323–375

Archibald, A. R. & Coapes, H. E. (1971) *Biochem. J.* **123**, 665–667

Archibald, A. R., Baddiley, J. & Blumsom, N. (1968) *Advan. Enzymol. Relat. Areas Mol. Biol.* **30**, 223–253

Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956) *J. Chem. Soc. London* 2818–2823

Beinert, H., Green, D. E., Hele, P., Hiftz, H., von Korff, R. W. & Ramakrishman, C. G. (1953) *J. Biol. Chem.* **203**, 35–45

Brundish, D. E., Shaw, N. & Baddiley, J. (1966) *Biochem. J.* **99**, 546–549

Burger, M. M. & Glaser, L. (1964) *J. Biol. Chem.* **239**, 3168–3177

Burton, K. (1956) *Biochem. J.* **62**, 315–323

Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758

Dos Santos Mota, J. M., Op Den Kamp, J. A. F., Verheij, H. M. & Van Deenen, L. L. M. (1970) *J. Bacteriol.* **104**, 611–619

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956) *Anal. Chem.* **28**, 350–356

Fischer, W. (1970) *Biochem. Biophys. Res. Commun.* **41**, 731–736

Frear, D. S. & Burrell, R. C. (1955) *Anal. Chem.* **27**, 1664–1665

Hanahan, D. J. & Olley, J. N. (1958) *J. Biol. Chem.* **231**, 813–828

Hanes, C. S. & Isherwood, F. A. (1949) *Nature (London)* **164**, 1107–1112

Hay, J. B., Wicken, A. J. & Baddiley, J. (1963) *Biochim. Biophys. Acta* **71**, 188–190
Heptinstall, S., Archibald, A. R. & Baddiley, J. (1970) *Nature (London)* **225**, 519–521
Hughes, A. H., Stow, M., Hancock, I. C. & Baddiley, J. (1971) *Nature New Biol. (London)* **229**, 53–55
Ishizuka, I. & Yamakawa, T. (1968) *J. Biochem. (Tokyo)* **64**, 13–23
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951) *Anal. Chem.* **23**, 415–420
Knox, K. W. & Wicken, A. J. (1971) *J. Gen. Microbiol.* **63**, 237–248
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Marinetti, G. V. (1962) *J. Lipid Res.* **3**, 1–20
Minnikin, D. E. & Abdolrahimzadeh, H. (1971) *J. Chromatogr.* **63**, 425–454
Ogur, M. & Rosen, G. (1950) *Arch. Biochem. Biophys.* **25**, 262–276
Roberts, W. K., Buchanan, J. G. & Baddiley, J. (1963) *Biochem. J.* **88**, 1–7
Shattock, P. M. F. & Smith, D. G. (1963) *J. Gen. Microbiol.* **31**, iv
Shaw, N. & Baddiley, J. (1968) *Nature (London)* **217**, 142–144
Shaw, N., Smith, P. F. & Verheij, H. M. (1970) *Biochem. J.* **120**, 439–441
Shockman, G. D. & Slade, H. D. (1964) *J. Gen. Microbiol.* **37**, 297–305
Smith, D. G. & Shattock, P. M. F. (1964) *J. Gen. Microbiol.* **34**, 165–175
Snyder, F. & Stephens, N. (1959) *Biochim. Biophys. Acta* **34**, 244–245
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950) *Nature (London)* **166**, 444–445
Vorbeck, M. L. & Marinetti, G. V. (1965) *J. Lipid Res.* **6**, 3–6
Walker, B. L. (1971) *J. Chromatogr.* **56**, 320–321
Wicken, A. J. & Baddiley, J. (1963) *Biochem. J.* **87**, 54–62
Wicken, A. J. & Knox, K. W. (1970) *J. Gen. Microbiol.* **60**, 293–302
Wicken, A. J., Elliott, S. D. & Baddiley, J. (1963) *J. Gen. Microbiol.* **31**, 231–239