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A Membrane-Associated Lipomannan in Micrococci

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Membranes of *Micrococcus lysodeikticus*, *Micrococcus flavus* and *Micrococcus sodonensis* contain acidic lipomannans. Lipoteichoic acids could not be detected in these organisms, and the suggestion that they are substituted for by the lipomannans is strengthened by the chemical and physical resemblances between the two polymers. The mannans contain glycerol, ester-linked fatty acids and mono-esterified succinic acid residues, giving them both hydrophobic and charged properties. The *M. lysodeikticus* mannan has a chain of about 60 hexose units with two branch points, and is joined at its reducing end to the 1-position of a glycerol moiety bearing two fatty acid residues. Succinic acid on the mannan enables it to bind Mg^{2+} efficiently, and the polymer is firmly associated with the cytoplasmic membrane, probably by intercalation of its fatty acids with those of the membrane lipids.

The widespread occurrence of lipoteichoic acids as a structurally uniform class of polymers in Gram-positive bacteria (Coley *et al.*, 1972) suggests a vital role of these cell-membrane components. Lipoteichoic acids have been implicated in the biosynthesis of wall teichoic acids (Fiedler & Glaser, 1974) and in the control of Mg^{2+} concentration at the cell membrane (Hughes *et al.*, 1973). Until recently, all Gram-positive bacteria examined had been found to contain these polymers, but in a preliminary report (Powell *et al.*, 1974) we showed that *Micrococcus lysodeikticus* lacked a lipoteichoic acid. The present study confirms this result and also shows the absence of lipoteichoic acid from *Micrococcus flavus* and *Micrococcus sodonensis*. By analogy with wall teichoic acids, which in some bacteria can be replaced by acidic polysaccharides under defined growth conditions (Ellwood & Tempest, 1972), we have examined the possibility that these micrococci contain membrane-bound acidic polymers as substitutes for lipoteichoic acids. We have thus found a new class of bacterial polysaccharides which bear some resemblance to lipoteichoic acids; they have a hydrophobic portion, which resembles the organism's membrane glycolipid, attached to a longer charged hydrophilic chain. Similar findings have just been published by Pless *et al.* (1975) and by Owen & Salton (1975).

In *M. lysodeikticus* this polysaccharide has been identified (Powell *et al.*, 1974; Schmit *et al.*, 1974) with the carbohydrate found in cell lysates by Hawthorne (1950). Work by Macfarlane (1964) and Scher & Lennarz (1969) established the presence of a membrane-associated mannan in this organism, and some unusual properties of the polymer have been described (Powell *et al.*, 1974; Schmit *et al.*, 1974); these have led us to make further structural investigations on the polymer. The description of the mannan as a polysaccharide (Macfarlane, 1964) has proved incorrect with the discovery of non-sugar components that can be related to the acidic and lipid properties of the polymer.

Further structural features of the mannan, its isolation, purification and association with the cytoplasmic membrane are described. The mannan binds Mg^{2+} efficiently, and this property enables a functional analogy between it and teichoic acids to be made.

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Experimental

Materials

NaB^3H_4 was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Deoxyribonuclease and ribonuclease were obtained from Miles Chemical Co., Clifton, N.J., U.S.A., and lysozyme and other biochemicals were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-50, G-75, Sepharose 6B, concanavalin A–Sepharose 4B and Blue Dextran were products of Pharmacia Fine Chemicals, Uppsala, Sweden, and Visking dialysis tubing was obtained from Visking Co., Chicago, Ill., U.S.A. All other chemicals and solvents used were of analytical grade and were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Growth of organisms

Micrococcus lysodeikticus A.T.C.C. 4698 was purchased as freeze-dried cells from Miles Chemical Co. They had been grown in a medium containing yeast extract, glucose and salts, and harvested at late-exponential phase. As described previously (Powell *et al.*, 1974) these cells were indistinguishable from cells grown in our laboratory.

Micrococcus lysodeikticus N.C.I.B. 9278, *Micrococcus flavus* N.C.I.B. 8166 and *Micrococcus sodonensis* N.C.I.B. 8854 were grown in batch culture at 37°C (Oxoid Nutrient Broth, 25g/l; Difco Yeast Extract, 10g/l; K_2HPO_4, 10g/l; glucose, 10g/l) and harvested in the late-exponential phase.

Cell fractionation

Washed bacteria were disrupted in an MSK Braun homogenizer with Ballotini no. 11 beads for 2.5min with cooling. Beads were removed by filtration, and unbroken cells and walls removed by centrifugation (16000*g*, 20min). The supernatant containing fragmented membranes and cytoplasmic material was freeze-dried.

Extraction of crude polysaccharides

Polysaccharides were extracted either from freeze-dried material from disrupted cells, or from whole cells. In each case material was first extracted twice overnight with chloroform–methanol (2:1, v/v) to remove lipids, and then dried. The defatted product was suspended in water and extracted with an equal volume of aq. 80% (w/v) phenol (30min, 20°C). The phases were separated by centrifugation (16000*g*, 30min) and the upper aqueous phase was removed and dialysed overnight against running tap water to remove phenol. The volume was adjusted to 50ml and adjusted to 100ml with sodium phosphate buffer (0.1M, pH6.9); nucleic acid was partially degraded by incubation with nucleases (10μg/ml, 37°C, 16h) and this was followed by a further phenol extraction to remove enzymes and residual protein. Analysis of the aqueous phase after overnight dialysis showed it to contain nucleic acid, carbohydrate and a small amount of protein.

Purification of crude extracts

Crude phenol extracts (100mg) were fractionated on a column of Sepharose 6B as described previously (Powell *et al.*, 1974). The contents of every third fraction were analysed for hexose, phosphate and material absorbing at 260nm. Appropriate fractions were combined, dialysed against water and freeze-dried.

Analytical methods

Hexose was determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956) by using mannose as a standard. Phosphorus was determined by the method of Chen *et al.* (1956), protein as described by Lowry *et al.* (1951), and nucleic acids by their absorption at 260nm. For the latter an approximate conversion factor of 5.6μg of phosphate/ml per E_{260} unit for degraded nucleic acid was used. Glycerol was determined with glycerol kinase in a Biochemica Test Kit (Neutral Fats), with a glycerol standard that had been standardized against erythritol by the method of Hanahan & Olley (1958). After hydrolysis of samples (4M-HCl, 100°C, 16h) amino acids were determined with a JEOL amino acid analyser. Magnesium was determined by atomic absorption spectroscopy by using a Unicam SP.90 series 2 instrument (Pye Unicam Ltd., Cambridge, U.K.).

Formaldehyde

Formaldehyde produced from the terminal glycerol residue in the mannan by oxidation with periodate was detected by Conway diffusion (Hollander *et al.*, 1951). Lipomannan and deacylated mannan (10mg) were each dissolved in water (1.2ml) and samples removed for hexose determination. Samples (0.8ml) were diluted to 1ml; 0.1M-$NaIO_4$ (2ml) was added to the solution. After 10min the reaction was stopped by the addition of $SnCl_2$ [0.3ml of a 30% (w/v) solution in 0.1M-HCl]. After centrifugation, samples (0.4ml) were placed in the outer well of a Conway cell, and chromotropic acid [0.8ml of an 0.2% (w/v) solution in 15M-H_2SO_4] was added to the inner well. The cells were sealed and kept overnight for distillation of formaldehyde. The contents of the inner well were transferred to a small tube, heated at 100°C for 30min, and absorbance at 570nm was measured after cooling.

Fatty acids

Fatty acids were identified as their methyl esters. These were prepared by treatment of either the lipomannan or the free fatty acids with methanolic HCl (0.5M, 2h, 70°C) in sealed ampoules. After the addition of water, the esters were extracted into ether, dried over anhydrous Na_2SO_4 and identified by g.l.c. on a column of 15% polyethylene glycol succinate on Celite (2.1m×3.5mm) at 170°C; N_2 was the carrier gas (15ml/h). For quantification of fatty acids, a standard of nonadecanoic acid in chloroform was included before the esterification step, and the amount was determined by integration of the trace from g.l.c.

Acyl residues attached to mannan

Standard hydroxamic acids of succinate, glutarate and malonate were prepared from the mixed anhydride of the parent acid and ethyl chloroformate (Seubert, 1960). A mixture of mono- and di-hydroxamates was obtained for each acid. The dihydroxamate of succinic acid was prepared by reaction of dimethyl succinate with aqueous hydroxylamine. Hydroxamates, corresponding to ester-linked acyl residues, were prepared from the lipomannan by treatment with alkaline 1M-hydroxylamine and were desalted on small columns of Dowex 50 (H^+ form) and by removal of HCl *in vacuo*. Hydroxamates were identified by comparison with standards on paper chromatography in solvents D and E (see under 'Chromatographic methods') and paper electrophoresis in pyridinium acetate buffer, pH5.3 (buffer B).

Total acyl groups were determined by the hydroxamic acid method described by Lee (1966). Ethyl acetate was used as a standard.

Attempted reduction of the lipomannan with NaB^3H_4

Reductions were carried out by using portions of a solution containing KB^3H_4 (12mCi; specific radioactivity 308mCi/mmol) in a carrier of $NaBH_4$ (0.1M). The lipomannan (7mg) from the Sepharose 6B column and a standard of glucose 6-phosphate (2mg) were incubated in the NaB^3H_4 solution (22h, 20°C). The reaction mixtures were adjusted to 0.2M with NaOH and kept for 1h. The reduction was stopped by acidification with acetic acid, and volatile radioactive compounds were removed from the samples *in vacuo*. The product was purified on a Sephadex G-75 column (Fig. 4), and glucose 6-phosphate was purified on a DEAE-cellulose column. From the latter the specific radioactivity of reduced hexose (under the conditions used) was calculated. Samples were counted for radioactivity in Triton–toluene scintillant as described by Hughes *et al.* (1973).

Methylation of the mannan

A modification of the Hakomori (1964) method was used; a solution of methyl sulphinyl carbanion in dimethyl sulphoxide was prepared by the method of Conrad (1972). Deacylated mannan (10mg), prepared by treatment of lipomannan with NaOH (0.2M, 20°C, 1h), was dried *in vacuo* and dissolved in dimethyl sulphoxide (1.5ml) at 20°C. Carbanion solution (0.5ml = 1mequiv.) was added to the solution, and the mixture stirred under N_2 (20°C, 2h). Methyl iodide (0.5ml = 8mmol) was added slowly to the alkoxide solution; after stirring for 30min the reaction mixture was dialysed overnight against running tap water and extracted with chloroform. The extract was evaporated to dryness at 35°C under decreased pressure, giving a white residue of permethylmannan.

Methanolysis of permethylmannan

The permethylated mannan was degraded by methanolysis in methanolic HCl (2M, 8h, 100°C) in sealed ampoules. The reagent was removed in a desiccator over KOH and anhydrous $CaCl_2$ at atmospheric pressure to prevent loss of volatile methyl tetra-*O*-methylmannoside (Stewart *et al.*, 1968). Ethers were dissolved in chloroform and analysed by g.l.c. on a column (4m) of 2% neopentyl glycol succinate polyester on 80–100mesh Chromosorb W. N_2 was used as carrier gas (15ml/h). Standards of di-*O*-methylglycerol and methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside were also run.

Sedimentation and molecular-weight studies

Ultracentrifugation studies were carried out in a Beckman model L2-65B ultracentrifuge with an AN-A-P rotor and schlieren optics. Sedimentation coefficients were determined by using solutions of the lipomannan (1% in 0.2M-ammonium acetate, pH6.9) and deacylated mannan (0.5% in 10mM-sodium phosphate buffer, pH7.0), by velocity runs at 20°C at a rotor speed of 60000rev./min.

A similar solution of deacylated mannan was used for molecular-weight determination by the sedimentation-equilibrium method. Runs were at 25000rev./min at 20°C, and the results analysed as described by Schachman (1959). Values of dc/dr (c = concentration at the radial distance from the centre of rotation, r) for different values of r were measured from the schlieren photograph. A value for the partial specific volume ($\bar{v}$) of 0.613 for hexose units (Gibbons, 1966) was used for the calculation of the molecular weight of the mannan.

Ion-binding to the lipomannan

The ion-binding properties of the lipomannan were investigated by using methods described by Lambert *et al.* (1975). The mannan was converted into its sodium salt by passage down a small column of Dowex 50 (Na^+ form), and freeze-dried. Solutions of the polymer (containing approx. 1μequiv. of free carboxyl group/ml) were prepared in buffer (sodium phosphate, 10mM with respect to Na^+, pH7.0), and 1ml portions in bags made from Visking dialysis tubing (20/32in) were placed in Universal (25ml) screw-cap bottles containing 10ml of similar buffer to which was added $MgCl_2$ at concentrations from 0.1 to 1.0mM. The contents of the bottles were equilibrated overnight by repeated inversions, and

the concentrations of Mg^{2+} inside and outside the bag were measured by atomic absorption spectroscopy. The concentration of polymer inside the bag was calculated from the hexose content.

Chromatographic methods

Solvent systems used were: A, butan-1-ol–pyridine–benzene–water (5:3:1:3, by vol.); B, ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.); C, butan-1-ol–pyridine–water (6:4:3, by vol.); D, propan-1-ol–aq. NH_3 (sp.gr. 0.88)–water (6:3:1, by vol.); E, propan-2-ol–aq. NH_3 (sp.gr. 0.88) (2:1, v/v); F, propan-1-ol–aq. NH_3 (sp.gr. 0.88) (3:2, v/v); G, benzene–methanol–acetic acid (45:8:4, by vol.). Paper chromatography was on Whatman no. 1 paper, and t.l.c. was on plates of Kieselgel H.

Sugars were detected with the $AgNO_3$ reagent (Trevelyan *et al.*, 1950), glycerol with the periodate–Schiff reagent (Baddiley *et al.*, 1956), and dicarboxylic acids by spraying with 0.05% Bromophenol Blue in 0.2% citric acid (Kennedy & Barker, 1951). Hydroxamates were detected on paper by spraying with aq. 10% (w/v) $FeCl_3$ (Thompson, 1951).

Electrophoresis was carried out in buffer B (pyridinium acetate, pH 5.3), at 50 V/cm on Whatman 3MM paper. Column chromatography on Sepharose 6B was in buffer at pH 6.9 (0.2 M-ammonium acetate containing 0.01% of NaN_3), and Sephadex columns were irrigated with buffer containing pyridine (4ml/litre) and acetic acid (10ml/litre). Void volumes were determined with Blue Dextran. Affinity chromatography on concanavalin A–Sepharose was in buffer A containing 0.1 M-sodium acetate, 1 M-NaCl, and $MnCl_2$, $MgCl_2$ and $CaCl_2$ (each 1 mM, containing 0.01% NaN_3, pH 6.0).

Results

Purity of material from the Sepharose 6B column

Fractionation of crude extracts from *M. lysodeikticus*, *M. flavus* and *M. sodonensis* on Sepharose 6B in each case gave a hexose fraction (peak 1), emerging just after the void volume of the column. Degraded nucleic acid and other carbohydrates were included further into the gel (Fig. 1). The yield of peak-1 material from *M. lysodeikticus* (25g dry wt.) was 310mg.

Acid hydrolysis of material corresponding to peak 1 for each organism showed mannose to be the major component on paper chromatography (solvent system B). Material from both *M. sodonensis* and *M. lysodeikticus* was contaminated with variable but small amounts of a component containing glucose and glucosamine, but the material from *M. flavus* contained mannose as the only sugar. Since the mannan from *M. lysodeikticus* was to be subjected to detailed structural analysis, it was further purified as described below. Results for the other micrococci were obtained directly from the material obtained by chromatography on Sepharose 6B.

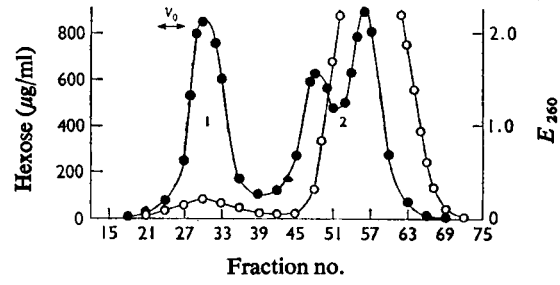

Fig. 1. *Chromatography of the crude phenol extract from whole cells of M. lysodeikticus on Sepharose 6B*

Peak 1 represents lipomannan. Similar elution profiles were obtained with extracts from broken-cell preparations of *M. lysodeikticus*, *M. flavus* and *M. sodonensis*. ●, Hexose; ○, E_{260}.

The nature of the contaminant in *M. sodonensis* is uncertain, but the presence of muramic acid in the polysaccharide from *M. lysodeikticus* indicated that it might be of wall origin. Contamination occurred in *M. lysodeikticus* irrespective of whether the phenol extraction was on whole or broken cells. Occasionally very clean preparations were obtained, such as the one used in previous studies (Powell *et al.*, 1974).

Analysis at this stage showed that the mannan from *M. lysodeikticus* contained about 1% of protein and 1.5% of phosphate, all of which could be accounted for as nucleic acid. No glycerol phosphates, the expected hydrolysis products of teichoic acids, were detected after acid hydrolysis.

Material corresponding to peak 2 from the Sepharose 6B fractions (Fig. 1) was heavily contaminated with nucleic acid and in all cases contained glucose, glucosamine and mannose; it was not examined further.

Further purification of the lipomannan from M. lysodeikticus

Attempts to free the lipomannan from contaminating polysaccharide by rechromatography on Sepharose 6B were only partly successful, showing that contamination was not due to overlap of fractions. Since we showed (Powell *et al.*, 1974) that the lipomannan has a strong affinity for concanavalin A, a purification step using concanavalin A–Sepharose 4B was attempted. This method has been used successfully in the purification of lipoteichoic acids containing glucose (Chiu *et al.*, 1974), so the fact that

the lipomannan is not a simple polysaccharide was not expected to cause difficulties.

The lipomannan (90mg) from the Sepharose column was applied to a column of concanavalin A–Sepharose (21cm×2.5cm) in buffer A. The chromatogram was developed with 2 column-volumes of buffer A and gave 12mg of material containing mainly glucose and glucosamine. Elution with buffer A containing 0.1M-methyl α-mannoside gave pure lipomannan (25mg), but complete recovery of polymer required the subsequent addition of Triton X-100 (1%, w/v) to the eluting buffer. Triton was removed from the lipomannan by repeated extraction with toluene and chromatography on Sepharose 6B. Material purified in this way contained mannose as the only sugar constituent.

Properties of the micrococcal lipomannans

Polysaccharides from each of the micrococci emerged just after the void volume on Sepharose 6B, consistent with a mol.wt. of about 1×10^6. Lipoteichoic acids, in micellar form, extracted from other organisms (Coley *et al.*, 1975) chromatograph in the same position, indicating a similar micellar size.

Chromatography of the lipomannan from *M. lysodeikticus* on DEAE-cellulose (acetate form) showed it to have acidic properties (Fig. 2); it was not removed from the column by elution with water, but in a pyridinium acetate gradient (0–1M) the lipomannan was partly recovered between 0.1 and 0.3M-acetate. Complete recovery required the addition of Triton X-100 (1%, w/v) to the final eluting buffer, in a similar way as was observed in the chromatography on concanavalin A–Sepharose.

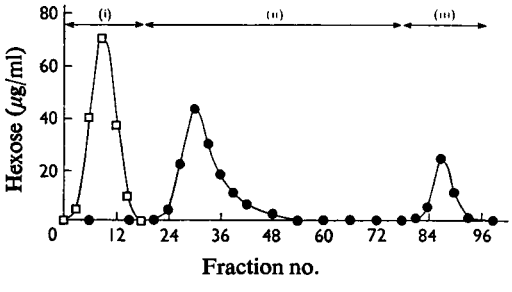

Fig. 2. *Chromatography of lipomannan and deacylated polysaccharide from M. lysodeikticus on a column of DEAE-cellulose (acetate form)*

The column (30cm×10cm) was irrigated with (i) water; (ii) a linear gradient of 0–1M-pyridinium acetate; (iii) 1M-pyridinium acetate containing 1% of Triton X-100. Fractions (4ml) were collected. □, Deacylated mannan; ●, lipomannan.

No chemical differences were found between forms having different affinities for either the ion-exchange or concanavalin A–Sepharose columns; the effect of detergent suggests that the lipomannan may exist in solution in two physically distinguishable forms, each having a different affinity for the column. These observations are consistent with our suggestion (Powell *et al.*, 1974) that it forms micelles in solution. The high apparent molecular weight, shown by its characteristics on Sepharose 6B, also supports this conclusion.

On treatment of *M. lysodeikticus* mannan with mild base, marked differences in its size and charged properties were observed. The mannan treated in this way had no affinity for DEAE-cellulose (Fig. 2) and showed an apparent decrease in size sufficient for it to be included into Sepharose 6B. Chromatography on Sephadex G-50 and G-75 indicated a molecular weight of less than 50000 and probably about 10000.

Paper electrophoresis of the mannan (buffer B, pH5.3, 50V/cm) before and after deacylation also illustrated the change in its charged properties; the lipomannan moved towards the anode whereas the treated material had no mobility.

Ultracentrifugation studies

Sedimentation-velocity studies at 20°C and 60000rev./min gave a sedimentation coefficient, $s_{20,w}$, of 12.4S for purified lipomannan from *M. lysodeikticus*. This is consistent with the micellar properties of the polymer. After base treatment similar experiments gave a $s_{20,w}$ value of 1.1S; this is about the value expected for material with the observed gel-filtration properties of deacylated mannan. In both runs the schlieren pattern was symmetrical during its passage across the cell, indicating probable homogeneity of the materials.

Composition of the lipomannan from M. lysodeikticus after purification by affinity chromatography

The properties described so far suggest that the charged properties and micellar structure of the polysaccharide in solution are imposed by components attached through alkali-labile linkage to the sugar residues. When the lipomannan was examined by paper chromatography after acid hydrolysis (1M-HCl, 100°C, 3h) by using solvent systems A, B and C, the only sugar detected was mannose; in solvents B and D glycerol was detected by the periodate–Schiff reagents. No uronic acids were found.

After alkali hydrolysis (0.1M-NaOH, 100°C, 1h) the mannan was examined for non-sugar components. Acidification and ether extraction of the hydrolysate yielded fatty acids identified by g.l.c. as their methyl

esters. Fatty acids (anteiso C_{15}, 37.2%; isoC_{16}, 7.5%; C_{16} 13.4%; anteiso C_{17}, 13.1%; C_{18}, 10.3%; $C_{18:1}$, 6.3%) were qualitatively similar to those found in whole-cell lipids.

Treatment of the lipomannan with alkaline hydroxylamine converted the acyl substituents into their hydroxamates. Paper chromatography of these derivatives (solvent systems D and E) showed a major component indistinguishable from *N*-hydroxysuccinamic acid (R_F 0.23 in system E) and fatty acid hydroxamates running near the solvent front. Paper electrophoresis in buffer B (pH5.3, 50V/cm) showed only one charged component with the same rate of migration as the monohydroxamate of succinic acid. This suggests that succinic acid residues are attached through monoester linkage to the mannan and that they are responsible for the charged properties of the polymer. Fatty acid hydroxamates had no electrophoretic mobility under the experimental conditions used. The identity of the charged acyl group was confirmed by t.l.c. in systems F and G. In both systems the acyl group was identical with succinic acid and was well separated from other short-chain dicarboxylic acids.

The aqueous hydroxylamine method gave a molar ratio of total acyl groups/mannose of 1:3.9. Fatty acids were determined by treatment of the lipomannan with methanolic HCl after inclusion of a C_{19} acid as internal standard. G.l.c. and integration of the trace obtained gave a ratio fatty acid/mannose of 1:26.

Examination of the polysaccharides from *M. flavus* and *M. sodonensis* gave similar qualitative results; in both polymers, mannose, glycerol, succinic acid and fatty acids were detected. The acyl group/hexose ratio for *M. flavus* was 1:6.98 and for *M. sodonensis* 1:4.88. Detailed structural analysis was not carried out on the lipomannans from these organisms.

Some structural features of the mannan from M. lysodeikticus

Structural analysis of the polysaccharide part of the mannan was carried out on deacylated material; since purification of lipomannan by affinity chromatography was laborious, an alternative procedure was used. The lipomannan (50mg) from the Sepharose 6B column was treated with $NaBH_4$ (0.2M, 20°C, 30min) to protect reducing ends if present, and deacylation was achieved by addition of NaOH to the treated material (0.2M, 20°C, 1h). After neutralization, the deacylated mannan was fractionated on a column (55cm×3cm) of Sephadex G-75. Pure deacylated mannan was included into the column (Fig. 3); it was completely deacylated, and gave no colour in the hydroxylamine assay. Acid hydrolysis showed that glycerol was still associated with the mannan. The contaminating material containing glucose and glucosamine was separated from the mannan and eluted at the column void volume.

The mannan was examined for reducing end groups by treatment with NaB^3H_4. Mannan from the Sepharose 6B column was deacylated after treatment overnight with NaB^3H_4 (see the Experimental section). Chromatography on Sephadex G-75 showed that most of the incorporated radioactivity was excluded from the column and was associated with the polysaccharide contaminant (Fig. 4). By comparison with the specific radioactivity of a glucose 6-phosphate standard reduced under the same conditions it was concluded that the mannan preparation had not more than one reducing end group

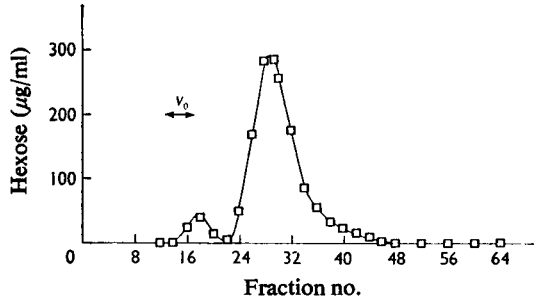

Fig. 3. *Purification of deacylated mannan from M. lysodeikticus on Sephadex G-75*

Contaminating polysaccharide is at the column void volume (V_0), and deacylated mannan is included into the gel. Fractions (2ml) were collected from the column (35cm×1.5cm) and analysed for hexose (□).

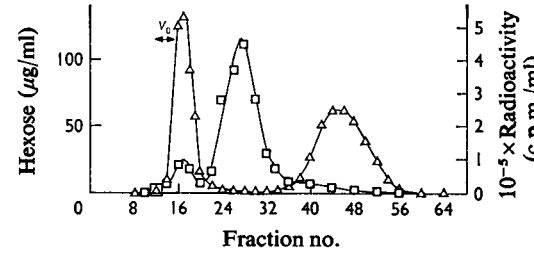

Fig. 4. *Chromatography on Sephadex G-75 of mannan from M. lysodeikticus after treatment with NaB^3H_4 and deacylation in 0.2M-NaOH*

Incorporation of radioactivity is confined to the material emerging at the column void volume. Fractions (2ml) from the column (35cm×1.5cm) were monitored for hexose (□) and radioactivity (△). The mannan has not been labelled.

for 960 hexose units. We conclude therefore that the molecule does not in fact possess a reducing end, and that one end of the chain is terminated by glycerol; this conclusion is confirmed by the methylation studies described below.

Chain-length studies

(*a*) *Ultracentrifugation.* Sedimentation-equilibrium studies on the deacylated mannan showed, from a plot of log $[1/r(dc/dr)]$ (related to the fringe displacement) against r^2, that the material was homogeneous; deviation from linearity in this plot would indicate heterogeneity. Calculation of molecular weight (see the Experimental section) gave a value of 10400 for the deacylated mannan, indicating a chain length of about 64 hexose units.

(*b*) *Chemical methods.* A glycerol analysis was carried out by using glycerokinase. An acid hydrolysate of the lipomannan contained 1 μmol of glycerol/69.5μmol of hexose, indicating one glycerol residue in each chain of about the same length as that found by ultracentrifugation.

The linkage between glycerol and the mannan was examined by periodate oxidation. Lipomannan and deacylated material were treated with sodium metaperiodate and the resulting formaldehyde was detected by the chromotropic acid method. Deacylated mannan yielded formaldehyde, but quantification by this method was not possible since the presence of large amounts of oxidizable hexose interfered. However, oxidation followed by Conway diffusion gave 1 mol of formaldehyde for 65 mol of hexose units with the deacylated mannan; lipomannan itself gave no detectable formaldehyde. These results indicate that glycerol is attached through its 1-position to the mannan and that there is one glycerol on each chain. Further, the glycerol is not oxidizable in the intact polymer, as it is substituted by acyl groups.

Methylation

Deacylated mannan was exhaustively methylated by the Hakomori (1964) method, and the permethyl product treated with methanolic HCl to yield methyl ethers of mannose. Samples were examined by g.l.c. and peaks identified by comparison of their retention times with methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside and the retention times reported by Jones (1972) for other mannose derivatives (Table 1). Methyl tri-*O*-methyl-D-mannoside and methyl tetra-*O*-methyl-D-mannoside were separated on the column at 160°C, and corresponded to sugars from within the chain and from the non-reducing-end positions respectively. Methyl di-*O*-methyl-D-mannosides arise from chain branch points; as these are more polar, a temperature of 190°C was required to elute them from the column. Tri-*O*-methyl derivatives corresponding to 1→2, 1→3 and 1→6 linkages were observed in the approx. proportions 1.88:1:2.36. No evidence for 1→4 links in the main chain was found. Methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside from the non-reducing ends was found in a ratio of 1 to every 21 methyl tri-*O*-methyl ethers, which on the above chain-length indicates two branch points in each mannan chain. A ratio of 1 methyl di-*O*-methyl ether per 31 methyl tri-*O*-methyl ethers again is consistent with two branches in each chain. The linkages at the branch points appear to be 1→2 and 1→4, since the di-*O*-methyl ether was identified as methyl 3,6-di-*O*-methyl-α-D-mannoside. A peak corresponding to methyl 3,5-di-*O*-methyl-α-D-mannoside was detected, but its size was negligible in comparison with the major branch-point derivative, and quantification was not possible. 1,2-Di-*O*-methylglycerol was also found among the methanolysis products and provides further evidence that glycerol is attached to the mannan through its 1-position. It was not possible to determine accurately the amount of this product, since it was incompletely separated from the solvent peak, but the

Table 1. *Partially methylated mannose derivatives from permethylmannan*

Retention times are relative to methyl tetra-*O*-methyl-α-D-mannoside (see the text for temperatures of column). Peak areas are also expressed relative to methyl tetra-*O*-methyl-α-D-mannoside.

Mannose derivative (*O*-methyl mannose)	Relative retention times: Observed	Relative retention times: From Jones (1972)	Relative peak areas
1,2,3,4,6-	1.00	1.00	1.00
1,2,3,4-	2.01	1.88	9.48
1,3,4,6-	2.13	1.93	7.56 } 21.06
1,2,4,6-	2.44	2.30	4.02
1,3,6-	3.46	3.50	0.67
1,3,5-	4.59	4.60	trace peak
Di-*O*-Me-glycerol	0.53	0.53*	≈0.4

* Retention time of a standard run on the same column.

approximate ratio of di-*O*-methylglycerol to methyl 2,3,4,6-tetra-*O*-methyl-α-D-mannoside of 1:2.5 again is consistent with the proposed chain length, and with the presence of one glycerol group in each chain.

Binding of Mg^{2+} to lipomannan

The binding of Mg^{2+} to the lipomannan from *M. lysodeikticus* was measured at various concentrations of Mg^{2+} under conditions of constant ionic strength (10mM-Na^+) at pH7. Binding data are presented as a binding isotherm (Fig. 5*a*), which shows the number of Mg^{2+} ions bound/ml of lipomannan solution ($\bar{r}$) against the equilibrium concentration of Mg^{2+} ions in the system (*A*) (see Lambert *et al.*, 1975). The isotherm shows that the compound progressively binds more Mg^{2+} ions as the concentration of applied Mg^{2+} increases, even though Na^+ ions are present in a 10–100 molar excess.

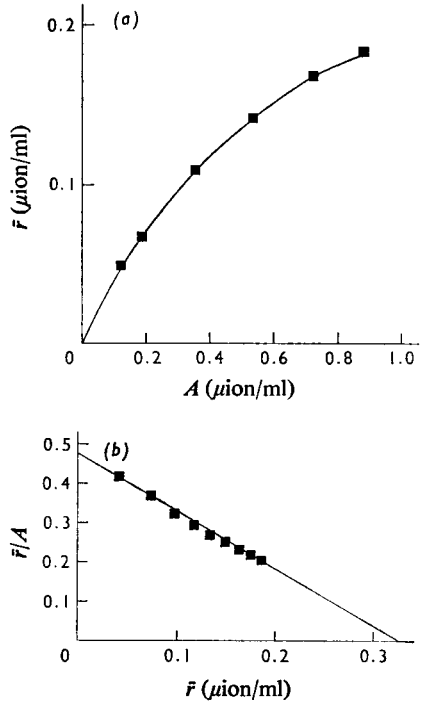

Fig. 5. *Binding of Mg^{2+} to lipomannan at pH7.0*

(*a*) Isotherm for Mg^{2+} binding to lipomannan from *M. lysodeikticus*. The amount of Mg^{2+} bound/ml of mannan solution ($\bar{r}$) increases with equilibrium concentration of Mg^{2+} (*A*) in the system. Binding was measured by equilibrium dialysis (see the text) at pH7 in the presence of 10mM-Na^+. (*b*) Binding data for lipomannan expressed as a Scatchard (1949) plot. Extrapolation of the plot to its intercept with the $\bar{r}$ axis gives the apparent number of binding sites/ml of solution.

Analysis of the results using the Scatchard (1949) plot of $\bar{r}/A$ against $\bar{r}$ (Fig. 5*b*) gives an indication of the nature and number of the binding sites for Mg^{2+} (Lambert *et al.*, 1975). The linearity of the Scatchard plot indicates that all binding sites on the lipomannan are equivalent. The total number of available sites (*n*) is obtained from the intercept of the Scatchard plot with the $\bar{r}$ axis, and is equivalent to one Mg^{2+} ion bound per 3–4 succinic acid residues. The apparent association constant, $K_{assoc.}$ (from the slope of the Scatchard plot), is $1.47\times10^3\,M^{-1}$ at pH7.0, and is of the same order as that calculated for the binding of Mg^{2+} to glycerol teichoic acids ($2.7\times10^3\,M^{-1}$, at pH5.00) (Lambert *et al.*, 1975).

Affinity for the cytoplasmic membrane

Lipoteichoic acid can be released from isolated cell membranes by washing to remove Mg^{2+} ions (Hughes *et al.*, 1973; Coley *et al.*, 1975); by the application of similar methods to the lipomannan its relative affinity for membranes can be established.

Cells (*M. lysodeikticus*, 20g wet wt.) were suspended in Tris buffer (100ml, 0.05M, containing 0.03M-$MgCl_2$ and 0.01M-EDTA, pH7.5) and treated with lysozyme (1mg/ml) and nucleases (deoxyribonuclease and ribonuclease, each 10μg/ml) at 37°C for 16h to effect complete cell lysis and degradation of nucleic acids. Membranes were centrifuged (27000*g* at 0°C) and washed again in the same buffer to remove wall material. After further washing with the same Tris buffer to remove excess of Mg^{2+}, the membranes were incubated twice at 0°C for 1h in Tris buffer containing 3mM-EDTA. Phenol extraction of all of the buffer washings, and chromatography on Sepharose 6B, showed that negligible amounts of the lipomannan had been removed from the membrane. However, at each washing stage, material corresponding to peak 2 (Fig. 1) was released. Lipomannan could be extracted with phenol from the membrane pellet remaining after the above procedure. Chromatography on Sepharose 6B showed a peak as in Fig. 1 corresponding to lipomannan, but peak 2 was very small. Hence the lipomannan appears to have a greater affinity for membranes than do lipoteichoic acids.

Discussion

The micrococcal lipomannans described here add another class of polymers with both hydrophobic and hydrophilic properties to the envelope components of bacterial cells. These substances vary in structural complexity, but all seem to be associated through their lipid parts with hydrophobic regions of the cell envelope. In the outer membrane of Gram-negative bacteria lipopolysaccharides occur together with lipid and protein, and in Gram-

positive organisms lipoteichoic acids are common membrane components. Less widespread are the mycobacterial polysaccharides (Ballou, 1968) containing succinic acid in half-ester linkage, among other acyl substituents; these have been implicated in fatty acid biosynthesis (Machida & Bloch, 1973). In *Thermoplasma acidophilum* an interesting polysaccharide containing long-chain alcohols in ether linkage has been isolated (Mayberry-Carson *et al.*, 1974). In the present paper the occurrence of lipomannans in membrane extracts of *M. lysodeikticus*, *M. sodonensis* and *M. flavus* is described. Some structural features of the compound from *M. lysodeikticus* have been elucidated.

The properties of the lipomannan from *M. lysodeikticus* are inconsistent with those of a simple polysaccharide. It has a negative charge and exists as micelles in solution, the latter property inferring a lipid character similar to that described for lipoteichoic acids (Coley *et al.*, 1972). Treatment with dilute alkali gave a polysaccharide having no acidic properties and a markedly decreased apparent size. The molecule is thus composed of a carbohydrate portion and alkali-labile groups, giving it both a charged and a hydrophobic character. These groups are succinic acid and long-chain fatty acids which are in ester linkage to the mannan.

Ultracentrifugation of the deacylated mannan indicated a molecular weight of 10400, corresponding to a chain length of about 64 sugar units. The reducing end of the mannan is substituted by an alkali-stable, acid-labile group, and the detection of glycerol in acid hydrolysates in quantities consistent with there being one molecule per chain suggests that it is the terminal group. The deacylated mannan gave one molar proportion of formaldehyde per chain on periodate oxidation, and 1,2-di-*O*-methylglycerol on methanolysis of the permethyl polymer; the mannan–glycerol linkage therefore involves a primary hydroxyl group on glycerol. Two fatty acid residues are present for 52 hexose units, and it is likely that these fatty acids are in ester linkage to glycerol because the glycerol is stable towards periodate in the lipomannan itself. Succinylmonohydroxamate was formed when the polymer was treated with alkaline hydroxylamine, indicating the presence of succinic half-esters on sugar hydroxyl groups; one of these half-ester residues is present for about four sugar residues. The identity of succinic acid as the charged substituent on the mannan was also found by Owen & Salton (1975) in a report which appeared during final preparation of this paper.

Methylation studies on the deacylated mannan show the glycosidic linkages to be a mixture of 1→2, 1→3 and 1→6 in the proportions 1.88:1:2.36, and that there are two branch points in each chain; the linkages at the branch points are 1→2 and 1→4. It is likely that a high proportion of the glycoside bonds have the α-configuration, since the mannan has a high affinity for concanavalin A (Powell *et al.*, 1974) and a high positive optical rotation (Macfarlane, 1964; Scher & Lennartz, 1969).

While we were preparing this paper, a report appeared (Pless *et al.*, 1975) in which a closely similar set of experiments on the structure of the lipomannan from *M. lysodeikticus* had been carried out. The data presented are in broad agreement with our own, the major differences being in fatty acid composition and the total acyl/hexose ratio, both of which could be a consequence of the different growth conditions used. The conclusions reached from our data and the results of Pless *et al.* (1975) are consistent with the structure proposed for the lipomannan in Fig. 6.

The similarity between the proposed lipomannan structure and that of lipoteichoic acids may be significant, since in the three organisms studied here lipoteichoic acids were not detected. Both molecules have similar properties, and it is likely that the association of lipomannan with membranes is due at least in part to intercalation of its fatty acids with hydrophobic membrane components. Experiments *in vitro* (Pless *et al.*, 1975) have shown that the deacylated mannan does not bind to membranes, supporting the suggestion that acyl residues are responsible for its affinity. The lipomannan is a true membrane component, and we have shown that it remains associated with cytoplasmic membranes under conditions which have been found to remove lipoteichoic acids in other organisms. Treatment of membranes with high salt concentrations and detergents (Pless *et al.*, 1975) also fails to remove it. It is noteworthy that work by Owen & Freer (1972) has shown, by comparison of the distribution of hexose in membrane fractions, that mannan may be concentrated in mesosomal vesicles of *M. lysodeikticus*.

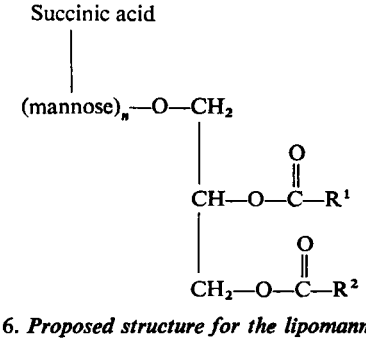

Fig. 6. *Proposed structure for the lipomannan*

R^1 and R^2, fatty acids; $n = 52$–70. Ratio succinic acid/mannose, 1:4.

From earlier studies it was apparent that lipoteichoic acids are bound to the cell membrane through two structural features: (1) non-bonded interactions involving the long-chain acyl substituents in association with the hydrocarbon chains of other lipids in the bilayer structure (Toon *et al.*, 1972; Van Driel *et al.*, 1973) and (2) bonds through bivalent cations, e.g. Mg^{2+}, between phosphate groups in the teichoic acid and unidentified groups (possibly phosphate) in other membrane components. Thus removal of cations through washing of membranes with EDTA causes the gradual removal of lipoteichoic acid (Hughes *et al.*, 1973), and the preparation of membrane particles from disrupted cells in the presence of only low concentrations of Mg^{2+} results in loss of most of the teichoic acid to the supernatant (Hay *et al.*, 1963), whereas in higher concentrations of Mg^{2+} it remains with the particles (Shockman & Slade, 1964). The lipomannan, however, is more firmly bound to the membrane, as it remains attached even after extensive washing with EDTA solutions.

It appears to be essential for organisms to have a charged polymer in the region of the membrane; hence it is not surprising that the ion-binding properties of the lipomannan are closely similar to those of lipoteichoic acids. The latter, together with wall teichoic acids, have been implicated in the maintenance of the correct cationic environment for membrane-bound enzymes (Hughes *et al.*, 1973). Glycerol teichoic acids have a higher binding capacity (Lambert *et al.*, 1975) for Mg^{2+} than does the lipomannan, but the binding constants are of the same order, so the proposal that both polymers have similar functions is reasonable.

In view of our suggestion that the lipomannans represent lipoteichoic acid substitutes in these micrococci, it is interesting that the presence of teichoic acids in wall preparations from *M. flavus* and *M. sodonensis* has also been precluded (Partridge *et al.*, 1973). Similarly *M. lysodeikticus* completely lacks a teichoic acid in its cell envelope; its wall contains a teichuronic acid polymer of alternating glucose and 2-acetamido-2-deoxymannuronic acid residues (Perkins, 1963).

The demonstration of the presence of an acylated polysaccharide containing succinic acid residues in *M. sodonensis* is interesting in view of the observation that secretion of an alkaline phosphatase from this organism is dependent on the simultaneous secretion of an acidic polysaccharide (Braatz & Heath, 1974).

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