AN ACYLATED MANNAN IN THE MEMBRANE OF *MICROCOCCUS LYSODEIKTICUS*

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Received 22 February 1974

1. Introduction

Membrane teichoic acids of Gram-positive bacteria are covalently attached to glycolipid [1—3]. These lipoteichoic acids are situated on the outside of the cytoplasmic membrane [4, 5] and, unlike the wall teichoic acids which are absent from some species, the membrane lipoteichoic acids were thought to occur in all Gram-positive bacteria. As studies by others [6, 7] on the composition of membranes from *Micrococcus lysodeikticus* did not refer to the presence of a membrane teichoic acid this possibility has now been examined. It was already known that the wall of this organism contains a polymer of equimolar amounts of *N*-acetylaminomannuronic acid and glucose [8] and the membrane has associated with it a mannan [9]. Various structural features of this mannan have been described [10].

We report here that using an 80% aqueous phenol extraction procedure [3] no lipoteichoic acid has been found. However these studies have shown that the membrane-associated mannan has properties in common with lipoteichoic acids [3, 11]. It forms micelles in aqueous solution and possesses a negative charge that makes it capable of binding cations. The mannan is acylated and is presumably attached to the cytoplasmic membrane by intercalation of its fatty acid residues in a similar manner to that suggested for lipoteichoic acids [2].

2. Results

Micrococcus lysodeikticus ATCC 4698 (purchased as freeze-dried cells from Miles Chemical Co., USA) and *Micrococcus lysodeikticus* NCIB 9278 grown in this laboratory in batch culture (Oxoid Nutrient Broth 25 g/l, Difco yeast extract 10 g/l, dipotassium hydrogen phosphate 10 g/l, glucose 10 g/l) were studied and found to give closely similar results. They were morphologically indistinguishable, sensitive to lysozyme [12], contained only traces of phosphorus in their cell walls [13] and lipid analysis indicated that both conformed to the pattern reported by DeSiervo et al. [14].

Washed bacteria (30 g dry weight) were disrupted in a Braun disintegrator with cooling; unbroken cells and walls were removed by centrifugation (17 000 *g* for 20 min) and the supernatant solution was freeze-dried. The residue was extracted twice with $CHCl_3$—MeOH (2:1 v/v) to remove lipids and was then dried (yield 620 mg). Analysis of the resultant material showed it to contain hexose [15], fatty acids [16] and nucleic acids (estimated by absorption at 260 nm). This defatted membrane material was extracted with 80% aqueous phenol, and the nucleic acid in the extract was degraded by incubation with nucleases as previously reported [3]. The crude membrane extract was fractionated on a column of Sepharose 6B (25 mm × 450 mm) using upward flow (15 ml/hr) with 0.2 M-ammonium acetate containing 0.01% of sodium acetate as eluant. The elution diagram (fig. 1) showed a peak in the void volume (hexose as parameter) and also a second peak included into the gel. This second peak represented material containing both hexose and degraded nucleic acid.

The fractions from the void volume were evaporated to a small volume, dialysed and freeze-dried (yield 380 mg). A sample of freeze-dried powder was subjected to acid hydrolysis (1 M HCl, 100°C, 2 hr) and the products were examined by paper chromatography

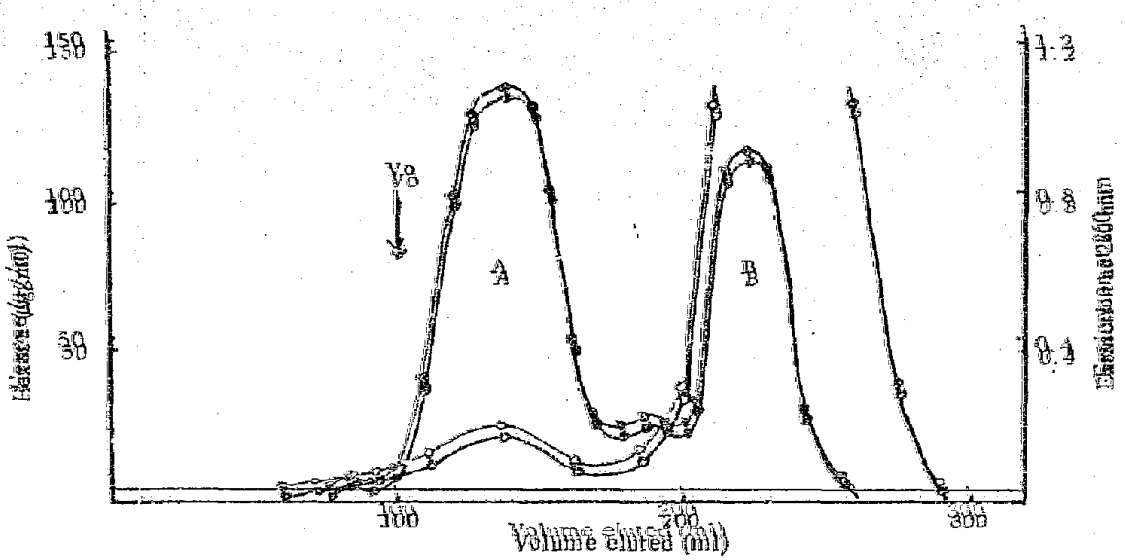

Fig. 1. Chromatography of phenol extracts of membranes on Sepharose 6B using 0.2 M ammonium acetate containing 0.01% of sodium azide. Peak A is the void volume peak and represents mannan. Peak B is included in the gel. (•——•) Represents hexose; (○——○) Represents extinction at 260 nm.

using a solvent system giving a good separation of sugars (ethyl acetate–pyridine–acetic acid–water, 5:5:1:3 by volume); mannose (determined as hexose [15]) and glucosamine (detected by amino acid analysis of a 2 M acid hydrolysate) were present in molar ratio 220:1. Examination by gas-liquid chromatography (on a column of 3% ECNSS-M on Gas-Chrom. Q [17]) of the alditol acetates prepared from an acid hydrolysate of this material confirmed this hexose composition. We therefore conclude that the material emerging from the column in the void volume is the mannan previously described [9, 10].

Further analysis of this sample revealed traces of glycerol-containing material (detected on paper chromatography), protein (1%) [18] and organic phosphates (1.5%) [19]. All of the phosphorus could be accounted for as nucleic acid and no α-glycerol phosphate, a characteristic product of a glycerol teichoic acid, was detected in hydrolysates. Acyl group analysis by the hydroxamate method showed that the mannan contained 1 acyl residue in 22 hexose units. Treatment of the mannan with methanolic HCl yielded fatty acid methyl esters, shown by gas-liquid chromatography (on a column of 3% PEGS on celite) to occur in the following proportions: *Anteiso* C_{15} 37.2; *iso* C_{16} 7.5%, C_{16} 13.4%, C_{19} 13.1%, C_{18} 10.3%, $C_{18:1}$ 6.3%. Agarose gel double diffusion of the mannan against concanavalin A gave a single sharp line which was dissolved by α-methyl mannoside at concentrations above 20 mg/ml.

Surprisingly the purified mannan bound readily to a column of DEAE-cellulose and could be eluted in a gradient of pyridinium acetate (fig. 2a). This ability to bind to ion-exchange columns was destroyed by controlled treatment of the mannan with dilute alkali (0.1 M NaOH, 100°C, 1 hr). Loss of binding capacity was accompanied by the formation of free fatty acids which were extracted into ether, emthylated and examined by g.l.c. as before. They were qualitatively similar to the fatty acids produced by hydrolysis of lipids extracted from whole cells.

The mannan was examined by paper electrophoresis to assess its ionic properties. At pH 6.5 (pyridinium acetate buffer at 50 volts/cm for 1 hr) the intact mannan migrated towards the anode. Under the same conditions deacylated mannan behaved as a neutral molecule. Fatty acid methyl esters, the product of methanolic HCl treatment of mannan were examined by t.l.c. (in petroleum ether [60–40]; ether, 60:40 by volume). Two classes of methyl esters were found; one travelling near the solvent front (typical of neutral esters), the other travelling in a position (R_f 0.39) indicative of more polar character. Treatment of mannan with hydroxylamine at pH 8.0 and

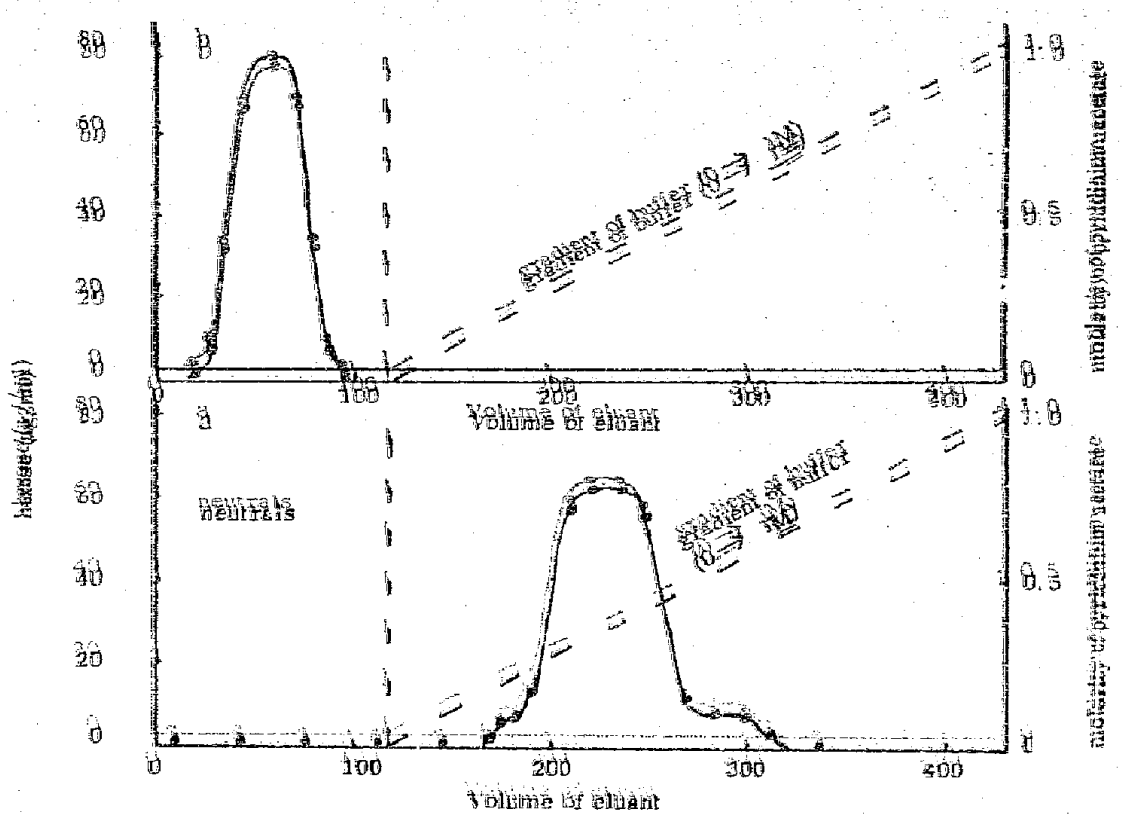

Fig. 2(a). Chromatography of intact mannan on DEAE-cellulose. Material was eluted with water followed by a linear gradient of pyridinium acetate (0 → 1 M). (●——●) Represents hexose. (b) Chromatography on DEAE-cellulose of mannan after deacylation with 60% HF. (●——●) Represents hexose.

methylation of the resultant acyl hydroxamates, gave a similar methyl ester composition. The presence of the second class of fatty acids may explain the charged properties of the polymer.

Treatment of the mannan with 60% hydrogen fluoride at 0°C for 24 hr followed by neutralisation with lithium hydroxide, centrifugation and addition of an equal volume of chloroform to the supernatant yielded free fatty acids in the chloroform phase and the deacylated mannan in the aqueous phase. This deacylated mannan did not bind to DEAE-cellulose (fig. 2b) and was included in a column of Sepharose 6B (fig. 3). Under these conditions, which are normally used for the dephosphorylation of phosphodiesters, partially acylated sugars are deacylated whereas acyl esters of acyclic alcohols, e.g. glycerol, are unaffected (personal communication by Dr N. Shaw).

The mannan was examined for its ability to bind cations; control experiments were carried out using yeast mannan and a neutral polysaccharide, amylopectin (both purchased from Sigma Chemical Co., USA). Samples of polysaccharides (20 mg) were equilibrated with magnesium ions by incubation in $MgCl_2\ 6H_2O$ (25 mM, 1 hr, 37°C). Material was recovered by the addition of 3 volumes of ethanol, and the supernatant discarded. Unbound Mg^{2+} was removed by washing (3 times) with water, followed by ethanol precipitation. Mg^{2+} was extracted from dried material (10 mg) into 1 M $HClO_4$ (5 ml) [11] and determined by atomic absorption spectrometry (Unicam SP 20 series 2). Hexose was determined by the phenol-sulphuric acid method of Dubois et al. [15]. Binding occurred to the following extent (milli-equivalents of Mg^{2+}/mole of hexose): amylopectin 8, yeast mannan 17 and mannan from *M. lysodeikticus* 92.

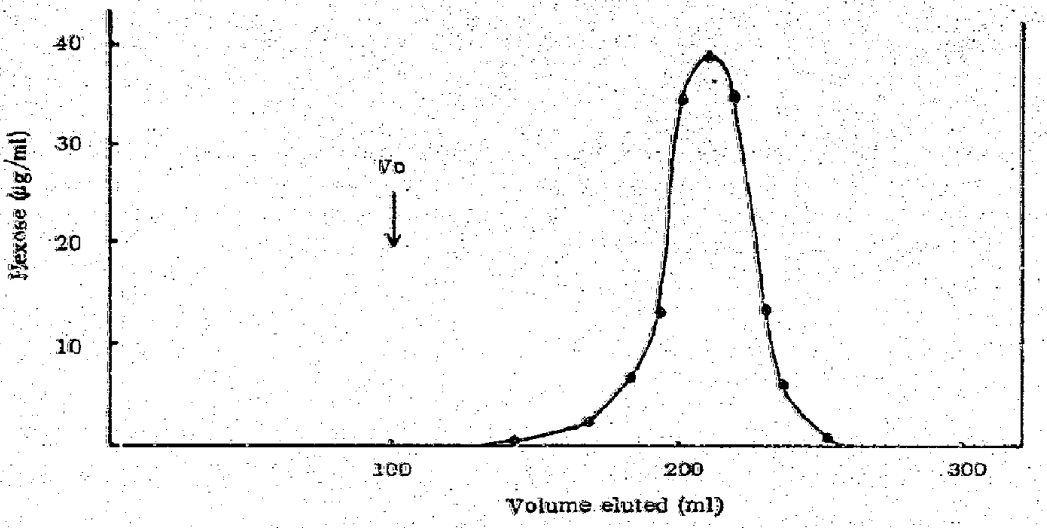

Fig. 3. Chromatography on Sepharose 6B of mannan after deacylation with 60% HF. (●——●) Represents hexose.

3. Discussion

Extraction of the membrane of *M. lysodeikticus* by a standard procedure (80% aqueous phenol) has failed to reveal the presence of a lipoteichoic acid. This is the first reported absence of lipoteichoic acid from the membrane of a Gram-positive bacterium. The membrane does, however, possess a mannan. This mannan contains acyl residues which can be removed from the polymer by gentle hydrolysis in alkali. Exclusion of the acylated polymer from Sepharose 6B suggests that it could have a molecular weight of at least 1×10^6; this high apparent molecular weight however, is probably due to the formation of micelles, as has been observed with lipoteichoic acids [3].

The presence of fatty acid ester groups in the mannan presumably accounts for its ability to form micelles and for its association with the cell membrane; the hydrophobic acyl chains would intercalate with lipids in the membrane in the same way as has been proposed for lipoteichoic acids [2]. The presence of polar acyl residues accounts for the charge on the polymer and its ability to bind to DEAE-cellulose; after removal of acyl groups by hydrolytic procedures the charge and binding capacity are lost.

The small amounts of protein which have been consistently found in mannan preparations may be a contaminant (as is the nucleic acid); there is no direct evidence for a mannan-protein linkage. Various structural features of the mannan have been determined by exhaustive methylation [10]; the polymer was shown to contain 1→2, 1→3 and 1→6 mannosidic linkages. We suggest that a high proportion of these linkages must be α as the polymer has a very high affinity for concanavalin A. A revaluation of the methylation data is now necessary as the hitherto undetected acyl residues might affect the degree of methylation and consequently the apparent number of branch points.

Biosynthetic studies on mannan [10] indicate that mannose is added to the nonreducing end of endogenous mannan via GDP-mannose and mannosyl-l-phosphoryl-undecaprenol. De novo synthesis has not been demonstrated and again the presence of acyl groups may explain this by requiring the participation of an additional factor during the addition of mannosyl units.

Recently it has been shown that membrane lipoteichoic acids can act as acceptors for the biosynthesis of wall teichoic acids of the poly(ribitol-phosphate) or poly(glycerol-phosphate) types [20]. It may be significant that the biosynthesis of the wall polymer of *N*-acetylaminomannuronic acid and glucose in *M. lysodeikticus* needs an acceptor within the membrane [21]. This acceptor is not a lipid intermediate but its described properties are consistent with those of the acylated mannan.

The distribution of acyl groups along the mannan has not been determined, but if the analogy with lipo-

teichoic acids (where the acyl groups are attached to the glycolipid end) can be extended, then the non-polar fatty acids would occur towards one end of the molecule for anchorage of mannan to membrane, whereas the charged fatty acids would be distributed along the chain where their function would be similar to that of charged phosphate groups along a lipoteichoic acid chain. An important aspect of teichoic acid function is their ability to bind cations and thereby control particularly the availability of divalent cations at the membrane [22, 11]. The mannan from *M. lysodeikticus* probably assumes a similar role since the polar acyl substituents enable it to bind cations effectively. It is eleven times more efficient in binding Mg^{2+} as is amylopectin and five times more efficient than is yeast mannan; the significant ability of the yeast mannan to bind cations is presumably due to the known presence of phosphate groups in that polymer [23].

We thank the Science Research Council for a studentship to D. A. Powell.

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