The Characterization of Mannan of *Micrococcus lysodeikticus* as an Acidic Lipopolysaccharide*

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**SUMMARY**

Ghosts of *Micrococcus lysodeikticus* contain a mannan that is not removed by intensive washing procedures. Purified mannan, isolated by extraction of whole cells with hot, aqueous phenol, binds to membranes in vitro. Mannan also binds to DEAE-cellulose and migrates toward the anode in neutral and sodium dodecyl sulfate disc gel electrophoresis. In aqueous solution mannan has an apparent molecular weight of 10, but in the presence of sodium dodecyl sulfate its apparent molecular weight is 50,000 to 100,000; removal of the detergent results in reaggregation. Purified mannan contains mannose, succinate, fatty acid, and glycerol in a ratio of 50:4.9:2.1:1.0. Treatment of mannan with mild base produces a neutral, hydrophilic polysaccharide of relatively low molecular weight that has no affinity for membranes. At least 90% of the reducing termini are blocked in a base-stable linkage. Based on these results a tentative structure is proposed for the mannan.

Previous investigations in this laboratory have shown that incubation of the crude membrane fraction of *Micrococcus lysodeikticus* with GDP-[14C]mannose results in the incorporation of mannose into four compounds, mannosyl-(1→3)-diglyceride, mannosyl-((1→3)-mannosyl-(1→3)-diglyceride (1), mannosyl-phosphoryl-undecaprenol, and mannan (2→4). It was established that mannosyl-phosphoryl-undecaprenol is an intermediate in the transfer of mannose from GDP-mannose to the nonreducing termini of mannan. Structural studies showed that mannan contains α(1→2), (1→3), and (1→6) linkages in a ratio of 2:2:1 and acetylation results in the formation of trisaccharide, disaccharide, and monosaccharide in nearly equal amounts. However, it has not yet been possible to define the structure of the mannan in terms of a small repeating unit.

These earlier studies had suggested that mannan is associated with the crude membrane fraction of *M. lysodeikticus* and that it has a relatively high molecular weight (≥2 × 10^6). In addition, it was found that mannosyl is the sole glycosyl moiety in mannan and that it accounts for at least 85% of the dry weight. Because it was difficult to account for the association of a simple, hydrophilic homopolysaccharide with the bacterial membrane, and because we ultimately wished to investigate the de novo biosynthesis of this macromolecule, further structural analyses of mannan have been undertaken. The results demonstrate that, indeed, mannan is tightly associated with purified ghosts of *M. lysodeikticus*. The absence of a reducing terminus and the presence of both a glycosyl moiety and acyl moieties in mannan have been established. The acylated mannan exists as a multimolecular aggregate with a molecular weight of approximately 10^8 in aqueous solution; upon removal of the acyl groups with mild base, mannan exhibits a much lower molecular weight. It appears that the base-labile groups are responsible for the affinity of mannan for the membrane. A preliminary account of some of this work has been presented (5).

**EXPERIMENTAL PROCEDURE**

**Materials**—All chemicals were analytical reagent grade and were purchased from commercial sources unless otherwise indicated. Sterile, uniformly labeled [14C]glucose (268 mCi per mmol) was obtained from Amersham-Searle, and sodium[H]borohydride (164 mCi per mmol) was obtained from New England Nuclear Corp. [γ-32P]ATP (4.5 mCi per mmol) was the gift of Dr. S. Polakis of this department. Glycerokinase (Candida mycoderma) and N-hydroxysuccinimide were the products of Worthington and Nutritional Biochemicals, respectively. Sucinic acid monomethyl ester and Diazald were purchased from Aldrich, and long chain fatty acid methyl esters were obtained from Applied Science Laboratories. Deoxyribose T19, T40 and T110 as well as Sephadex G-75, Sepharose 4B and 6B, and concanavalin A Sepharose were purchased from Pharmacia. DEAE-cellulose was the product of Schleicher and Schuell, Inc.

**Preparation of Uniformly Labeled [14C]Mannan**—The preparation of uniformly labeled [14C]mannan was similar to that previously reported (3). For a typical preparation, 5 liters of media containing 1% Bacto-peptone (Difco), 0.5% NaCl, 0.1% yeast extract, and 250 μCi of uniformly labeled [14C]glucose were inoculated with *M. lysodeikticus* ATCC 4608 and incubated with shaking at 30°C until the cell density reached 500 Klett units (66 filter). After harvesting and washing with 100 ml of 20% Triton X-100, 1% HCl, pH 7.2, the cells had a wet weight of 38.5 g. Washed cells (37.5 g) were suspended in 375 ml of water, and 375 ml of 10% phenol were added. The suspension was heated to 70°C and main-

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tained at that temperature with stirring for 2 hours. The suspension was cooled and centrifuged at 5000 × g for 10 min at 0°C. The phenol layer was washed with 200 ml of water saturated with phenol, and then the aqueous phenol was dialyzed for 17 hours against 60 ml of water at room temperature. The dialyze was centrifuged for 10 min at 12,000 × g to remove insoluble material and then concentrated by rotary evaporation to 60 ml. After making the solution 0.02 M in Tris-HCl, pH 7.5, and 10-4 M MgCl2, 2.1 mg of deoxyribonuclease, and 2.3 mg of ribonuclease were added, and digestion was carried out for 4 hours at 37°C. The sample was concentrated by rotary evaporation to 4.5 ml and applied to a Sepharose 6B column (1.9 × 63 cm). Fractions containing 14C were pooled and analyzed for both 14C and 3H. A control sample containing 1.8 pmol of mannan in 100 μl of H2O was treated with a second 20-μl aliquot of the basic borotritide solution. After removal of methyl borate as described above, the sample was chromatographed on Whatman No. 3MM paper in Solvent System B. Both radioactivity and periodate-benzenidine-positive material were detected exclusively in the position corresponding to mannotetul under conditions where separation of mannoose and mannotetul was achieved. These results demonstrate that the reaction conditions used for the mannan sample were sufficient for quantitative conversion of 1.0 pmol of mannoose to mannotetul. In addition, radioactive analysis of the 14C-mannan control product of the control sample confirmed the specific radioactivity calculated from the data supplied with the [14C]-NaBH4.

Identification and Quantitative Determination of Acyl Groups—Analyses for ester were performed by a modification of the methods described by Lee (10) and Stern and Shapiro (11). To 200 μl of a freshly prepared solution of basic hydroxylamine (1 N NaOH-HCl containing 1.75 N NaOH in water), 15 μl of the ester solution were added. After incubation for 20 min at room temperature, 500 μl of ethanol-ether (3:1) were added, and the reaction was allowed to proceed for 30 min. Then 100 μl of 3.7 M FeCl3 in 0.1 N HCl were added, and the optical density at 520 nm was measured. Monomethyl succinate was used as a standard. Preformed hydroxamic acid was determined in the same way except that water was substituted for the basic hydroxylamine and strong acid solutions, and sodium succinyl monohydroxamate was used as a standard.

Acetyl hydroxamate was synthesized and recrystallized using the method reported by Fishbein et al. (12). Sodium succinyl monohydroxamate was obtained by reacting sucinic anhydride with neutral hydroxylamine (13). Caprylic hydroxamate and stearoyl hydroxamate were prepared from the corresponding methyl esters (14). Dimethyl succinate, formed by treatment of succinic acid with diazomethane, was dissolved in benzene and treated as an equal volume of basic hydroxylamine. After removing the solvent by rotary evaporation, sucinyl dihydroxamate was extracted from the dry residue with methanol. For the preparation of the unknown hydroxamates, an aliquot of mannan purified by ethanol precipitation (52 pmol of hexose) was lyophilized, and 100 μl of basic hydroxylamine were added and allowed to react for 20 min at room temperature. Ethanol-ether (3:1, 250 μl) was added, and the reaction was allowed to proceed for 30 min at room temperature. Ether and excess methyl borate were removed in a stream of nitrogen, and the sample was dissolved in 3.7 M FeCl3 in 0.1 N HCl with stirring. The optical density at 520 nm was measured by the method reported by Novak (15) using myristic acid as standard. For identification of the long chain fatty acids, a solution of diazomethane in ether was added to the ether extract of base-treated mannan and allowed to react for 30 min at room temperature. Ether and excess diazomethane were removed in a stream of nitrogen, and the methylester of the esters was dissolved in 25 μl of benzene. Identification of the methyl esters by gas chromatography was carried out using the conditions reported previously (1).

Quantitative determination of succinate was carried out with succinic dehydrogenase using a cytoplasmic membrane preparation of Bacillus subtilis isolated by the method of Ferrandes et al. (16) as enzyme. The assay reported by these authors for succinic dehydrogenase was modified as follows. The incubation mixture contained 5 μl of 0.025 M Tris-HCl (pH 7.0), 0.6 μl of neutralized KCN, 20 μl of dichlorophenolindophenol, and substrate. The reaction was initiated by the addition of membranes containing 45 μg of protein. After incubation at 30°C for 30 min, the optical density was determined at 600 nm. The content of free succinate in mannan and in a neutralized base hydrolysate of mannan was established by comparison to a standard curve using 0 to 10 μmol of succinate.

Glycerol Determination—Glycerol determinations were carried out by modification of the assay reported for glycero kinase by
Bulbitz and Wieland (17) using the method of Thormer and Paulus (18) to isolate glycerophosphate. The incubation mixture contained 10 μmol of glycerine adjusted to pH 8.0 with KOH, 0.1 μmol of MgCl₂, 0.4 μg of glycerokinase (81 units per mg), 614 pmol of [γ-³²P]ATP (25.0 cpm per pmol) and 0 (v) 100 pmol of glycerol in a total volume of 60 μl. The reaction was allowed to proceed for 30 min at 30° and terminated by the addition of 0.5 ml of 1 N HClO₄ containing 1 μM H₂PO₄. The tubes were placed in boiling water for 40 min to hydrolyze unreacted ATP. After cooling, inorganic phosphate was precipitated by the addition of 0.25 ml of 0.05 M ammonium molybdate and 0.125 ml of 0.2 M triethyamine and centrifugation. The amount of glycerophosphate formed was determined by counting an aliquot of the supernatant solution.

Appropriate aliquots of mannan or mannan hydrolyzed with mild base or strong acid were analyzed after adjusting the pH to about 9. Controls indicated that the mannan contained no components inhibitory to the assay under the conditions used, and paper chromatography of the reaction mixtures in solvents other than glycerol, inorganic phosphate, or ATP, and that no mannosyl ATP was formed.

**Chromatographic Methods**—Solvent systems employed were: A, ethyl acetate-pyridine-water (12:5:3); B, ethyl acetate-pyridine-water (8:2:1); C, isopropyl alcohol-concentrated NH₄OH (2:1); D, benzene-methanol-formic acid (60:40:2); and E, isobutyric acid-concentrated NH₄OH-water (37:4:39). Carbohydrates were detected by periodate benzenediazonium (10), and hydroxamic acids were visualized by spraying with 5% FeCl₃ in ethanol (20).

**Spectroscopy**—Optical density measurements were performed on a Gifford Spectrophotometer 240. Nuclear magnetic resonance measurements were kindly performed by Dr. D. W. Cochran of the Division of Biophysics at The Johns Hopkins University School of Hygiene and Public Health, were carried out on a Varian HA-100 modified with a Digilab Pulse Unit and a Data General Computer to operate in the Fourier Transform mode.

**Results**

**Association of Mannan with Ghosts**—To determine the affinity of mannan for the membrane, ghosts of *M. lysodeikticus* were prepared by lysozyme treatment in hypotonic medium. The resulting crude ghosts were then diluted extensively, collected by centrifugation, and washed three times. This process of dilution, centrifugation, and washing was repeated a second time. Aliquots of the crude ghosts, as well as the ghost after each dilution and multiple washing stage, were analyzed for total protein and mannan content. The latter was determined by a method of hydrolysis of the membranes followed by paper chromatography, elution of the mannan, and quantitative estimation of it by the Park-Johnson method (9). The results, shown in Table 1, reveal that the mannan content of the membrane preparation remains constant throughout the ghost isolation procedure. Moreover, although there is extensive loss of protein during the first dilution and washing steps, thereafter the mannan to protein ratio of the membranes is virtually constant.

**Comparison of Methods for Isolation of Mannan**—The firm association of the mannan with the membrane fraction of *M. lysodeikticus* suggested that the mannan molecule contained constituents other than mannan which could account for its affinity for the membrane. In previous studies (3), uniformly labeled [³⁵C]mnanan was prepared by growing *M. lysodeikticus* in the presence of [³⁵C]glucose. The cells were harvested and incubated at 70° in 41% phenol. After centrifugation of the cooled suspension, the aqueous phase was incubated with deoxyribonuclease, ribonuclease, and pronase. Mannan prepared in this manner was extensively characterized and found to contain [³⁵C]mnanan as the sole radioactive compound after acid hydrolysis.

To examine the possibility that the phenol procedure might cleave linkages of mannan residues to protein or lipid, an alternative method not expected to rupture covalent bonds was used to isolate mannan. Membranes were suspended in 20 volumes of chloroform-methanol (2:1). After the addition of 1 volume of saline (0.9% NaCl solution) and centrifugation, mannan was isolated in the aqueous phase. This preparation and the mannan isolated by the phenol extraction procedure were compared by gel filtration on Sepharose 4B. The two preparations showed no apparent difference in molecular weight. In addition, digestion by pronase had no effect on the elution profile of either sample. Both preparations bound to DEAE-cellulose in 0.05 M Tris-HCl, pH 7.5, and neither pronase digestion nor incubation with bacterial alkaline phosphatase influenced the affinity of the mannan for the anion exchange resin. For subsequent studies, therefore, mannan was isolated by the phenol procedure except that the digestion with pronase was omitted.

**Properties of Mannan**—Mannan eluted from Sepharose 4B near the exclusion volume indicating an apparent molecular weight on the order of 10⁶ (Fig. 1). In the presence of sodium dodecyl sulfate, however, the apparent molecular weight of mannan is reduced by about 10-fold. Mannan which had been preincubated in sodium dodecyl sulfate for 2 hours at 37° was still more retarded by the column, indicating that the disaggregation is time-dependent. As shown, this disaggregation of mannan is

![Fig. 1. Gel filtration of [³⁵C]mannan in the presence and absence of sodium dodecyl sulfate. An aliquot of [³⁵C]mannan purified by ethanol precipitation was applied to a Sepharose 6B column (1.25 × 38.4 cm) and eluted with 0.04 M NaCl (O—O). A second aliquot of [³⁵C]mannan (4.7 × 10⁵ cpm, 170 μmol of mannan) was incubated in 1% sodium dodecyl sulfate for 10 min at 37° and applied to the column which had been equilibrated with 0.04 M NaCl containing 0.1% sodium dodecyl sulfate and eluted with the same solvent (O—O). Fractions containing the peak of radioactivity (17.7 to 25.4 ml) were pooled and dialyzed against 4 × 9 liters of water for a total of 114 hours at 4°. The dialyzed solution was concentrated by rotary evaporation, redissolved in H₂O, and rechromatographed on the same Sepharose 6B column which had been washed with 0.04 M NaCl to remove sodium dodecyl sulfate (Δ—Δ). The [³⁵C]mannan (14.8 to 18.1 ml) was concentrated and analyzed. The arrow labeled X indicates the elution position of a mannan sample incubated for 2 hours at 37° in the presence of 1% sodium dodecyl sulfate prior to gel filtration in 0.1% sodium dodecyl sulfate. B.D., blue dextran.**

### Table 1

| Stage of purification | Mannan | Protein |
|-----------------------|--------|---------|
| I. Crude              | 17.5   | 600     |
| II. Ghosts after first osmotic shock and three washes | 17.5 | 240     |
| III. Ghosts after second osmotic shock and three washes | 17.5 | 220     |
Fig. 2. Gel filtration of untreated and base-hydrolyzed [14C]mannan on Sepharose 6B. The Sepharose 6B column (2 × 26.5 cm) was equilibrated with 0.05 M NaCl, and either [14C]mannan (O—O) or a neutralized base hydrolysate of [14C]mannan (●—●) was applied to the column.

Table II

DEAE-cellulose chromatography of [14C]mannan before and after mild base hydrolysis

Each sample contained 2000 cpm (157 nmol of mannose) and NaOH as indicated in a total volume of 40 µl. After incubation at 37° for 20 min, the reaction mixtures were placed on ice and 2 µmol of Tris- HCl, pH 7.5, 20 µmol of HCl, and sufficient NaOH to adjust the total to 20 µmol were added. After dilution with H2O to a total volume of 1.0 ml, each sample was applied to a column (0.6 × 3 cm) of DEAE-cellulose equilibrated with 0.05 M Tris- HCl, pH 7.5, and eluted sequentially with three 1-ml portions of each of the three eluants.

| Concentration of NaOH in hydrolysate | % [14C]mannan eluted |
|-------------------------------------|----------------------|
| Eluant                              | 0 0.02 0.10 0.50     |
| 0.05 M Tris-HCl                     | 0 2 68 92            |
| 1 M NaCl in 0.05 M Tris-HCl         | 62 58 16 3           |
| 1% Triton X-100 and 1 M NaCl in 0.05 M Tris-HCl | 38 40 16 5 |

The ionic properties of the mannan were also examined by disc gel electrophoresis. As shown in Fig. 3A, intact mannan migrated toward the anode in a standard, neutral gel system. After mild base hydrolysis, however, mannan appeared to be neutral since radioactivity was detected only in the sample and stacking gels. When analyzed by disc gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 3B), intact mannan migrated toward the anode but, as in the neutral gel system, base-hydrolyzed mannan was detected only at the top of the gel. These results suggested that intact mannan is acidic and binds sodium dodecyl sulfate and that both of these properties are lost after mild hydrolysis.

The infrared spectrum of intact mannan indicated the presence of a carbonyl moiety (data not shown). Analysis of mannan before and after base hydrolysis by nuclear magnetic resonance spectroscopy revealed that resonances characteristic of aliphatic groups (<3 ppm) were present in intact mannan but absent after base hydrolysis (Fig. 4). The resonances of the sugar ring protons (3.4 to 4.3 ppm) are similar in the two samples. Both preparations show peaks characteristic of α-linked anomeric C1 protons (4.9 to 5.4 ppm) (1, 23). The only difference in this region of the spectrum between intact mannan and base-treated mannan is the presence of a shoulder at 5.4 ppm in the former sample. Acylation of a hydroxyl group has been shown to deshield the

![Fig. 3A](image1.png)

**Fig. 3A** Disc gel electrophoresis of [14C]mannan before and after base hydrolysis. A, untreated [14C]mannan (5000 cpm, 0.39 µmol of mannose, O—O) or [14C]mannan which had been hydrolyzed in NaOH, neutralized and dialyzed (8000 cpm, 0.63 µmol of mannose, ●—●) were prepared as sample gels for disc gel electrophoresis using the neutral system of Davis (21). Electrophoresis was carried out at 1.5 ma per gel for 3 hours (the tracking dye ran off the end of the gel in 3½ hours). For each experiment, the sample gel, the stacking gel, and 0.25 cm slices of the resolving gel containing the sample were counted to determine radioactivity after treating with NCS solubilizer (22). B, sodium dodecyl sulfate gel electrophoresis was carried out by the method reported previously (22) for 18 hours at 5.5 ma per gel. The samples of either intact [14C]mannan (O—O) or a neutralized base hydrolysate which had been purified by gel filtration and dialysis (●—●) contained 2000 cpm (0.16 µmol of mannose), 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 5 M urea. Radioactivity was determined as indicated above.

![Fig. 3B](image2.png)

**Fig. 3B** Sodium dodecyl sulfate gel electrophoresis of [14C]mannan before and after base hydrolysis. A, untreated [14C]mannan (5000 cpm, 0.39 µmol of mannose, O—O) or [14C]mannan which had been hydrolyzed in NaOH, neutralized and dialyzed (8000 cpm, 0.63 µmol of mannose, ●—●) were prepared as sample gels for disc gel electrophoresis using the neutral system of Davis (21). Electrophoresis was carried out at 1.5 ma per gel for 3 hours (the tracking dye ran off the end of the gel in 3½ hours). For each experiment, the sample gel, the stacking gel, and 0.25 cm slices of the resolving gel containing the sample were counted to determine radioactivity after treating with NCS solubilizer (22). B, sodium dodecyl sulfate gel electrophoresis was carried out by the method reported previously (22) for 18 hours at 5.5 ma per gel. The samples of either intact [14C]mannan (O—O) or a neutralized base hydrolysate which had been purified by gel filtration and dialysis (●—●) contained 2000 cpm (0.16 µmol of mannose), 1% sodium dodecyl sulfate, 1% mercaptoethanol, and 5 M urea. Radioactivity was determined as indicated above.
The methods for the phenol extraction and nucleic digestion (I) as well as gel filtration on Sepharose 6B (II) and ethanol precipitation (III) are described under "Experimental Procedure." Table III contains data on the purification process. Mannan purified through Step III was subjected to gel filtration in the presence of sodium dodecyl sulfate followed by exhaustive dialysis and rechromatography in the absence of detergent (IV) as described in Fig. 1. [14C]Mannan purified through Step IV was hydrolyzed in 0.5 N NaOH. After neutralization the radioactively labeled material was isolated by gel filtration on Sepharose 6B (V).

| Purification step | Ester | Protein |
|-------------------|-------|---------|
|                   | υμmol/50 μmol | [14C]mannose | mg |
| I                 | 9.7   |         | 1.69 |
| II                | 5.9   |         | 0.22 |
| III               | 6.4   |         | 0.24 |
| IV                | 6.2   |         | 0.25 |
| V                 | N.D.* |         | 0.02 |

* N.D., not determined.

Composition of Mannan—The methods described above, together with the previous observation that mannos is the only hexose present (>95%) (3), suggested that the mannan is bound to one or more constituents that contain aliphatic protons, a carbonyl group, and a negative charge at neutral pH. To provide evidence that the non-hexose components are covalently bound, mannan was analyzed for ester, protein, phosphate, and radioactivity as a function of two purification procedures designed to remove noncovalently associated impurities.

In the first experiment, [14C]mannan was isolated by the phenol procedure and gel filtration on Sepharose 6B. After precipitation with 80% ethanol, mannan was chromatographed on concanavalin A Sepharose in 1% Triton X-100 followed by extraction with chloroform-methanol (2:1) and gel filtration on Sepharose 4B to remove the detergent. The ratio of ester to mannan determined for the sample isolated from the initial Sepharose 6B fractionation was unchanged by the subsequent treatments, and the protein content was reduced only by 25% (data not presented).

Analyses of a second preparation of mannan carried through an alternate purification procedure are presented in Table III. Mannan obtained by phenol extraction, dialysis, and nucleic digestion was isolated by gel filtration on Sepharose 6B and further purified by ethanol precipitation. This preparation was subjected to gel filtration in the presence of sodium dodecyl sulfate followed by extended dialysis and rechromatography on Sepharose 6B in the absence of detergent. In spite of the apparent dissociation and reaggregation of the mannan, both the ester and protein contents are essentially unchanged.

For comparison, the composition of the mannos-containing polymer obtained by base hydrolysis and purified by gel filtration is also shown in Table III. In addition to cleavage of the ester linkages, mild base hydrolysis results in a 10-fold reduction in the ratio of protein to mannos. For both untreated and base-

proton attached to the same carbon atom resulting in its absorption appearing in the region from 4.9 to 5.6 ppm (24). Thus, the presence of the shoulder at 5.4 ppm only in the spectrum of intact mannan is consistent with the presence of a small proportion of acetylated mannos residues (approximately 1 per 10 residues).

Identification of the Acyl Groups Linked to Mannan—After treatment of mannan with basic hydroxylamine, paper chromatography of the reaction mixture indicated the formation of both a polar (Rf = 0.12) and a nonpolar (Rf = 0.95) hydroxamic acid. The partially purified hydroxamates were eluted from the paper, acidified, and analyzed by mass spectrometry. The results suggested that the nonpolar component was a mixture of long chain fatty acyl hydroxamic acids and that the slower moving product was succinyl monohydroxamic acid or N-hydroxy succinimide. The reaction mixture was also analyzed by thin layer chromatography (Fig. 5). The major component (Rf = 0.32) co-chromatographed with succinyl mono-

hydroxamic acid. The second product (Rf = 0.68) had a mobility characteristic of the hydroxamic acids of long chain fatty acids. The nonpolar product selectively extracted from the acidified reaction mixture with ether comprised about one-third of the total hydroxamic acid.

The presence of succinyl residues in the mannan was confirmed by assaying for succinate released by base hydrolysis with a membrane-bound succinate dehydrogenase. The quantitative assay indicated that the base hydrolysate of mannan contained 4.9 μmol of succinate per 50 μmol of mannos whereas untreated mannan contained less than 0.5 μmol of free succinate per 50 μmol of mannos. Parallel incubations containing either succinate or the succinate-containing hydrolysate were inhibited to the same extent by an 8-fold excess of malonate (Fig. 6), thus confirming that the enzymatic assay was detecting succinate.

Gas chromatography was used to identify the ether soluble products of the base hydrolysate of mannan. The hydrolysate was acidified and extracted with ether. Fatty acids in the extract were treated with diazomethane and the resulting esters were analyzed. As shown in Fig. 7, the major fatty acids (86%) were a mixture of 12- and 13-methyltetradecanoic acid which have been found to be the major lipid-linked fatty acids in this organism (1). Quantitative analyses for long chain fatty acid were carried out by the method of Novak (15). The results indicated the presence of 2.1 μmol of fatty acid per 50 μmol of
FIG. 5. Analyses of hydroxamic acids by thin layer chromatography. Thin layer chromatography was carried out on Silica Gel H prepared in 2% EDTA, pH 7.2, and developed with Solvent System D. The samples were: 1, acetyl hydroxamate; 2, stearoyl hydroxamate; 3, decyl hydroxamate; 4, ether extract of the acidified reaction mixture after treatment of mannan with hydroxylamine; 5, complete reaction mixture after treatment of mannan with hydroxylamine; 6, succinyl monohydroxamate; 7, succinyl dihydroxamate; 8, α-hydroxysuccinimide.

FIG. 6. Comparison of succinate and base-treated mannan as substrates for succinic dehydrogenase. Reaction mixtures (total volume 600 μl) contained 0.05 M potassium phosphate, pH 7.0, 20 μmol of neutralized KCN, 20 nmol of dichlorophenol indophenol, 77.5 pg of Bacillus subtilis plasma membrane protein, and either no substrate (O-O), 15 nmol of sodium succinate (A, A--A), or a neutralized base hydrolysate of mannan containing 141 nmol of mannose (B, ■—■, ■—■). Sodium malonate (120 nmol) was added to parallel incubation mixtures (■—■). The reaction was initiated by the addition of enzyme, and the optical density at 600 nm determined as a function of time.

Mannose in a base hydrolysate of mannan. Prior extraction of the mannan with 20 volumes of chloroform-methanol (2:1) did change this value. Mannan appears to contain little or no amide-linked fatty acid since the fatty acid content of a strong acid hydrolysate of mannan was not greater than the fatty acid content of a mild base hydrolysate.

Molecular Weight and End Group Analyses—The quantity of reducing end group estimated by the method of Park and Johnson (9) indicated that mannan contained 0.050 μmol of reducing sugar per 50 μmol of mannose. Since the procedure involves boiling the samples for 15 min at 100° in 0.05 M Na₂CO₃, this value would reflect the sum of reducing sugar, if any, in intact mannan as well as reducing sugar released by base hydrolysis. As an alternative method to determine reducing end groups, [¹⁴C]mannan was treated with base in the presence of [³H]NaBH₄. The [¹⁴C]mannan was reisolated by gel filtration on Sepharose 6B. The pooled [¹⁴C]mannose-containing fractions had a mole ratio of [¹⁴C]/[³H] of 0.046:50, confirming the value of about 0.05 reducing terminus per 50 mannose residues. The molecular weight of base-treated [¹⁴C]mannan was estimated to be no more than 15,000 by gel filtration (see below). These results suggest, therefore, that a maximum of 10% of the mannose polymers released by base hydrolysis terminate in a reducing sugar; in at least 90% of the polymannose chains the reducing end is blocked by a base-stable linkage.

In considering possible compounds linked to the reducing terminus of most of the polymannose chains, acidic groups such as phosphate, glycerophosphate, or glyceric acid were considered unlikely as the mannan has no acidic character after base hydrolysis. On the other hand, a linkage of the mannose polymer to glycerol would be consistent with the chemical and physical properties of mannan and, moreover, this type of linkage is known to occur in dimannosyl diglyceride found in large quantities in the membrane of M. lysodeikticus.

Mannan was analyzed for glycerol by the formation of [³²P]-glycerophosphate in the presence of glycerokinase and [γ-³²P]-ATP. Acid hydrolysis of intact mannan or of an unfractionated base hydrolysate of mannan released 1.0 μmol of glycerol per 50 μmol of mannose. Extraction of an aqueous solution of mannan with 20 volumes of chloroform-methanol (2:1) did not change the ratio of glycerol to mannose. Control assays containing samples of untreated mannan or a base hydrolysate of mannan contained no free glycerol prior to acid hydrolysis.

Base-treated mannan was subjected to gel filtration on Sepharose 6B, and analyses of acid hydrolysates of the effluent established that a single peak of glycerol eluted just after the peak of radioactivity (Fig. 8). The elution profile would be consistent with the release by base hydrolysis of a polydispersity population of mannan-containing polymers each covalently bound to a single glycerol and ranging in size from as few as 10 to more than 100 mannose residues per polymer. The discrepancy in the elution positions of the peak of radioactivity and the peak of
glycerol would arise because the maximal glycerol would reflect a number average molecular weight (approximately 9,000) whereas the higher specific radioactivity of the longer polymers would shift the radioactive profile toward higher apparent molecular weight (approximately 15,000). The absence of a second peak of glycerol at the inclusion volume as well as the failure of chloroform-methanol extraction to reduce the glycerol content argues against significant contamination of the mannan with glycerolipids.

Influence of Base Treatment on Binding of Mannan to Membranes—After incubation of purified [14C]mannan with M. lysodeikticus membranes, mannan was found to sediment with the particulate fraction (Fig. 9). At the highest concentration of purified mannan tested, the amount of bound mannan represents a 17% increase over the amount of endogenous mannan of purified mannan tested, the amount of bound mannan represents a 17% increase over the amount of endogenous mannan (0.54 μmol of mannose per mg of protein (3)). To a lesser extent, mannan also sedimented with membranes from B. subtilis. In contrast, base-treated mannan has no affinity for the membranes from either organism.

**DISCUSSION**

The recent discovery that several bacterial polysaccharides are covalently linked to protein or lipid indicates a new dimension in both the structural complexity and potential physiological significance of these macromolecules. Braun and Rehn (23) have demonstrated that the peptidoglycan of *Escherichia coli* is covalently linked to a lipoprotein, and the structure of the lipoprotein has been fully characterized (20). The lipopolysaccharide of the outer membrane of *E. coli* has also been shown to be covalently bound to protein (27). Lipoamylchoic acids have recently been isolated from a number of gram-positive bacteria (28-30). Synthesis of the cell wall teichoic acid of *Staphylococcus aureus* H, polyribitol phosphate, is dependent on an acceptor which contains fatty acids as well as glycerol, phosphate, and glucose. This acceptor is indistinguishable in its composition and properties from the membrane-bound lipoamylchoic acid of the same organism (31). Thus, all three of these major polysaccharides of the bacterial cell envelope have been shown to have a lipophilic region. In addition, a novel class of *O*-methylglucose-containing lipopolysaccharides has been found in *Microbacterium phlei*. These are of particular interest because of the finding that they stimulate fatty acid biosynthesis (32). The structural studies of Gray and Ballou (33) have established that the backbone of the lipopolysaccharide is composed of 7 glucose and 11 *O*-methylglucose residues linked at the reducing terminus to a glyceric acid moiety. The lipopolysaccharide molecule contains six short chain fatty acids and zero to three succinyl groups in ester linkage.

The results reported in this communication establish that the mannan of *M. lysodeikticus* is an additional example of a membrane-bound polysaccharide containing covalently linked lipid and permit its chemical composition to be correlated with its physical properties. The composition of mannan is summarized in Table IV. The presence of esterified succinate is consistent with the observation that mannan binds to DEAE cellulose and migrates toward the anode in neutral disc gel electrophoresis. In addition, mannan contains long chain fatty acid esters which provide a lipophilic portion of the molecule available for interaction with the corresponding hydrophobic portion of another mannan molecule or with membrane lipids. The lipophilic

**Figure 8.** Gel filtration of base-treated mannan on Sepharose 6B. A sample of mannan (115,000 cpm, 4.48 μmol of mannose) was treated with mild base, neutralized with HCl, and applied to a column (33 × 1.05 cm) of Sepharose 6B, and 0.77-ml fractions were collected. Aliquots of each fraction were analyzed for radioactivity (---O). A second aliquot was hydrolyzed with acid, neutralized, and analyzed for glycerol (● ● ●). The elution positions of blue dextran, dextrins T10 (MW = 9,000), T40 (MW = 39,500), and T110 (MW = 105,000); and glucose were determined in parallel experiments.

**Figure 9.** Affinity of untreated and base-treated mannan for bacterial membranes. Each sample contained *Micrococcus lysodeikticus* membranes (O—O) or *Bacillus subtilis* membranes (● ● ●, 1.92 mg of protein) or *B. subtilis* membranes (△ △ △, 1.74 mg) in 0.05 M Tris-HCl, pH 7.2. After the addition of untreated [14C]mannan (O—O, △ △ △) or base-treated mannan purified by gel filtration (● △ △, ● △ △), the samples were incubated at 30° for 30 min in a total volume of 1.0 ml, centrifuged for 30 min at 100,000 × g, 4°, and the radioactivity in the pellet was determined.

**Table IV**

| Component        | Intact mannan | Base-treated mannan after gel filtration |
|------------------|---------------|----------------------------------------|
|                  | μmol/50 μmol [14C]mannose | N.D.*       |
| Total ester      | 6.4           | N.D.*                                  |
| Succinyl ester   | 4.9           | N.D.*                                  |
| Fatty acyl ester | 2.1           | N.D.*                                  |
| Glycerol         | 1.0           | 0.70*                                  |
| Protein (mg)     | 0.24          | 0.02                                   |
| Phosphate        | 0.08          | 0.02                                   |
| Reducing termini | ≤0.050        | 0.052                                  |

*a N.D., not determined
dThis value is lower than that of intact mannan because only the fractions containing maximal radioactivity were pooled (cf. Fig. 8).
nature of mannan is indicated by the observation that mannan exists as an aggregate in aqueous solution. In the presence of sodium dodecyl sulfate the mannan dissociates but it is not clear that the product of dissociation is the monomer. In a short report, published while this manuscript was in preparation, evidence for the presence of long chain fatty acyl groups in mannan was reported (34). It was also demonstrated that the mannan was acidic, but the presence of sucreinyl groups was not established.

Three types of evidence indicate that succinate and long chain fatty acids are covalently linked to mannan. The ratio of ester to mannose is constant through diverse purification procedures employing organic solvents, detergents, and affinity chromatography. In addition, hydrolysis of the esters with mild base results in a concomitant alteration of the properties of mannan including loss of acidic character, loss of affinity for the membrane, and a dramatic reduction in molecular weight. Finally, comparison of the nuclear magnetic resonance spectra of untreated and base-treated mannan provides evidence for a small proportion of acylated mannose residues, indicating that at least some of the acyl moieties may be linked directly to the polysaccharide.

Base-treated mannan contains a very low content of reducing termini (about 0.05 per 50 mannose residues), and a maximum of 10% of the mannose polymers may terminate in a reducing sugar or in an alkali-labile glycosidic linkage to a hydroxyaminoglycero acid. Glycerol was identified as a constituent of mannan, and it is present in sufficient quantity to be the residue blocking the reducing termini. In addition, the linkage of mannan to glycerol is stable to mild base but is cleaved by strong acid, which are properties consistent with a glycosidic ether linkage of the polysaccharide to glycerol. However, direct proof for such a glycosidic linkage has not been obtained. The ratio of fatty acid to glycerol in untreated mannan (2:1:1) suggests that the long chain fatty acyl groups may be linked to glycerol.

Purified mannan preparations contain a small amount of protein, which constitutes about 3% of the dry weight. Preliminary amino acid analyses of the proteins released by base indicated a short peptide of constant composition is unlikely, the attachment protein, which constitutes about 3% of the dry weight. Preliminary saccharide to glycerol. However, direct proof for such a glycosidic linkage has not been obtained. The ratio of fatty acid to glycerol in untreated mannan (2:1:1) suggests that the long chain fatty acyl groups may be linked to glycerol.

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Purified mannan preparations contain a small amount of protein, which constitutes about 3% of the dry weight. Preliminary amino acid analyses of the proteins released by base indicated a typical spectrum of amino acids. The quantity of protein present would be equivalent to only 3 amino acids per 50 mannose residues. Thus, whereas linkage of each mannose polymer to a short peptide of constant composition is unlikely, the attachment of a small proportion of the polymers to a larger polypeptide cannot be ruled out.

The data presented are consistent with the tentative, partial structure of mannan shown in Fig. 10. An average length of the polysaccharide chain of 60 mannose residues may be calculated from the number average molecular weight determined by gel filtration (about 9000). This value is in reasonable agreement with an average chain length of 50 mannose residues (calculated MW 8000) based on the ratio of glycerol to mannose, assuming a single glycerol per polymer. However, it should be noted that determination of the molecular weight by gel filtration is of limited value because both the calibration standards and the base-treated mannan appear to be polydispersed. Further structural features of the polysaccharide chain remain to be established. As noted in the introductory section, it was not possible to deduce the structure of a short oligosaccharide repeating unit in mannan from the results of the permethylation and acetylation of mannan. Analogous studies on purified, de-acylated mannan might, however, be more fruitful.

A possible functional analogy between membrane-bound mannan of M. lysodeikticus and the lipoteichoic acid found in most gram-positive membranes but absent in M. lysodeikticus has been suggested (34). It is of interest that recent preliminary reports conclude that the level of both polymers is higher in the mesosomal membrane than in the plasma membrane (35, 36). Although no attempt has been made in our study to separate these two membrane fractions, the strong affinity of mannan for the membrane is established by the observation that the mannan content of the ghosts remained constant despite extensive washing steps. Additional experiments (data not shown) involving treatment of membranes with a high concentration of salt or mild detergents corroborate this conclusion; the finding that purified, untreated mannan binds to membranes in vitro, whereas base-treated mannan does not, indicates that the covalently bound acyl groups are responsible for the association of the polysaccharide with the membrane. Finally, it should be noted that the partial structure proposed for mannan, the lipophilic portion of the molecule is identical with the major glycolipid of M. lysodeikticus, mannosyl-mannosyl-diglyceride, thus suggesting a possible biosynthetic relationship between these two membrane-associated compounds.

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Fig. 10. Proposed structure of the mannan of Micrococcus lysodeikticus.
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