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ISOLATION AND CHARACTERIZATION OF A MANNAN FROM MESOSOMAL MEMBRANE VESICLES OF *MICROCOCCUS LYSODEIKTICUS*

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SUMMARY

The carbohydrate content of mesosomal membranes of *Micrococcus lysodeikticus* has been shown to be consistently higher (about four times) than that of corresponding plasma membrane preparations. Analysis of washed membrane fractions by gas-liquid chromatography indicated that mannose was the major neutral sugar of both types of membrane (accounting for 95 and 89 %, respectively, of the mesosomal and plasma membrane carbohydrate). Small amounts of inositol, glucose and ribose were also detected.

We have shown by polyacrylamide gel electrophoresis in sodium dodecylsulphate and by precipitation and agar gel diffusion experiments with concanavalin A that a mannan is the major carbohydrate component of both types of membrane. This polymer can be selectively released from mesosomal membranes by a simple procedure involving low ionic strength-shock and heating to 80 °C for 1 min, and purified by ultrafiltration and ethanol precipitation.

The mannan contains mannose as the only neutral carbohydrate, is not phosphorylated and does not contain significant amounts of amino sugars or uronic acids. Agar gel electrophoresis experiments, however, indicate an anionic polymer whose acidic properties are eliminated upon mild base hydrolysis. Analysis of native mannan by infrared spectroscopy reveals absorption bands attributable to ester carbonyl groups and to carboxylate ions, consistent with the presence of succinyl residues in the polymer (Owen, P. and Salton, M.R.J. (1975) Biochem. Biophys. Res. Commun. 63, 875–880).

A sedimentation coefficient of 1.39 S was obtained by analytical ultracentrifugation in 1.0 M NaCl and a value of one reducing equivalent per 50 mannose residues by reduction with NaB^3H_4. The polysaccharide was only slightly degraded (2 %) by jack bean α-mannosidase and could precipitate 15 times its own weight of concanavalin A.

The acidic polymer was also detected in the cell "periplasm" and was secreted from cells grown in defined media during the period of decelerating growth.

INTRODUCTION

Mesosomes are intracytoplasmic membranous structures of bacteria, the

mesosomal vesicles of which can constitute over 13 % of the dry weight of the total membrane system [1]. Many functions have been proposed for these membranous organelles which have been found in a wide variety of Gram-positive and Gram-negative bacteria (for reviews see refs 2–4). The most direct approach to the elucidation of mesosome function has been to isolate the vesicles and to compare their chemical and biochemical properties with those of corresponding plasma membrane fractions. Although many investigators have adopted this approach with a variety of organisms, the results have frequently been confusing and no firm conclusions as to their functions have been drawn. Indeed, attempts to demonstrate the presence of mesosome specific enzymes or components have generally been unsuccessful and in only a few instances have the mesosomes been reported to be enriched in certain components [1, 5–9].

In a comparative study of the morphological, chemical and biochemical properties of the two types of membranes isolated from *Micrococcus lysodeikticus*, Owen and Freer [1] observed several years ago that the carbohydrate content of the mesosomal vesicles was some 4–5 times greater than that of corresponding plasma membrane fractions. The carbohydrate, which was shown to be composed mainly of mannose, accounted for approx. 20 % of the dry weight of the mesosomal membrane. Mannose had been found in earlier analyses of cell lysates of *M. lysodeikticus* by Hawthorne [10] and in protoplast membrane fractions by Gilby et al. [11]. The latter authors reported that carbohydrate constituted 15–20 % of the dry weight of the membranes but more recent estimations on plasma membrane fractions have placed this value at a much lower level [1, 12–15]. Mannose polymers have since been isolated from whole cells of *M. lysodeikticus* [16–18] and also from supernatant fractions from physically disrupted cells [19] by procedures usually involving hot phenol extraction [15, 17–20].

In searching for clues to the function of mesosomes our investigations have been directed towards identifying the carbohydrate components present in washed membranes obtained from *M. lysodeikticus*, determining their distribution between and mode of association with both the mesosomal and plasma membranes and towards an understanding of the role of these compounds in the bacterial membrane system. This communication presents some of the properties of a mannan isolated from mesosomal vesicles of *M. lysodeikticus* by a simple and essentially non-degradative procedure.

MATERIALS AND METHODS

Bacterial growth. Cells of *M. lysodeikticus* (NCTC 2665) were cultivated on peptone/water/yeast extract medium as previously described [21], harvested by centrifugation after incubation for 17–24 h at 30 °C and washed twice in 50 mM Tris · HCl buffer, pH 7.5, at room temperature (approx. 23 °C).

For growth of *M. lysodeikticus* on defined medium the synthetic medium described by Salton [22] was supplemented with amino acids arginine, tyrosine, leucine and phenylalanine at 0.002 % (w/v), proline at 0.0026 % (w/v) and methionine at 0.00175 % (w/v) (Cooper, P. and Salton, M.R.J., unpublished results). Bacterial growth was followed by measuring the absorbance of cultures at 625 nm.

Preparation of membrane fractions. Total membranes (i.e. plasma plus meso-

somal) and isolated mesosomal and plasma membrane fractions from *M. lysodeikticus* were prepared as described elsewhere [1, 21]. Washed membrane fractions were either used immediately following preparation or stored at − 70 °C until required.

Release of mannan from mesosomal membranes. Washed mesosomal membranes (about 20 mg dry weight/ml in 50 mM Tris · HCl buffer, pH 7.5) were diluted with 100 volumes of either buffer or distilled water, 8.0-ml portions subjected to various extraction procedures and subsequently centrifuged at 125 000 × *g* for 2 h at 4 °C. Supernatant fractions were carefully decanted and centrifuged again under similar conditions. Membrane pellets or "shock residues" obtained from the first centrifugation were resuspended in 8.0 ml of buffer or distilled water.

The following extraction procedures were performed upon mesosomal membrane preparations (a) heating to 100 °C for varying periods of time; (b) autoclaving at 121 °C for 150 min; (c) sonication for a total of 8 min at 0 °C; (d) freeze-thawing. The latter procedure entailed freezing membrane suspensions in an alcohol/solid CO_2 bath followed by thawing in a water bath at 25 °C. The process was repeated either five or ten times.

Comparison of the effects of "ionic-heat shock" on mesosomal and plasma membrane fractions. Membrane fractions were diluted to 1 mg dry weight/ml with 50 mM Tris · HCl buffer (pH 7.5) and centrifuged in stainless steel centrifuge tubes at 125 000 × *g* for 2 h at 4 °C. Supernatant fractions were carefully decanted and membrane pellets made to original volume with distilled water. The suspensions were then heat shocked by immersion of the centrifuge tubes in a boiling water bath for 1 min. (This combined low ionic strength-heat shock treatment will subsequently be referred to simply as "ionic-heat shock".) On cooling, the suspensions were centrifuged at 125 000 × *g* for 2 h at 4 °C and the supernatant fractions carefully decanted. This procedure was repeated twice, after which residual membrane pellets were made to original volume with distilled water. All supernatant fractions were centrifuged again at 125 000 × *g* for 2 h at 4 °C, any small membrane pellets discarded and the extracts set aside for analysis.

Mannan purification from isolated mesosomal membrane preparations. Mesosomal membrane suspensions were adjusted to 25 mg dry weight/ml and poured into 100 volumes of vigorously stirred distilled water at 80 °C. After cooling to room temperature, a mannan-rich supernatant fraction was obtained by centrifugation at 150 000 × *g* for 2 h. Following concentration to about 10 ml in an Amicon ultrafiltration unit fitted with a PM-10 filter, the fraction was centrifuged at 150 000 × *g* for 2 h to remove residual membrane contamination and the clear supernatant fraction was lyophilized. The resultant white powder was dissolved in 1 ml of distilled water at 0 °C and mannan precipitated by dropwise addition of ethanol to a final concentration of 75 % (v/v). After holding for 1 h at 0 °C the mannan was recovered by centrifugation at 10 000 × *g* for 30 min at 0 °C, washed once with 1 ml 75 % ethanol (v/v) at 0 °C and the pellet dissolved in distilled water and lyophilized. Approx. 19 mg dry weight of mesosomal membrane yielded 1 mg of purified mesosomal mannan.

Preparation of "periplasmic fractions". Sequential removal of intact protoplasts and mesosomal vesicles from lysozyme-digested cells of *M. lysodeikticus* yielded a soluble "periplasmic" fraction together with cell-wall digest. Crude supernatant devoid of membranes for the preparation of a mannan-enriched "periplasmic" fraction was concentrated 15-fold by ultrafiltration and acidifed [23]. Following centrifugation

(10 000 × *g*, 30 min, 4 °C) the supernatant fraction was neutralized and lyophilized. The resultant powder was dissolved in distilled water and the 75 % (v/v) ethanol-insoluble fraction obtained as described above.

Culture supernatant fractions. Cell-free culture filtrates of *M. lysodeikticus* grown on defined medium were obtained by centrifugation (4000 × *g*, 10 min, 4 °C) and filtration of supernatant fluids through a Millipore filter (0.45 μm). Total hexose was determined and filtrates concentrated 50-fold by ultrafiltration through PM-10 Amicon filter. Insoluble material was removed by centrifugation.

Chemical analysis. Dry weights of membrane fractions were determined after dialysis against three changes of distilled water at 4 °C, lyophilization and desication in vacuo over P_2O_5.

Protein was determined by the method of Lowry et al. [24] using bovine serum albumin or concanavalin A as standard and total hexose by the anthrone method of Morris [25] with mannose as standard. The carbazole reagent [26] with β-D-mannuronic acid-γ-lactone as standard was used for the detection of uronic acids in mannan preparations. Absorption at 530 nm was corrected for non-specific color formed by equivalent amounts of mannose reacted with the sulphuric acid-carbazole reagent.

Phosphorus was determined by the method described by Chen et al. [27] using the ashing technique of Ames and Dubin [28] with α-glycerophosphate disodium salt as standard. Carotenoid content of membrane preparations was estimated by measurement of the extinction value at 446 nm of fractions solubilized with 0.1 % (w/v) sodium dodecylsulphate assuming $A\ {}^{1\%}_{1\,cm} = 3 \cdot 10^3$ [29]. Lipids were extracted from membranes and from whole cells using the method of Bligh and Dyer [30].

Quantitative determination of reducing equivalents. Reducing equivalents were determined in triplicate by the method of MacLean et al. [31]. To solutions of carbohydrate in 100 μl of distilled water was added 50 μl 0.2 M tritiated $NaBH_4$ (2.5 Ci/mol) in 0.1 M NaOH and reduction allowed to proceed at 4 °C for 24 h. Excess NaB^3H_4 was decomposed by addition of 150 μl of 1 M HCl, contents of reaction tubes frozen as slants and lyophilized. Dried samples were reconstituted to 200 μl with distilled water and lyophilized again, a procedure which was repeated. The final product was dissolved in 100 μl of distilled water and 20-μl volumes subjected to scintillation counting. The mannose standard gave a linear curve over the concentration range of 0–0.5 μmol. All values were corrected for a reproducible acid stable NaB^3H_4 blank value of approx. 0.05 %.

Hydrolysis procedures. (a) Amino sugars: Mannan preparations (4 mg) were hydrolyzed in 300 μl 6 M HCl for 2 h at 100 °C under N_2 gas [32] and examined in a Beckman Model 120 C amino acid analyzer for amino sugars.

(b) Neutral sugars: For the analysis of neutral sugars by gas-liquid chromatography, membrane fractions (2–10 mg dry weight membrane) were hydrolyzed by either of two methods: (1) Hydrolysis was performed using 1 ml 20 % (w/v) Dowex AG 50W-X_2 (H^+) resin (200–400 mesh) in 0.01 M HCl at 100 °C for 65 h under N_2 gas [33]. The course of hydrolysis (Fig. 1) had indicated 65 h to be the optimal time under these conditions. 50 μl of an aqueous solution of α-methyl-D-mannoside (2.01 mg/ml) was then added as internal standard and the hydrolysate mixed thoroughly. In control experiments α-methyl-D-mannoside did not coelute with any membrane carbohydrate components under the conditions used for gas chromatography of

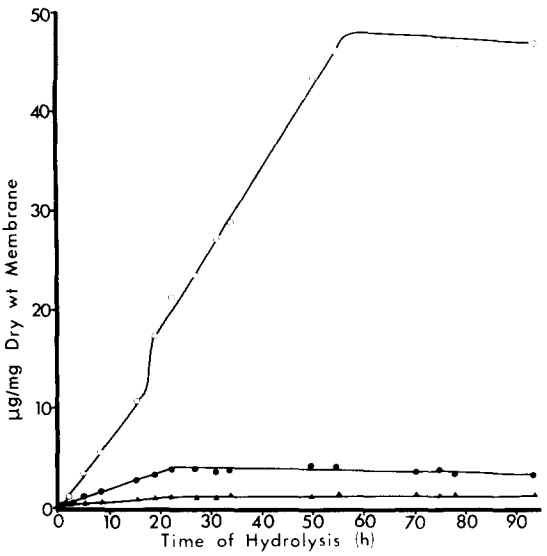

Fig. 1. Time course hydrolysis of plasma membrane by 0.01 M HCl-Dowex (H^+) resin. Plasma membranes from *M. lysodeikticus* were hydrolyzed for various time intervals and carbohydrates present in neutralized hydrolysates analyzed as their trimethylsilylether derivatives by gas-liquid chromatography. ○—○, mannose; ●—●, inositol; ▲—▲, glucose,

their trimethylsilylether derivatives. Neutralization and concentration of samples was achieved as previously described [33]. (2) Hydrolysis was performed with 1 ml 1 M H_2SO_4 for 80 min at 105 °C under N_2 gas after determination that these were optimal conditions. α-Methyl-D-mannoside was added as internal standard and the hydrolysates were transferred quantitatively to beakers containing 10 ml 50 % (w/v) Dowex AG-X8 ($HCO^-{}_3$) resin (200–400 mesh) and held overnight at room temperature. Neutralized hydrolysates were eluted through a small ion-exchange column containing 0.5 ml 50 % (w/v) Dowex AG-50W-X_2 (H^+) resin (200–400 mesh) and worked up as described in (1).

(c) Alkaline hydrolysis: Mild alkaline hydrolysis of mesosomal mannan was achieved by heating solutions of mannan, at concentrations of 1 mg/ml in 0.1 M NaOH, for 1 h at 100 °C under N_2 gas. Following neutralization with 1 M HCl, hydrolysates were extracted three times with one-third volumes of diethylether. Combined ether extracts were held at −70 °C for analysis.

Gas-liquid chromatography. Gas-liquid chromatography was performed using an F and M Model 400 Biomedical gas chromatograph equipped with a flame ionization detector. For the identification and quantitation of neutral sugars, hydrolysates were converted to their trimethylsilylether derivatives [34] and analyzed as previously described [33, 35]. Peak areas were considered a product of peak height and peak width at half peak height.

Fatty acids were analyzed at 165 °C on a 6-ft stainless steel column containing 10 % EGSS-X (ethylenesuccinate-methyl silicone copolymer) on 100/120 Gas Chrom P following conversion to their corresponding methyl esters with BF_3/methanol at 100 °C for 30 min [36]. Peak areas were estimated by weighing photocopies of the peaks recorded on chart paper.

Infrared spectroscopy. Infrared spectra were recorded using a Perkin-Elmer grating spectrophotometer Model 421. 1 mg of lyophilized sample was thoroughly mixed with 200 mg dry KBr and pellets, approx. 0.3 mm in thickness, obtained by compression.

Polyacrylamide gel electrophoresis. Electrophoresis was performed in 10 % (w/v) polyacrylamide gels in the presence of sodium dodecylsulphate and molecular weights determined as described by Weber and Osborn [37]. Following fixation, gels were stained for protein with Coomassie blue and for carbohydrates with periodic acid-Schiff base reagent as described by Glossman and Neville [38].

Action of α-mannosidase on mannan preparations. Mannan preparations (60–150 μg in 50 μl distilled water) were incubated with 0.13 unit [39] of dialyzed α-mannosidase (EC 3.2.1.24) in 50 μl 0.1 M citrate buffer, pH 4.4, at 37 °C for varying time intervals. Reactions were terminated by heating to 100 °C for 5 min, the pH adjusted to alkalinity by the addition of 10 μl 1 M NaOH and reducing equivalents determined as described above using tritiated $NaBH_4$. Results were corrected for control reactions containing citrate buffer and α-mannosidase alone.

Immunochemical analysis. Immunochemical diagnosis of the linkages of the mannose residues in mesosomal mannan was performed as described by Heidelberger and Cordoba [40].

Precipitation of mannan with concanavalin A. Quantitative precipitation of purified mesosomal mannan was performed essentially as described by So and Goldstein [41]. The washed precipitates were dissolved in 2 ml 1 M NaOH and protein determined [24] using concanavalin A as standard.

Gel diffusion and gel electrophoresis. Agar gel diffusion was performed in borate-buffered 0.85 % (w/v) Bacto agar gels containing 0.25 % (w/v) NaCl and 0.01 % (w/v) merthiolate. Membrane preparations were sonicated for 5 min prior to use. Where samples were dispersed with detergent, the same detergent was also incorporated into the gels at the required concentration.

Agar gel electrophoresis was performed in buffered 1 % (w/v) Bacto agar gels on microscope slides. The following buffers of constant ionic strength, 0.05, were used: (a) borate buffer, pH 8.6; (b) barbital buffer, pH 8.2; (c) phosphate buffer, pH 7.4; electrophoresis was performed at 5 mA/gel for 1–3 h at 4 °C. Mannans were then developed by diffusion against concanavalin A (10 mg/ml); dextran (average molecular weight 73 200) was precipitated by direct immersion of the slide in 75 % (v/v) ethanol.

Ultracentrifugation. Analytical ultracentrifugation was performed on 0.3–0.4 % (w/v) solutions of mesosomal mannan in 1.0 M NaCl using a Spinco Model E analytical ultracentrifuge fitted with an analytical D rotor and 0.35 ml capacity double sector cells. Sedimentation velocity runs were performed at 20 °C and followed by Schlieren optics. Photographic recordings were taken at 8-min intervals upon attaining maximum speed (48 000 rev./min).

The L-plate agar diffusion method [42] was used for the determination of the diffusion coefficient of mannan. Mannan (600 μg/ml) reacted against concanavalin A (5 mg/ml) gave a linear precipitin band which allowed the computation of the diffusion coefficient of the former. The diffusion coefficient of concanavalin A was assumed to be $D = 5.6 \cdot 10^{-7}$ cm/s [43].

Sonication. Sonic dispersion of samples was achieved using an MSE sonifier

fitted with a $\frac{3}{8}$-inch probe. Sonication was performed at 0 °C using 30-s pulses and 1-min cooling intervals [44].

Radioactive measurements. Radioactive measurements were made in a Nuclear Chicago Mark I liquid scintillation counter, using the xylene-based scintillation cocktail Aquasol.

Chemicals and reagents. Tritiated $NaBH_4$ and the scintillation cocktail, Aquasol, were purchased from New England Nuclear and gas chromatography columns from Applied Science Labs., Inc. Mannan (from *Saccharomyces cerevisiae*), dextran B-512 (average molecular weight 73 200, from *Leuconostoc mesenteroides*) were obtained from Sigma Chemical Co. and concanavalin A from Miles-Yeda Ltd. α-Mannosidase (EC 3.2.1.24) was supplied by Boehringer Mannheim Corp.

The antisera, antityphoid 646 and antiparatyphoid B 1137 were from the Institut Pasteur, Paris, and kindly supplied by Dr Michael Heidelberger.

RESULTS

Membrane-bound carbohydrate

The distribution of membrane-bound hexose between isolated mesosomal and plasma membrane fractions prepared from cells of *M. lysodeikticus* harvested at various stages in the growth cycle is shown in Table I. The hexose content of mesosomal membranes rose steadily from approx. 20 % during late logarithmic phase of growth (10 h) to above 35 % in mid-stationary phase (24 h). A corresponding rise was also noted for plasma membrane hexose although the absolute value was some four times less than that observed for mesosomal membranes. It is unlikely that the hexose content of the plasma membranes can be accounted for simply by contamination with mesosomal membranes for this would require a level of heterogeneity that is not supported by electron-microscopic observation of the fractions [1].

Carbohydrate analysis by gas-liquid chromatography (Table II) revealed that approx. 95 and 89 %, respectively, of the neutral carbohydrate of mesosomal and plasma membranes was mannose. Small amounts of glucose and inositol together with traces of ribose were the only other sugars detected. Following lipid extraction the

TABLE I

HEXOSE COMPOSITION OF MEMBRANE FRACTIONS ISOLATED FROM *M. LYSODEIKTICUS* FOLLOWING GROWTH ON PEPTONE/WATER/YEAST EXTRACT MEDIUM

Membrane preparation No.	Age of culture (h)	Percent (w/w) hexose	
		Mesosomal membrane	Plasma membrane
1–4*	10	n.d., 16.8, 21.9, 19.3	4.8, 3.6, 5.8, 4.7
5, 6	17	25.4, 24.3	n.d.
7	18	28.5	7.0
8	19	34.5	12.9
9, 10	24	33.3, 44.6	n.d., 12.0

* Ref. 45.
n.d., not determined.

TABLE II

CARBOHYDRATE ANALYSIS OF MEMBRANE FRACTIONS AND RESIDUES

Defatted and total lipid extracts were obtained by extraction of 2–5 mg dry weight of membranes by the method of Bligh and Dyer [30]. Hydrolysis was performed by method 1 and neutral sugars analyzed by gas-liquid chromatography. Results are expressed as μg/mg dry weight of membrane.

Membrane fraction	Mesosomal membrane				Plasma membrane			
	Mannose	Glucose	Inositol	Ribose	Mannose	Glucose	Inositol	Ribose
Whole membranes	250	10.3	3.9	Trace	49.0	1.8	4.6	Trace
Defatted membrane residues	209	9.1	3.1	Trace	39.8	1.3	2.8	Trace
Membrane lipid extract	9.7	0.9	0.6	0.0	5.6	0.6	1.2	0.0

bulk of the mannose was detected in the defatted residues. Surprisingly, 80 and 60 % of the inositol content of mesosomal and plasma membranes, respectively, was also retained in the "delipidated" residue.

Mannan extraction

Various extraction procedures were tested in an attempt to purify the polymer (s) containing mannose by essentially non-degradative procedures. Mesosomal membranes were chosen for study because of the enriched localization of hexose in this fraction. Simple dilution in buffer to a concentration of approx. 200 μg dry weight membrane/ml released 11 % of the mesosomal hexose, with a further 19 % release upon heating at 121 °C for 150 min. A more dramatic solubilization (47 %) occurred following dilution of mesosomal membranes in distilled water. Subsequent treatment of water-diluted membranes with ultrasound or by freeze-thawing did not increase hexose release but brief heating (1 min) to 100 °C solubilized an additional 13 % of the membrane carbohydrate (Fig. 2). Prolonged heating solubilized increasing levels of protein and lipid phosphorus but little additional hexose.

A comparison of the effect of successive "ionic-heat shock" washes on the release of components from mesosomal and plasma membrane fractions is shown in Table III. The period of heating at 100 °C was limited to 1 min in all cases. It should be noted that the total recovery of mesosomal and plasma membrane components was less than 100 %. This incomplete recovery can in large part be attributed to the

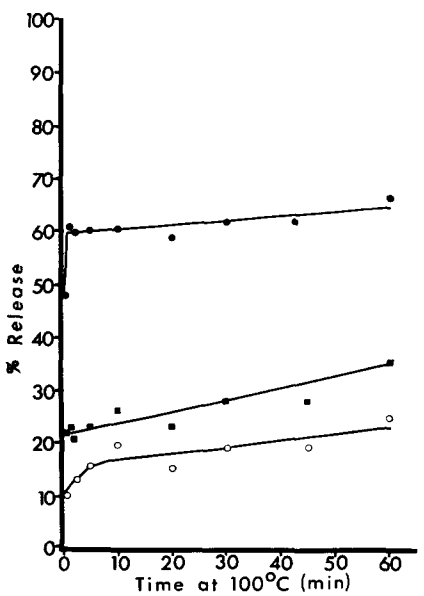

Fig. 2. Time course release of mesosomal membrane components following ionic-shock treatment and heating to 100 °C. Mesosomal membranes at 18.9 mg dry weight/ml were diluted with 100 volumes of distilled water and aliquots heated to 100 °C for 0–60 min. Supernatant fractions obtained following subsequent centrifugation of shocked membranes were assayed for total hexose (●—●), for protein (■—■), and for lipid phosphorus (○—○).

TABLE III

RELEASE OF MEMBRANE COMPONENTS FOLLOWING "IONIC-HEAT SHOCK" TREATMENT

Six times-washed mesosomal and plasma membrane preparations were each resuspended to 1 mg dry weight/ml in 50 mM Tris · HCl buffer (pH 7.5) and sedimented at 125 000 × *g* (wash 7). Membrane fractions were then subjected to three successive "ionic-heat shock" treatments as described in Materials and Methods (washes 8, 9 and 10, respectively). Values given for washes 7–10 refer to supernatant fractions obtained from each, and values in parentheses to the percentage of the total membrane hexose present in each fraction.

Fraction	Mesosomal membrane					Plasma membrane				
	Hexose (μg)	Protein (μg)	Total phosphorus (μmol)	Lipid phosphorus (μmol)	Carotenoid (μg)	Hexose (μg)	Protein (μg)	Total phosphorus (μmol)	Lipid phosphorus (μmol)	Carotenoid (μg)
Initial analysis	3931 (100 %)	3377	4.381	3.557	0.973	924 (100 %)	5547	7.54	5.51	1.033
Wash 7	395 (10 %)	286	0.094	0.000	0.027	351 (38 %)	821	1.131	0.183	0.020
Wash 8	1368 (35 %)	628	0.484	0.328	0.040	187 (20 %)	724	0.380	0.289	0.040
Wash 9	187 (5 %)	100	0.100	0.030	0.027	27 (3 %)	106	0.052	0.067	0.013
Wash 10	48 (1 %)	72	0.070	0.017	0.023	8 (1 %)	52	0.062	0.031	0.006
Residue	1482 (38 %)	1767	2.910	2.650	0.616	328 (35 %)	3453	5.286	4.08	0.790
Total	3480 (89 %)	2781	3.658	3.025	0.733	900 (97 %)	5156	6.911	4.65	0.870

residual membrane pellets discarded following centrifugation of the wash supernatant fractions (Table III, washes 7–10).

As shown in Table III a total of 51 and 62 %, respectively, of the mesosomal and plasma membrane hexose was released by the full wash sequence. Most of the hexose released from the mesosomal membrane was recovered in the first "ionic-heat shock" wash. In contrast most of the hexose (and non-lipid phosphorus) released from plasma membranes was solubilized by the initial dilution in buffer. Additional hexose and non-lipid phosphorus was released from plasma membranes by the first "ionic-heat shock" wash but very little in subsequent washes. In addition, approx. 30 % of the total protein content of both mesosomal and plasma membranes was solubilized by the washing procedure, the distribution of released protein between the various washes closely paralleling that for released hexose.

Phospholipids and carotenoids are integral components of the membrane system [13], hence their solubilization may be regarded as an indication of the extent of membrane disruption. It should be noted that less than 12 % of the total carotenoid

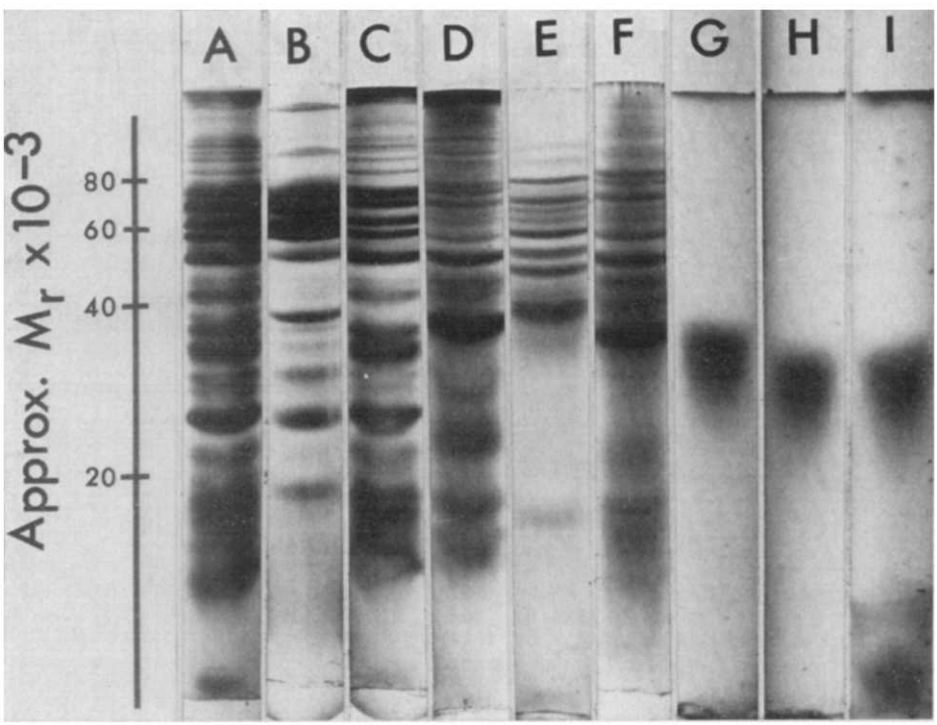

Fig. 3. Sodium dodecylsulphate-polyacrylamide gel electrophoresis of membrane fractions and residues. Gels A–F are stained for proteins with Coomassie blue and gels G–I for carbohydrate with periodic acid-Schiff base reagent. Gel A, plasma membrane (32 μg protein and 6 μg hexose); gel B, material solubilized from plasma membrane following "ionic-heat shock" treatment (19 μg protein and 5 μg hexose); gel C, residual plasma membrane following "ionic-heat shock" treatment (25 μg protein and 2.3 μg hexose); gels D and G, mesosomal membrane (22 μg protein and 28 μg hexose); gels E and H, material solubilized from mesosomal membrane following "ionic-heat shock" treatment (14 μg protein and 28 μg hexose); gels F and I, residual mesosomal membrane following "ionic-heat shock" treatment (27 μg protein and 23 μg hexose).

or lipid-phosphorus was released by the full washing sequence outlined in Table III.

Gel electrophoresis in the presence of sodium dodecylsulphate was used as an additional monitoring procedure for the release of membrane components induced by the above washing sequence (Fig. 3). Protein staining revealed that "ionic-heat shock" treatment solubilized a broad spectrum of polypeptides from mesosomal membrane, whereas several proteins were selectively solubilized from plasma membrane, notably three polypeptides of approximate molecular weights 70 000, 61 000 and 59 000. The latter two components probably correspond to the subunits of ATPase [46]. Periodic acid-Schiff base staining revealed the presence of a strong broad band (apparent molecular weight 25 000–35 000) in gels of material solubilized from mesosomal membranes. This band, which was also detected in residues obtained after three consecutive "ionic-heat shock" treatments, corresponded to a diffuse region staining for protein detectable in native mesosomal membranes. Similar periodic acid-Schiff base-positive bands were also found in plasma membrane fractions but the gels had to be heavily loaded with protein to visualize them.

Periodic acid-Schiff base-positive material was also noted in the region of the tracking dye, and probably represents the lipids, dimannosyl diglyceride [47], phosphatidylglycerol [48] and phosphatidylinositol known to be present in the membranes of this organism. One additional minor component staining positive with both periodic acid-Schiff base stain and also with Coomassie blue was observed in both mesosomal and plasma membranes. This component (molecular weight 67 000) did not appear to be selectively released upon "ionic-heat shock" treatment.

The observation that mesosomal membranes subjected to a single "ionic-heat shock" wash yielded a soluble preparation with hexose/protein ratio of 1.93 (corresponding value for plasma membrane was 0.35) provided the basis for the procedure adopted for the isolation and purification of the mannan as described in Materials and Methods. Such preparations were used for the following characterization of the mannan.

Characterization of purified mesosomal mannan

Chemical analysis. The chemical composition of polysaccharide fractions isolated from three different mesosomal membrane preparations is shown in Table IV. Hexose accounted for over 92 % of all the fractions, mannose being the sole neutral sugar detected by gas-liquid chromatography. No glucose was detectable in the purified mannan preparations (Fig. 4). We were unable to confirm the presence of glucosamine [19] in mannan isolated from the mesosomes of *M. lysodeikticus*. Only trace amounts of this amino sugar were detected (molar ratio of glucosamine to

TABLE IV

CHEMICAL COMPOSITION OF MESOSOMAL MANNAN

Property	Preparation No.		
	1	2	3
Total hexose (%)	96.5	92.4	92.9
Protein (%)	4.4	2.7	2.0
Phosphorus (%)	0.00	0.27	0.05
Nucleic acid (%)	< 0.1	1.0	0.2

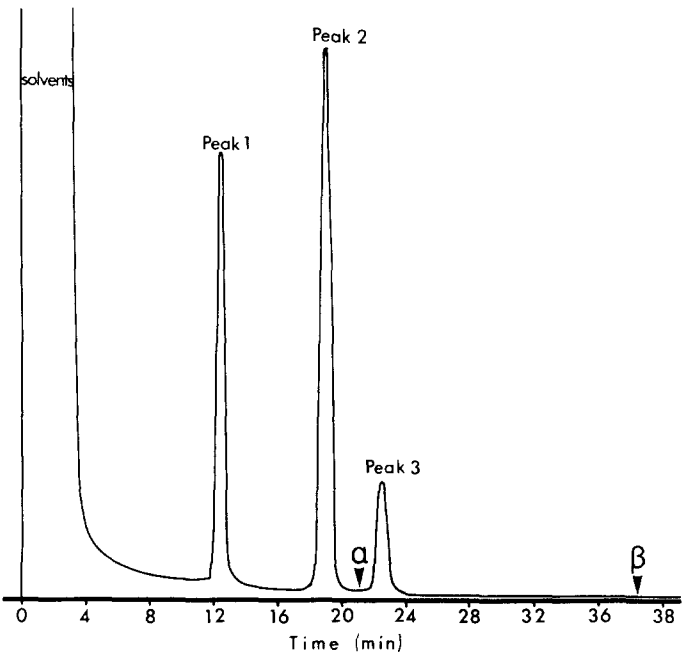

Fig. 4. Gas-liquid chromatographic analysis of purified mesosomal mannan. Mannan was hydrolyzed in 1 M H_2SO_4 for 80 min as described in Materials and Methods. Neutral carbohydrates were analyzed as their trimethylsilylether derivatives on a 15 % Carbowax 20 M column. Peaks 1, 2 and 3 correspond to trimethylsilylether derivatives of α-mannose, α-methyl-D-mannoside (internal standard) and β-mannose, respectively. Arrows labelled α and β indicate the expected positions of trimethylsilylether derivatives of α and β-glucose, respectively.

mannose 1 : 10 000). Uronic acids do not appear to be present in mesosomal mannan as determined by the carbazole color reaction [26].

Fatty acid analysis of the ether-soluble products of mild alkaline hydrolysis of mesosomal mannan revealed a spectrum of fatty acids very similar to that obtained for a total lipid extract of *M. lysodeikticus*. The C_{15} branch-chain fatty acid accounts for over 82 % of the total fatty acid content. Small amounts of C_{13} (1.1 %) and C_{17} (2.3 %) branch-chain fatty acids together with *n*-C_{16} (1.8 %) and *n*-C_{18} (1.7 %) fatty acids were also detected. Quantitative measurements indicated a fatty acid content for the intact mannan of approx. 1.0 %.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate revealed the presence of one broad band stainable with periodic acid-Schiff base reagent (similar to gel H in Fig. 3). When similar gels were stained for protein with Coomassie blue no counterpart to this band was detected (cf. ref. 15). However, at loadings exceeding 50 μg hexose two faint components were found (molecular weights 52 000 and 34 000). It is possible that these components account for the small amount of protein found in mannan preparations (Table IV).

Two of the three mannan preparations contained virtually no phosphorus (see Table IV) and the small amount detected in the remaining preparation probably

originated from contaminating nucleic acid in that fraction since it could be partially removed by preferential adsorption onto charcoal.

We have already shown from an analysis of mannan isolated from total membranes grown in the presence of [1,4-$^{14}C_2$]succinic acid that this polymer possesses ester-linked succinyl residues [49]. Semi-quantitative analysis of the water-soluble product of an extract of base-hydrolyzed mesosomal mannan indicated a succinate content for this polymer of 2.5 %. Thus analysis shown in Table IV together with detection of fatty acid (1 %) and succinyl residues brings the recovery of identifiable components of purified mesosomal mannan to 100.5 %.

Infrared spectroscopy. Analysis of purified mesosomal mannan by infrared spectroscopy showed various adsorption bands that could be attributed to the chemical characteristics of mannan. In addition two weak bands were seen at 1716 and at 1568 cm^{-1}. The former is characteristic of the carbonyl stretching mode in saturated aliphatic ketones and aldehydes. Normally ester carbonyls show absorption bands at slightly higher wave number, i.e. 1735 cm^{-1} but this value can decrease depending on the degree of hydrogen bonding. The weak absorption band centered at 1568 cm^{-1} may represent the anti-symmetrical stretching mode of the carboxylate ion. The corresponding symmetrical stretching mode could be accounted for in an absorption band centered 1406 cm^{-1}.

Structural analysis. Reduction of mannan preparations (1.48 μmol mannose) with tritiated $NaBH_4$ revealed the presence of one reducing equivalent per 50 sugar residues. It seems quite unlikely from the recovery of identifiable components of mannan (about 100 %) that this value represents contamination. However, the existence of an alkali labile O-glycosidic bond to protein cannot be ruled out.

The sensitive NaB^3H_4 reduction procedure was also used to monitor the effect of jack bean α-mannosidase on mesosomal mannan. Whereas relatively short incubation periods (2 h) doubled the number of available reducing equivalents, prolonged incubation had little additional effect. These results confirm the earlier observation that this polysaccharide was not substantially degraded by α-mannosidase [18].

Scher and Lennarz [18] concluded from exhaustive methylation analysis that a mannan isolated from whole cells of *M. lysodeikticus* contained mannosyl units linked at 3-, 2-, and 6-positions in the ratio 1 : 1.26 : 0.53. We have attempted to confirm similar linkages for mesosomal mannan using the simple and rapid immunochemical techniques developed by Heidelberger and Cordoba [40] and Heidelberger [50]. Surprisingly, mesosomal mannan gave only weak reactions (±) in antityphoid serum and none at all (−) in antiparatyphoid serum [40, 51]. Mild alkaline hydrolysis and ether extraction of mannan did not alter the precipitation reactions observed.

Reactions with concanavalin A. We have shown that both mesosomal and plasma membrane fractions isolated from *M. lysodeikticus* can be agglutinated by the lectin concanavalin A, even in the presence of detergents [33]. In the present study gas-liquid chromatographic analysis of the membrane fractions precipitated with concanavalin A in the presence of 1 % (v/v) Triton X-100 has shown mannose to be the major neutral carbohydrate (about 96 %). Only small quantities of glucose (about 4 %) were detected and no inositol or ribose. Under our experimental conditions, however, concanavalin A precipitated only about 60 % of the membrane mannose. This feature of the reaction may in part reflect the inhibitory properties of dimannosyl diglyceride [47].

Fig. 5 presents the precipitation curves for concanavalin A reacted with purified mesosomal mannan and indicates maximal precipitation at concanavalin A/polysaccharide ratios of 15 : 1 (w/w). Plateau precipitation of the lectin occurred over a wide range of mannan concentrations (at least 15-fold) with a small linear increase in the total amount of mannan precipitated. These results are compatible with a mannan molecule of relatively low molecular weight [52] possessing a number of terminal non-reducing mannopyranosyl residues and/or of internal 2-*O*-linked mannopyranosyl residues in an α-D-configuration [53, 54]. The interaction at polysaccharide excess between concanavalin A and increasing numbers of mannan molecules may result in part from utilization of fewer determinants per mannan molecule [55].

Purified mesosomal mannan gave a single precipitin line in agar gel diffusion tests with concanavalin A (Fig. 6). This band showed a line of identity with diffusable material from sonicated plasma membranes, mesosomal membranes (but not with yeast mannan) and was absent from mesosomal membrane residues obtained after three "ionic-heat shock" washes. It is interesting that a "periplasmic" fraction obtained from *M. lysodeikticus* also revealed the presence of this component. Two additional precipitin lines, which were sometimes difficult to resolve, were also detected for mesosomal membranes and for mesosomal shock residues. Corresponding precipitin lines were present, but to a much lesser extent, in plasma membranes. As expected for specific carbohydrate-concanavalin A interactions, no precipitin lines were observed in the presence of the inhibitor α-methyl-D-mannoside (Fig. 6b). Incorporation of various detergents into both samples and gels resulted in the appearance of one congruent precipitin band for all membrane fractions and

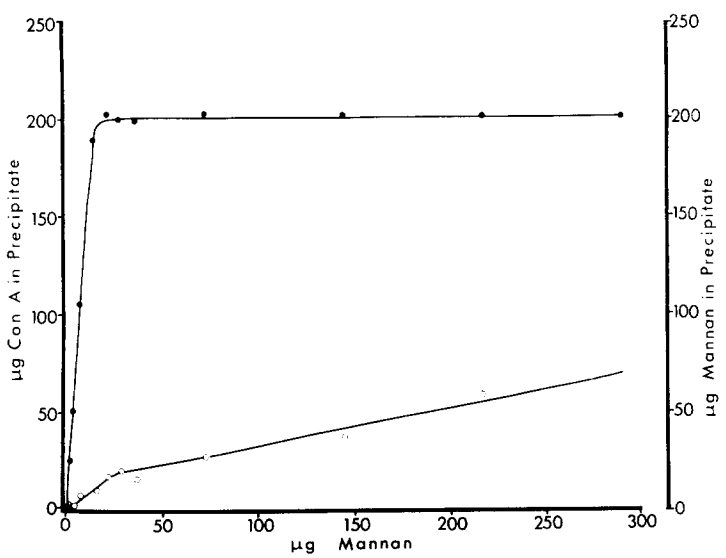

Fig. 5. Quantitative precipitation of purified mesosomal mannan with concanavalin A. Reaction mixtures (1.0 ml) contained 225 µg concanavalin A and mesosomal mannan in 0.018 M phosphate buffer, pH 7.2, made 1.0 M with respect to NaCl. Resultant precipitates were washed [41] and analyzed for protein [24] and for mannan [25] following overnight incubation at 23 °C.

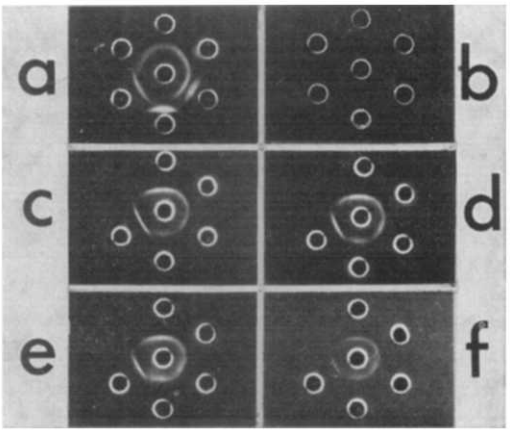

Fig. 6. Agar gel diffusion of membrane fractions against concanavalin A. Wells are numbered in clockwise order from the the uppermost well. Well 1, mesosomal mannan; well 2, plasma membrane; well 3, mesosomal membrane; well 4, residual mesosomal membrane following "ionic-heat shock"; well 5, yeast mannan; well 6, "periplasmic fraction". The central well in each case contains concanavalin A. Both sample and agar in gels b, c, d, e and f were supplemented with 0.2 M α-methyl-D-mannoside, 1 % (v/v) Tween 80, 1 % (v/v) Nonidet P-40, 1 % (v/v) Triton X-100 and 0.1 % (w/v) sodium dodecylsulphate, respectively.

residues tested. Occasionally a second faint precipitin line was observed (Fig. 6e). However, this component was only present in membrane fractions and never in purified mannan preparations.

Electrophoretic properties. Mild alkaline hydrolysis of mesosomal mannan produced a polysaccharide with differing electrophoretic properties (Fig. 7). Gel electrophoresis of intact mannan showed a polymer with ionic properties not unlike those of yeast mannan, a polysaccharide which is known to be phosphorylated [56]. Indeed, analysis of the yeast mannan used indicated a phosphorus content of 0.37 %. Furthermore the "streaking" behavior of intact mesosomal mannan (cf. hydrolyzed mannan) suggested a degree of charge heterogeneity that was eliminated upon hydrolysis. These properties were also exhibited when electrophoresis was performed in the presence of BO_3^{3-}, which are known to form negatively charged complexes with carbohydrates [57]. Further experimentation is necessary to determine whether differences observed in the electrophoretic properties of hydrolyzed mesosomal mannan and neutral dextran are due to intrinsic charge differences or simply to differences in the molecular weight.

Molecular weight. The apparent molecular weight estimated from polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate must be regarded as an overestimate in view of the known anomalous migration of carbohydrates in that system [58]. Attempts to obtain ultracentrifuge sedimentation patterns of mesosomal mannan in distilled water were unsuccessful as only concentration redistribution was observed. However, sedimentation profiles were obtained if centrifugation was performed in 1.0 M NaCl (cf. ref. 59). A sedimentation coefficient of 1.39 S was obtained. Using a calculated diffusion coefficient of $D = 7.15 \cdot 10^{-7}$ cm/s and assuming a partial specific volume $\bar{v} = 0.658$ cm^3/g (yeast mannan, ref. 60) or $\bar{v} = 0.57$ cm^3/g (teichoic acid, ref. 59) approximate molecular weights of 14 900 or

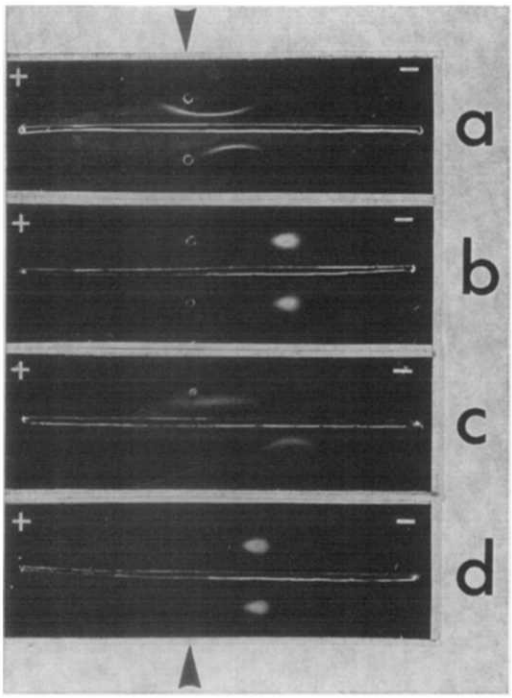

Fig. 7. Agar electrophoresis of mesosomal mannan. Purified mesosomal mannan before (lower well in gel a, upper well in gel c) and after mild alkaline hydrolysis (lower well in gel c) and yeast mannan (upper well in gel a) and dextran (upper and lower wells in gels b and d) were electrophoresed in agar containing barbital buffer (pH 8.2) for 1 h (gels a and b) and 1.5 h (gels c and d). Mannans were visualized by diffusion against concanavalin A and dextran by precipitation with ethanol. The line of normalized electrophoretic origins is indicated by arrow heads.

of 11 600 can be calculated. The latter is in reasonable agreement with the value of 9600 obtained by assuming one reducing terminal per mannan molecule.

Mannan secretion

The possibility that mannan is secreted from cells of *M. lysodeikticus* was investigated by monitoring culture supernatant fractions obtained from cells grown on defined medium. The overall growth curve (Fig. 8) is very similar to that obtained for cells grown in peptone/water/yeast extract medium. However, in the latter instance the generation time is about 2 h, cf. 3.5 h for growth on defined medium. Secretion of material containing hexose was observed mainly in the period between the end of logarithmic phase and the beginning of stationary phase of growth. The fact that little additional hexose production occurred during stationary phase of growth would argue against it being a product of cell lysis. The ability of mannan to coprecipitate with concanavalin A in gels has enabled us to demonstrate the presence of this polysaccharide in the hexose-enriched culture supernatant fractions. Examination of the diffusion pattern illustrated in Fig. 8 (insert) verified that active production of mannan which can be shown to be (represented by the major precipitin band) occurred during the period of decelerating growth. Several minor precipitin bands were also observed.

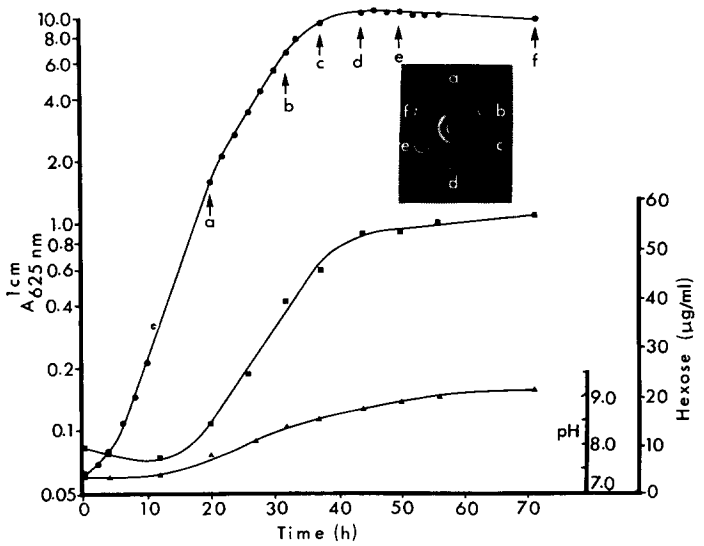

Fig. 8. Release of hexose-containing material from *M. lysodeikticus* during growth on defined medium. ●—●, $A^{1\,cm}_{625\,nm}$; ■—■, total hexose; ▲—▲, pH. Insert, Agar diffusion tests of concentrated culture supernatant fractions against concanavalin A. Fractions a–f were obtained from cultures harvested at the absorbancies indicated.

DISCUSSION

Gas-liquid chromatographic analysis of isolated membranes and mesosomes of *M. lysodeikticus* indicate that mannose is the principal monosaccharide. Lipid-extracted membranes also contain substantial quantities of mannose suggesting that mannan may account for about 80 % of the membrane-bound carbohydrate. Our investigations indicate that mesosomal vesicles consistently contain higher levels of membrane-associated mannan in comparison with corresponding plasma membrane fractions. Since mesosomal membrane fractions represent at least 13 % of the total membrane [1] it follows that about 40 % of the membrane mannan is localized in this vesicle fraction. However, this value is probably an underestimate since the yield of mesosomal vesicles can be increased by reducing the Mg^{2+} concentration of the protoplasting fluid, an effect which also causes loss of protoplast integrity [1]. It should be noted that our studies clearly demonstrate that mannan is also present in "periplasmic" fractions and is secreted into the medium during the late logarithmic phase of growth.

We were unable to confirm the results of Schmit et al. [15] that glucose is the major carbohydrate of solubilized membranes of this organism. Although a glucan has been reported [15] it has not been specifically identified as a membrane component. Indeed, no glucose was found in purified mesosomal mannan preparations analyzed by the sensitive gas-liquid chromatography procedure (see Fig. 4). Inositol has been conclusively identified in *M. lysodeikticus* membranes, thus supporting the chromatographic detection of phosphatidylinositol as one of the membrane phospholipids of this organism [61].

Our investigations indicate that the membrane mannan of *M. lysodeikticus* can be extracted from both plasma and mesosomal membranes by relatively mild procedures ("ionic-heat shock" treatment) in contrast to phenol extraction used by other investigators [15, 17–20]. Residual mannan is found in the membrane after maximal release by the "ionic-heat shock" washes and its presence can be demonstrated by solubilizing the membrane residues in detergent and reacting against concanavalin A. This fraction of the mannan is clearly more firmly bound to the membrane. The mannan released from the mesosomes by the "ionic-heat shock" method and purified as described in this paper has about one-sixth the fatty acid content of the polymer recently isolated by Pless et al. [20]. This fact, together with our observation of the presence of one reducing end group per 50 sugar residues (cf. ref. 20) suggests that the mesosomal mannan preparations may contain a higher proportion of deacylated polymer compared to the preparations isolated by Pless et al. [20]. The loss of hydrophobic ends to the molecules may render it more readily extractable by the "ionic-heat shock" treatment and mannan retained in the membrane residues may represent the acylated form of the polymer. Indeed, there is precedence for such a situation with the lipoteichoic acid of *Streptococcus faecalis* which Joseph and Shockman [62] have recently shown to be largely in the deacylated form in the extracellular product in contrast to the predominantly acylated cellular form. The extension of the latter studies to the *M. lysodeikticus* "lipomannan" would help to clarify the existence of the two forms of the polymer and differences in their extractability from the membranes.

Although we have not examined the mode of attachment of the fatty acid moiety to the mannan, Pless et al. [20] have suggested attachment of the polysaccharide to diglyceride. Such a structure would be basically similar to lipoteichoic acid and provide a hydrophobic anchoring portion for association with the membrane. Indeed it has been suggested that acylated mannan of *M. lysodeikticus* may be an analogous structure to the membrane lipoteichoic acids [19, 49] which have been shown to reside largely in the mesosomal vesicle fraction of some organisms [7].

The function of the membrane mannan is still uncertain at the present time. Its precise characterization will, however, be of importance in determining its role in both mesosome and plasma membranes of this organism. In this study we have described procedures for selective release of mannan from membranes, for its purification and chemical characterization by a variety of techniques. The preferential localization of the mannan in the mesosomes and the secretion of this polymer into the growth medium may provide some clues as to the functions of this polysaccharide and its role in the biochemical significance of the enigmatic mesosome structures [3]. Of relevance is the interesting observation that the secretion of alkaline phosphatase from the related organism *Micrococcus sodonensis* is closely linked to the secretion of an acidic polysaccharide [63]. However, further experimentation will be necessary to determine whether the mannan of *M. lysodeikticus* plays a role in protecting secreted enzymes from proteolysis during a vulnerable stage in their synthesis and/or secretion. The detection of succinyl residues in this mannan may also lead to localization of unique enzymes involved in the transfer of these residues to the mannan.

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