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DISTRIBUTION OF ENZYMES INVOLVED IN MANNAN SYNTHESIS IN PLASMA MEMBRANES AND MESOSOMAL VESICLES OF *MICROCOCCUS LYSODEIKTICUS*

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SUMMARY

The distribution of membrane-bound enzymes involved in mannan biosynthesis in plasma and mesosomal membranes of *Micrococcus lysodeikticus* has been investigated.

Isolated mesosomal vesicles, unlike plasma membrane preparations, cannot catalyze the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose into mannan. This appears to result from the inability of this membrane system to synthesize the carrier lipid [^{14}C]mannosyl-l-phosphorylundecaprenol. In contrast, this is the major mannolipid synthesized from GDP-[^{14}C]mannose by isolated plasma membranes. The possibility that substrate inaccessibility could account for the failure to detect the enzyme in isolated mesosomal vesicles appears unlikely from the lack of activity following disruption of the vesicles with ultrasound or with surface active agents.

Both membrane preparations possessed the ability to catalyse the transfer of [^{14}C]mannose from purified [^{14}C]mannosyl-l-phosphorylundecaprenol into mannan, Furthermore, free mannan and mannan located on both unlabeled mesosomal and unlabeled plasma membranes could act as acceptors of [^{14}C]mannosyl units from ^{14}C-labeled carrier lipid located in prelabeled plasma membranes. The possibility that the juxtaposition of mesosomal vesicles and enveloping plasma membrane (i.e. the mesosomal sacculus) in vivo allows mannan, located on mesosomal vesicles, to accept mannosyl units from carrier lipid located in the sacculus membrane is discussed.

INTRODUCTION

It has been known for some time that *Micrococcus lysodeikticus* possesses a mannan in its membrane [1, 2]. The mannan has generally been extracted from whole cells of this Gram-positive organism and some of its chemical characteristics have been reported [2–5]. The occurrence of this mannan in bacterial membranes is rather unusual and it has been suggested that, in this organism, it may represent a "functional" analogue of the lipoteichoic acid commonly found in the membranes of many

Gram-positive organisms [5, 6]. The important observation that a mannose polymer is localized in the mesosomal membranes of *M. lysodeikticus* [7] has served as a stimulus for the characterization of this unique bacterial membrane component as reported in the accompanying communication [8] and to attempt to gain further information on the significance of this component in the mesosomes and to elucidate the functions of the latter structure. With the precise characterization of this succinylated mesosomal mannan [6, 8] isolated from *M. lysodeikticus*, it was tempting to explore the possibility that its synthesis may also be localized in the mesosomes, thereby providing a unique enzymatic function for these structures.

The development of suitable procedures for the isolation of homogeneous preparations of mesosomal vesicles and plasma membranes from *M. lysodeikticus* [7] has enabled us to examine the distribution of enzymes catalyzing the incorporation of labeled mannose from GDP-[^{14}C]mannose into mannan. It should be recalled that the important studies of Lennarz and his coworkers [3, 9, 10] established the role of the carrier lipid mannosyl-l-phosphorylundecaprenol as an obligatory intermediate in the synthesis of mannan by *M. lysodeikticus*. Synthesis involves the transfer of mannosyl groups from GDPmannose to carrier lipid and is followed by subsequent transfer of hexose from mannosyl-l-phosphorylundecaprenol to mannan acceptor [3]. In addition to investigating the distribution of enzymes involved in mannan synthesis, we have also attempted to resolve the question of permeability problems which may have accounted for the absence of phospho-*N*-acetylmuramylpentapeptide translocase and of teichoic acid synthetase in mesosomal vesicles isolated from *Bacillus subtilis* and *Bacillus licheniformis* [11].

MATERIALS AND METHODS

Bacterial growth and membrane preparations. The methods for isolation of mesosomal and plasma membrane fractions and also "total" (i.e. mesosomal and plasma) membrane fractions from cells of *M. lysodeikticus* (NCTC 2665) have been described in detail elsewhere [7, 12]. Mesosomal mannan was selectively released and purified as described in the preceding paper [8].

Chromatographic procedures. Identification and separation of the various labeled and unlabeled components was achieved with the following chromatographic systems. Descending chromatography was performed on Schleicher and Schuell green ribbon paper No. 589 at room temperature (approximately 23 °C) with Solvent I, ethanol/1.0 M ammonium acetate, pH 7.3 (5 : 2, v/v). Ascending paper chromatography was performed on Whatman SG-81 silica gel-impregnated paper using the following solvents: Solvent II, chloroform/methanol/7 M NH_4OH (66 : 17 : 3, by vol.); Solvent III, chloroform/methanol/diisobutylketone/pyridine/0.5 M NH_4Cl, pH 10.4 (30 : 17.5 : 25 : 35 : 6, by vol.); Solvent IV, diisobutylketone/pyridine/water (25 : 25 : 4, by vol.); Solvent V, chloroform/methanol/diisobutylketone/pyridine/water (30 : 25 : 25 : 35 : 8, by vol.); Solvent VI, chloroform/methanol/water (130 : 50 : 8, by vol.); Solvent VII, chloroform/methanol/water (12 : 6 : 1, by vol.); Solvent VIII, diisobutylketone/acetic acid/water (120 : 90 : 12, by vol.); Solvent IX, diisobutylketone/acetic acid/water (40 : 25 : 5, by vol.); Solvent X, chloroform/methanol/diisobutylketone/acetic acid/water (60 : 20 : 40 : 27 : 5.3, by vol.); Solvent XI, chloroform/methanol/acetic acid/water (120 : 80 : 6 : 10, by vol.). In instances when ^{14}C-

labeled mannolipids were subjected to two-dimensional chromatography it was found necessary to dry chromatograms in N_2 gas between solvent runs (usually Solvent X and Solvent III) in order to prevent the hydrolysis of [^{14}C]mannosyl-l-phosphoryl-undecaprenol to [^{14}C]mannose l-phosphate and free prenol.

Monosaccharides were detected with the aniline hydrogen phthalate reagent [13] and sugar phosphates by the periodate-benzidine method [14]. Rhodamine 6G (ref. 15) and 8-anilino-l-naphthalene sulphonic acid [16] were used as general lipid stains. Phospholipids were detected using the molybdenum blue stain [17] or Zinzade's reagent [18] and polyisoprenol alcohols by the semispecific spray reagent acidic *p*-anisaldehyde [19] and also by fluorescein [20].

Lipid extraction. Lipids from whole cells and isolated membrane fractions were extracted by Bligh and Dyer's method [21]. Individual phospholipids, dimannosyl diglyceride and neutral lipid fractions were isolated from total cellular lipid of *M. lysodeikticus* by preparative paper chromatography on silica gel-impregnated paper using Solvent X [22, 23].

Enzyme preparation. As the source of enzyme for the synthesis and subsequent purification of [^{14}C]mannosyl-l-phosphorylundecaprenol, acetone powders were prepared from washed "total" membrane fractions [9]. A yield of 4.06 g of acetone powder was obtained from 4.8 g dry weight of membrane and such preparations were stored at -70 °C until use. Treatment of the acetone powder with 106 ml of 20 mM Tris · HCl buffer, pH 7.6, for 45 min followed by centrifugation at $6000 \times g$ for 15 min yielded a clear supernatant fraction which was used immediately as the enzyme source in the synthesis of [^{14}C]mannosyl-l-phosphorylundecaprenol.

Synthesis and purification of [^{14}*C*]*mannosyl-l-phosphorylundecaprenol.* Synthesis of [^{14}C]mannosyl-l-phosphorylundecaprenol was performed by a modification of Scher and Lennarz' procedure [24]. To a sonic dispersion of 1.45 g total lipid from cells of *M. lysodeikticus* in a solution containing 79 ml distilled water, 15.6 ml 0.8 M Tris/maleate buffer, pH 8.5, 10 ml 50 mM Tris · HCl buffer, pH 7.5, and 10 ml 1 M $MgCl_2$ was added 11.24 μmol GDP-[^{14}C]mannose (10 μCi) in 40 ml distilled water followed by 60 ml of acetone powder extract (697 mg). The final pH of the reaction mixture was 8.35. The reaction was terminated after 2 h at 37 °C by pouring the mixture into 3900 ml chloroform/methanol (2 : 1, v/v) and the lipid extracted [24].

Subsequent purification of [^{14}C]mannosyl-l-phosphorylundecaprenol from the lipid extract of the incubation mixture followed that described by Scher et al. [9]. All fractions were monitored in our purification procedure by chromatography on silica gel-impregnated paper in Solvents II or X followed by staining with Rhodamine 6G or 8-anilino-l-naphthalenesulphonic acid and also by either autoradiography or strip counting of the chromatograms. Authentic marker lipids were always run in parallel.

Fractionation of the crude ^{14}C-labeled lipid extract ($6.5 \cdot 10^6$ cpm, 4.96 μmol) on a Unisil silicic acid column (8×3.5 cm internal diameter, ref. 9) gave a product ($5.6 \cdot 10^6$ cpm, 4.27 μmol) essentially free of dimannosyl diglyceride ($0.95 \cdot 10^6$ cpm, 0.95 μmol) and neutral lipid. Subsequent chromatography on a Whatman DE-52 cellulose column (34×4.5 cm internal diameter) gave a product in the fraction eluted with solvent, chloroform/methanol/ammonia (50 : 25 : 9, by vol.) which chromatographed as a single radioactive spot in Solvent X ($5.1 \cdot 10^6$ cpm, 3.98 μmol). All the major phospholipids, also present in this fraction, were removed upon alkaline

hydrolysis, yielding a product ($3.9 \cdot 10^6$ cpm, 2.98 μmol) consisting mainly of neutral lipid and fatty acids. Chromatography of this product on a second DE-52 cellulose column followed by fractionation upon a second silicic acid column (6.5×1.2 cm internal diameter) removed all the detectable fatty acid and neutral lipid and yielded a product ($2.9 \cdot 10^6$ cpm, 2.21 μmol) which chromatographed as a single radioactive spot in Solvents II and X.

Although appearing homogeneous by analytical paper chromatography, this preparation was still faintly yellow in color. The product was therefore purified further by chromatography on Whatman SG-81 papers in Solvent II, dried for 1 h in a stream of N_2 gas and a narrow central strip stained with Rhodamine 6G to localize the lipid. Corresponding regions were then cut from the chromatograms and eluted [23] consecutively with the following volumes of solvents: two times 1 ml of chloroform, two times 1 ml of acetone, three times 1 ml of chloroform/methanol (2 : 1, v/v) and finally with two times 1 ml of methanol. All the radioactivity ($2.73 \cdot 10^6$ cpm, 2.08 μmol) was eluted in the methanolic fractions and the residual carotenoid pigment in the chloroform fraction.

The final product (2.2 mg, theoretical value based on radioactive measurements 2.1 mg) ran as a single component staining with Rhodamine in Solvents II–XI. Autoradiograms of the chromatographed lipid gave one radioactive spot corresponding to the stained component, although hydrolysis was noted in acidic solvents as indicated by considerable tailing of spots (Fig. 1). It gave a positive reaction for phosphorus [18] and also with the semispecific spray for polyisoprenols [19] and upon mild acid hydrolysis gave a radioactive substance with the mobility of [^{14}C]mannose 1-phosphate in Solvents V and X. Analysis by mass spectrometry, kindly performed by Dr A. J. Baker, Department of Chemistry, University of Glasgow, indicated that under a variety of ionization conditions spontaneous decomposition of [^{14}C]mannosyl-1-

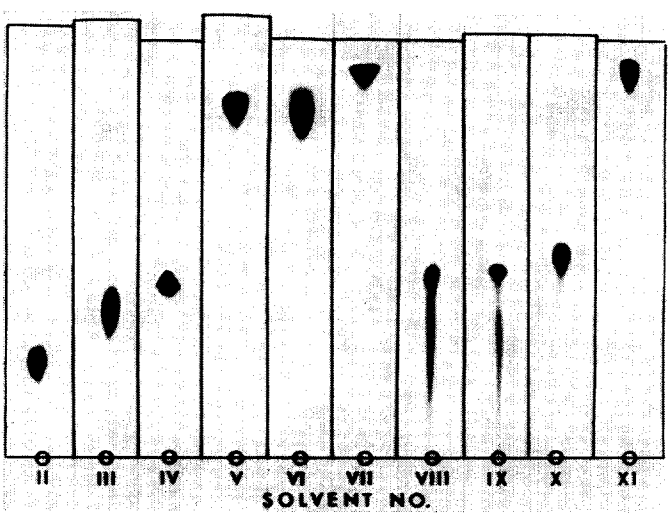

Fig. 1. Autoradiograms of purified [^{14}C]mannosyl-1-phosphoryl undecaprenol following chromatography in various solvents. Purified [^{14}C]mannosyl-1-phosphorylundecaprenol ($5 \cdot 10^3$ cpm, 3.8 nmol) was chromatographed on silica gel-impregnated paper in Solvents II–XI (as listed in Materials and Methods).

phosphorylundecaprenol occurred to give mannosyl l-phosphate and free undecaprenol. The fragmentation pattern of the free prenol was identical to published data for this compound [10] and also indicated the presence of small amounts (about 3 %) of dodecaprenol.

Enzyme assays. Mannan synthetase activity of membrane preparations was determined by a modification of the method of Scher and Lennarz [3]. Reaction mixtures contained 1.2–3.0 mg dry weight of fresh membrane preparation, 50 μl 0.8 M Tris/maleate buffer, pH 8.5, and 150 μl 50 mM Tris · HCl buffer, pH 7.5, in a total volume of 240 μl (final pH 8.30). Reactions were initiated by the addition of GDP-[^{14}C]mannose (26–29 nmol, 834–4063 cpm/nmol) or by addition of purified [^{14}C]mannosyl-l-phosphorylundecaprenol (10.1 nmol, 1310 cpm/nmol) as a liposome suspension. Following incubation at 37 °C for 1 h, the contents of the reaction tubes were rapidly frozen at −20 °C. Individual reaction mixtures were then thawed and spotted as three-inch bands on Schleicher and Schuell green ribbon paper No. 589 and eluted at room temperature with Solvent I for 24 h. ^{14}C-labeled mannan synthesis was estimated by cutting zones at the origin into 1 inch × 0.5 inch strips and counting in toluene scintillation fluid. Results were corrected for origin counts obtained from control assays using equal amounts of boiled (10 min) membrane preparations. In all assays equal weights of corresponding mesosomal and plasma membrane were employed.

The ability of both mesosomal and plasma membranes to synthesize [^{14}C]-mannosyl-l-phosphorylundecaprenol was compared by incubation of membranes with GDP-[^{14}C]mannose under the conditions described above. Reactions were terminated by the addition of 900 μl of chloroform/methanol (1 : 2, v/v) and lipids extracted by the method of Bligh and Dyer [21]. Dried lipids were made to 0.5 ml with chloroform and samples subjected directly to scintillation counting. The percentage of the total radioactivity due to [^{14}C]mannosyl-l-phosphorylundecaprenol was estimated by chromatography of portions of extracted ^{14}C-labeled mannolipids in Solvent X. Regions of the chromatograms corresponding to labeled carrier lipid were localized either by autoradiography or from the relative migration of authentic [^{14}C]mannosyl-l-phosphorylundecaprenol and of known marker lipids which had been cochromatographed in parallel. Pertinent regions of the chromatograms were cut out and radioactive determinations made directly by scintillation counting. Results were compared with radioactive measurements obtained for equivalent amounts of ^{14}C-labeled mannolipids which had been counted under identical quenching conditions. In most instances it was found that [^{14}C]mannosyl-l-phosphorylundecaprenol accounted for about 90 % of the total radioactivity extracted in the lipid fraction.

Preparation of prelabeled plasma membranes. Plasma membranes (41.2 mg dry weight) were labeled with [^{14}C]mannose by incubation with 2.25 ml of 50 mM Tris · HCl, pH 7.5, 0.75 ml 0.8 M Tris/maleate, pH 8.5, 0.75 ml distilled water and 161 nmol GDP-[^{14}C]mannose (36 570 cpm/nmol) for 1 h at 37 °C. Two volumes of Buffer mixture I (50 mM Tris · HCl, pH 7.5; 0.8 M Tris/maleate, pH 8.5; distilled water; 3 : 1 : 1, by vol.) at 0 °C were added and membranes sedimented at 12 000 × g for 30 min at 0 °C. Pellets were washed a further three times in Buffer mixture I to remove residual GDP-[^{14}C]mannose. Chromatography of washed prelabeled membranes in Solvent I followed by strip counting failed to reveal any peak corresponding

to authentic GDP-[^{14}C]mannose. Washed prelabeled membranes were finally suspended to a final concentration of 15 mg dry weight/ml in Buffer mixture I.

Transfer of [^{14}C]mannose from prelabeled plasma membranes to unlabeled membrane fractions. Prelabeled plasma membranes were incubated at 37 °C for 1 h with an equal volume of either 50 mM Tris · HCl, pH 7.5, or unlabeled plasma membranes (20 mg dry weight/ml) or unlabeled mesosomal membranes (20 mg dry weight/ml) or boiled mesosomal membranes (20 mg dry weight/ml) or purified mesosomal mannan (4.0 mg dry weight/ml). To facilitate centrifugation, four volumes of Buffer mixture II (Buffer mixture I; 50 mM Tris · HCl, pH 7.5; 1 : 1, v/v) were then added at 0 °C and the reaction mixtures centrifuged at 12 000 × *g* for 15 min at 0 °C to sediment plasma membranes. (Control studies using plasma and mesosomal membranes had established that 95 % of the plasma membranes and only 10 % of mesosomal membranes were sedimentable under these conditions). The supernatant fractions were carefully decanted and centrifuged at 87 000 × *g* at 0 °C for 2 h to pellet the mesosomal membranes and to give a membrane-free supernatant fraction. Both the pellets obtained following centrifugation at 12 000 × *g* (Fraction A, largely plasma membranes) and at 87 000 × *g* (Fraction B, largely mesosomal membranes) together with the 87 000 × *g* supernatant fraction (Fraction C) were applied quantitatively onto Schleicher and Schuell paper No. 589, subjected to chromatography in Solvent I and the quantity of [^{14}C]mannan present in each fraction determined as described previously.

Chemical analysis. Protein was estimated by the method of Lowry et al. [25] and total hexose by the method of Morris [26] using bovine serum albumin and mannose, respectively, as standards. Dry weights were determined on membrane suspensions as previously described [8].

Sonication. Dispersion of membranes and of dried lipid films was achieved by ultrasound using an MSE sonifier fitted with a 3/8 inch probe [8, 27].

Radioactive measurements. Radioactive measurements were made in a Nuclear Chicago Mark I liquid scintillation counter, using a toluene-based scintillation fluid containing 100 mg 2,2′-*p*-phenylenebis (5-phenyloxazole) and 4 g 2,5-diphenyloxazole per l of toluene. Radioactive peaks on chromatograms were located either by autoradiography or by cutting 1/8 inch strips from the chromatograms and counting in 4 ml vial inserts with 1 ml of scintillant. Visible absorption due to carotenoid pigments was first destroyed by the addition of a drop of bromine [28] and excess bromine removed under vacuum.

Chemicals. GDP-[U-^{14}C]mannose (160 Ci/mol and 246 Ci/mol) and $H_3{}^{32}PO_4$ (carrier free) were purchased from New England Nuclear. Autoradiograms were made by exposure to Cronex 4 medical X-ray film purchased from DuPont de Nemours and Co. Inc. Poly Tergent S305LF was a gift of Olin Chemicals.

RESULTS

A comparison of the mannan synthetase activities of isolated mesosomal and plasma membrane preparations is given in Table I. For four different membrane preparations it was observed that the mannan synthetase activity of mesosomal membranes was negligible in comparison with corresponding plasma membrane fractions when GDP-[^{14}C]mannose was used as substrate. However, when purified

TABLE I

COMPARISON OF THE MANNAN SYNTHETASE ACTIVITIES OF MESOSOMAL AND PLASMA MEMBRANES USING GDP-[^{14}C]MANNOSE AND [^{14}C]MANNOSYL-1-PHOSPHORYLUNDECAPRENOL AS SUBSTRATES

Membrane preparation	^{14}C-labeled mannan formed (pmol/h per mg dry weight membrane)			
	GDP-[^{14}C]mannose		[^{14}C]Mannosyl-1-phosphorylundecaprenol	
	Mesosomal membrane	Plasma membrane	Mesosomal membrane	Plasma membrane
1	3	263	38	170
2	0	335	n.d.	n.d.
3	11	178	89	128
4	7	226	45	124

n.d., not determined.

[^{14}C]mannosyl-l-phosphorylundecaprenol was used as substrate a dramatic increase was observed in the ability of mesosomal membranes to catalyze the incorporation of labeled [^{14}C]mannose into mannan (see Table I). On a dry weight basis of the fractions, the activity present in the plasma membranes exceeded that of corresponding mesosomal membrane preparations by a factor of 1.4–4.5. However, plasma membranes possess over 1.5 times the protein content of mesosomal membranes [7] so that the specific activities of the two fractions are more comparable.

It would appear from these results that the inability of mesosomal membranes to synthesize mannan is due to failure to synthesize the lipid intermediate, mannosyl-l-phosphorylundecaprenol. This was confirmed by comparison of the abilities of both mesosomal and plasma membranes to synthesize [^{14}C]mannosyl-l-phosphorylundecaprenol from GDP-[^{14}C]mannose. That the capacity of plasma membranes to synthesize [^{14}C]mannosyl-l-phosphorylundecaprenol far exceeds that of equivalent amounts of mesosomal membrane is demonstrated in Fig. 2 (A and B). The ratio of labeled carrier lipid synthesized by the plasma and by the mesosomal membranes was about 34 : 1 as determined by scintillation counting. Two minor mannolipids were also detected in autoradiograms of lipids extracted from plasma membranes. One cochromatographed with authentic dimannosyl diglyceride, the second (M_1) remained unidentified. Control experiments revealed that the latter was not a lipid contaminant of GDP-[^{14}C]mannose.

A control chromatogram showing the positions of the major phospholipids of *M. lysodeikticus* is presented in Fig. 2C. A spot corresponding to mannosyl-l-[^{32}P]-phosphorylundecaprenol (or to M_1) was not detected in lipid extracts from whole cells grown in the presence of $H_3{}^{32}PO_4$. This probably reflects the relatively small quantities of the phosphoryl lipid carrier present in the membranes of viable cells at the time of harvesting. Even under the optimised conditions of in vitro assay [^{14}C]-mannosyl-l-phosphorylundecaprenol formed accounted for no more than 2 % of the total membrane lipid.

Since mesosomal membranes are largely in the form of membrane vesicles [7] it was conceivable that the enzyme-transferring mannose units from GDP-[^{14}C]-

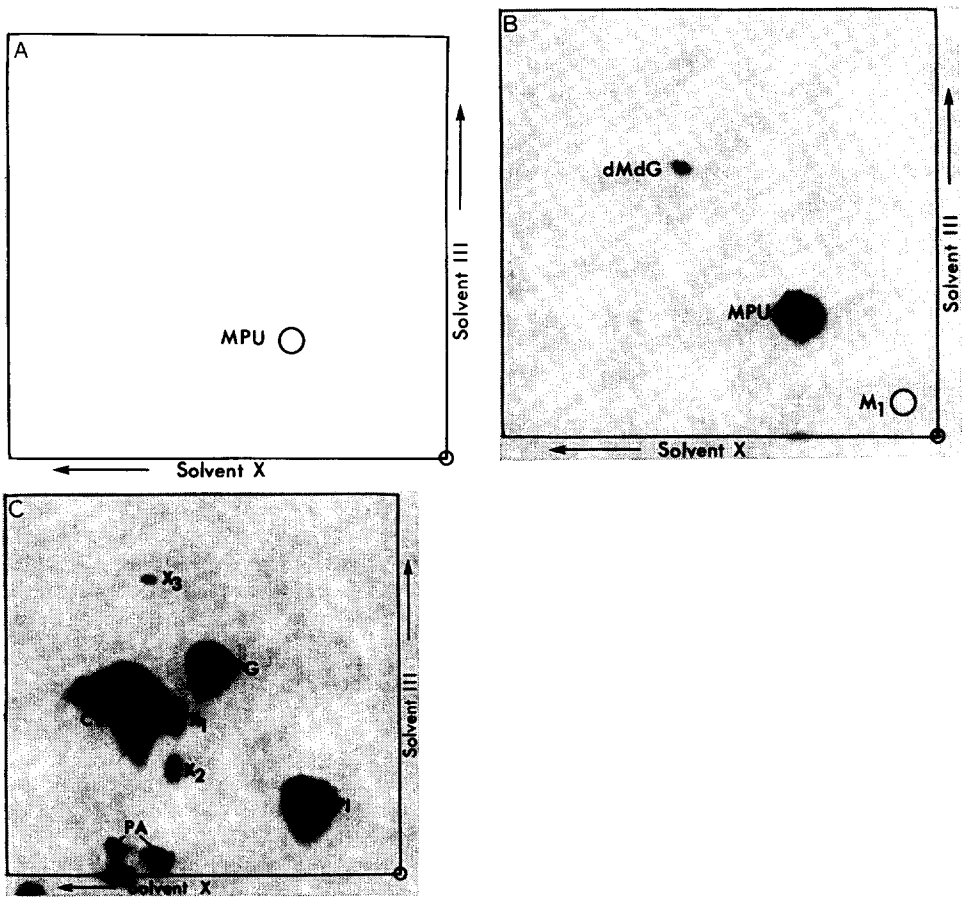

Fig. 2. Autoradiograms of chromatographed ^{14}C-labeled mannolipids following incubation of mesosomal and plasma membrane preparations with GDP-[^{14}C]mannose. 1.89 mg dry weight of mesosomal and plasma membranes were incubated with 6.1 nmol GDP-[^{14}C]mannose (96 000 cpm/nmol) for 1h at 37 °C. Lipids were extracted from reaction mixtures as described in Materials and Methods and equivalent amounts subjected to two-dimensional chromatography in Solvents X and V. A and B are autoradiograms of chromatographed lipids extracted from mesosomal and plasma membrane preparations, respectively. C is an autoradiogram of chromatographed lipids extracted from whole cells of *M. lysodeikticus* following growth in the presence of $H_3{}^{32}PO_4$. The identity of individual lipids was determined by comparison of their chromatographic properties in Solvents II–X with those of authentic marker compounds. dMdG, dimannosyl diglyceride; MPU, mannosyl-1-phosphorylundecaprenol; M_1, unknown mannolipid; PL, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid; X_1, X_2 and X_3, unknown phospholipids. Circles represent compounds which were marginally detectable by autoradiography.

mannose to undecaprenol phosphate resided on the inner surface of the vesicle thereby presenting a permeability barrier for the substrate. Washed plasma membranes, on the other hand, are largely in the form of open sheets [7] and should be freely accessible to substrate. Sonication fragments membrane preparations [27] and would thus be expected to disrupt closed vesicles and expose components of the inner-membrane surface. No detectable incorporation of [^{14}C]mannose from GDP-[^{14}C]mannose into

mannan was observed for mesosomal membranes subjected to sonication for periods of 30 s to 5 min. Plasma membranes sonicated under identical conditions did, however, exhibit a 43 and 60 % reduction in mannan synthetase activity after 1 min and 2 min exposure to ultrasound, respectively. Lack of activity on sonicating mesosomal vesicles could thus be due to inactivation.

Other methods of perturbation were tested in an attempt to obtain full expression of mannosyl-l-phosphorylundecaprenol synthetase and of mannan synthetase activities associated with the mesosomal vesicles. Triton X-100 is known to dissociate membranes and stimulate activities of many membrane-bound enzymes (see ref. 29). Furthermore, previous workers have shown that mannosyl-l-phosphorylundecaprenol synthesis in *M. lysodeikticus* is virtually unaffected by low concentrations of this detergent [3]. Another surfactant, Poly Tergent S305LF, has been used to optimize galactose-diphosphoglycosylundecaprenol synthetase activity in membrane fractions isolated from *Salmonella typhmurium* LT2 [30]. Activation of partially purified undecaprenol pyrophosphate synthetase from *Lactobacillus plantarum* by cardiolipin has also been observed [31]. However, as shown in Table II under all of the conditions tested there was no increase in either mannan synthetase or mannosyl-l-phosphoryl-undecaprenol synthetase activities of isolated mesosomal vesicles from *M. lysodeikticus*. Indeed, the activities remained negligible compared with those of plasma membranes. From these results (Table II) it would appear that mesosomal membranes cannot synthesize mannosyl-l-phosphorylundecaprenol carrier lipid. However, this membrane fraction can catalyze the transfer of mannose residues into mannan when supplied with mannosyl-l-phosphorylundecaprenol (see Table I).

Thus it seemed conceivable that mannan located on mesosomal membrane vesicles could act as acceptor of mannose units from carrier lipid in plasma membranes when they are in juxtaposition to one another. To test this possibility plasma membrane fractions were prepared containing ^{14}C-labeled carrier lipid (see Materials and Methods). Such preparations contained [^{14}C]mannosyl residues present in mem-

TABLE II

EFFECT OF SURFACE-ACTIVE AGENTS ON THE SYNTHESIS OF MANNOSYL-1-PHOSPHORYLUNDECAPRENOL AND OF MANNAN BY MEMBRANE FRACTIONS

Triton X-100, Poly Tergent S305LF and cardiolipin were incorporated into the assay mixtures at the final concentrations shown. [^{14}C]Mannosyl-1-phosphorylundecaprenol synthesis and ^{14}C-labeled mannan synthesis were then determined for mesosomal and plasma membrane fractions as described in Materials and Methods. The results are expressed as

Surface-active agent	Final concentration	pmol/h per mg dry weight membrane. The results are expressed as:			
		Mannosyl-1-phosphoryl-undecaprenol synthetase		^{14}C-labeled mannan formed	
		Mesosomal membrane	Plasma membrane	Mesosomal membrane	Plasma membrane
None		82	3423	17	294
Triton X-100	0.1 % (w/v)	10	2086	9	62
Poly Tergent	0.1 % (w/v)	97	4078	19	174
Cardiolipin	1 mM	74	3023	2	165

brane mannan, in mannosyl-l-phosphorylundecaprenol, in dimannosyl diglyceride and in mannolipid M_1 in the ratios 1.00 : 7.20 : 0.43 : 0.33. The membranes were, therefore, predominantly labeled in the [^{14}C]mannosyl-l-phosphorylundecaprenol carrier lipid which could then be available for enzymatic transfer to suitable acceptor sites.

The effect of incubation of prelabeled membrane with unlabeled membrane fractions is shown in Table III. Following incubation of prelabeled membranes with buffer the amount of total label incorporated into mannan increased by 21 %, a phenomenon not unexpected since prelabeled membranes still have mannan acceptor sites available to the pool of endogenous carrier lipid. However, incubation of prelabeled plasma membranes with either unlabeled fractions (mesosomal, boiled mesosomal or plasma membranes) or with purified mesosomal mannan resulted in a further reproducible increase in the total label incorporated into mannan (Table III). Thus it seems probable that mannan present in the fractions tested can act as acceptors for mannosyl residues present in the pool of [^{14}C]mannosyl-l-phosphorylundecaprenol in prelabeled plasma membranes. The fact that mesosomal membranes are better acceptors than plasma membranes probably reflects the larger amounts of mannan in the former fraction [8].

Whereas most of the sedimentable ^{14}C-labeled mannan produced from incubation of prelabeled plasma membranes with unlabeled plasma membranes resided, as expected from control centrifugation studies, in the 12 000 $\times g$ sedimentable fraction (A), the additional ^{14}C-labeled mannan synthesized following incubation with unlabeled mesosomal membranes (or with boiled mesosomal membranes) was distributed between Fractions A, B and C. Furthermore, when purified mesosomal mannan was used as acceptor most of the additional ^{14}C-labeled mannan synthesized

TABLE III

TRANSFER OF [^{14}C]MANNOSE FROM PRELABELED PLASMA MEMBRANES TO UNLABELED MEMBRANE FRACTIONS

100 μl of prelabeled plasma membranes (1.5 mg dry weight membrane; 122 700 cpm, 3.36 nmol) were incubated at 37 °C for 1 h with (a) 100 μl 50 mM Tris · HCl buffer, pH 7.5; (b) 100 μl plasma membranes (2.0 mg dry weight membrane); (c) 100 μl mesosomal membranes (2.0 mg dry weight membrane); (d) 100 μl boiled mesosomal membranes (2.0 mg dry weight membrane); (e) 100 μl purified mesosomal mannan (400 μg). Following incubation reaction mixtures were diluted with buffer and centrifuged first at 12 000 $\times$ g to give membrane Fraction A and subsequently at 87 000 $\times$ g to give membrane Fraction B and supernatant Fraction C. The quantity of ^{14}C-labeled mannan in each fraction was then determined as described in Materials and Methods.

Unlabeled test component	Time of incubation (h)	[^{14}C]Mannose incorporation into mannan (pmol)			
		A	B	C	Total
None	0	–	–	–	350
Buffer	1	415	3	6	424
Plasma membranes	1	450	12	28	490
Mesosomal membranes	1	471	45	29	545
Boiled mesosomal membranes	1	469	54	32	555
Mesosomal mannan	1	519	3	17	539

was recovered in the plasma membrane fraction (A). Whether or not these results simply reflect the ability of extensively washed prelabeled membranes to redistribute mannan, or to more complex and specific events involving insertion and integration of the mannan into the membrane will require further exploration.

DISCUSSION

In a recent freeze-etch study of membrane fractions isolated from *M. lysodeikticus* it was observed that the total population of mesosomal vesicles was orientated right-side in ref. 7. These results appear to be consistent with electron-microscopic observations on negatively stained preparations [7, 32, 33]. Thus, substrate accessibility to enzyme complexes localized on the inner surface of mesosomal membrane vesicles could be a major factor limiting activity in this type of membrane. However, our results on the effect of ultrasound and of surfactants on the activities of the membrane-associated enzymes involved in mannan biosynthesis in *M. lysodeikticus* suggest that substrate inaccessibility is not a major factor responsible for the low activity observed for mesosomal membrane preparations obtained from this organism.

The inability of mesosomal membranes to transfer mannose units from GDP-mannose to mannan appears to result from the inability of this membrane system to synthesize the carrier lipid mannosyl-l-phosphorylundecaprenol. By comparison, this is the major mannolipid synthesized from GDPmannose by washed plasma membrane preparations. It seems unlikely that the apparent absence of mannosyl-l-phosphorylundecaprenol synthetase in the mesosomal membranes is due to suppression or latency of this enzyme, since low concentrations of detergents, which are known to activate certain membrane-bound enzymes [29], do not result in expression of activity. Moreover, in view of the current evidence of membrane fluidity [34] it seems improbable that mesosomal membranes would not possess any undecaprenol phosphate. Furthermore undecaprenol has been tentatively identified in both mesosomal and plasma membrane fractions isolated from *Staphylococcus aureus* [35] and also shown to be present at similar concentrations in mesosomal and plasma membrane fractions isolated from *L. plantarum* and *Lactobacillus casei* [36]. Thus it would seem more likely that the mesosomal membranes, unlike plasma membranes, do not possess the enzyme mannosyl-l-phosphorylundecaprenol synthetase.

Our experiments clearly show, however, that mannan located on both mesosomal and plasma membranes can act as acceptor for mannosyl units either from dispersions of purified mannosyl-l-phosphorylundecaprenol isolated from membranes or from this same carrier lipid as it exists in situ in the plasma membrane. To what extent the activities of the two preparations can be directly compared is difficult to assess, since mesosomal membranes have been shown to consistently contain 4–5 times the acceptor (mannan) content of the corresponding plasma membrane preparations [7, 8]. Furthermore, it has been shown that de novo synthesis of ^{14}C-labeled mannan does not occur under the conditions of assay, the majority of the [^{14}C]-mannose units incorporated from [^{14}C]mannosyl-l-phosphorylundecaprenol being located on the non-reducing termini of ^{14}C-labeled mannan [3]. Clearly further investigations are necessary to clarify the role of mannosyl-l-phosphorylundecaprenol in the biosynthesis of this succinylated polymer [6].

It is tempting to speculate, however, that the juxtaposition of mesosomal vesicles and of mesosomal sacculus (i.e. the region of the plasma membrane surrounding the mesosomal vesicles) in vivo allows mannan located on the mesosomal vesicles to accept mannosyl units either from carrier lipid located in the sacculus membrane or by direct carrier lipid exchange between the two membrane systems [37]. It should be noted that carrier lipid (doubly labeled with ^{3}H-labeled diaminopimelic acid and [^{14}C]mevalonic acid) involved in cell wall biosynthesis was found in both mesosomal and plasma membrane fractions isolated from *L. casei* and *L. plantarum* [36]. The ability of isolated membrane fractions to synthesize the carrier lipid, however, was not investigated.

Thus the plasma membrane with its ability to synthesize the carrier lipid may be the principal control site in polymer synthesis for both plasma and mesosomal membrane. It is perhaps significant that newly synthesized bactoprenol has been shown to be localized largely in the septal region of cells of *L. plantarum* that have just undergone cell division [39] and that in many bacteria mesosomes are closely associated with the growing cross wall (for review see ref. 40).

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