Isolation and Properties of Mesosomal Membrane Fractions from *Micrococcus lysodeikticus*

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1. A method is described for the isolation of pure mesosomal membrane fractions from *Micrococcus lysodeikticus*. 2. Plasmolysis of cells, before wall digestion, was necessary for effective mesosome release. 3. The effect of mild shearing forces, temperature and time upon the release of mesosomal membrane from protoplasts was investigated. 4. The optimum yield of mesosomal membranes from stable protoplasts was achieved at 10 mM-Mg²⁺. 5. Mesosomal membrane vesicle fractions prepared at differing Mg²⁺ concentrations above 10 mM were similar in chemical composition. 6. Comparison of the properties of peripheral and mesosomal membrane fractions revealed major differences in the distribution of protein components, membrane phosphorus, mannose and dehydrogenase activities between the two fractions. 7. Only cytochrome *b*₅₅₆ was detected in mesosomal membranes, whereas peripheral membranes contained a full complement of cytochromes. 8. Preliminary investigations suggested the localization of an autolytic enzyme(s) in the mesosomal vesicles. 9. The anatomy of mesosomal and peripheral membrane have been compared by the negative-staining and freeze-fracture technique. 10. The results are discussed in relation to a plausible role for the mesosome.

Since the first description of intracellular membrane systems in *Bacillus subtilis* (Chapman & Hillier, 1953), the vast majority of bacteria have subsequently been shown to contain membranous invaginations (mesosomes) of varying degrees. The widespread occurrence of these organelles in bacteria has tempted many investigators to speculate on their possible function, and evidence for the proposed roles has been adequately reviewed by Salton (1967). The aim of our current research is to isolate pure mesosomal membrane from *Micrococcus lysodeikticus*, to characterize it in chemical and functional terms, and from these results to assess the significance of this organelle in relation to the previously suggested cellular functions.

Several investigators have reported on the properties of 'mesosome fractions' isolated from a variety of Gram-positive bacteria. The methods of isolation of such fractions involve detergent treatment of total cell lysates (Fitz-James, 1967, 1968), density-gradient separation of total cell lysates (Ghosh & Murray, 1969; Patch & Landman, 1971) and a variety of methods involving separation of mesosomes via a protoplast stage (Ferrandes *et al.*, 1966; Rogers *et al.*, 1967; Ellar, 1969; Ellar & Freer, 1969; Reaveley & Rogers, 1969; Sargent *et al.*, 1969; Ferrandes *et al.*, 1970; Sargent & Lampen, 1970; Daniels, 1971; Ellar *et al.*, 1971; Thorne & Barker, 1971, 1972). The rationale for our method is based upon observations of van Iterson (1961), who showed that mesosomes in *Bacillus subtilis* were extruded into

the periplasmic space upon plasmolysis of whole cells, and of Fitz-James (1964), who subsequently showed that extruded membranous vesicles were released into the suspending buffer after removal of cell wall with lysozyme. These observations have formed the basis of methods developed for the isolation of mesosomal fractions from *Bacillus* species (Ferrandes *et al.*, 1966; Ryter *et al.*, 1967; Ferrandes *et al.*, 1970), *Staphylococcus* spp. (Popkin *et al.*, 1971) and *Micrococcus lysodeikticus* (Ellar, 1969; Ellar & Freer, 1969; Ellar *et al.*, 1971).

Reported differences in distribution of membrane components between mesosomal and peripheral membranes have in no case been absolute. However, in most organisms that have been examined a general feature of the mesosomes appears to be the lack of oxidative enzymes associated with respiration (for reviews see Patch & Landman, 1971; Rogers, 1970). Earlier reports (Fitz-James, 1967, 1968) suggested that mesosomes may function as the site of membrane synthesis, but recent data from several laboratories do not support such a proposed role (Ghosh & Murray, 1969; Daniels, 1971; Ellar *et al.*, 1971; Patch & Landman, 1971). Indeed, of the functions that have been proposed for the mesosome, only a role in secretion of penicillinase in *Bacillus licheniformis* (Lampen, 1965) has been substantiated with isolated mesosome fractions (Sargent *et al.*, 1969; Sargent & Lampen, 1970). The investigations on bactoprenol distribution in membrane fractions from *Lactobacillus casei* (Thorne & Barker, 1971,

1972) provide evidence for peptidoglycan synthesis (Salton, 1956) in both the mesosomal and peripheral membrane of this organism.

Many of the suggested functions of the mesosome were based upon its cellular location and appearance in electron micrographs. More recently the internal structure of peripheral and mesosomal membranes has been revealed by using the freeze-etching technique on whole cells. In some bacteria examined by this technique, the intracellular membranes have appeared smooth (Nanninga, 1968; Beaman & Shankel, 1969; Ghosh *et al.*, 1969; Wildermuth, 1971), whereas the peripheral membrane invariably appeared particle-studded. In other instances both mesosomal and peripheral membranes appeared particle-studded (Fiil & Branton, 1969; Sleytr, 1970; Sleytr & Kocur, 1971; Sleytr & Krebs, 1971) and, in some reports, mesosomes with either smooth or particle-studded fracture faces were observed in the same organism (Remsen, 1968; Holt & Leadbetter, 1969). With a view to clarifying the function of the mesosome, we now report the results of a comparative study of some biochemical and anatomical (freeze-fracture) features of mesosomal and peripheral membrane fractions isolated from *Micrococcus lysodeikticus*.

Materials and Methods

Chemicals

Egg-white lysozyme (EC 3.2.1.17), bovine serum albumin and yeast hexokinase were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), deoxyribonuclease (EC 3.1.4.6) from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), ovalbumin, sperm whale myoglobin and horse heart cytochrome *c* from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.) and bovine pancreatic chymotrypsinogen from Miles-Seravac (Pty) Ltd. (Maidenhead, Berks., U.K.). All other chemicals and reagents were of analytical grade.

Preparation of membrane fractions

Cultivation. Cells of *Micrococcus lysodeikticus* (N.C.T.C. 2665) were cultivated as previously described (Owen & Freer, 1970) and harvested after 10h growth by centrifugation at 2500g for 25 min. Sedimented cells were washed twice in 50mm-tris-HCl buffer, pH 7.5, at room temperature (21°C). (This buffer, referred to subsequently as 'tris buffer', was always used, unless otherwise stated.)

Plasmolysis. In procedures involving plasmolysis, washed cells were resuspended to one-tenth of the culture volume in tris buffer, which was made 2.0M with respect to sucrose, and contained MgCl₂ at the desired concentration (see the Results section). Cells

were allowed to equilibrate in this solution for 1.5h at room temperature.

Protoplast formation. Washed cells were resuspended to one-tenth of the original culture volume in tris buffer, 0.8M with respect to sucrose and containing MgCl₂ at the desired concentration. Cells were then equilibrated for 1.5h at room temperature. After equilibration in buffered 0.8M-sucrose or, for plasmolysed cells, in buffered 2.0M-sucrose, lysozyme was added to a final concentration of 250 µg/ml and suspensions were incubated for 45 min at 30°C, unless otherwise stated. The resulting protoplast suspension was then diluted with 1.5 vol. of tris buffer containing sufficient sucrose to give a final molarity of 0.8M. MgCl₂ was incorporated in the diluent buffer at the desired concentration.

Swirling. Protoplast suspension (60ml) was subjected to swirling in a 250ml Erlenmeyer flask at 85 rev./min in an orbital shaker-incubator at 30°C for 2h.

Sedimentation of mesosomal and peripheral membrane fractions

Protoplasts were sedimented from the suspending medium by centrifugation at 12000g for 2h. Peripheral membranes were prepared from protoplasts by osmotic lysis in tris buffer and washed as previously described (Owen & Freer, 1970). The 12000g supernatant was centrifuged again at 12000g for 2h to remove any residual protoplasts. This second supernatant fraction, subsequently referred to as the 'protoplast supernatant fraction', contained the released mesosomal membranes. These could be sedimented from the protoplast supernatant fraction by centrifugation at 280000g for 2h. Mesosomal membrane fractions were washed six times in tris buffer before chemical analysis. For estimations of dry weight, membrane suspensions were dialysed exhaustively against distilled water at 4°C, before freeze-drying and weighing.

Monitoring procedures

Protoplast leakage. An estimation of leakage of protoplast contents during the release of mesosomal membranes was achieved by monitoring the E_{260} of the protoplast supernatant fraction. The extinction value was compared with the E_{260} of an osmotic lysate of the protoplast suspension. This method, although not totally satisfactory, offers a rapid and convenient method of assessing the intactness of protoplasts (Reaveley & Rogers, 1969).

Release of mesosomal membrane. Convenient marker molecules for membrane fractions of *M. lysodeikticus* are the carotenoid pigments (see Salton & Ehtisham-ud-din, 1965). The E_{446} values for carotenoid absorption correlate directly with dry

weight of membrane in suspensions, and as such offer a convenient method for assaying the concentration of membrane in aqueous suspensions. The carotenoid content per unit dry wt. of membrane does not differ appreciably between peripheral and mesosomal membranes. Before extinction values were measured, membrane suspensions were clarified by addition of sodium dodecyl sulphate to a final concentration of 0.1%. The E_{446} values of the protoplast supernatant fractions and those of the total protoplast lysates were compared and expressed as a percentage.

Electron microscopy. All membrane preparations were examined in a Philips EM 300 electron microscope by the negative-staining procedure (Brenner & Horne, 1959) with 2.0% ammonium molybdate. Before examination, mesosomal membranes were washed three times in tris buffer.

Chemical analysis

Carotenoids. Fresh membrane suspensions were solubilized with 0.1% sodium dodecyl sulphate and the extinction value was measured at 446nm in a Pye Unicam SP. 600 spectrophotometer. Values for carotenoid content were obtained by assuming a molar extinction coefficient of 3×10^3 (Salton & Schmitt, 1967).

Lipids. Extractable lipids were estimated gravimetrically by the method of Salton & Schmitt (1967) with acetone-methanol (7:2, v/v) as solvent. Total lipid was extracted with diethyl ether after mild acid hydrolysis (refluxing for 1 h with methanolic 5%, v/v, HCl) and estimated gravimetrically (Yudkin, 1967).

Protein estimation. Protein was estimated on freeze-dried membrane preparations by the methods of Lowry *et al.* (1951) and Gornall *et al.* (1949) after dispersion and solubilization with 0.1% sodium dodecyl sulphate. Dry bovine serum albumin fraction V (fatty acid-free) in the presence of 0.1% sodium dodecyl sulphate was used as a standard in both instances. In estimations with the biuret reagent, the extinction obtained at 560nm was corrected for the absorption due to membrane pigments.

Polyacrylamide-disc-gel electrophoresis. The procedure for electrophoresis in the presence of sodium dodecyl sulphate was a modification (McNiven *et al.*, 1972) of the method of Davis (1964). The wide range in molecular weight of membrane components necessitated the use of separating gels with differing pore sizes. Lower-molecular-weight components were best resolved in a separating gel containing 11.7% (w/v) acrylamide and 0.153% *NN'*-methylenebisacrylamide, whereas resolution of higher-molecular-weight constituents was best obtained with 7.0% (w/v) acrylamide and 0.184% *NN'*-methylenebisacrylamide. The gels and the buffer systems were made 0.1% with respect to sodium dodecyl sulphate. For comparative purposes, a mixture of proteins of known molecular

weights was included in each electrophoresis run. Membrane samples were dispersed by ultrasound and solubilized in 0.2% sodium dodecyl sulphate before application to the gel.

Cytochromes. Difference spectra were recorded at 21°C in a Pye-Unicam SP. 800 spectrophotometer on fresh membrane preparations clarified by ultrasound. Reduction was achieved by the addition of sodium dithionite to a final concentration of 1.0mg/ml or in some instances by the addition of NADH₂ (to a final concentration of 2.0mM) after treatment of membrane with 1.0% (v/v) Triton X-100 (Gel'man *et al.*, 1970).

Nucleic acid. Nucleic acid was estimated by measurement of E_{260} and E_{280} (Warburg & Christian, 1941) in membrane preparations clarified by ultrasound.

Carbohydrate. Total hexose was estimated in solubilized membrane preparations with the anthrone reagent by using the method of Morris (1948), with glucose as the standard. Individual sugars were separated from acid hydrolysates (1M-H₂SO₄ at 105°C for 2h) of membrane preparations on one-dimensional descending paper chromatograms (Whatman no. 4) run in ethyl acetate-pyridine-water (8:2:1, by vol.) and identified by comparison with known standards. For separation of hexose sugars, chromatograms were run for 18h, and for pentose sugars, for 5h. Sugars were visualized by the aniline hydrogen phthalate reagent (Cummings & Harris, 1956). Mannose was identified by its R_F value on paper chromatograms and differentiated from fructose by a negative reaction for keto sugars (Ikawa & Nieman, 1949).

Enzyme assay

All enzyme assays were carried out at 25°C on freshly prepared membrane fractions. Succinate dehydrogenase (EC 1.3.99.1) activity was determined by a modification (Owen & Freer, 1970) of the spectrophotometric method of Ells (1959). NADH₂ dehydrogenase (EC 1.6.99.3) and malate dehydrogenase (EC 1.1.1.37) were determined spectrophotometrically as described by Gel'man *et al.* (1970). All the above enzyme activities were determined in a Pye-Unicam SP. 800 spectrophotometer and expressed as $\Delta E_{600}^{1\text{cm}}/\text{min}$ per mg dry wt. of membrane.

Autolytic enzyme activity. The lytic activity of membrane preparations against whole cells of *M. lysodeikticus* was estimated in the following way. Washed exponential-phase cells were suspended in tris buffer to $E_{450} = 0.4$. The reaction cuvette contained 2.9ml of cell suspension and 0.1ml of freshly prepared membrane suspension. The blank cuvette contained 2.9ml of cell suspension and 0.1ml of tris buffer. The assay was performed at 30°C and $\Delta E_{450}^{1\text{cm}}/\text{min}$ was determined in a Pye-Unicam SP. 800

spectrophotometer over the linear portion of the plot. One unit of activity is defined as $\Delta E_{450}^{1\text{cm}}/\text{min} = 0.001$ in a suspension of *M. lysodeikticus* of $E_{450}^{1\text{cm}} = 0.4$ at 30°C, pH 7.5, in a total volume of 3 ml.

Freeze-etching

Fresh membrane preparations were suspended in 20% glycerol in 0.01M-tris buffer, pH 7.5, before freezing. For whole cells, impregnation with glycerol was carried out for 3 h at room temperature. Drops of suspension (approx. 10 μ l) were placed on gold specimen supports and rapidly frozen by immersion in Freon 22 at liquid N₂ temperature for 2–3 s. Specimens were then rapidly transferred into liquid N₂ and stored until used (always less than 24 h). Freeze-fracturing was performed in a Balzer's 360 M Freeze Etching Unit with a specimen temperature of –100°C. Platinum–carbon replicas were floated on water and cleaned by transfer into 5% (w/v) sodium dodecyl sulphate for approx. 18 h, followed by transfer into 70% (v/v) H₂SO₄ for approx. 5 h. After washing in water, replicas were transferred to grids covered with carbon-coated Formvar, and examined in a Philips EM 300 electron microscope operated at 60 kV.

Results

Protoplast stability

Numerous investigators have reported upon the requirement for bivalent cations, in particular Mg²⁺,

for stability of bacterial protoplasts (Reaveley & Rogers, 1969). Table 1 illustrates the effects of different Mg²⁺ concentrations upon protoplast stability. Although there is clearly a requirement for Mg²⁺, this is apparently satisfied at a concentration of Mg²⁺ greater than 2.0 mM.

Membrane morphology

In all the experiments reported, membrane preparations derived from both protoplasts and protoplast supernatant fractions were examined by negative staining in the electron microscope. The characteristic small smooth-surfaced vesicular structures, which are typical of extruded mesosomal membranes (Ryter *et al.*, 1967; Ryter, 1968; Fitz-James, 1968; Ghosh & Murray, 1969; Ferrandes *et al.*, 1970; Frehel *et al.*, 1971; Patch & Landman, 1971; Popkin *et al.*, 1971; Burdett & Rogers, 1972), accounted for all membranous material in protoplast supernatant fractions (Plate 1b) except in those prepared below 10 mM-Mg²⁺. In contrast to the mesosomal vesicles, peripheral membrane appeared as flat, particle-studded sheets (Munoz *et al.*, 1968) of variable shape and size (Plate 1a). Occasional mesosomal vesicles were seen contaminating peripheral membrane fractions.

Release of mesosomal membrane

Effect of plasmolysis. From Table 2 it can be seen that plasmolysis, before digestion of the cell wall,

Table 1. *Effect of Mg²⁺ concentration on protoplast stability*

Protoplasts of *M. lysodeikticus* were prepared in 0.8M-sucrose–tris buffer containing various Mg²⁺ concentrations. Suspensions were swirled for 2 h at 85 rev./min at 30°C and then sedimented. The E_{260} values of the protoplast supernatant fractions were subsequently determined.

Concn. of MgCl ₂ (mM)	0	1	2	5	10	20	40
Leakage (% E_{260} released)	19.0	6.1	5.6	5.9	5.8	5.7	5.7

Table 2. *Effect of plasmolysis on stability and membrane release from protoplasts*

Protoplast suspensions were prepared from cells plasmolysed in 2.0M-sucrose–tris buffer and from control cells kept in 0.8M-sucrose–tris buffer. Suspensions were kept at 30°C for 1 h after cell-wall digestion. E_{260} and E_{446} values were then determined on the protoplast supernatant fractions. A similar experiment was performed with cell-wall digestion omitted. The concentration of Mg²⁺ throughout the experiments was 10 mM.

	Protoplasts		Whole cells	
	Plasmolysed	No plasmolysis	Plasmolysed	No plasmolysis
Membrane release (% E_{446} released)	8.0	4.1	0.0	0.0
Leakage (% E_{260} released)	4.2	2.7	1.2	1.5

significantly increased the release of mesosomal membrane from protoplasts. At 10mM-Mg²⁺ the amount of membrane released from plasmolysed cells was approximately twice that of control cells. In a similar experiment carried out at a concentration of 40mM-Mg²⁺, there was even more marked membrane release in plasmolysed cells, compared with control cells, although the amount of membrane released was in fact less than that at 10mM-Mg²⁺. Although there was an increased *E*₂₆₀ value in protoplast supernatant fractions derived from plasmolysed cells, the degree of leakage was nevertheless extremely small. Indeed, leakage of a similar order occurred in cells that were not subjected to digestion of the cell wall (see Table 2).

Effect of temperature, swirling, time and Mg²⁺ concentration. Ionic shock involving a decrease in Mg²⁺ concentration has been used previously in isolation procedures for mesosomes from *M. lysodeikticus* (Ellar & Freer, 1969). However, over a range of Mg²⁺ concentrations (40–5mM) a sudden 50% decrease in concentration did not alter the amount of membrane released when compared with suspensions held at the lower value throughout. Ionic shock did appear to increase the leakage from protoplasts, as evidenced by increased *E*₂₆₀. In proto-

Fig. 1. *Effect of Mg²⁺ concentration on membrane release and protoplast stability*

Protoplast suspensions were prepared from plasmolysed cells at various Mg²⁺ concentrations and kept for 2h at 30°C. *E*₂₆₀ (■) and *E*₄₄₆ (●) values were determined for each supernatant fraction.

plast suspensions, the amount of released membrane was inversely related to the concentration of Mg²⁺ used in the preparation procedure (Fig. 1). Although membrane release increased appreciably below 10mM-Mg²⁺, it appeared that protoplast stability was considerably decreased below this value (Fig. 1). The occurrence of peripheral membrane fragments in negatively stained mesosome fractions prepared below 10mM-Mg²⁺ supports this view.

The possibility that mild shearing forces may

Scheme 1. *Procedure used to obtain pure mesosomal membrane preparations from protoplast suspensions*

promote the release of attached mesosomal membrane from protoplasts was investigated. From the results obtained it was clear that swirling of protoplast suspensions under the conditions described had no effect on membrane yield. In instances where rotation speed was increased to 150 rev./min, the only observable effect was to elevate the E_{260} values of protoplast supernatant fractions.

Membrane release was optimum at 30°C, although in experiments involving ionic shock a slightly higher optimum was observed. However, over the temperatures tested (25°, 30°, 35° and 40°C), the maximum difference in membrane yield was only 20%.

The time-course of membrane release was investigated by utilizing protoplast suspensions prepared from cells plasmolysed in the presence of 10 mM-Mg²⁺. Protoplast supernatant fractions from suspensions held for up to 2 h after wall digestion all showed less than 3% increase in membrane release over supernatant fractions prepared immediately after wall digestion. However, sedimentation of protoplasts necessitates centrifugation for 2 h, and therefore it is not possible to say whether or not release occurs spontaneously with wall digestion.

Properties of mesosomal membrane fractions prepared at different Mg²⁺ concentrations

Results described above show that pure mesosomal membrane preparations can be obtained from protoplast suspensions by utilizing the procedure outlined in the flow diagram (Scheme 1). At concentrations of Mg²⁺ greater than 10 mM, no detectable contamination with peripheral membrane was evident (Plate

1b). It was necessary, however, to consider the possibility that a population of mesosomal vesicles released at a particular Mg²⁺ concentration may differ functionally, yet be morphologically indistinguishable, from that released at a different concentration of Mg²⁺. The results of analysis of mesosomal membranes prepared at 10, 20 and 40 mM-Mg²⁺ are shown in Table 3. The yield of membrane increases with decreasing Mg²⁺ concentration, and probably reflects a decrease in the stability of mesosomal membrane attachment to the protoplasts. Similar observations have been also reported for *Bacillus* spp. by Reaveley & Rogers (1969), Rytter *et al.* (1967) and Fitz-James (1967). However, no significant differences were detected in the protein, nucleic acid, membrane phosphorus, carotenoid pigments or total hexose content of the different preparations. By contrast, significant differences in the activity of succinate dehydrogenase and an unspecified autolytic enzyme(s) were detectable in the different preparations. Although the activity of succinate dehydrogenase and the autolytic enzyme(s) increased with decreasing Mg²⁺ concentration, it is noteworthy that in both cases the ratio of the specific activity of mesosomal membrane to that of corresponding peripheral membrane preparation remains practically constant. Thus the partition of enzyme activity between mesosomal and peripheral membrane is independent of the concentrations of Mg²⁺ in the preparation procedures described. We have shown previously that the observed activity of succinate dehydrogenase in membrane preparations from *M. lysodeikticus* is a function of the Mg²⁺ concentration (Owen & Freer, 1970).

Table 3. *Comparison of some properties of mesosomal membranes prepared at various Mg²⁺ concentrations*

The results in this table are those from a single experiment. Protein was estimated by two methods: (a) Lowry *et al.* (1951) and (b) Gornall *et al.* (1949). Results for gross chemical composition are expressed as percentages of the total weight of dry membrane. Values given in parentheses refer to corresponding activity found in peripheral membrane prepared at the same Mg²⁺ concentration.

	Concn. of Mg ²⁺ (mM) ...	10	20	40
Total membrane (% dry wt.)		13.2	10.2	6.3
Protein { Method (a)		29.3	28.4	27.8
Method (b)		38.5	40.9	40.1
Carotenoids		0.14	0.13	0.13
Total hexose		19	20	19
Total membrane phosphorus		1.38*	1.20	1.14
		(1.63)	(1.60)	(1.57)
Succinate dehydrogenase		0.077	0.067	0.059
($\Delta E_{600}^{\text{cm}}$ /min per mg dry wt. of membrane)		(1.35)	(1.13)	(0.79)
Autolytic enzyme(s)		110	42	43
(units/mg dry wt. of membrane)		(7.2)	(2.9)	(2.6)

* In this preparation the value for total membrane phosphorus was unusually high. An average value derived from five similar preparations is 1.21 ± 0.07 .

EXPLANATION OF PLATE I

Morphology of isolated peripheral (a) and mesosomal (b) membranes

Negatively stained (a) peripheral membrane fragments and (b) mesosomal membrane vesicles prepared at 10 mM- Mg^{2+} . In (a) the arrow points to enzyme particles. Magnification $\times 97\,000$.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of peripheral and mesosomal membranes from M. lysodeikticus

Separating gels A, B and C contain 11.7% (w/v) acrylamide and 0.153% *NN'*-methylenebisacrylamide whereas gels A', B' and C' contain 7% (w/v) acrylamide and 0.184% *NN'*-methylenebisacrylamide. A and A', Peripheral membrane (140 μ g dry wt. of membrane); B and B', mesosomal membrane (250 μ g dry wt. of membrane); C and C', marker proteins: (i) artifact band; (ii) bovine serum albumin (mol.wt. 67000); (iii) ovalbumin (mol. wt. 45000); (iv) hexokinase (mol.wt. 45000); (v) chymotrypsinogen (mol.wt. 25000); (vi) myoglobin (mol.wt. 17800); (vii) cytochrome *c* (mol.wt. 12400); (viii) Bromophenol Blue. Mechanically strengthened stacking gels (McNiven *et al.*, 1972) are opaque and contain a negligible percentage of the total staining material.

EXPLANATION OF PLATE 3

Replicas of freeze-fractured isolated peripheral (a, b) and mesosomal (c) membranes

Thin arrows point to intra-membrane particles and thick arrows to rod-like structures on convex fractures of peripheral membrane (a) and to linear depressions on concave fractures of peripheral membrane (b). Encircled arrow heads in the bottom left-hand corner of each micrograph indicate the direction of shadow. Magnification $\times 121\,000$.

EXPLANATION OF PLATE 4

Replica of frozen fractured cells of M. lysodeikticus

Thick arrows point to rod-like structures on convex fractures of the peripheral membrane (upper cell) and to linear depressions on concave fractures of the peripheral membrane (lower cell). Thin arrows point to intra-membrane particles and to similar particles in the cross-fractured cell wall. The encircled arrow head in the bottom left-hand corner of the micrograph indicates the direction of shadow. Magnification $\times 115000$.

Table 4. Comparison of the properties of mesosomal and peripheral membranes from *M. lysodeikticus*

Results for gross chemical composition are expressed as percentages of the total weight of dry membrane, and for dehydrogenase activity as $\Delta E_{460}^{1\text{cm}}/\text{min}$ per mg dry wt. of membrane. Protein was estimated by two methods: (a) Lowry *et al.* (1951) and (b) Gornall *et al.* (1949). Each value in the table, with the exception of values for autolytic enzyme activity (means of two experiments), represents the average of values obtained from at least three separate experiments.

Property	Mesosomal membrane (A)	Peripheral membrane (B)	Ratio A/B
Protein { Method (a)	28.0	42.8	0.65
Protein { Method (b)	38.0	50.0	0.76
Protein (b/a ratio)	1.36	1.17	1.16
Carotenoids	0.12	0.15	0.80
Extractable lipid	26.1	26.4	0.99
Total membrane phosphorus	1.21	1.62	0.75
Phosphorus/lipid ratio	0.046	0.061	0.75
Total hexose	19.3	4.7	4.1
Succinate dehydrogenase	0.08	0.79	0.10
Malate dehydrogenase	0.04	0.85	0.05
NADH ₂ dehydrogenase	0.38	6.44	0.06
Autolytic enzyme(s) (units/mg of membrane)	110	7	15.7

Comparison of the properties of mesosomal and peripheral membrane preparations

Mesosomal membranes isolated in the presence of 10mM-Mg²⁺ account for between 7% and 13% (w/w) of the total membrane fractions of *M. lysodeikticus*. The results of the chemical analysis and values for selected enzyme activities of both peripheral and mesosomal membranes are given in Table 4. The protein/lipid ratio for plasma membrane is greater than that of mesosomal membrane; in large part this is a consequence of a low protein content in the latter. The very low dehydrogenase activities found in mesosomal membranes correlates with the lower protein content, which is a feature of the mesosomal membrane. Apart from this quantitative difference between peripheral and mesosomal membranes, there also exist qualitative differences, which can be demonstrated by disc-gel electrophoresis (see Plate 2). Qualitative variation of protein in the two fractions would also account for the different ratios observed when values for protein obtained by the biuret method (Gornall *et al.*, 1949) or the Folin-Ciocalteu reagent (Lowry *et al.*, 1951) are compared.

Although mesosomal membranes are consistently lower in carotenoid content, the value for extractable lipid in mesosomal membrane fractions compares closely with that of peripheral membrane. The figure for total phosphorus, however, would suggest appreciable differences in the phospholipid components of the two types of membranes. Preliminary studies showed that the hexose component of mesosomal membrane consists mainly of mannose, a

sugar not detectable in the extractable lipid fraction. It should be noted, however, that figures for extractable lipid do not necessarily represent the total lipid content of membrane fractions. Indeed peripheral membrane fractions have been shown to contain 20% (w/w) of bound lipid (P. Owen, unpublished work).

Mesosomal membrane preparations possessed an autolytic enzyme activity, which was demonstrated by decrease in extinction values for whole-cell suspensions and confirmed by observing cell disruption under phase-contrast optics. The differences in distribution of autolytic enzyme activity shown in Table 4 correlate with the observations of D. J. Ellar (personal communication). Mesosomal membranes showed an increase of approximately 15-fold in autolytic enzyme activity in comparison with that found in peripheral membrane fractions. The contribution of lysozyme to this activity is not known at present. When assayed under identical conditions, crystalline lysozyme was shown to have an activity of 96500 units/mg. If one assumes no binding to membrane, then the extensive washing procedure adopted would result in a murelytic activity due to residual lysozyme of less than 10⁻³ units/mg of membrane assayed. However, if the autolytic activity is a consequence of exogenous lysozyme, then preferential binding to mesosomal membrane must occur, and the extent of the binding is inversely related to the Mg²⁺ concentration used in the preparative procedure (see Table 3).

A most striking and interesting difference between

Fig. 2. Cytochrome difference spectra of peripheral and mesosomal membranes

(a) Sonicated peripheral membrane (18.4 mg dry wt. of membrane/ml) reduced with sodium dithionite. (b) Mesosomal membrane (17.4 mg dry wt. of membrane/ml) reduced with sodium dithionite. (c) Peripheral membrane (18.4 mg dry wt. of membrane/ml) reduced with NADH_2 after treatment of membrane suspension with Triton X-100.

the two membrane preparations was found in their cytochrome content. It can be seen from Fig. 2 that cytochromes a_{601} , b_{560} and c_{550} , clearly present in peripheral membranes, are not detectable in mesosome fractions. Cytochrome b_{556} was the only component evident in the latter, although the confirmation of this observation would require the increased sensitivity of low-temperature spectroscopy. In peripheral membrane preparations the

absorption due to cytochrome b_{556} is masked by the absorption peaks of cytochrome b_{560} and cytochrome c_{550} . This problem can be overcome by selective reduction with NADH_2 in the presence of detergent (Gel'man *et al.*, 1970).

Freeze-etching

The investigations of Branton and his colleagues (for review see Branton, 1969) have demonstrated that membrane faces revealed after freeze-fracturing represent the topography of the interior of the membrane, the fracture plane occurring in the region of the methyl end groups of fatty acids. Isolated peripheral membranes subjected to the freeze-fracture technique showed fracture faces remarkably similar to those seen in peripheral membranes of whole cells subjected to the same procedure. Plate 3(a) shows a convex fracture of isolated peripheral membrane and reveals the outer surface of the inner half of the membrane. Many intra-membrane particles are evident, together with numerous rod-like structures distributed over the surface. Plate 3(b) shows a concave fracture of peripheral membrane, and reveals the inner surface of the outer half of the membrane. Intra-membrane particles are also evident on this fracture face, although in much smaller numbers than those on the convex fracture face (Plate 3a). Linear depressions, possibly resulting from the presence of the rod-like structures seen in the convex fracture face, are clearly visible on concave fractures. Identical features are seen in the convex and concave fractures of peripheral membranes when whole cells are freeze-fractured (see Plate 4). The rod-like structures in this case show a clear continuity between the interior region of the membrane and the cell wall. Particles of similar dimensions to those seen in the membrane interior also appear to be embedded in the cell wall. Rod-like structures and linear depressions were never observed on the fracture faces of mesosomal membranes, but the convex and concave faces both revealed a distribution of intra-membrane particles closely similar to that observed on corresponding fracture faces in isolated peripheral membranes (Plate 3c).

Discussion

From previous studies on the isolation of total membrane fractions from lysates of *M. lysodeikticus* (Salton & Chapman, 1962) it was apparent that extensive fragmentation and vesicle formation occurred. Consequently, we expected that an attempt to isolate mesosomal vesicles from such total lysates would prove to be a difficult and probably unsatisfactory procedure. To minimize contamination of mesosomal membrane fractions with peripheral membrane vesicles, a method of isolation that retained proto-

plasts in the intact state was devised. The release of mesosomal membranes was greatly enhanced if cells were plasmolysed before wall digestion, although protoplasts from cells treated in this way required a higher Mg^{2+} concentration for stability than un-plasmolysed cells (compare Table 1 with Fig. 1). Plasmolysis has been shown to cause the extrusion and release of mesosomes into the periplasmic space (van Iterson, 1961; Fitz-James, 1964; Ferrandes *et al.*, 1966; Frehel *et al.*, 1971), a process which probably involves breaks in the membrane, the resealing of which is facilitated by Mg^{2+} . However, the release of extruded mesosomes is enhanced at lower Mg^{2+} concentrations; thus the choice of Mg^{2+} concentration during the protoplast-forming stage is critical. By experiment the optimum concentration for concomitant mesosome release and protoplast stability was found to be 10mM. It is not surprising that this critical Mg^{2+} concentration differs for different micro-organisms. In *B. licheniformis* (Rogers *et al.*, 1967) the optimum concentration was 20mM, whereas in *B. subtilis* (Ryter *et al.*, 1967) and *Lactobacillus casei* (Thorne & Barker, 1971) the critical value appeared to be much lower.

Of other parameters tested for their effect on the amount of membrane released from protoplasts, i.e. time, mild shear, ionic shock and temperature, only variations in temperature affected yield. Optimum temperature for release appeared to be between 30°C and 35°C.

Mesosomal vesicles from *M. lysodeikticus* retained integrity and appeared unchanged after washing in buffer free from Mg^{2+} . The presence of Mg^{2+} in the protoplast-forming medium may have been sufficient to stabilize mesosomal vesicles during the subsequent washing procedure. Lastras & Munoz (1971) showed that Mg^{2+} , associated with membranes in *M. lysodeikticus*, was retained after washing in buffer free from this cation. The instability of staphylococcal mesosomal membranes prepared in the absence of Mg^{2+} (Popkin *et al.*, 1971) would support this suggestion.

Characteristic differences in anatomy have been noted (Ferrandes *et al.*, 1966; Fitz-James, 1968; Reaveley, 1968; Sargent *et al.*, 1969; Ghosh & Murray, 1969; Patch & Landman, 1971; Popkin *et al.*, 1971) between isolated mesosomal vesicles released from intact protoplasts and peripheral membrane fragments from lysed protoplasts. These consistent differences in appearance in negatively stained preparations provided a convenient means of monitoring for cross-contamination in different membrane fractions. Utilizing the preparation method outlined in Scheme 1, it is possible to prepare mesosomal membrane fractions free from detectable contamination with peripheral membrane. It is apparent that mesosomal membrane is not quantitatively released from protoplasts under these conditions, since occas-

ional mesosomal vesicles can be detected in peripheral membrane preparations.

Mesosomal membrane fractions of identical morphology are also very similar in their chemical composition. The only noticeable effect of increasing the Mg^{2+} concentrations (all greater than 10mM) in the procedure for preparation of mesosomes was to decrease the yield of membrane. However, major differences in constitution were observed between peripheral and mesosomal membrane fractions. In addition to having a lower protein content than that of peripheral membranes, mesosomal membranes also displayed polypeptides which differed from those found in peripheral membranes (see Plate 2). These observations contrast with those of Reaveley (1968) and of Patch & Landman (1971), who showed only minor quantitative and qualitative differences in the protein components of peripheral and mesosomal membranes from *B. licheniformis* and *B. subtilis* respectively. Our observations of qualitative differences were not unexpected in view of the demonstrated partition of both oxidative enzyme systems and respiratory pigments between the two membrane fractions. The very low dehydrogenase activities found in the mesosomal membranes is in agreement with numerous earlier studies (Ghosh & Murray, 1969; Reaveley & Rogers, 1969; Rogers, 1970; Ferrandes *et al.*, 1970).

Four cytochromes have previously been demonstrated in total membrane preparations from *M. lysodeikticus* (Lukyanova & Tapytkova, 1968; Gel'man *et al.*, 1970), namely $b_{556} \rightarrow b_{560} \rightarrow c_{550} \rightarrow a_{601}$. Our results show that only one cytochrome, cytochrome b_{556} , is detectable in mesosomal membranes. This finding could explain, in part, the partition of cytochromes reported by Gel'man *et al.* (1970) with a detergent fractionation procedure on total (peripheral and mesosomal) membrane preparations from *M. lysodeikticus*. The occurrence of cytochrome b_{556} in peripheral membrane fractions is in keeping with a complete respiratory chain, and appears unlikely to be accounted for in full by contamination of peripheral membrane fractions with mesosomal vesicles. However, the demonstration of a single cytochrome component in mesosomal membrane fractions, together with the very low dehydrogenase activity, serve to eliminate this organelle as a centre of respiratory activity in this organism. The possibility that the mesosome represents the starting point for assembly of the cytochrome chain depends upon membrane components being assembled within or adjacent to the mesosome. Recent data from pulse-labelling experiments in Gram-positive organisms (Ghosh & Murray, 1969; Daniels, 1971; Ellar *et al.*, 1971; Patch & Landman, 1971), however, do not implicate the mesosomal vesicles themselves as the site of new membrane synthesis. Whether or not the membrane of the mesosomal sacculus differs in

functional terms from that of the mesosomal vesicle and peripheral membrane is not known.

The deficiency in components of the electron-transport system in mesosomal membranes contrasts with the relative enrichment of autolytic enzyme found in these fractions. The mesosome would appear to be a logical site for localization of a muralytic enzyme capable of opening up peptidoglycan to facilitate insertion of new wall material during septum formation, and also to function as a cell-separating enzyme. The demonstration of relatively high autolytic activity in mesosomal membrane fractions provides additional circumstantial evidence for the involvement of mesosomes in septation, and also provides data in support of the theoretical model of cell-wall synthesis proposed by Thompson (1971).

Mesosomal membrane fractions contain approx. 20% (w/w) of hexose, shown to be predominantly mannose. This observation is noteworthy in the light of the reports by Thorne & Barker (1971) that more than 50% of the membrane bactoprenol is found in the mesosomal membrane fractions of *Lactobacillus casei*, and that of Scher *et al.* (1968), who showed that bactoprenol is involved in polymannan biosynthesis in *M. lysodeikticus*.

The differences in the appearance of peripheral and mesosomal membranes in negatively stained preparations has already been noted. Further morphological differences were also seen in the details of the internal fracture planes of the two types of membranes when subjected to freeze-fracturing. In *M. lysodeikticus*, corresponding fracture faces in peripheral membrane in whole cells, isolated peripheral membrane and isolated mesosomal membrane all appeared to carry a similar distribution of intra-membrane particles. Rod-like structures and linear depressions in convex and concave fracture faces were evident in the peripheral membrane, both in the isolated state and in fractured whole cells. These latter features, however, were never observed in isolated mesosomal membranes. Rod-like structures apparently connecting wall and membrane have been reported by numerous investigators in freeze-fractured whole cells of a variety of micro-organisms (Remsen & Lundgren, 1966; Hoeniger *et al.*, 1968; Nanninga, 1968, 1971; Staehelin, 1968; Streiblova, 1968; Fiil & Branton, 1969; Ghosh *et al.*, 1969; Holt & Leadbetter, 1969; Abram & Davis, 1970; De Voe *et al.*, 1971; Wildermuth, 1971), and these may play a role in the anchoring of cell wall and membrane (see Rogers, 1970). The absence of these structures in the mesosomal membrane vesicles would be in keeping with such a role.

From previous studies, the fracture face of mesosomal membranes *in situ* appear to be either particle-studded or smooth depending upon the organism in question (see the introduction). However, it is not

apparent whether or not the fracture face represents the membranes of the mesosome contents or that of the mesosome sacculus. In many instances the lack of membrane cross-fractures would suggest that the fracture face is in fact that of the mesosomal sacculus. In our experiments, the mesosomal membrane fractions probably represent mesosome contents and not the mesosome sacculus, and such membranes show fracture faces revealing numerous intra-membrane particles. We have been unable to compare the fracture faces of isolated mesosomal membrane with those occurring *in situ* in whole cells, as only fractures through the sacculus membrane were observed. However, the identical topography of fracture faces of isolated peripheral membrane and of peripheral membrane *in situ* would suggest that little structural rearrangement occurs during the isolation and washing procedure.

This investigation of the properties of mesosomal and peripheral membrane fractions from *M. lysodeikticus* has revealed clear qualitative and quantitative differences between the two fractions. The high mannose and autolytic enzyme contents of the mesosomal membrane indicate functions associated with the assembly of surface polymers in this organism. The activity of these membrane fractions in suitable cell-free synthesizing systems should yield valuable information relevant to the role of the mesosome.

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