Some Enzymic Activities and Chemical Properties of the Mesosomes and Cytoplasmic Membranes of *Bacillus licheniformis* 6346

By D. A. REAVELEY AND H. J. ROGERS

National Institute for Medical Research, Mill Hill, London N.W.7

(Received 3 January 1969)

1. The liberation of the vesicles of the mesosomes from protoplasts of *Bacillus licheniformis* strain 6346 is controlled by the Mg^{2+} concentration present during the removal of the walls by lysozyme. 2. The functioning of the cytoplasmic membranes is also critically controlled by the Mg^{2+} concentration. 3. The isolated mesosomes and the cytoplasmic membranes differ in their enzymic activities. Both succinate dehydrogenase and NADH oxidase activities are very low or absent from the mesosomes. 4. The cytoplasmic membranes can also be separated into materials of different density. 5. Distribution of the membranes between these fractions is controlled by the Mg^{2+} concentration and the growth conditions of the micro-organisms.

The cytology of bacterial cells is slowly becoming clarified as a result of the combined application of high-resolution electron microscopy, improved sectioning and staining methods, and physiological experimentation. As far as can be seen at the moment, all bacteria have an exterior-supporting region usually called the wall making up 15–20% of the dry weight of the cell and underlying this a closely applied membrane usually referred to as the cytoplasmic membrane. This membrane can be seen to be continuous with internal membranous structures, more prominent in Gram-positive and acid-fast bacteria than in Gram-negative species; these internal membranes are now usually called mesosomes. They sometimes have the form of bag-like structures that appear to be filled with membranous beads of about 300–600 Å diameter and tubules [e.g. in *Bacillus megaterium* (Fitz-James, 1960, 1964*a,b*; Ellar, Lundgren & Slepecki, 1967), *Bacillus subtilis* (van Iterson, 1961; Ryter & Jacob, 1964; Ryter, Fréhel & Ferrandes, 1967) and *Lactobacillus plantarum* (Kakefuda, Holden & Utech, 1967)]. In other examples the mesosomes seem to consist of a long and complicated system of tubules (e.g. in *Lactobacillus corinoides*; Schötz, Abo-Efnaga & Kandler, 1965) or of onion-like whorls of membrane (e.g. *Micrococcus lysodeikticus*; Salton & Chapman, 1962). Not infrequently, more than one arrangement of the mesosomal membranes can be found, even in the same cell. Gram-negative bacteria have less well-developed internal membrane systems, although even in these species they can be shown to be present (Ryter & Jacob, 1966; Schnaitman & Greenawalt, 1966; Steed & Murray, 1966; Ryter, 1968). In Gram-positive species, the

total membrane system accounts for 11–31% of the dry weight of the cells according to species and growth conditions (Salton & Freer, 1965).

The membranes at least from Gram-positive bacteria appear to have a similar crude chemical composition to those from mammals and plants, consisting of 15–30% of lipid material and 50–75% of protein (see Salton & Freer, 1965). They account for almost the whole of the phospholipid in the cell, and a number of enzymes have been shown to be entirely associated with them (for a summary of this work see Rogers & Perkins, 1968).

On the basis of cytological and other evidence, a number of special functions has been claimed for the mesosomes in the Gram-positive bacteria. These are: (1) associations with the DNA and concern with the division of the nuclear material (Ryter, 1968); (2) association with the formation of septa (Ellar *et al.* 1967); (3) oxidative function (van Iterson & Leene, 1964; Ferrandes, Chaix & Ryter, 1966) [in particular the preparation of mesosomes made by Ferrandes *et al.* (1966) from *B. subtilis* (N.C.T.C. no. 3610) contained a very high proportion of the cytochromes in the bacteria]; (4) mesosome-like material has been isolated from *B. licheniformis* strain 749/C to which penicillinase is attached. Although these latter vesicles and tubules are closely similar in appearance to mesosomes seen in other bacteria, their exact relationship is not clear since they only appear in *B. licheniformis* 749/C and 749 when penicillinase is being formed (Sargent, Gosh & Lampen, 1968*a,b*; Gosh, Sargent & Lampen, 1968). From this it seems possible that at least some mesosome-like structures might be concerned with the export of proteins from bacteria,

as originally suggested by Lampen (1965). Also, on the basis of radioactive labelling experiments, it has been claimed (Fitz-James, 1967, 1968) that [^{14}C]acetate and ^{59}Fe are preferentially incorporated into mesosomes rather than into the cytoplasmic membranes of *B. megaterium*. This does not happen when radioactively labelled amino acids are supplied to the micro-organisms. These results might suggest either that the mesosomes are the site of synthesis for lipids and porphyrins but not for membrane proteins, or that the former are in a more dynamic state in the organelles than elsewhere in the membrane.

The aim of the present work was to separate mesosomes and any other recognizable fractions of membrane from *B. licheniformis* strain 6346 and to compare the composition and enzymic activities of these various fractions. This micro-organism was chosen because we already knew a good deal about the wall (Hughes, Pavlik, Rogers & Tanner, 1968) and one of our long-term aims is to study the topology of the biosynthesis of wall macromolecules. Some work was also done with *B. subtilis*. Preliminary accounts of parts of this work have already appeared (Rogers, Reaveley & Burdett, 1967; Reaveley, 1968).

MATERIALS

Chemicals. Cytochrome *c* (from horse heart and said to be 90–100% pure) and *p*-nitrophenol phosphate were from the Koch-Light Laboratories Ltd., Colnbrook, Bucks.; NADH was from Boehringer Corp. (London) Ltd., London W.5; ATP was from Sigma Chemical Co., St Louis, Mo., U.S.A.; other chemicals were A.R.-grade materials where these were available. Two batches of polyethylene glycol were used, one of average molecular weight 400 and the other of 600, and were obtained from British Drug Houses Ltd., Poole, Dorset; deoxyribonuclease was from Worthington Biochemicals Corp., Freehold, N.J., U.S.A., and lysozyme from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

METHODS

Growth of micro-organisms

The bacteria were kept at 0–4° as spore suspensions in water. Cultures were grown by first inoculating 10 ml. of casein-hydrolysate medium (Janczura, Perkins & Rogers, 1961), supplemented by 3.3 mM-MgSO₄ and 0.5% glucose, with spore suspension and incubating this culture overnight at 35° without shaking. The bulk culture was grown by inoculating the same medium, warmed to 35°, with 5% (v/v) of the primary culture and shaking at 35° until growth had reached the required extinction value measured with a Spekker absorptiometer. These readings were related to dry weight of bacteria by the use of a calibration curve constructed in the usual way. In constructing these curves bacteria from exponentially growing cultures and from cultures incubated until growth had ceased were used. The

curves obtained from these two sorts of culture were not identical, presumably largely because of the different shapes and associations of the cells together under these two different conditions.

When a synthetic medium was used, as for example during the study of synchronous cultures, it had the following composition: KH₂PO₄ (13.6 g.), NH₄Cl (1 g.) and (NH₄)₂SO₄ (1 g.), dissolved in 1 l. of water, adjusted to pH 7.0 and autoclaved at 120° for 20 min. Glucose (5 g./l.) and MgSO₄·7H₂O (0.822 g./l.) were then added as separately sterilized solutions.

Synchronized cultures. Cultures were synchronized by a starvation method (Williamson & Scopes, 1962; Donachie, 1965; Masters, Kuempel & Pardee, 1964). Spores were inoculated into casein-hydrolysate medium containing 0.2% of glucose and 3.3 mM-MgSO₄ and incubated without shaking for 16 hr. at 37°. The bacteria were centrifuged and resuspended in minimal medium containing 0.05% of glucose and 3.3 mM-MgSO₄ to an extinction value of 0.6. The cells were incubated with shaking at 35° until the density of the suspension ceased to increase (about 4 hr.) and then for a further hour. The bacteria were then collected and resuspended in minimal medium containing 0.5% of glucose and 3.3 mM-MgSO₄ to an extinction value of 0.6. Incubation of this culture with shaking at 37° showed that the bacteria divided synchronously for two divisions. Cell growth and division were monitored by extinction, by DNA estimations (Burton, 1956), by plate counts and by electron-microscopic examination.

Large-scale preparation of membranes and mesosomes

Preparation of protoplasts. Cells from 1 l. of culture (approx. 800 mg. dry wt. from stationary-phase culture) were cooled, collected by centrifuging and washed once with buffer [50 mM-sodium hydrogen maleate-NaOH buffer, pH 6.5, containing 20 mM-MgSO₄ and 20% (v/v) of polyethylene glycol 600]. The washed cells were resuspended in buffer to a concentration of 10 mg. dry wt./ml. and, after the addition of lysozyme (20 µg./ml.), the suspension was incubated at 35° until the rod-shaped cells were completely converted into spherical protoplasts (about 1–3 hr., depending on growth phase). The protoplasts were collected by centrifuging at 10000g for 1 hr. at 2°.

Preparation of mesosomes. The protoplast supernatant was centrifuged at 100000g for 2 hr. at 2° and the resultant pad washed twice with m-NaCl containing 20 mM-MgSO₄. The pad was finally taken up in 2 ml. of m-NaCl containing 20 mM-MgSO₄ and the suspension layered on top of a CsCl density gradient. The linear gradient was prepared by mixing 5 ml. of 33% (w/v) CsCl with 5 ml. of 35% (w/v) CsCl in a suitable automatic apparatus. On centrifuging at 100000g for 16 hr. at 2° two bands of membrane and a pad were produced. The bands were normally recognized by inspection. A hole was then pierced through the tube with a hot wire and samples were taken. In some cases 0.5 ml. samples were taken successively from the top of the gradient. The *E*₂₆₀ and *E*₂₈₀ values and the refractive index of the samples were recorded. The density of the fractions was calculated from the refractive indices by using the formula of Vinograd (1963). For chemical analysis the bands were exhaustively dialysed at 2° against water and freeze-dried; for electron-microscopic examination samples were dialysed against 20 mM-MgSO₄. For enzyme estima-

tions the samples were dialysed against 20 mM-MgSO₄ and not dried from the frozen state.

Preparation of cytoplasmic membranes. The protoplasts were resuspended to a concentration of 10 mg./ml. in buffer. Buffer (2 vol.; without added polyethylene glycol) was added to rupture the protoplasts osmotically. A trace of deoxyribonuclease was added and the mixture shaken for 20 min. at room temperature. Membranes were collected by centrifugation at 30000g for 20 min. at 2° and washed three times with M-NaCl containing 20 mM-MgSO₄ by centrifuging at 20000g for 20 min. at 2°. The membranes were finally taken up in 6 ml. of M-NaCl containing 20 mM-MgSO₄. Linear sucrose density gradients (total vol. 10 ml.) containing 20 mM-MgSO₄ were prepared from equal volumes of 2.05 M-sucrose and 1.76 M-sucrose. The membrane suspension in NaCl solution (2 ml.) was layered on to the top of each of three such gradients. Gradients were centrifuged and material was isolated as described for mesosomes. Densities were calculated from measurement of refractive index by using data in the International Critical Tables. With membranes from stationary-phase cells two bands (light and heavy) and a pad were normally produced.

Chemical determinations

RNA. This was separated from DNA by the Schmidt-Thannhauser procedure as described by Hutchison & Munro (1961) and determined by the method of Ceriotti (1955).

Protein. This was determined, after disintegration of the samples in a sonic oscillator, by the method of Lowry, Rosebrough, Farr & Randall (1951); bovine serum albumin was used as standard.

Lipids. These were extracted from samples (20 mg.) by three successive extractions (1 hr. at room temperature with chloroform-methanol, 2:1, v/v). Extracts were passed through a no. 4 sintered-glass filter into a tared tube.

Cytochromes. These were determined from oxidized-reduced difference spectra obtained with a Cary spectrophotometer. The samples were first disaggregated (at concentrations of 5 mg./ml.) with detergent (0.5% sodium deoxycholate in 0.1 M-tris-HCl, pH 7.6).

Enzyme assays

NADH oxidase. This was assayed by following the disappearance of NADH measured by continuously recording the E_{340} value with a Beckman DB recording spectrophotometer. The substrate concentration was arrived at from experiments described in the Results section. The reaction mixture was as follows: one 1 cm. cuvette of the spectrophotometer contained 0.1 ml. of 1.5 mM-NADH, 2.65 ml. of 0.1 M-tris-HCl buffer, pH 7.9, and 0.1 ml. of enzyme preparation; the other cuvette contained the same ingredients without NADH. The solutions without enzyme were warmed to 30° and maintained at this temperature. The reaction was started by rapidly mixing 0.1 ml. of the preparations with the substrate solution and was followed for 2-3 min.

Succinate dehydrogenase. This enzymic activity was measured by the principle of Singer & Kearney (1957) as applied by Ellis (1959) and by Arrigoni & Singer (1962). Each 1 cm. cuvette of the Beckman DB recording spectrophotometer contained 0.3 ml. of 0.5 M-sodium

potassium phosphate buffer, pH 7.6, 0.3 ml. of 0.2 M-sodium succinate, 0.3 ml. of 10 mM-KCN and sufficient water and enzyme samples to make the final volume to 2.7 ml. in the test cuvette and 3.0 ml. in the other. This mixture was warmed to 30° and kept at this temperature; 0.15 ml. of 0.4 mM-2,6-dichlorophenol-indophenol solution was then added to the test cuvette followed by 0.15 ml. of 0.2% phenazine methosulphate solution and mixed as rapidly as possible. The decrease in E_{600} was then followed for 2-3 min. Over this period the decrease in E_{600} was linear when a preparation of suitable enzymic activity was included. The results are expressed as percentage decrease in E_{600} /min.

Potassium ferricyanide-NADH reductase. The test cuvette of the Beckman DB spectrophotometer contained 1.9 ml. of 0.1 M-tris-HCl buffer, pH 8.0, 0.5 ml. of 3 mM-K₃Fe(CN)₆ and 0.3 ml. of 7.5 mM-NADH. The other cuvette contained 2.4 ml. of 0.1 M-tris-HCl buffer, pH 8.0, and 0.5 ml. of 7.5 mM-NADH preparation. Both solutions were warmed to 30° and maintained at this temperature. The reaction was started by adding 0.1 ml. of the enzyme preparation to each cuvette with rapid mixing. The reaction was followed by continuously measuring the decrease in E_{420} . The reaction was usually linear for 2-3 min., but in any case only the slope of the line during the first minute of measurement was used. The results are expressed in percentage decrease in E_{420} /min.

NADH-cytochrome c reductase. This was measured by following the increase in E_{550} , which indicates the formation of reduced cytochrome c. The mixtures used in the cuvettes were as follows. The test cuvette contained 0.2 ml. of 1.08 mM-cytochrome c, 0.45 ml. of 7.5 mM-NADH, 0.2 ml. of 10 mM-KCN and sufficient 0.1 M-tris-HCl buffer, pH 7.9, and enzyme preparation to make the final volume 2.85 ml. The contents of the other cuvette were the same except that the NADH solution was omitted. The reaction was performed at 30° and was started by the addition of the membrane preparations. The results are recorded as percentage increase in E_{550} /min.

Alkaline phosphatase. A volume (0.2 ml.) of 50 mM-p-nitrophenol phosphate was mixed with 1.0 ml. of 0.1 M-tris-HCl buffer, pH 9.0. This mixture was warmed to 35° and 0.1 ml. of the enzyme preparation added. After 10 min. incubation 1.0 ml. of M-K₂CO₃ was added and the tests, along with blanks containing no enzyme, were read on a Unicam SP.600 spectrophotometer at 420 nm. The extent of hydrolysis of the substrate was measured from a standard curve constructed with various concentrations of pure p-nitrophenol.

Acid phosphatase. This was measured in the same way as the alkaline phosphatase except that the alkaline tris buffer was replaced by 1.0 ml. of 0.1 M-sodium acetate buffer, pH 5.0, and the incubation time increased to 30 min.

Estimation of leakage by protoplasts

A number of methods for assessing the intactness of the outer membranes of protoplasts can be used and all presumably measure different aspects of degeneration. For the present work, a method was required that was reasonably rapid and sensitive and at the same time would give a good general indication of damage or its absence to the membrane. The method finally chosen was to measure the E_{260} value of the supernatant fluid remaining after protoplasts had been removed by centrifuging at 10000g for

Table 1. *Effect of various conditions for forming protoplasts on their apparent leakiness*

Bacteria from an exponentially growing culture were washed once and resuspended at 12.1 mg./ml. in 0.1 M-sodium-potassium phosphate buffer, pH 6.8. The various additions were then made from solutions twice the finally required strength. The suspensions were then warmed to 30° and lysozyme was added. Observations were made under the phase-contrast microscope every 5–10 min., and as soon as 90–100% of the rods had been converted into round protoplasts the suspensions were centrifuged. It should be noted that a suspension of the micro-organisms in buffer alone without lysozyme also lysed almost completely in 60 min. and therefore the natural lysis acting at the same time as the lysozyme must play its part in removing the walls from the bacteria.

Concn. of sucrose (M)	Concn. of poly- ethylene glycol (%, w/v)	Concn. of MgSO ₄ (M)	Time to form protoplasts (min.)	Lysozyme added (μg./ml.)	Leakage (% increase in <i>E</i> ₂₆₀)
0.5	—	0	< 15	100	87
0.5	—	0.02	< 15	100	73
—	20	0	< 15	100	87
—	20	0.02	< 15	100	25
—	5	0.02	—	20	66
—	10	0.02	45	20	68
—	15	0.02	50	20	49
—	20	0.02	65–75	20	19

Table 2. *Effect of Mg²⁺ on protoplasts of B. licheniformis 6346*

The values given are for the proportion of the various entities that appeared in the supernatant fluids remaining after removal of the protoplasts by centrifuging. The MgSO₄ concentrations were those present during the action of 40 μg. of lysozyme/ml. for 1 hr. at 30°. Also present in all the suspension fluids were 20% (w/v) polyethylene glycol and 0.05 M-tris-HCl buffer, pH 6.5.

Concn. of MgSO ₄ (mm)	Ferricyanide reductase activity (%)	Alkaline phosphatase activity (%)		Leakage (% increase in <i>E</i> ₂₆₀)
		Expt. I	Expt. II	
15	69	95	100	46
20	62	88	73	35
25	47	—	64	21
30	39	79	—	1
40	39	54	—	5

15 min. The value obtained indicates the approximate concentration of soluble nucleotides and nucleic acid that have either leaked out of protoplasts or been released from those that have been disrupted. The value obtained was compared with that of a suitable dilution of a total lysate of the cells made by acting on a suspension of cells in buffer with 100 μg. of lysozyme/ml. It is, of course, subject to a number of errors, not least of which is the amount and intactness of ribosomal material and the amount of this that adheres to the membranes. The values obtained can only be accepted as indicative of the degree of membrane damage, not as meaningful absolute values.

RESULTS

Various conditions for forming protoplasts

In initial experiments the degree of protoplast formation and leakage from these bodies was compared by using various concentrations of

sucrose and polyethylene glycol of average molecular weight 400. Bacteria from the middle of the exponential phase of growth were used in these experiments. As shown in Table 1 the minimum concentrations of polyethylene glycol appeared to be 20% (w/v) and Mg²⁺ appeared to be essential (see also Table 2). In the absence of Mg²⁺ very few protoplasts survived, as was indicated both from the appearance of large amounts of material absorbing u.v. light at 260 nm. in the supernatant fluid from them (see under 'Leakage' in Table 1) and from direct observation under the phase-contrast microscope. Surprisingly, 20% (w/v) polyethylene glycol of average molecular weight 400 was very much more effective in preventing leakage of material absorbing u.v. light at 260 nm. than an equivalent concentration of sucrose. In view of these results, 20% polyethylene glycol was used as a stabilizing agent in all the further work described below. The lower concentration of lysozyme was also used because it was found that at higher concentrations the basic protein of the enzyme tended to be precipitated with any nucleic acid liberated from severely damaged protoplasts, thus vitiating leakage tests and possibly enmeshing enzymes in the precipitate.

Effect of Mg²⁺ concentration on protoplasts

Protoplasts were made from bacteria harvested in mid-exponential phase with 20% (w/v) polyethylene glycol and 50 mM-tris-maleate buffer, pH 6.5, and including various concentrations of magnesium sulphate. Incubation with 40 μg. of lysozyme/ml. at 30° was continued until the rods were converted into round protoplasts, which usually occurred within 60 min. The amount of enzyme in the supernatant fluid was compared

with that present in a total lysate of the bacterial suspension prepared by allowing lysis by lysozyme of a suspension of the micro-organisms in buffer to proceed to completion. It is shown in Table 2 that, even when the Mg^{2+} concentration was raised to a point where no significant leakage of nucleotide material could be detected, 30–40% of the ferricyanide reductase was still found outside the protoplast. Measurements of NADH oxidase and succinate dehydrogenase showed that neither of these entities was liberated from the protoplast. When the supernatant fluids were centrifuged at 100 000g, 30–40% of the ferricyanide reductase was deposited but none of the alkaline phosphatase. This provides some support for the suggestion (Rogers *et al.* 1967) that the part of the reductase enzyme that is liberated is related to the mesosomes that are extruded during the formation of protoplasts. Various unsuccessful attempts were made to obtain a higher proportion of the enzyme in such a state that it could be deposited at 100 000g. In view of these unsuccessful attempts, the situation must remain that all the secreted ferricyanide reductase may have been in some way or at some time associated with the liberated mesosomes, but no direct evidence exists, except for the 30–40% that is deposited under the same conditions as the mesosomes and that some can be demonstrated in the isolated mesosome beads (see below). Examination of sections of protoplasts prepared with different Mg^{2+} concentrations showed increasing association of mesosome beads with the exterior of the protoplasts as the Mg^{2+} concentration was raised beyond 20mM (Rogers *et al.* 1967).

A proportion of some of the other enzymes not mentioned in Table 2 also occurs in the supernatant fluid from the protoplasts. Among these are acid phosphatase and NADH-cytochrome *c* reductase, about 30–40% of the former and 50% of the latter being liberated from the protoplasts when the Mg^{2+} concentration was 25mM. A small proportion of both enzymes was found associated with the mesosomes and the liberation of these enzymes, too, could be related to the extrusion of the organelles. Succinate dehydrogenase and NADH oxidase were never found in the supernatant fluid.

When non-leaky but agglutinated protoplasts are prepared by having 30mM- Mg^{2+} present during the action of the lysozyme, they do not seem to be altered by transferring them to 10mM- Mg^{2+} as judged either by the appearance of nucleotide or of enzymes in the soluble phase. Very brief treatment with low concentrations (0.02–0.1mM) of EDTA, however, leads to rapid extrusion of ferricyanide reductase but a comparatively small loss of nucleotide material (see Table 3). It is possible to replace Mg^{2+} by Ca^{2+} at about the same molarity to stabilize the protoplasts. Likewise, Mn^{2+} successfully

Table 3. *Effect of decreasing the Mg^{2+} concentration in the membranes of protoplasts*

Protoplasts were made in the usual way from bacteria suspended in polyethylene glycol-tris-HCl buffer containing 35mM- $MgSO_4$. The treatment with EDTA was carried out by centrifuging the protoplasts down and resuspending them in buffer and polyethylene glycol containing 0.12mM-EDTA. They were then spun down again within 5–6min. at room temperature and resuspended in buffer alone containing 35mM- $MgSO_4$ and 100 μ g. of deoxyribonuclease/ml. After lysis of the protoplasts and removal of the membranes by centrifuging, about 30% of the total cell ferricyanide reductase was found to be soluble in the diluted cytoplasmic contents of the cell and 10% was associated with the membrane.

	Ferricyanide reductase activity (%)	Leakage (% increase in E_{280})
Liberated after lysozyme action	23	0–5
Liberated after subsequent treatment with EDTA	44	22
Total liberated	67	22–27
Liberated after transfer from 30mM- into 10mM- Mg^{2+}	10	0
Liberated when protoplasts made directly in 10mM- Mg^{2+}	79	60

prevented leakage of nucleotide material, but the higher concentrations (above 10mM) could not be tested for their effect on the extrusion of enzymes owing to interference of this ion in the estimations of enzymic activity.

The effect of Mg^{2+} on protoplasts is clearly dependent on the species and probably the strain. Examination of a closely related species, *B. subtilis* (strains W23 and 168 Ind⁻), showed protoplasts of these micro-organisms to require much lower concentrations of Mg^{2+} to be present during the making of protoplasts.

Fractionation of protoplasts

Protoplasts were suspended in the 20% (w/v) polyethylene glycol buffer containing the appropriate concentration of Mg^{2+} and the mixture was centrifuged at 10 000g for 15min. The protoplasts were then taken up in more of the same mixture and centrifuged again in the usual way. They were then resuspended in half the original volume of 50mM-tris-maleate buffer, pH 6.5, containing the same concentration of magnesium sulphate as used in the formation of protoplasts, and 100 μ g. of deoxyribonuclease was added. This mixture was kept for 20min. at room temperature. It was then diluted with the same buffer-magnesium sulphate mixture, but containing no nuclease, to twice the original volume and centrifuged at 20 000g for 15min. at

Table 4. *Distribution of enzymic activities between cell fractions according to the Mg²⁺ concentrations*

Protoplasts were made, lysed and the membranes prepared as described in the text (p. 71-72). The indicated concentration of Mg²⁺ was present throughout the procedure.

	% of enzyme not liberated from protoplast					
	In cytoplasmic contents			In unwashed membrane		
Concn. of Mg ²⁺ (mM)	20	25	30	20	25	30
Ferricyanide reductase	77	64-74	68	23	26-36	32
Alkaline phosphatase	97-98	96	91	2-3	4	9
Acid phosphatase	80	79	68	20	21	32
NADH-cytochrome c reductase	20-30	—	—	70-80	—	—
Succinate dehydrogenase	0-5	0-5	0-5	95-100	95-100	95-100
NADH oxidase	0-5	0-5	0-5	95-100	95-100	95-100

room temperature. The separated membranes were resuspended for testing without further washing. This was deliberate since the first need was to know what can be present in membranes without presuppositions as to how it was held there. The contrast is between alkaline phosphatase, which is almost wholly soluble, appearing either in the fluid surrounding the protoplasts or in the soluble cytoplasmic contents, and on the other hand NADH oxidase and succinate dehydrogenase, which are both located entirely in the insoluble part of the cell, called here membranes (Table 4). The situation of the remaining enzymes is less clear since 20-30% is associated with the membrane whereas the rest appears to be soluble in the cytoplasm. An obvious explanation would be that these are really soluble enzymes, but are adsorbed on the membranes during preparation. To check this, a membrane preparation was prepared and washed ten times with 50mM-tris-hydrochloric acid buffer, pH 6.5, containing 20mM-magnesium sulphate. At each wash the specific activity of ferricyanide reductase and succinate dehydrogenase in the membrane was tested. The specific activity of the former enzyme steadily increased up to about 150% of its initial value. That of the latter remained constant to within $\pm 15\%$. Likewise, when washes with M-sodium chloride were included no evidence for the elution of either enzyme was obtained. Thus within the circumstances tested the enzymes were both firmly fixed to the membrane. The apparent increase in the activity of ferricyanide reductase is presumably due either to the removal of inhibitors or to alteration in the activity of the enzymic protein itself.

Examination of two strains of *B. subtilis* (W23 and 168 Ind⁻) and one other strain of *B. licheniformis* (749) showed general similarities, but also certain quantitative differences. All the succinate dehydrogenase and NADH oxidase were again found to be firmly fixed to the cytoplasmic-membrane preparations, but a higher proportion of some of the other enzymes such as ferricyanide reductase and

Fig. 1. Density-gradient centrifugation of a mesosome preparation. Measurements were made on consecutive 0.5 ml. samples from a CsCl density gradient after the initial application of the deposit from the supernatant fluid remaining after the removal of protoplasts. ○, *E*₂₈₀; ▲, *E*₂₆₀; —, density gradient.

acid phosphatase were in the membrane fraction from some strains. For example, in the two *B. subtilis* strains, 25% of the last-named enzyme was fixed to the membranes in strain W23 and 50% in strain 168 Ind⁻; alkaline phosphatase, on the other hand, was never significantly associated with the membranes.

Mesosome preparation

The gradient pattern of a mesosome preparation from a culture in the early stationary phase is shown in Fig. 1. This pattern was very reproducible. Material with a density of approx. 1.25 was present in the upper band, and when mixed with phosphotungstic acid and examined under the electron microscope it showed flattened spheres and a few flagella. In thin section (Plate 1) the spheres were seen to consist of unit membranes. In some other preparations small spheres were found inside larger ones, and occasionally a preparation consisted of spheres of different sizes (extremes of diameter were

EXPLANATION OF PLATE I

Section of mesosomes from the upper band obtained on a CsCl density gradient. The sections were fixed with glutaraldehyde and treated by the Kellenberger-Ryter technique. The magnification is $\times 97\,000$.

EXPLANATION OF PLATE 2

Section of mesosomes from the lower band obtained on a CsCl density gradient. The sections were fixed with glutaraldehyde and then treated by the Kellenberger-Ryter procedure. The magnification is $\times 97000$.

D. A. REAVELEY AND H. J. ROGERS

EXPLANATION OF PLATE 3

Section of the cytoplasmic membranes from the heavier band obtained on a sucrose density gradient. The sections were fixed with glutaraldehyde and then treated by the Kellenberg-Ryter procedure. The magnification is $\times 97000$. No significant differences could be seen between sections of the material in the heavier and light bands from the gradients.

D. A. REAVELEY AND H. J. ROGERS

Table 5. *Analysis of mesosome fractions*

The upper and lower bands are those obtained when the mesosome preparation was centrifuged in a CsCl density gradient. The values are given as percentages of the dry weights of the specimens. N.D., Not determined.

Sample Expt. no.	RNA			Protein	Lipid
	1	2	3	3	3
Upper band	3.5	10.3	6.3	44	17
Lower band	16.1	28.9	7.3	48	21
Pad (ribosomes)	72.1	55.8	N.D.	N.D.	N.D.

300 and 3000 Å). Amorphous material was sometimes present inside the spheres.

The lower band on the gradient (density approx. 1.28) consisted of flagella and mesosomes. These mesosomes tended to be larger than those in the upper band and were often associated with amorphous material (Plate 2).

The pad from the gradient consisted almost entirely of ribosomes; RNA estimations (Table 5) on the other fractions (upper and lower bands) showed that contamination by ribosomes, assumed to give rise to the RNA, was relatively low; moreover, the variability of the RNA content of these bands suggests it is present merely as a contaminant and not as an integral component.

Analysis and fractionation of cytoplasmic-membrane preparations

Morphology and composition. Protoplasts lysed in the absence of Mg^{2+} even when polyethylene glycol was present. Consequently, a Mg^{2+} concentration of 20 mM was used as a routine to prepare protoplasts; above this concentration protoplasts aggregated (see above) and mesosomes adhered to the protoplasts. The greater part of the work was carried out on membranes from early stationary-phase cultures grown on casein-hydrolysate medium. These membranes (see Plate 3) when applied to sucrose gradients (Fig. 2) produced three principal bands (light band, density 1.21; heavy band, density 1.25; pad, density > 1.26). In thin sections the material present in the different bands was indistinguishable and consisted of unit-membrane sacs to which various numbers of ribosomes and some mesosome spheres were attached.

Effect of Mg^{2+} concentration on the density of fractions. The gradient pattern shown in Fig. 2 was not changed by extended centrifugation (for 88 hr.), but was altered by changing the concentration of Mg^{2+} used in the preparation and washing of the membranes. By using the results obtained with a 20 mM- Mg^{2+} concentration for comparison, it was found that on lowering the Mg^{2+} concentration to 10 mM the positions of the light band and the pad

Fig. 2. Density-gradient centrifugation of a cytoplasmic membrane preparation. Measurements were made on consecutive 0.5 ml. samples of a sucrose density gradient after the initial application of a washed membrane preparation made in the presence of 20 mM- $MgSO_4$. ○, E_{280} ; ▲, E_{260} ; ●, density gradient.

remained the same, but the intermediate band moved very slightly further down the gradient. Conversely, on increasing the Mg^{2+} concentration to 30 mM, this intermediate band was situated somewhat closer to the light band (density 1.245). Total yield of membrane and the distribution of the amounts of membrane material between the various bands were much affected and composition of material in the bands was also slightly affected (Tables 6 and 7) by Mg^{2+} concentration. Briefly, lowering the Mg^{2+} concentration slightly decreases the total yield of membrane but increases the relative amount of light membrane. Omission of Mg^{2+} completely from the solutions used to prepare the membranes yielded material that sedimented almost entirely as a light band.

Examination of Table 7 shows that the difference between the analysis of the membrane material in the bands is not striking. The significant result would appear to be a higher lipid content in the light band as compared with the heavy band, a result that might well account for the lower density of the material. Comparison of the results for each

Table 6. *Effect of Mg^{2+} concentration on the total yield and distribution of membrane between the various fractions*

Membranes were prepared from stationary-phase cells as described in the Methods section and washed three times with m -NaCl before being applied to the density gradient. All solutions contained the appropriate amounts of Mg^{2+} . The terms light, heavy and pad are used to designate the bands obtained on sucrose density gradients. The results given are averages for three separate experiments, with the ranges shown in parentheses.

Concn. of Mg^{2+} (mM) ...	Total yield or distribution (%)			
	0	10	20	30
Membrane*	14 (14)	15 (13-17)	22 (18-25)	19 (16-24)
Light membranes†	74 (54-91)	62 (59-64)	32 (11-63)	31 (22-38)
Heavy membranes†	22 (0-43)	34 (31-37)	47 (25-80)	55 (36-70)
Pad†	4 (0-9)	4 (4)	20 (4-40)	20 (4-36)

* Expressed as a percentage of the dry weight of the bacteria.

† Expressed as a percentage of the total membrane.

Table 7. *Compositions of membranes of different density when produced in the presence of different Mg^{2+} concentrations*

Results are expressed as percentages of the total weight of dry membrane.

Concn. of Mg^{2+} (mM) ...	Composition (%)											
	0	10			20					30		
	1	2	3	4	5	6	7	8	9	10	11	12
Prep. no. ...	...	...	...	...	...	...	...	...	...	...	...	...
Protein												
Light membranes	78	59	57	54	41	50	50	42	41	59	59	62
Heavy membranes	—	59		54	46	52	48	41	45	49	56	54
RNA												
Light membranes	2	8	8	7	15	6	12	16		11	11	18
Heavy membranes	—	9	12	10	15	10	8	9		11	15	18
Chloroform-methanol-soluble												
Light membranes	28	34	37	31	35	33		28	29			31
Heavy membranes	—		34			28	25	23	13		31	38
Chloroform-soluble*												
Light membranes	23		31	25	22	27						20
Heavy membranes	—					17					20	26

* Chloroform-methanol-soluble material that is also soluble in chloroform. It has been alleged (Green, Haard, Lenaz & Silman, 1968) that some membrane proteins are soluble in chloroform-methanol as well as other solvents containing hydrophilic components.

Table 8. *Effect of washing on membrane preparations*

Membranes were prepared from early stationary-phase cells with a $MgSO_4$ concentration of 20 mM.

No. of washes and composition of washing fluids		Relative amounts in bands in sucrose gradients		
m -NaCl + 20 mM- $MgSO_4$	Other washes	Light	Heavy	Pad
0	—	+	(+)	+++
3	—	++(+)	++(+)	++(+)(+)
8	—	++(+)	++(+)	++(+)(+)
3	10 × 20 mM- $MgSO_4$	+++	++	+
3	10 × water	+++	—	+
0	3 × 0.1 M-tris-HCl, pH 7.6 + 20 mM- $MgSO_4$	+	(+)	+++
0	3 × 0.1 M-tris-HCl, pH 7.6	+++	+	—
0	3 × 20 mM- $MgSO_4$	+	(+)	+++
0	8 × 20 mM- $MgSO_4$	+	—	+++

band made in the presence of the various Mg^{2+} concentrations shows that there may be a tendency for the protein content to decrease somewhat between concentrations of 0 and 20 mM- Mg^{2+} and, as might be expected, for the RNA content to increase.

Effect of washing. The influence of the nature of the solutions and number of washes given to the membrane preparation on the pattern obtained from subsequent density-gradient centrifuging was investigated. The results are summarized in Table 8. Membranes were prepared from stationary-phase cells and solutions before washing contained 20 mM-magnesium sulphate. The following conclusions may be drawn from Table 8.

(a) If the membrane material is not washed at all, or is washed with Mg^{2+} (20 mM) contained in solutions of relatively low ionic strength, large yields of pad and relatively low yields of light and heavy material are obtained. (b) Washing with solutions not containing Mg^{2+} produces mainly light material. (c) Washing with M-sodium chloride in the presence of Mg^{2+} diminishes the pad considerably and produces light and heavy bands in variable amounts. (d) Repeated washing with any one of the solutions does not alter the results obtained in subsequent density-gradient fractionation compared with the standard procedure using three washes.

Effect of different methods of protoplast rupture. In some experiments the protoplast suspension, instead of being disrupted osmotically, was disrupted either by ultrasonic treatment (for 2 min. at 0°) or by use of a Braun homogenizer (20 ml. of polystyrene beads plus 25 ml. of suspension shaken for 3 min. at 0°). The membranes were then diluted with 2 vol. of buffer without polyethylene glycol and prepared by the normal procedure. The gradient patterns for these fragmented membranes differed from those of osmotically lysed protoplasts.

Mechanically broken protoplasts yielded smaller amounts of light material and pad and produced more heavy material. Such effects were more

pronounced with ultrasonically treated protoplasts. The heavy region in both cases differed significantly from that produced from osmotically disrupted protoplasts. When mechanically broken protoplasts were mixed with osmotically broken protoplasts the gradient pattern produced was not simply an addition of the two separate patterns.

Effect of growth conditions. With casein-hydrolysate media, cells from the exponential phase had a much lower content of membrane material (approx. 6% compared with 22%) than cells from the early stationary phase. Preliminary results suggest that such a phenomenon does not occur for cells grown on the minimal medium described in the methods section.

The gradient pattern obtained with the membranes was markedly affected by growth conditions used for the bacteria. Results with a Mg^{2+} concentration of 20 mM during protoplast production and membrane preparations are summarized in Table 9.

Synchronous cultures. Cultures were synchronized by glucose starvation (see the Methods section). One well-defined step-growth curve was obtained and bacteria were harvested for membrane preparation immediately after cell division. Examination of the membrane preparation by density-gradient centrifugation showed material of the same density distribution as was found in membrane from cells harvested in the early exponential phase from minimal growth medium (see Table 9).

Cellular distribution of cytochromes

Membranes and mesosomes were prepared from stationary-phase cells grown on casein-hydrolysate medium. The concentration of cytochromes in both preparations was low and attempts to raise this by varying the growth conditions for the micro-organisms were not successful. Reduced-oxidized difference spectra for membranes were very similar to those obtained for *B. licheniformis* by Salton & Ehtasham-ud-din (1965). Spectra for mesosomes and membranes were basically similar. Small

Table 9. *Effect of media and growth phase on membrane preparations*

Membranes were prepared with a $MgSO_4$ concentration of 20 mM. The results are given in qualitative forms for the weights of material in the light, heavy and pad bands produced after density-gradient centrifugation in sucrose solutions.

No. of experiments	Media	Growth phase	Relative amounts		
			Light	Heavy	Pad
10	Casein hydrolysate + glucose	Early stationary	+++	++	+
3	Casein hydrolysate + glucose	Mid-exponential	+	—	+++
1	Minimal medium + glucose	Early stationary	++++	—	+
4	Minimal medium + glucose	Early exponential	+++	(+)	+

differences, however, were noted. There was a peak at approx. 500nm. in the spectra from the mesosome preparations and the 'a' type cytochrome peak was at a slightly higher wavelength (615nm. compared with 605nm.).

Comparison of the enzymic activities of cytoplasmic membranes and mesosomes

It had been found that all the NADH oxidase and the succinate dehydrogenase of the bacterial cell occurred in the protoplast membranes and none in the supernatant fluid remaining after centrifuging out the protoplasts. Therefore it seemed unlikely that the mesosomes would contain a high proportion of these two membrane-bound enzymes. In fact, as shown in Table 10, both of these activities are very much lower in the mesosome bead fractions on a specific-activity basis than in the fractions from the cytoplasmic membranes. The results for succinate dehydrogenase are particularly impressive, the specific activity of the mesosomes being only about 5% of that of the heavy membrane band. The mesosome preparations had been fractionated by using a density gradient of caesium chloride whereas the membranes were separated on sucrose density gradients. To check that caesium chloride did not inactivate or remove the succinate dehydrogenase or the NADH oxidase, membrane preparations were divided in two samples after preparation, which included the standard washing procedures. One was centrifuged in a density gradient of sucrose, the other in a density gradient of caesium chloride. In caesium chloride, a band and a pad were obtained. In sucrose two bands and a pad were produced. Enzymic estimations showed that the same total amount of enzyme was present when the activities in the separate bands were added together after both sucrose and caesium chloride treatment. Therefore there was no evidence that caesium chloride could inactivate or remove the membrane-bound succinate dehydrogenase or NADH oxidase. For the other enzymes no very significant differences were to be seen between the various membrane bands. The activity of alkaline phosphatase in the membrane preparations was too low for meaningful results to be obtained from these cells grown under the repressed conditions used. The question of the distribution of the NADH oxidase between cytoplasmic membrane and mesosomes is complicated by the instability of the enzyme system (see below), which is likely to account for the large variation in activity from batch to batch of the membrane preparations (Table 5). Consistent differences between the activities in the three fractions produced by density-gradient separations of the cytoplasmic membranes were not marked. The most variable

Table 10. *Comparison of the enzymic activities of cytoplasmic-membrane and mesosome fractions*

Prep. no.	Activity (units/mg.)																													
	Succinate dehydrogenase						NADH oxidase						Ferricyanide reductase						NADH-cytochrome <i>c</i> reductase						Acid phosphatase					
	Membranes			Mesosomes			Membranes			Mesosomes			Membranes			Mesosomes			Membranes			Mesosomes			Membranes			Mesosomes		
Bands ...	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3			
13	106	94	75	6	—	—	—	—	—	17	6	8	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
14	18	84	71	5	—	—	—	—	—	13	5	6	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
15	14	53	13	3	—	—	—	—	—	13	9	5	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
16	84	112	47	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
17	57	110	50	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
18	167	227	86	17	10	42	33	25	8	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
19	156	150	73	9	4	18	19	15	6	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				
20*	122	34	68	0	3	6	4	4	0, 7	0, 0†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—				

* These bacteria were harvested in the middle of the exponential phase of growth. The remainder were from cultures grown until the end of the exponential phase and just entering the resting phase.

† Four bands were recognized in the CsCl density gradients of mesosomes from these cells.

results were obtained from a minor light band and it was shown that the amount of enzyme in this band could be greatly influenced by introducing small differences in the Mg^{2+} concentration used in making the protoplasts. The heavy band had the highest specific activity for succinate dehydrogenase and probably for NADH-cytochrome *c* dehydrogenase.

Properties of the membrane-bound NADH oxidase activity

Substrate concentration. This enzyme has been suggested as a marker for bacterial membranes. In our initial studies we were troubled by the kinetics shown by the disappearance of NADH. Repeatedly, and with all amounts of membrane preparations examined, the rate accelerated sharply over the

first 1–2 min. It was subsequently found that this was due to strong inhibition of the enzyme by higher substrate concentrations (see Table 11). A substrate concentration of about 0.06 mM is as high as can be used without inhibiting the membrane-bound enzyme.

Stability. The activity of the enzyme decayed at a rate of about 20–30%/day when aqueous buffered magnesium sulphate suspensions were stored at 0–4°.

Inhibitors. In view of the finding (Ferrandes *et al.* 1966) that almost all the cytochromes of *B. subtilis* N.C.T.C. 3610 are contained in the mesosome fraction, and of the relatively high proportion of the NADH oxidase contained in the cytoplasmic membranes (see above), it became important to show whether or not the cytochrome chain was involved in the disappearance of NADH in our system. Table 12 shows the effect of various inhibitors on the NADH oxidase in membrane preparations. The inhibition by cyanide was shown to be a true inhibition and not to be due to the formation of the potassium cyanide–NAD⁺ complex, which, of course, also absorbs at 340 nm. In general, the concentrations of the inhibitors mentioned in Table 12 are high compared with those required to inhibit known systems involving the cytochromes. For example, cytochrome oxidase is 50% inhibited by 0.01 μ M-cyanide and with less than 10 mM-azide, whereas concentrations of about 0.1 mM-rotenone inhibit mitochondrial NADH-cytochrome *c* reductase and 1 mM-Amytal inhibits respiration of the subcellular organelles. It is therefore probably unjustifiable to draw the firm conclusion from these studies of the action of inhibitors that the cytochromes are definitely involved here in the disappearance of the NADH, although this is made probable.

Table 11. *Effect of different substrate concentrations on the disappearance of NADH*

The test system was as described in the Methods section except that the total volume in the cuvettes was adjusted by the addition of buffer. An 0.1 ml. volume of a cytoplasmic-membrane preparation was used as source of enzyme. The results are expressed as change in $E_{340}/\text{min.}$ measured over the first minute after adding the enzyme.

Concn. of NADH (mM)	$\Delta E_{340}/\text{min.}$	
	<i>B. subtilis</i> W23	<i>B. licheniformis</i> 6346
0.0078	6.0	—
0.053	6.5	—
0.063	6.0	24.0
0.107	3.0	—
0.120	—	9.0
0.214	0.5	1.7

Table 12. *Effect of inhibitors on the NADH oxidase in membrane preparations from B. licheniformis 6346 and B. subtilis W23*

Inhibitor	Concn. (M)	Inhibition (%)	
		<i>B. licheniformis</i> 6346	<i>B. subtilis</i> W23
KCN	0.000059	15	—
	0.00056	25	38
	0.0029	31	56
	0.0058	43	69
	0.0290	84	—
	0.0580	92	—
NaN ₃	0.070	20	—
	0.350	50	—
	0.700	62	—
Amytal	0.007	65	—
Rotenone	0.018	38	—
	0.088	75	—

DISCUSSION

One of the primary aims of this work appears to have been fulfilled, namely that of separating mesosomal components from cytoplasmic membranes. Unlike the preparations of mesosomes made from *B. megaterium* by Fitz-James (1967, 1968) or from *B. subtilis* by Ryter *et al.* (1967) and by Ferrandes *et al.* (1966), our best preparations consisted of separate vesicles homogeneous in size, although in some less good preparations these were mixed with vesicles of a much larger diameter. The small vesicles had about the same diameter as those seen in sections of whole bacteria both before and after extrusion of the mesosomes under the wall by the presence of hyperosmotic solutions surrounding the cells. Also, their different enzymic activities and protein contents (Reaveley, 1968) as compared with the cytoplasmic membrane gives

further confidence that they are indeed the mesosomes rather than cytoplasmic membrane material that has been partly disaggregated and re-formed as small vesicles, a well-known property of all membranes. It would seem possible that the larger vesicles seen in our less good preparations may have this latter origin. The preparations described by the other authors were different from ours in that they consisted of unevenly inflated tubules and other membranous material. This difference probably depends on the strain of micro-organism we have used and may be related to the particularly critical effects of Mg^{2+} on the behaviour of the membranes of strain 6346. Mg^{2+} was also shown to be important in the relationships between the mesosomes and protoplasts in *B. subtilis* strain 6310 (Ryter *et al.* 1967), but in this case the spherules did not completely separate from each other even when Mg^{2+} was low in concentration. If the Mg^{2+} concentration that was present with *B. licheniformis* strain 6346 while the walls were being removed by the action of lysozyme was greater than 20 mM then the mesosomes appeared as strings of beads, fixed usually at one point to the protoplast membrane. As the concentration of Mg^{2+} was raised from about 25 mM to 40 mM the protoplasts, together with the mesosomes, first agglutinated and at still higher concentrations the bacteria failed to change into spheres despite the removal of the walls (Rogers *et al.* 1967). At concentrations much lower than 20 mM- Mg^{2+} excessive leakage of nucleotide from the protoplasts occurred, indicating damage to the membranes. Even at 20 mM- Mg^{2+} some leakage took place, and therefore presumably some damage was done. The presence of considerable amounts of RNA in the most dense fraction during the density-gradient centrifugation of the mesosomes (see Table 5) suggests that in fact a few protoplasts must have ruptured completely under these conditions, liberating their ribosomes. However, this damage did not seem to be sufficient to solubilize any of the enzymic activities known to be firmly bound to the membranes.

The distribution of the cytoplasmic membranes during density-gradient centrifugation was also controlled by the Mg^{2+} concentration present during the formation of the protoplasts and the washing of the membrane preparations. For example, if no Mg^{2+} was added to the suspension during the action of lysozyme, then no whole protoplasts could be seen and a very high proportion of the membrane had a density of 1.21 or less. When the concentration of Mg^{2+} was raised this proportion fell to about 30% at 20 or 30 mM, but it never disappeared entirely. By using a constant concentration of Mg^{2+} during preparation of the membranes, the proportion of the light material was also under the control of the growth conditions. It was much

higher, for example, when the cells were grown with ammonium salts as a source of nitrogen rather than acid-hydrolysed casein. It was decreased in amount when rapidly dividing cells, rather than cells not so dividing, were harvested from the latter medium. The material in the light band on sucrose density gradients seems to be distinguished by a slightly higher lipid content, which might explain its relative density.

An obvious possible explanation of the presence of various bands of membrane differing in their density would be that they arose from cells in the culture at different points in their division cycle. This explanation, however, would not seem to be true since membrane preparations from cultures of synchronously dividing cells also produced more than one band when centrifuged in sucrose density gradients. A number of authors have studied the effects of Mg^{2+} on the aggregation of membranes (e.g. Razin, Morowitz & Terry, 1965; Wallach, Kamat & Gail, 1966; Butler, Smith & Grula, 1967; Grula, Butler, King & Smith, 1967; Rottem, Stein & Razin, 1968; Wallach & Zahler, 1968). From all these studies it is clear that the aggregation of membrane subunits is dependent on the presence of bivalent cations, and Mg^{2+} has usually been studied in particular. At lower concentrations of this ion, the pH and ionic strength of the suspending fluid are also important (Rottem *et al.* 1968). Of particular interest, however, are the observations of Grula *et al.* (1967), who showed that, even when the lipids have been almost completely removed from the membranes of *M. lysodeikticus*, the resulting material can be disaggregated by anionic detergents and that reaggregation is dependent on the presence of Mg^{2+} . Also relevant are the observations by Wallach *et al.* (1966), who showed that the cytoplasmic membranes and endoplasmic reticulum differed greatly in their response to Mg^{2+} and concluded that "turbidity increment produced by the addition of salts to ER suspensions represents primarily a 'structural' change in these membranes". Our own results, so far of a rather preliminary nature, would be consistent with a change that can be brought about in the organization of the membrane proteins and lipids either by deficiency in Mg^{2+} or by the conditions used for growth of the micro-organisms. Whether or not these different growth conditions lead to different concentrations of Mg^{2+} inside the cell, or to a difference in the distribution of Mg^{2+} , say between ribosomes and membrane, has not been examined. Nevertheless, this does not seem to be wholly probable as an explanation since the effects due to application of a low concentration of Mg^{2+} at one stage in the preparation of the membranes can be largely reversed by application of a different concentration at a later stage. We have no evidence whether the

whole or only parts of the membrane of an individual cell are involved in these effects.

Unfortunately, we are not in a position to comment with certainty on the quantitative distribution of cytochromes between cytoplasmic membrane and mesosomes, and therefore to confirm the most clear-cut claim, namely that by Ferrandes *et al.* (1966), who found these pigments to be continued to the mesosome fraction. The NADH oxidase activity in our cytoplasmic-membrane preparations, however, was inhibited by those substances usually thought of as acting on the cytochrome chain, namely potassium cyanide, sodium azide, Amytal and rotenone. Although the concentrations required to effect 50% inhibition were high compared with those required to inhibit electron transport in other systems, it would seem likely that the cytochrome chain is active in the membranes, which would imply the presence of cytochromes in them. At the same time, it must be pointed out that our membrane preparations were not completely free of mesosomes.

Our thanks are due to Mr I. Burdett for the electron-microscopic work recorded here, and to Mrs Barbara Gebhart, Mrs Anne Raine and Mr David Gunner for their conscientious technical assistance.

REFERENCES

- Arrigoni, O. & Singer, T. P. (1962). *Nature, Lond.*, **193**, 1256.
- Burton, K. (1956). *Biochem. J.* **62**, 315.
- Butler, T. F., Smith, G. L. & Grula, E. A. (1967). *Canad. J. Microbiol.* **13**, 1471.
- Cerioti, G. (1955). *J. biol. Chem.* **214**, 59.
- Donachie, W. D. (1965). *Nature, Lond.*, **205**, 1084.
- Ellar, D. J., Lundgren, D. G. & Slepecky, R. A. (1967). *J. Bact.* **94**, 1189.
- Ellis, H. A. (1959). *Arch. Biochem. Biophys.* **85**, 561.
- Ferrandes, B., Chaix, P. & Ryter, A. (1966). *C.R. Acad. Sci., Paris*, **263**, 1632.
- Fitz-James, P. (1960). *J. biophys. biochem. Cytol.* **8**, 507.
- Fitz-James, P. (1964a). *J. Bact.* **87**, 1202.
- Fitz-James, P. (1964b). *J. Bact.* **87**, 1483.
- Fitz-James, P. (1967). *Proc. 15th Symp. Protides of the Biological Fluids*, p. 289. Ed. by Peeters, H. Amsterdam, London and New York: Elsevier Publishing Co.
- Fitz-James, P. (1968). In *Microbial Protoplasts, Spheroplasts and L-Forms*, p. 124. Ed. by Guze, L. B. Baltimore: The Williams and Wilkins Co.
- Greene, D. E., Haard, N. F., Lenaz, G. & Silman, H. I. (1968). *Proc. nat. Acad. Sci., Wash.*, **60**, 277.
- Gosh, B., Sargent, M. & Lampen, J. O. (1968). *J. Bact.* **96**, 1314.
- Grula, E. A., Butler, T. F., King, R. D. & Smith, G. L. (1967). *Canad. J. Microbiol.* **13**, 1499.
- Hughes, R. C., Pavlik, J. G., Rogers, H. J. & Tanner, P. J. (1968). *Nature, Lond.*, **219**, 642.
- Hutchison, J. C. & Munro, H. N. (1961). *Analyst*, **86**, 768.
- Janczura, E., Perkins, H. R. & Rogers, H. J. (1961). *Biochem. J.* **80**, 82.
- Kakefuda, T., Holden, J. T. & Utech, N. M. (1967). *J. Bact.* **93**, 472.
- Lampen, J. O. (1965). *Symp. Soc. gen. Microbiol.* **15**, 115.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Masters, M., Kuempel, P. L. & Pardee, A. B. (1964). *Biochem. biophys. Res. Commun.* **15**, 38.
- Razin, S., Morowitz, H. J. & Terry, T. M. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 219.
- Reaveley, D. A. (1968). *Biochem. biophys. Res. Commun.* **30**, 649.
- Rogers, H. J. & Perkins, H. R. (1968). *Cell Walls and Membranes*, pp. 355-368. London: E. and F. N. Spon Ltd.
- Rogers, H. J., Reaveley, D. A. & Burdett, I. D. J. (1967). *Proc. 15th Symp. Protides of the Biological Fluids*, p. 303. Ed. by Peeters, H. Amsterdam, London and New York: Elsevier Publishing Co.
- Rottem, S., Stein, O. & Razin, S. (1968). *Arch. Biochem. Biophys.* **125**, 46.
- Ryter, A. (1968). *Bact. Rev.* **32**, 39.
- Ryter, A., Frehel, C. & Ferrandes, B. (1967). *C. R. Acad. Sci., Paris*, **265**, 1259.
- Ryter, A. & Jacob, F. (1964). *Ann. Inst. Pasteur*, **107**, 384.
- Ryter, A. & Jacob, F. (1966). *Ann. Inst. Pasteur*, **110**, 801.
- Salton, M. R. J. & Chapman, J. A. (1962). *J. Ultrastruct. Res.* **6**, 489.
- Salton, M. R. J. & Ehtasham-ud-din (1965). *Aust. J. exp. Biol. med. Sci.* **43**, 255.
- Salton, M. R. J. & Freer, J. H. (1965). *Biochim. biophys. Acta*, **107**, 531.
- Sargent, M., Gosh, B. & Lampen, J. O. (1968a). *J. Bact.* **96**, 1213.
- Sargent, M., Gosh, B. & Lampen, J. O. (1968b). *J. Bact.* **96**, 1239.
- Schnaitman, C. & Greenawalt, J. W. (1966). *J. Bact.* **92**, 780.
- Schötz, F., Abo-Efnaga, I. & Kandler, O. (1965). *Z. Naturf.* **20b**, 790.
- Singer, T. P. & Kearney, E. B. (1957). *Meth. biochem. Anal.* **4**, 307.
- Steed, P. & Murray, R. G. E. (1966). *Canad. J. Microbiol.* **12**, 263.
- van Iterson, W. (1961). *J. biophys. biochem. Cytol.* **9**, 183.
- van Iterson, W. & Leene, W. (1964). *J. Cell Biol.* **20**, 361.
- Vinograd, J. (1963). In *Methods in Enzymology*, vol. 6, p. 854. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Wallach, D. F. H., Kamat, V. B. & Gail, M. H. (1966). *J. Cell Biol.* **30**, 601.
- Wallach, D. F. H. & Zahler, P. H. (1968). *Biochim. biophys. Acta*, **150**, 186.
- Williamson, D. H. & Scopes, A. W. (1962). *Nature, Lond.*, **193**, 256.