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Some Features of the Bacterial Membrane Studied with the Aid of a New Fractionation Technique

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Membranes of *Bacillus megaterium* and other bacteria were bound to crystals of cadmium lauroylsarcosinate and were resolved into nine lipoprotein fractions by elution with potassium chloride and sodium deoxycholate solutions. The fractions differed widely in protein/lipid ratio. Some major protein species were probably common to all the fractions, but the phospholipid composition showed some variation. Electron-microscopic examination after negative staining revealed that material in certain fractions was in the form of particles of diameter about 12nm, and other fractions consisted of amorphous aggregates. The composition of the membrane in terms of the nine fractions was influenced markedly by the conditions of culture of the bacteria. Radioactive-labelling experiments suggested that some fractions served as precursors of others, and one fraction appeared to contain the attachment points of the DNA.

Biochemical analysis of the problems of membrane metabolism, and in particular membrane synthesis, has been hampered by the lack of simple methods for fractionating the material.

A novel technique for isolating a portion of the bacterial membrane was described by Tremblay, Daniels & Schaechter (1969), which utilized the property possessed by the membrane bearing the chromosome-attachment region of binding to crystals of magnesium lauroylsarcosinate. While attempting to study the mechanism of this interaction I have developed the fractionation method described in this paper, based on the different affinities of fractions of the membrane for crystals of cadmium lauroylsarcosinate. Nine fractions are obtained, containing lipid and protein in various ratios. Some appear to serve as precursors of others, and one seems to contain the chromosome attachment points.

METHODS AND MATERIALS

Bacteria. Two derivatives of *Bacillus megaterium* KM, one requiring threonine (KMthr; Daniels, 1969) and the other thymine and tryptophan (KMTT; Schaechter & McQuillen, 1966) were used for the experiments unless otherwise stated. The bacteria were grown with shaking at 37°C in the salts medium of Davis & Mingioli (1950), supplemented with glucose (0.2%), casein hydrolysate (Oxoid; 0.05%), L-tryptophan (1μg/ml) and thymine (1μg/ml), and, when necessary, with 20% (w/v) sucrose.

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Bacillus subtilis 168 was cultured in the casein hydrolysate medium described by Donellan, Nags & Levinson (1964). Sporulation was initiated by transferring bacteria to a minimal-salts medium (Sterlini & Mandelstam, 1969).

Escherichia coli BB and 3/62 were grown in the Davis & Mingioli (1950) medium supplemented with glucose (0.2%) and L-methionine (20μg/ml).

Radioactive labelling. [1-^{14}C]Glycerol (15.4 or 23.6mCi/mmol) and [2-^{3}H]glycerol (500mCi/mmol) were normally used as precursors of membrane lipid (Daniels, 1969). [^{35}S]Sulphate (carrier-free) was employed as a label for proteins. To secure maximum incorporation the Davis & Mingioli (1950) medium was modified by replacing $(NH_4)_2SO_4$ and Na_2SO_4 with equimolar quantities of the chlorides and the casein hydrolysate was replaced by a mixture of 18 of the 20 common L-amino acids (i.e. excluding methionine and cysteine), each at a concentration of 20μg/ml. When it was necessary to label the DNA of *B. megaterium* KMTT the non-radioactive thymine in the medium was replaced by [2-^{14}C]thymine (0.2μCi/ml, 60mCi/mmol). Unless otherwise stated cultures were incubated with labelled precursors for at least three generations to give approximate steady-state labelling patterns.

Samples were finally treated with trichloroacetic acid (final concentration 5%, w/v) and the precipitates collected on glass-fibre discs for scintillation-counting (Daniels, 1969).

Membrane preparation. Bacteria were rapidly harvested by centrifugation and were suspended at a density of 1–5mg dry wt./ml in TMK buffer (0.01M-tris–HCl buffer, pH7.4, 0.01M-magnesium acetate and 0.1M-KCl) containing sucrose (20%, w/v) and lysozyme (200μg/ml for the *Bacillus* spp. and 5mg/ml for *E. coli*). The suspensions were incubated at room temperature or at 37°C until

complete conversion into protoplasts or spheroplasts had occurred, as judged by the appearance under a phase-contrast microscope.

In cases where the nature of the experiment demanded a very rapid conversion of *B. megaterium* cells into protoplasts in the cold, the growth medium was supplemented with 20% (w/v) sucrose and the procedure of Schaechter (1967) was used.

The protoplasts or spheroplasts were collected by centrifugation (1000*g* for 15min), and were lysed by resuspending them in TMK buffer containing a few crystals of deoxyribonuclease (EC 3.1.4.5). The crude membranes ('ghosts') were pelleted by centrifugation at 16000*g* for 30min in a Servall centrifuge at 4°C. They were then resuspended in TMK buffer and again centrifuged, this procedure being repeated at least four times (Salton, 1967). The pellet was finally suspended in 0.5 or 1ml of water and stored at −20°C until required. Protein content of preparations was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with lysozyme as a standard.

Analysis of membrane proteins. Membrane fractions were dialysed for 24h at room temperature against three changes of 100 vol. of 0.01M-tris–HCl buffer, pH7.4, and then against water. Concentration of the samples was achieved by suspending the dialysis bags in a current of cold air. Sodium dodecyl sulphate and sucrose were then added to final concentrations of 1% (w/v) and 10% (w/v) respectively. Suitable portions were subjected to electrophoresis in slabs of polyacrylamide gel as described by Waites & Wild (1970), with the addition of 1% (w/v) sodium dodecyl sulphate to the buffer solution. At the end of the run the gel was immersed in acetic acid–methanol–water (2:5:13, by vol.) overnight to fix the proteins and to remove the sodium dodecyl sulphate before staining with Naphthalene Black 12B (Waites & Wild, 1970).

The distribution of radioactivity among the various protein bands in gels from ^{35}S-labelled membranes was examined by radioautography, as described by Daniels & Wild (1970).

Analysis of lipids. Radioactive membrane fractions were mixed with about 0.5mg of unlabelled membrane material, dialysed as described above and the lipids were extracted with chloroform and methanol (Bligh & Dyer, 1959) or with butan-1-ol (Wright, Dankert & Robbins, 1965). The extracts were washed with water and dried down *in vacuo*; the lipids were then dissolved in a few drops of chloroform and applied to thin layers of silica gel G (Merck) for t.l.c. The plates were developed with chloroform–methanol–water–acetic acid (46:29:3:6, by vol.). After the solvents had evaporated the position of the lipid species was revealed by exposure of the plate to iodine vapour. The areas of gel containing the spots were scraped off into glass vials, toluene scintillator solution was added and the radioactivity determined as described by Daniels (1969). Alternatively, the phospholipids were deacylated by the method of Dittmer & Wells (1969) and the water-soluble glycerophosphoryl compounds were applied to Whatman no. 3 paper and separated by descending chromatography with as a solvent phenol saturated with NH_3 (0.1ml of conc. NH_3, sp.gr. 0.88, in 100ml of water) (Kanfer & Kennedy, 1963). After excess of moisture had evaporated residual phenol was removed by dipping the paper several times in ether. The position of the several compounds was established by spraying a marker strip with the reagent of Hanes & Isherwood (1949), and corresponding areas of the paper were then cut out and immersed in toluene scintillator fluid in glass vials for radioactivity determination.

Sucrose-density-gradient centrifugation. Linear sucrose gradients (4.5ml) covering suitable density ranges were formed in cellulose nitrate tubes with a two-chamber mixing device. Samples of the material under investigation were carefully layered on the sucrose solutions and the tubes were centrifuged in a SW50L rotor for 17h at 130000$g_{av.}$ in a Spinco model L centrifuge. Fractions were collected after centrifugation by puncturing the bottoms of the tubes and allowing the contents to drip into glass vials. The density of the sucrose solution comprising each fraction was deduced from measurements of refractive index. A disc (2.5cm diam.) of Whatman GF/C glass-fibre paper was then inserted into the vial and the solution evaporated to dryness in a vacuum oven. Scintillator solution was added and the radioactivity measured.

Electron microscopy. Solutions containing membrane fractions were placed on grids covered with Formvar (TAAB Laboratories, Reading, Berks., U.K.) and coated with carbon, inverted for 5min on 0.25M-formaldehyde solution containing 1mM-$MgCl_2$ at room temperature, and subsequently stained with uranyl acetate solution (1%, w/v) before examination in a Philips EM200 electron microscope.

Enzyme assays. Alkaline phosphatase (EC 3.1.3.1) of *B. subtilis* was assayed by measuring the rate of hydrolysis of *p*-nitrophenyl phosphate (Sigma) as described by Sterlini & Mandelstam (1969).

Phospholipase A_1 (EC 3.1.1.4) of *E. coli* was assayed by determining the rate of formation of water-soluble lysophosphatidylglycerol from a preparation of lipids extracted from a culture of *E. coli* labelled with [^{14}C]-glycerol. The incubation mixtures contained ^{14}C-labelled lipid (0.05mg/ml) added as a solution of 1mg/ml in light petroleum (b.p. 60–80°C), tris–HCl buffer, pH8.6 (0.03M), $CaCl_2$ (0.05M) and Triton X-100 (Koch–Light Laboratories Ltd.) (0.5mg/ml), in addition to the membrane material under investigation. The mixtures were incubated at 37°C and at intervals samples were removed for lipid extraction by the method of Bligh & Dyer (1959). The aqueous phase was dried on to glass-fibre discs for radioactivity determination.

Reagents. Radioactive compounds were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., supplied sodium *N*-lauroylsarcosinate and phospholipids employed as chromatographic markers. Lysozyme (EC 3.2.1.17) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and deoxyribonuclease (EC 3.1.4.5) from Miles–Seravac Ltd., Maidenhead, Berks., U.K.

RESULTS

Development of the fractionation method

Preliminary experiments. Tremblay *et al.* (1969) showed that membranes (and also free phospholipids) will bind to crystals of magnesium *N*-lauroylsarcosinate. Further investigations of this

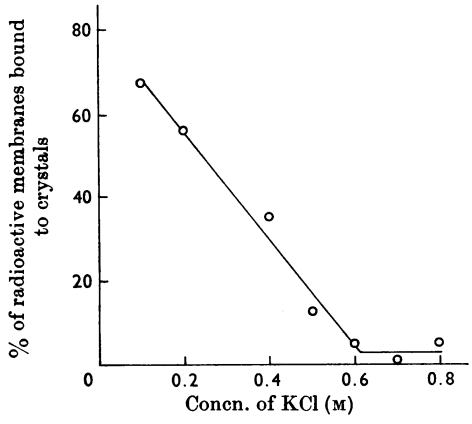


Fig. 1. Effect of potassium chloride on the binding of membranes to crystals of magnesium lauroylsarcosinate. *B. megaterium* membranes (approx. 10μg) labelled with [2-^{3}H]glycerol were suspended in 5ml of 0.01M-tris–HCl buffer, pH7.4, 0.01M-magnesium acetate and KCl at various concentrations. Sodium lauroylsarcosinate was added (final concentration 0.1%, w/v) and the mixture was left at 0°C for 1h. The tubes were then centrifuged at 5°C for 4min at 1000*g* and the radioactivity of the precipitated crystals and of the supernatant fluid was determined.

reaction revealed that the binding is markedly decreased by increasing the salt concentration of the medium. A typical experiment is illustrated in Fig. 1. *B. megaterium* membranes labelled with [2-^{3}H]glycerol were suspended in buffers containing 0.01M-tris–HCl, pH7.4, 0.01M-magnesium acetate, and potassium chloride at various concentrations, and sodium *N*-lauroylsarcosinate was added to give a final concentration of 0.1% (w/v). The mixtures were left at 0°C for 1h to allow the detergent to crystallize and the tubes were then briefly centrifuged. The proportion of the radioactivity bound to the crystals was determined.

One possible interpretation of this finding is that the membrane is composed of a number of easily dissociable 'subunits' with different affinities for the detergent crystals, and to study this possibility an alternative approach was used. Membranes were mixed with detergent crystals at low salt concentrations (i.e. potassium chloride was omitted from the tris–magnesium acetate buffer) so that almost all the material was bound. The crystal–membrane mixture was deposited on a piece of filter paper and a series of solutions of increasing potassium chloride concentration up to 0.5M were applied and allowed to run through the filter. Up to 60% of the membrane material could thus be eluted. The remainder could not be removed by using potassium chloride solutions of higher concentration, but further treatment with solutions of sodium deoxycholate (up to 0.4%) gave almost quantitative elution. Although these effects were always observed the actual degree of binding under given conditions varied considerably between experiments. This variability was ascribed to the unsatisfactory properties of magnesium lauroylsarcosinate. The crystals only form at low temperatures (4°C or less) and melt when the temperature is raised. They also dissolve to some extent when treated with the stronger potassium chloride and deoxycholate solutions. Further, the crystals are in equilibrium with an appreciable concentration of free detergent, which inhibits the binding of membranes.

To overcome these difficulties, magnesium lauroylsarcosinate was replaced by the corresponding cadmium compound, obtained by mixing sodium lauroylsarcosinate solution with cadmium chloride solution. This material was largely free of the characteristics mentioned above and was used for all subsequent experiments.

Fractionation technique. First 0.5ml of 0.05M-sodium *N*-lauroylsarcosinate and 0.5ml of 0.05M-cadmium chloride were mixed, and 0.5ml of a suspension of membranes in water was added, the mixture being allowed to stand for 1h in ice to permit adsorption of the membranes to occur.

The suspension was then deposited on a disc of Whatman no. 41 filter paper clamped in a Seitz filter assembly (internal diam. 2.1cm). Elution of membrane material was achieved by allowing five 4ml portions of each of a number of potassium chloride and sodium deoxycholate solutions in 0.01M-tris–HCl buffer, pH7.4, to pass through the filter. The solutions were used in the following order: potassium chloride, 0, 0.1, 0.2, 0.4, 0.5M; sodium deoxycholate, 0.005, 0.01, 0.05, 0.1, 0.2, 0.4% (w/v). Some 5–10% of the membrane could not usually be eluted and remained bound to the crystals at the end of the experiment. Measurement of radioactivity in the fractions was achieved either by drying a small portion (0.1 or 0.2ml) on a GF/C glass-fibre disc in a glass vial or by adding 0.4ml of 50% (w/v) trichloroacetic acid to the solution and collecting the precipitate on a GF/C disc, for scintillation-counting in a Beckman instrument (Daniels, 1969). When necessary, corrections were made for the effect of the precipitated deoxycholate on the radioactivity counting.

The fractionation patterns obtained, which are discussed in detail below, were unaffected by various modifications to the procedure. For example, the order of addition of the sodium lauroylsarcosinate, cadmium chloride and membrane suspension was unimportant, and the membranes could be disaggregated by ultrasonic oscillation (5min in an MSE 60W instrument) before adsorption if desired. In cases where the radioactivity was confined to the

membrane (i.e. cells labelled with [2-^{3}H]glycerol; Daniels, 1969) it was unnecessary to purify the membranes and the lysate obtained by suspending protoplasts or spheroplasts in water was used without further treatment.

Each experiment used membranes derived from a maximum of 1 mg (dry wt.) of bacterial cells. On one occasion the fractionation was scaled up so that membranes from about 1 g of cells were fractionated on 3.5 g (wet wt.) of cadmium lauroylsarcosinate crystals, but this experiment was rendered extremely tedious by the low flow rate of the eluants.

Physicochemical properties of the membrane fractions

Fractionation pattern. Fig. 2 shows the pattern obtained by fractionating purified membranes prepared from bacteria grown for many generations in the presence of [2-^{3}H]glycerol and [^{35}S]sulphate. Nine components containing both lipid (^{3}H) and protein (^{35}S), but in widely differing ratios, are resolved. The use of additional elution solutions at intermediate concentrations failed to reveal further components. In one experiment [^{14}C]-phenylalanine was used to label protein in place of [^{35}S]sulphate and essentially identical results were obtained.

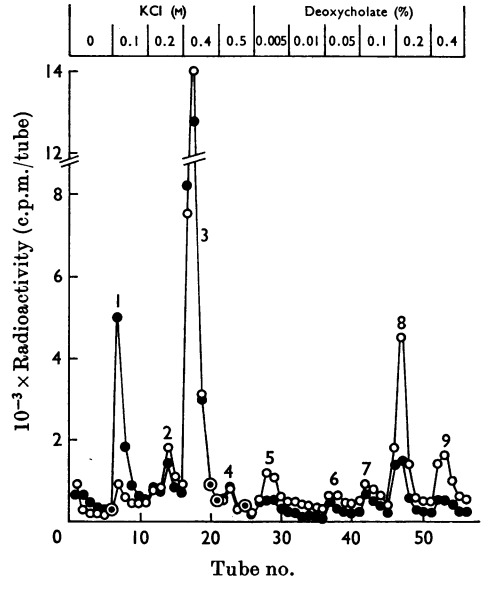


Fig. 2. Fractionation by elution from cadmium lauroylsarcosinate of membranes from *B. megaterium* grown in the presence of [2-^{3}H]glycerol and [^{35}S]sulphate. The concentrations of the elution solutions are indicated above the curve, and the numbering of peaks is also shown. ○, ^{3}H; ●, ^{35}S. Comparison with Fig. 6 illustrates the effect of growth medium on membrane composition.

Density-gradient centrifugation. When samples of the major separated fractions were centrifuged to equilibrium through sucrose density gradients, both lipid and protein labels were found in a single peak. The buoyant density was deduced from refractive-index measurements and the values are shown in Table 1. It can be seen that the density correlates roughly with the protein/lipid (^{35}S/^{3}H) ratio in Fig. 2. Unfractionated membranes disaggregated by ultrasound gave two peaks on centrifugation of density 1.21 and 1.071 g·cm^{-3}. The major component had a density (1.21) greater than any of the separated fractions, suggesting that the fractionation procedure had caused some modification of the structure of the membrane components.

Membrane proteins. ^{35}S-labelled membrane fractions mixed with unlabelled carrier membranes were analysed by polyacrylamide-gel electrophoresis. Fig. 3 shows the major protein bands revealed by staining with Naphthalene Black. When the distribution of radioactivity was studied by radioautography it was found that each of the resolved bands was labelled in all the membrane fractions. However, the relative intensity of labelling of certain bands varied somewhat between fractions.

Lipids. The major lipid species were found in all nine membrane fractions, but in different proportions. Analysis by t.l.c. or paper chromatography after deacylation yielded similar results and Table 2 records values from an experiment of the second type. Phosphatidylethanolamine is the major phospholipid. Particularly noteworthy are the larger amounts of cardiolipin in fraction 6, of phosphatidic acid in fractions 3 and 5 and of phosphatidylglycerol in fractions 4, 5 and 6.

Membrane enzymes. Some difficulty was encountered in finding membrane-bound enzymes of sufficient activity and stability to permit their assay in the membrane fractions. However, preliminary studies have shown that the alkaline phosphatase of *B. subtilis* is confined to fraction 1, although some caution is needed in interpreting this result, since the

Table 1. *Density of membrane fractions*

B. megaterium cells labelled with [2-^{3}H]glycerol were fractionated and 0.4 ml portions of the solutions containing the peaks of fractions 1, 2, 3, 8 and 9 were layered on 4.5 ml linear sucrose density gradients (15–60%, w/v) and centrifuged. The density of each fraction was determined from radioactivity and refractive-index measurements as described in the text.

Membrane fraction no.	1	2	3	8	9
Density (g·cm^{-3})	1.092	1.072	1.106	1.058	1.035

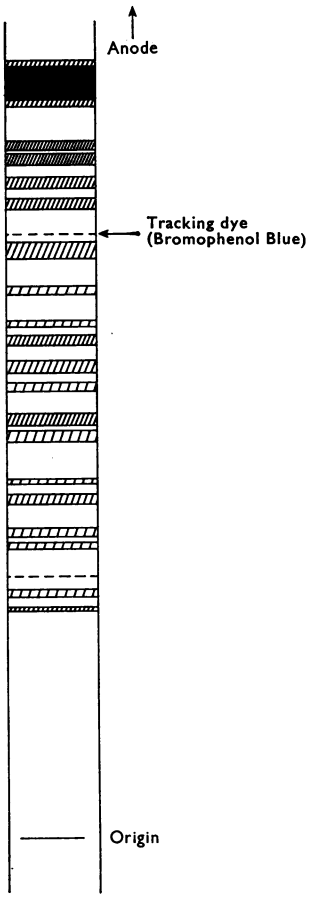


Fig. 3. Polyacrylamide-gel electrophoresis of *B. megaterium* membrane proteins in the presence of 1% (w/v) sodium dodecyl sulphate: sketch of a preparation after staining with Naphthalene Black.

enzyme is solubilized from membranes at potassium chloride concentrations above 0.3M. In addition, the phospholipase A_1 of *E. coli* BB appears to be confined to fraction 5.

Electron microscopy. Material obtained from a large-scale fractionation was examined with an electron microscope after negative staining. The fractions eluted with potassium chloride (fractions 1–5) all presented a similar appearance (Plate 1*a*), being composed of roughly spherical particles about 12nm in diameter. In contrast the later fractions eluted with deoxycholate (fractions 6–9) had an amorphous, aggregated appearance (Plate 1*b*).

Re-fractionation. When membrane fractions were dialysed against 0.01M-tris–HCl buffer, pH7.4), and then re-fractionated with cadmium lauroylsarcosinate crystals, about 20% of the material was recovered in the expected tubes, but the remainder could not be eluted from the crystals. This again suggests that the procedure has caused some change in the structure of the membrane material (cf. 'Density-gradient centrifugation' above).

Metabolic properties of the membrane fractions

Effects of growth conditions. In early experiments considerable variability was apparent in the relative proportions of the membrane fractions, and this led to the finding that the composition of the membrane (in terms of the nine fractions) is markedly influenced by the nature of the growth medium, and by the density at which bacteria are harvested from batch cultures.

The former effect is illustrated by comparing the lipid profiles in Fig. 2 (cells grown in a low-sulphate medium with a synthetic amino acid mixture) and Fig. 6 (cells grown in the normal casein hydrolysate medium).

The changes during the growth cycle are documented in Table 3 as the percentage distribution of the membrane material (as lipid) among the nine fractions. Four samples for membrane preparation and fractionation were taken from a culture at the points indicated on the growth curve in Fig. 4. The striking features are the increase of fractions 1, 5 and 6 and the decrease in fractions 3, 7, 8 and 9 as the culture reaches a higher density.

Radioactive-labelling kinetics. Preliminary experiments showed that fractions 8 and 9 were greatly enriched for pulse-labelled lipid as compared with steady-state labelled material. To analyse this feature the kinetics of labelling of the membrane fractions with radioactive glycerol were examined.

A culture of *B. megaterium* growing in a medium supplemented with 20% (w/v) sucrose was labelled for three generations with [1-^{14}C]glycerol, and [2-^{3}H]glycerol was then added. Six samples were taken over a period of 10min and protoplasts were rapidly prepared in the cold (Schaechter, 1967) and collected by centrifugation. They were lysed by suspension in water and the membranes were fractionated as described above. For the three or four tubes comprising each membrane fraction the $^{3}H/^{14}C$ ratio was calculated and the mean value is plotted in Fig. 5 against time of labelling for each fraction. Since the steady-state label (^{14}C) is proportional to the actual quantity of material, the $^{3}H/^{14}C$ ratio is a measure of the specific radioactivity of the newly made lipid.

It is clear that for short times of labelling the ratio is much higher in fractions 8 and 9, as mentioned above. The curves rise without a lag and begin to level off after 10min.

In contrast the curves for fractions 1, 2, 6 and 7 rise slowly after a lag of about 2min.

Table 2. *Lipid composition of membrane fractions*

Membranes from *B. megaterium* labelled with [1-^{14}C]glycerol were fractionated and lipids were extracted from each fraction, deacylated and subjected to paper chromatography. The radioactivity of each glycerophosphoryl compound was determined, and the percentage composition of each membrane fraction in terms of the parent phospholipids is tabulated. A blank entry indicates that the compound accounted for less than 2% of the radioactivity.

Membrane fraction no.	Phospholipid			
	Cardiolipin	Phosphatidic acid	Phosphatidylethanolamine	Phosphatidylglycerol
1	2	13	85	—
2	3	14	81	2
3	2	29	65	3
4	—	—	77	23
5	—	43	41	16
6	24	—	56	19
7	3	8	85	4
8	2	4	93	—
9	3	16	79	2

Table 3. *Changes in membrane composition during growth*

A culture of *B. megaterium* was labelled for many generations with [1-^{14}C]glycerol (0.1 μCi/ml) and samples were taken for membrane preparation and fractionation at the four points shown on the growth curve in Fig. 4. The radioactivity of each membrane fraction was determined and the table gives the percentage composition of each membrane sample in terms of the nine fractions. (The balance to 100% is accounted for by material retained on the crystals at the end of the fractionation.)

Membrane fraction no. / Sample no. ...	Composition (%)			
	1	2	3	4
1	3.9	6.2	10.2	14.0
2	2.7	3.2	4.7	3.4
3	24.3	25.4	15.6	12.3
4	8.7	8.1	8.2	8.5
5	2.3	3.5	12.6	13.4
6	0.8	0.9	3.5	2.6
7	7.2	5.7	3.6	2.8
8	25.5	26.7	21.5	23.2
9	20.8	15.7	17.2	16.8

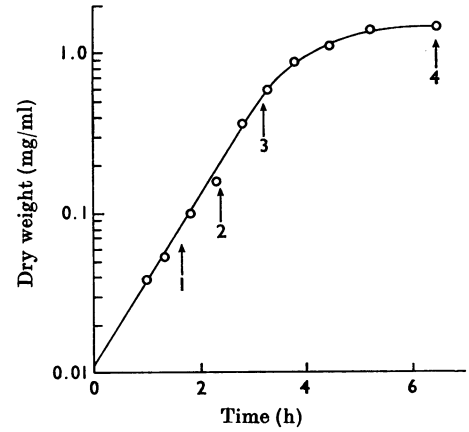
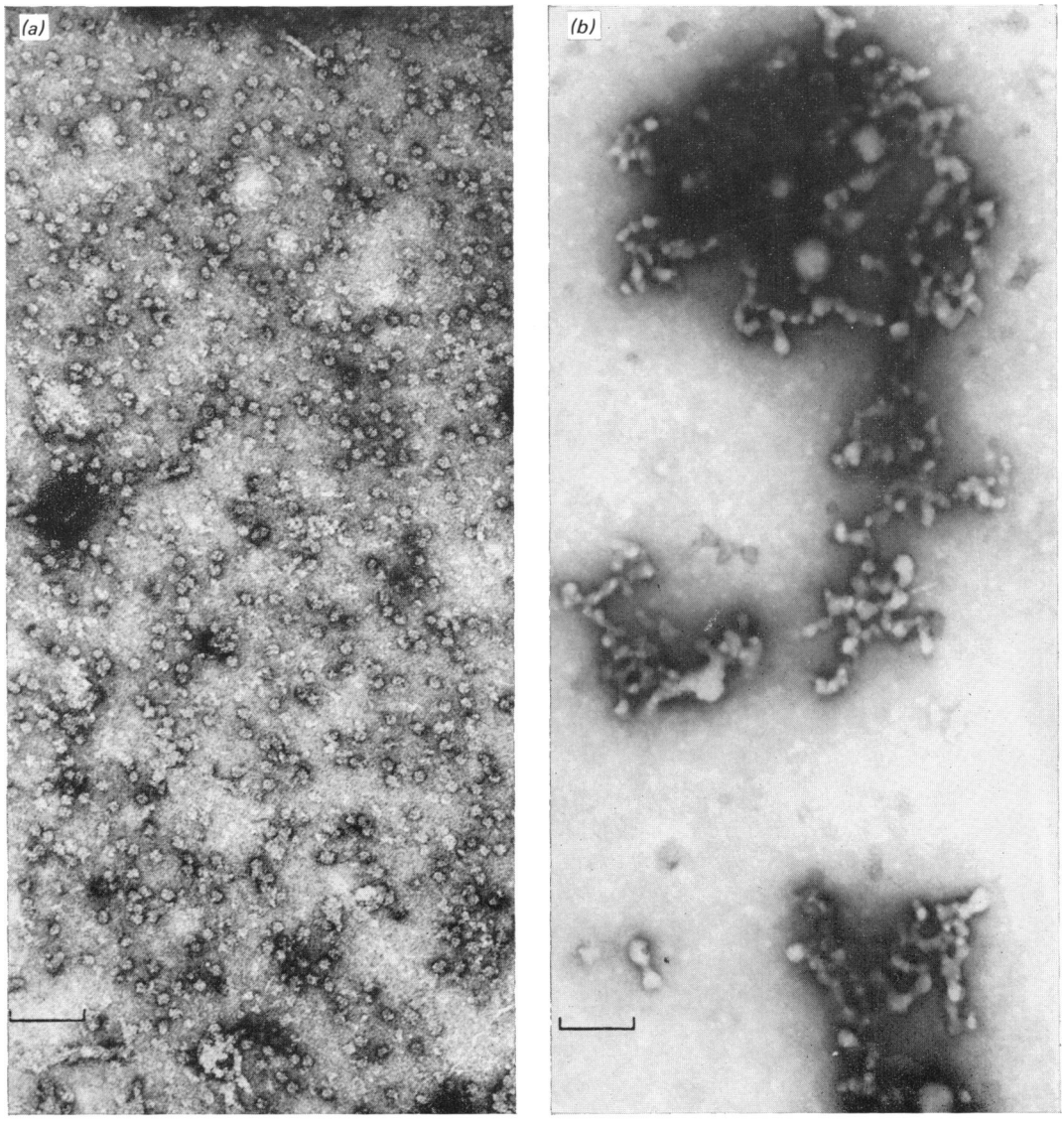


Fig. 4. Growth curve of *B. megaterium*. The cell density was determined by measuring the E_{600} with a Unicam SP. 600 spectrophotometer, the values being converted into mg dry wt./ml by means of calibration curves. Samples were taken for membrane preparation and fractionation at the points shown by the arrows (see also Table 3).

This kinetic behaviour is consistent with a model in which the lipid in fractions 8 and 9 might be a precursor for that in fractions 1, 2, 6 and 7. On the other hand the curves for fractions 3, 4 and 5 suggest that they are labelled independently.

'*Pulse–chase*' *experiments*. In a further set of experiments bacteria were labelled for three generations with [1-^{14}C]glycerol and then transferred to medium lacking radioactive material. When the cell density had doubled [2-^{3}H]glycerol was added, and 3min later further incorporation was stopped by the addition of a large excess of unlabelled glycerol. Samples were taken at the moment of addition of unlabelled glycerol, and 10min and 30min later. Protoplasts were rapidly prepared in the cold, lysed osmotically and the membranes fractionated. The ^{3}H/^{14}C ratio was calculated for each fraction and the values are shown in Table 4. The values for the unfractionated membrane were approximately constant. Some ^{3}H-labelled lipid synthesized during the pulse-labelling period had been lost from fractions 8 and 9 and some had been gained by fractions 1, 6, 7 and perhaps 2, but the remaining fractions showed no significant change.



EXPLANATION OF PLATE 1

Electron micrographs of negatively stained membrane fractions. (*a*) Fraction 3 (typical of fractions 1–5); (*b*) fraction 8 (typical of fractions 6–9). The bars represent 100 nm.

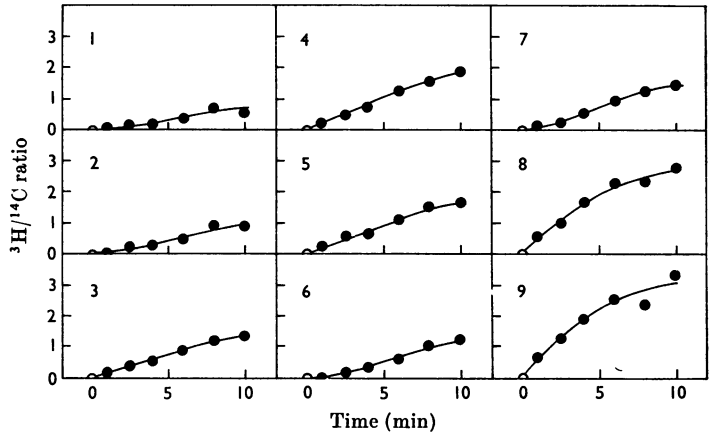


Fig. 5. Kinetics of labelling of membrane fractions with radioactive glycerol. A culture of *B. megaterium* in medium supplemented with 20% (w/v) sucrose was labelled for three generations with [1-^{14}C]glycerol (0.1 μCi/ml). [2-^{3}H]Glycerol was then added at a concentration of 1.5 μCi/ml, and six samples were taken at the times shown for rapid preparation of protoplasts and membranes. The membranes were fractionated and for each of the nine fractions the $^3H/^{14}C$ ratio was calculated in each sample.

Table 4. *Turnover of newly made lipid in the membrane fractions*

A culture of *B. megaterium* was labelled for three generations with [1-^{14}C]glycerol (0.3 μCi/ml) and then transferred to unlabelled medium and allowed to grow until a further doubling had occurred. [2-^{3}H]Glycerol was then added (4 μCi/ml), followed after 3 min by unlabelled glycerol (0.2%, v/v). Samples for membrane preparation and fractionation were taken on addition of the unlabelled glycerol, and 10 min and 30 min later. The mean value of the $^3H/C^{14}$ ratio was calculated for the nine membrane fractions in each case.

Membrane fraction no.	$^3H/^{14}C$ ratio		
	3 min pulse	10 min 'chase'	30 min 'chase'
Whole membrane	2.10	1.85	1.87
1	0.31	1.30	1.57
2	0.95	0.94	1.30
3	1.66	1.86	1.88
4	2.03	1.47	1.87
5	1.72	1.51	1.81
6	0.83	3.00	2.47
7	0.92	4.35	2.81
8	3.57	1.98	2.43
9	3.12	2.52	2.57

It should be mentioned that apart from the membrane lipid no appreciable pool of compounds labelled by glycerol could be detected that might complicate such experiments.

One experiment was also undertaken in which cells were labelled for three generations with [^{3}H]-glycerol, pulse-labelled with [^{35}S]sulphate and subsequently 'chased' with non-radioactive sulphate and cysteine and methionine. The results suggested that the proteins of membrane fractions obey similar precursor–product relationships to the lipids.

Mesosome vesicles. Fitz-James (1968) suggested that mesosome vesicles in Bacilli are the site of lipid synthesis, and in view of the results described above it was desirable to consider the possible relationship of the membrane fractions with the vesicles.

Protoplasts were prepared in media containing magnesium acetate at a range of concentrations between 2.5 and 40 mM, and were examined microscopically to check that lysis was insignificant. They were then collected by centrifugation at 10000*g* for 15 min at 4°C in a Sorvall centrifuge. Membrane material remaining in the supernatant fluid (up to 30% of the total lipid at the lower magnesium concentration) was assumed to be the mesosome vesicles and was recovered by high-speed centrifugation (Reaveley & Rogers, 1969) or the suspension was used directly for fractionation. In Fig. 6 a comparison is made of the fractionation patterns of the protoplast membrane and the vesicles of a typical preparation. The vesicles and the membranes have the same nine components, but in different proportions. I was unable to detect any difference in the ratio of pulse-labelled to steady-state-labelled lipid in the vesicles as compared with the protoplast membrane. D. J. Ellar (personal communication) has similarly been unable to confirm Fitz-James' (1968) finding.

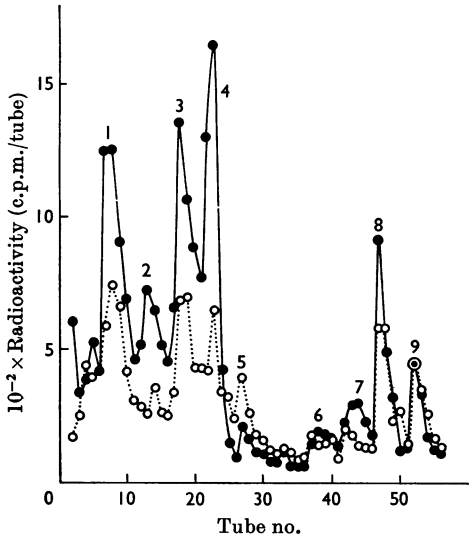


Fig. 6. Comparison of the fractionation patterns of membranes and mesosome vesicles. Protoplasts were prepared from *B. megaterium* cells labelled with [2-^{3}H]-glycerol (magnesium acetate concentration, 10mM). The protoplasts were collected by centrifugation for 15min at 10000*g* and the membranes fractionated. The supernatant fluid contained the mesosome vesicles, which were similarly fractionated. ○, Mesosome vesicles; ●, protoplast membrane.

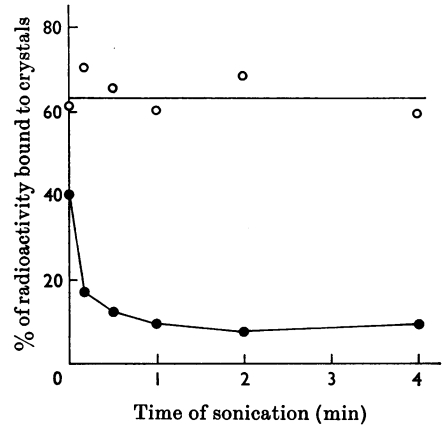


Fig. 7. Effect of ultrasonic oscillation on membrane-bound DNA. Membranes were prepared (without deoxyribonuclease treatment) from *B. megaterium* KMTT grown in the presence of [2-^{14}C]thymine (0.2μCi/ml) and [2-^{3}H]glycerol (1μCi/ml) and were washed six times so that only 5% of the original DNA remained associated with the membranes. They were suspended in TMK buffer and portions were sonicated for various times. Sodium lauroylsarcosinate was added (0.1%, w/v), the tubes were left at 0°C for 1h and centrifuged, and the radioactivity associated with the crystals was determined (cf. Fig. 1). ○, ^{3}H (membrane); ●, ^{14}C (DNA).

Attachment of DNA to the membrane. When deoxyribonuclease treatment was omitted, extensively washed membranes still contained up to 5% of the DNA of the original cell in terms of acid-insoluble [^{14}C]thymine residues. Ultrasonic oscillation did not affect the binding of the membranes to crystals of magnesium lauroylsarcosinate (in the experiment shown in Fig. 7), but removed most of the thymine residues from the membrane. The residue, equivalent to 0.6% of the total cell thymine, was resistant to prolonged sonication and was bound via the membrane to the crystals (Tremblay *et al.* 1969). The material was insoluble in cold 5% (w/v) trichloroacetic acid, was resistant to incubation with 1M-sodium hydroxide at 37°C for 16h, and was largely broken down to acid-soluble material by deoxyribonuclease. It was therefore considered to represent a fraction of the DNA bound to the membrane.

Sonicated membranes labelled with [2-^{3}H]-glycerol and [2-^{14}C]thymine were fractionated and the result shown in Fig. 8 was obtained. Free DNA sheared by ultrasonic oscillation was washed out in tubes 1–5. Almost all the bound DNA was associated with fraction 1, and much smaller quantities with fractions 2 and 3. The remaining six fractions were devoid of ^{14}C radioactivity. Identical results were obtained with *B. subtilis* 168 and *E. coli* BB and 3/62. These bacteria are prototrophic with respect to thymine and labelling of the DNA was achieved by growth with [^{14}C]thymidine and deoxyadenosine (250μg/ml; Boyce & Setlow, 1962). Evidence that the DNA is bound to the membrane fractions and not simply co-eluted from the crystals was obtained from density-gradient centrifugation. Samples of fraction 1 from membranes sonicated but not previously exposed to deoxyribonuclease were centrifuged to equilibrium and two peaks were obtained (Fig. 9), one having a density of 1.09g·cm^{-3} as found above (Table 1) and the other about 1.15g·cm^{-3}. If, however, the material was incubated with deoxyribonuclease (200μg/ml for 20min at room temperature in the presence of 5mM-magnesium acetate) before centrifugation, all banded at a density of 1.09g·cm^{-3}. In cases where the DNA was also labelled it was found associated only with the denser peak or, after deoxyribonuclease treatment, in the form of acid-soluble material at the top of the gradient.

Synchronous cultures. I have shown (Daniels, 1969) that the rate of lipid synthesis increases markedly around the time of cell division. To determine whether this represents a general increase

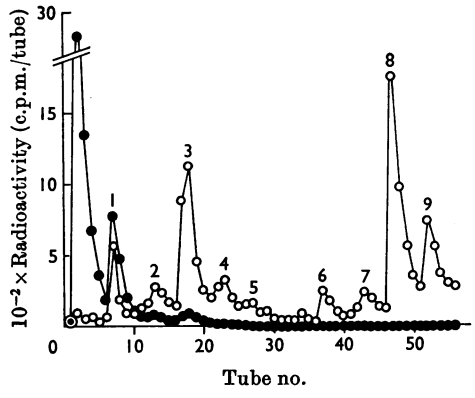


Fig. 8. Attachment of DNA to membrane fractions. Membranes were prepared and washed as described in the legend to Fig. 7 and suspended in 0.01 M-tris–HCl buffer, pH 7.4, and sonicated for 1.5 min. A suspension of cadmium lauroylsarcosinate crystals was added and the membrane fractionated. ○, ^{3}H (membrane); ●, ^{14}C (DNA).

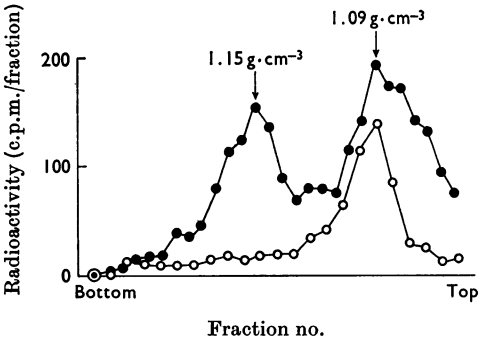


Fig. 9. Effect of attached DNA on the buoyant density of a membrane fraction. [2-^{3}H]Glycerol-labelled membranes were prepared and fractionated as described in the legend to Fig. 8. Two samples of fraction 1 (tube no. 7) were incubated at room temperature for 1 h in the presence of 5 mM-$MgCl_2$, one serving as a control and the other having deoxyribonuclease (250 μg/ml) added. They were then layered on 15–60% (w/v) sucrose density gradients, centrifuged to equilibrium and analysed as described in the text. ●, Control; ○, deoxyribonuclease-treated.

in the rate of membrane synthesis or preferential synthesis of lipid-rich components, *B. megaterium* cells were grown to give steady-state labelling of lipids with [1-^{14}C]glycerol and then synchronized by amino acid starvation (Daniels, 1969). At various points during the two subsequent division cycles, portions of the culture were pulse-labelled for 3 min with [2-^{3}H]glycerol and protoplasts were quickly prepared and the membranes fractionated. No differences in the labelling patterns were observed at any time.

Sporulation. Cultures of *B. subtilis* were labelled for 10 min with [2-^{3}H]glycerol during vegetative growth and at various times up to 5 h after sporulation had been initiated. Fractionation of the membranes failed to demonstrate any differences in the nature of the newly made membrane.

DISCUSSION

I have described a fractionation method that resolves bacterial protoplast membranes into nine lipoprotein fractions. The simplest explanation is that the membrane is composed of a number of such particles held together by non-covalent bonds. Fragments of membrane bind to crystals of magnesium or cadmium lauroylsarcosinate (Tremblay *et al.* 1969), but may then be dissociated into the particles by the small concentration of free detergent in equilibrium with the crystals (thus, prior disaggregation of membranes by sonication does not affect the subsequent fractionation). The particles have characteristic affinities for the crystals: some are primarily bound by forces affected by salt (fractions 1–5), and others by deoxycholate-sensitive forces (fractions 6–9). Thus if the elution with deoxycholate is carried out before that with potassium chloride, then the fractions are recovered in the order 6–9, then 1–5.

It is possible that fractions 1–5 are bound to the crystals by hydrophobic forces, for the work of Reynolds & Tanford (1970) shows that binding of detergents to proteins can under some conditions be influenced by ionic strength. Further characterization of the interaction would require detailed study of the properties of the crystals and the effect of salts on them.

The fractions differ notably in their relative proportions of lipid and protein, and this may be in part responsible for the differential affinity for the crystals, since isolated lipids are bound whereas proteins, in general, are not (Tremblay *et al.* 1969). Gross differences in composition of the fractions in terms of both lipid and protein appear to be quantitative rather than qualitative. However, it must be borne in mind that electrophoresis of proteins in the presence of sodium dodecyl sulphate (which is necessary to solubilize the membranes) is relatively insensitive in resolving species of similar molecular weight. Moreover the preliminary results on the localization of enzymes suggest that there are indeed absolute differences between the fractions, perhaps of proteins present in relatively small

amounts and therefore not detectable in the stained gels.

The membranes of the three bacterial species examined, including membranes made at all stages of the division cycle and at various points during the process of sporulation of *B. subtilis*, had similar qualitative compositions in terms of the nine fractions, suggesting that all are necessary for a fully functional membrane. It would be of great interest to examine the membranes of other types of cell and of mutants defective in some aspects of membrane function.

The observed variations in the relative proportions of the membrane fractions at various stages of growth in batch culture and after growth in different media may be compared with the well documented changes in lipid composition of bacteria under similar conditions (see, e.g., op den Kamp, van Iterson & van Deenen, 1967; Cronan, 1968).

A notable finding is that some of the fractions appear to serve as biosynthetic precursors of others. Since the putative precursor fractions are those with a high lipid content the process of maturation of the membrane may involve the addition of proteins to this material and in this connexion it may be noted that alkaline phosphatase seems to be confined to an 'end-product' fraction.

There is now radioautographic evidence that lipids are inserted into the membrane of *B. megaterium* in a specific region of the cell surface (Morrison & Morowitz, 1970). The present results therefore imply that the membrane differs chemically at various points on the cell surface.

DNA appears to be largely bound to the membrane through fraction 1, and to a smaller extent through fractions 2 and 3. Since both the replication points and the origin of replication of bacterial chromosomes are believed to be membrane-bound (Smith & Hanawalt, 1967; Sueoka & Quinn, 1968) the biochemical role of these membrane fractions in DNA metabolism merits further study. The membrane-bound DNA that is protected from shearing by ultrasonic oscillation represents 0.6% of the total DNA of the cell. If the genome of *B. megaterium* is of a similar size to that of *E. coli* this would represent a length of 6μm, and if the resistance to shearing derives from proximity to a membrane particle of diameter 12nm, multiple points of attachment for each chromosome are indicated. Similar conclusions follow from rough calculations from the buoyant-density results in Fig. 9. If the increased density (about 1.15g·cm^{-3}) is assumed to be caused by the attachment of a rod of DNA of diameter 2nm and density 1.7g·cm^{-3} to a membrane sphere of diameter 12nm and density 1.09g·cm^{-3}, then the piece of DNA would be about 31nm in length. Thus the shear-resistant portion of the DNA of the cell would consist of about 200 such pieces. Although this argument undoubtedly represents a great over-simplification of the true situation it is noteworthy that Rosenberg & Cavalieri (1968) deduced from experiments of a completely different nature that the chromosome of *E. coli* has at least 35 points of attachment to the membrane.

Patterson, Weinstein, Nixon & Gillespie (1970) raised the question of whether the attachment of DNA to the membrane might be an artifact caused by the presence of lysozyme. This can be ruled out because a parallel series of experiments in which cells were broken by sonication in the absence of lysozyme gave identical results.

Some caution should be exercised in attempting to relate the membrane fractions to the structure of the membrane *in vivo* because density determination and re-fractionation experiments suggest that some change of the structure may have occurred. In addition the detergent treatments may denature some proteins. However, the experiments on labelling kinetics, DNA-attachment points and enzyme localization suggest that the method breaks up the membrane in a highly specific manner. It remains to be seen whether it will prove to be useful in analysing the biochemistry of the membrane.

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