Comparison of the Biochemistry and Rates of Synthesis of Mesosomal and Peripheral Membranes in \textit{Bacillus subtilis}\(^1\)

CEPHAS T. PATCH\(^2\) AND OTTO E. LANDMAN

Department of Biology, Georgetown University, Washington, D.C. 20007

Received for publication 22 February 1971

The membrane vesicle (beaded chain) portion of the mesosomes and peripheral (ghost) membrane of \textit{Bacillus subtilis} were obtained by protoplast lysis and separated by differential and sucrose gradient centrifugation. Electron microscopy revealed that both fractions were satisfactorily homogeneous. Comparison of the two membrane preparations showed that they were similar with respect to total protein, total phosphorus, and lipid-soluble phosphorus content. Their protein patterns on acrylamide gel electrophoregrams did not differ significantly. A possible point of distinction was revealed by a difference spectrum analysis of their cytochromes. The two preparations showed clear quantitative differences in all five of the enzyme activities assayed. Acrylamide gel electrophoregrams of peripheral membrane stained for malate dehydrogenase showed four weak isozyme bands, whereas electrophoregrams of mesosome membranes exhibited a single strong peak. (A survey of published data on enzymes in mesosome fractions shows a marked lack of correspondence between different species of bacteria.) Comparison of \(^3\)H-acetate incorporation into the two membrane fractions showed that both were labeled at the same rate. Similarly, \(^{35}\)SO\(_4\) was taken up by both fractions at a comparable rate and was chased from both comparably. Lipid and protein labeling thus indicates that mesosome vesicle membrane is not a precursor or special growing point of peripheral membrane.

The intracytoplasmic membranous structures of the \textit{Bacillaceae}, called mesosomes by Fitz-James (9), were first observed in thin sections of \textit{Bacillus cereus} by Chapman and Hillier (5). In the intervening years, mesosomes have been observed in many gram-positive organisms and in a few gram-negative ones (33). In thin-section electron microscopy of intact bacteria, mesosomes appear as saclike invaginations of the peripheral cytoplasmic membrane. The internal structure of mesosomes appears to consist of a folded chain of membrane-bounded vesicles 40 to 60 nm in diameter. Bacteria placed in hypertonic media extrude their mesosomal vesicles into the periplasmic space (10, 36). When the cell wall is removed by the action of lysozyme in hypertonic media and the resulting protoplasts are examined by negative-staining electron microscopy, mesosomal vesicles appearing as beaded chains are seen to remain attached to the peripheral membrane (12, 14, 34).

The role of mesosomes has been the subject of much study in recent years, but it still remains to be fully elucidated. Functions that have been proposed for mesosomes include: principal sites of respiratory activity (33, 41, 46); the sites of attachment of the chromosomes to membrane (16, 35); sites of attachment of plasmids to the membrane (18); sites of cell wall synthesis (5, 7, 9); sites of incorporation of lipid into membrane (12) and of septation (36); sites of localization of cell-bound penicillinase (40) and other enzymes; and the sites of deoxyribonucleic acid penetration in transformation (45). The experimental evidence concerning these proposed functions was obtained from a variety of organisms. Some of the data are based on morphological studies of intact cells and others are derived from experiments with isolated mesosomes and protoplast ghosts. However, overriding the whole question of mesosomal function is the observation of Landman et al. (21, 37) that 75% of the cells in \textit{B. subtilis} cultures growing exponentially on gelatin medium are devoid of mesosomes yet appear to be fully functional. These authors concluded

---

\(^1\) This work was carried out by C.T.P. in partial fulfillment of the requirements of Georgetown University for the degree of Doctor of Philosophy.

\(^2\) Present address: Laboratory of Biology of Viruses, NIAID, National Institutes of Health, Bethesda, Md. 20014.
that, whatever functions mesosomes may perform in normal cells, these functions are either not essential to cell survival or can be carried out by peripheral membrane without a mesosome structure.

Fractions enriched for the beaded chain portion of mesosomes have been isolated from several gram-positive organisms and compared with peripheral membrane by a variety of techniques (8, 11, 12, 15, 27–29, 31). In the present work, membrane from mesosome vesicles of *B. subtilis*, released by osmotic shock, is compared with peripheral membrane by electron microscopy and by a number of enzymological, chemical, and physical criteria. In addition, the rate of tracer incorporation into these two membrane fractions is compared to ascertain whether mesosome vesicle membranes function as growing points for peripheral membrane.

**MATERIALS AND METHODS**

**Organisms.** Two strains of *B. subtilis* were used: *B. subtilis* 168 trp*–* and a nonflagellated strain, *B. subtilis* 108 trp mot*–* (43). Both were maintained as spores on Nutrient Agar (Oxoid) slants.

**Buffers.** MP buffer consisted of 0.1 M potassium phosphate and 0.001 M MgCl₂, pH 7.2. MPS buffer consisted of MP buffer made 0.6 M in sucrose. TG buffer contained 0.025 M tris(hydroxymethyl)aminomethane and 0.2 M glycine, pH 8.3.

**Growth of cells.** Cultures from which membranes were to be prepared were started from an inoculum grown overnight in Brain Heart Infusion broth (Difco). The cells were spun down, suspended in minimal medium (48) to a concentration of about 2 × 10⁷/ml, and grown to a concentration of about 2 × 10⁹/ml. Cultures were maintained in 1-liter volumes in Fernbach flasks on a rotary shaker at 37°C.

**Viability assays.** Bacteria were diluted in 0.85% saline and plated on low-tapecy Blood Agar Base (Difco) plates. Protoplasts were diluted in high-tapecy dilution fluid, plated on high-tapecy soft-agar plates, incubated at 30°C, and scored as L colonies (20). For comparisons of viability before and after protoplasting, high-tapecy media were used for both bacteria and protoplasts.

**Protoplasting.** Bacteria were harvested in late-log phase by centrifugation at 5,000 × g. The cells were washed once in MPS buffer and resuspended in one-tenth the original volume in MPS. Lysozyme was then added to a concentration of 500 µg/ml, and the suspension was incubated statically for 30 min at 33°C. Additional lysozyme was added to give a final concentration of 1,000 µg/ml, and incubation was continued for 30 min. Completion of protoplasting was observed by phase microscopy. Viability assays showed that fewer than 0.01% of the cells were capable of forming colonies on low-tapecy media.

**Density of sucrose gradient fractions.** In each experiment, sucrose solutions of known density were prepared and their refractive indexes were determined in an Abbe 3L refractometer. The refractive indexes of membrane-containing fractions of the sucrose density gradients were then measured and their densities read from the standard curve.

**Electron microscopy.** Membrane fractions were pelleted from MP buffer, suspended in either distilled water or 1% ammonium acetate, negatively stained with 1% phosphotungstic acid (4), and examined immediately in an RCA EMU-3G electron microscope at 100 kV with the use of a 50-µm objective aperture.

**Dry weight and chemical analyses.** Samples of membrane suspensions in 2 to 3 ml of MP buffer were dialyzed for 48 hr against four 1-liter volumes of distilled water. A sample of dialyzed membrane was dried to constant weight in a vacuum desiccator at 75°C, and other samples were assayed for protein (23) and for total phosphorus (19). A fourth sample was extracted three times with chloroform-methanol, 2:1 (13), and the extracts were assayed for lipid-extractable phosphorus (19).

**Difference spectra.** Membranes were dissolved in TG buffer containing 0.5% deoxycholate. Cytocromes were reduced by adding sodium hydroxide to a final concentration of 1 mg/ml. The spectrum of reduced membrane was read against air-oxidized membrane in a Beckman DB recording spectrophotometer.

**Gel electrophoresis.** Gel electrophoresis was carried out by the method of Davis (6) with equipment manufactured by Buchler Instruments, with 7.5% acrylamide gels and TG buffer. Electrophoresis was for 2 hr at 2°C with 2.5 mA/gel. Parallel electrophoregrams from a run were stained for protein (6), for nicotinamide adenine dinucleotide-dependent oxidative enzymes (2), for reduced nicotinamide adenine dinucleotide dehydrogenase (3), and for adenosine triphosphatase (1). Densitometric measurements of the stained gels were made on a Beckman Analytrol instrument equipped to read acrylamide gels. This instrument records stain density as a curve and also provides numerical data for the area under this curve. This area was assumed to be proportional, respectively, to protein concentration or to enzymatic activity.

**Radioactive tracer experiments.** 3H-sodium acetate (300 mCi/mmol) was purchased from International Chemical and Nuclear Corp., and carrier-free 35S-sulfuric acid was purchased from New England Nuclear Corp. Radioactive counting was carried out in a TriCarb scintillation counter (Packard Instrument Co.). Samples of labeled membranes were taken up in TG buffer containing 0.5% deoxycholate, and 0.1 to 0.2 ml was added to a scintillation fluid consisting of one-third volume of Triton X 100, two-thirds volume of toluene, and containing 0.55% 2,5-diphenyloxazole (PPO) and 0.0125% 1,4-bis-(4-methyl-5-phenyloxazoly)-benzene (dimethyl POPOP).

**RESULTS**

Several investigators have reported that mesosome material is released from several *Bacillus* species concurrently with cell wall removal by lysozyme (8, 27, 40). However, it has also been observed that mesosomes often remain attached to protoplasts as long, beaded chain appendages (12, 14, 33, 34). Depending on the medium,
more efficient liberation of mesosome vesicles may be accomplished by subjecting protoplasts to osmotic lysis (12, 15).

Preparation of membrane fractions: density and electron microscopic morphology of fractions. Originally it was our aim to collect mesosomal vesicles from the supernatant fluid of fully viable protoplast suspensions and to compare these "pure" mesosome-vesicle preparations with preparations of peripheral membrane from the protoplast pellet. Using strain 168, we found that a small mesosome vesicle fraction devoid of ghost membranes could indeed be obtained from the protoplasting supernatant fluid (see "Mesosomes" in Fig. 1, Scheme A); however, protoplast viability was only 70 to 80%. Consequently, one could not be certain that the vesicle pellet was truly detached from intact viable cells. Further, this pellet was heavily contaminated with flagellae even after banding in sucrose gradients (Fig. 3B and 3C). In attempting the same approach with the flagellless strain 108, we obtained a marked improvement in the viability of the protoplasts, close to 100%, but virtually no mesosome vesicles could be recovered from the protoplasting supernatants.

After these unsuccessful attempts to obtain a spontaneously detached mesosome vesicle fraction, we developed a procedure for recovery of such a fraction from lysed protoplasts. The yield of mesosome vesicles from this procedure was about three times that obtainable from protoplasting supernatant fluids of strain 168 (see Fig. 1, Scheme B).

Cells were grown, concentrated, and protoplasted in MPS buffer as described. The protoplasts were pelleted at 9,000 × g for 10 min and suspended in one-tenth volume of MPS buffer. Then nine volumes of distilled water were added rapidly to lyse the protoplasts. The viscous lysate was treated with 30 μg of deoxyribonuclease per ml at 37 C for 1 hr. The large ghost membranes were then pelleted at 9,000 × g for 30 min. The supernatant fluid was again centrifuged, first at 9,000 × g for 30 min to remove any remaining ghosts, and then at 105,000 × g for 60 min to collect the mesosome vesicle fraction.

Both the protoplast ghost fraction and the mesosome fraction were purified further by sucrose gradient centrifugation. The membrane pellets were suspended in 1 to 2 ml of MP buffer, layered onto gradients of 30 to 60% sucrose in MP buffer, and centrifuged at 90,000 × g in a Spinco SW 25.2 rotor at 4 C.

The distribution in sucrose gradients of membrane fractions from 3H-acetate-labeled cells is shown in Fig. 2. Figure 2A shows the distribution of a protoplast ghost preparation after 90 min of centrifugation at 90,000 × g. In this experiment, the density of the membrane preparation was about 1.18 g/cm³. (In the various experiments, equilibrium densities ranged from 1.16 to 1.18 g/cm³. However, in a given experiment, equilibrium densities were always the same in all fractions.) A small pellet consisting mainly of intact bacteria and partially protoplasted cells was discarded. The material in the radioactive band of density 1.18 g/cm³ consisted predominantly of protoplast ghosts. Figures 3A and 4A show electron micrographs of negatively stained ghosts purified in sucrose gradients. One sees mainly large membranes devoid of the characteristic mesosome structures.

Figure 2B shows the distribution of the labeled mesosome vesicle fraction after 90 min of centrifugation at 90,000 × g. At that time, a small band had formed in the 1.18 g/cm³ density region, but a larger amount of material remained near the top of the gradient. We call the lower band "rapidly sedimenting mesosomal vesicles" (RM) and the upper band "slowly sedimenting mesosome vesicles" (SM; Fig 1). Figure 2C shows the distribution of the same original mesosome vesicle fraction after 20 hr of centrifugation. Now all of the labeled membrane appears in a single band at equilibrium density; the RM and SM fractions have coalesced.

Electron micrographs of negatively stained preparations of such fractions are shown in Fig. 3 and 4. Comparison of purified RM fraction (Fig. 3B and 4B) with purified SM fractions (Fig. 3C, 3D and 4C) reveal no striking differences. The characteristic small vesicles of 40- to 60-nm diameter and short chains of vesicles appear in both types of preparation. Furthermore, there are no significant morphological differences between the mesosome vesicle preparations derived from protoplast supernatants (Fig. 3B and 3C) and those recovered from lysed protoplasts (Fig. 3D, 4B, and 4C). Few or no contaminating ghosts were seen in either types of preparation.

Although the electron microscope revealed no differences between RM and SM mesosome vesicles, these two fractions were examined separately in most experiments. The bands from the 90-min, 90,000 × g centrifugation were collected and banded again for 16 to 20 hr in a 30 to 60% sucrose gradient in a Spinco SW39 rotor at 135,000 × g. The peripheral membrane fraction was similarly purified. This second sucrose gradient centrifugation was included to insure improved separation of membrane material from particulates of different density (e.g., ribosomes). In all cases, the membrane fractions gave single, sharp, symmetrical bands in the equilibrium density range after the 16- to 20-hr centrifugation.
Fig. 1. Schematic diagram for the fractionation of membrane from Bacillus subtilis. Discard fraction (*) was pooled with the protoplast ghost fraction prior to banding to obtain dry weight composition data.
Difference spectra of membranes. Figure 5 shows difference spectra obtained with peripheral membrane and RM and SM mesosomal membrane fractions of strain SB108. All three spectra are qualitatively quite similar. All show absorption peaks at 425, 442, 523, and 555 nm. The only qualitative difference is that the spectrum of SM membranes does not show the faint maximum at 598 nm which was detected in the other two preparations. The spectra accord well with those reported earlier for B. subtilis by Smith (42), except that a peak at 564 nm was not seen in our experiments.

Quantitative scrutiny of the spectra discloses that the trough at 420 nm is distinctly less shallow in the peripheral membrane spectrum. This suggests that this preparation is richer in cytochrome c than the mesosomal membranes. A small amount of 410-nm-absorbing material was detected in the protoplast supernatant fluid, indicating that some membrane-associated cytochrome c was solubilized.

Acrylamide gel electrophoresis study of membrane proteins and enzymes. Acrylamide gel electrophoresis is a sensitive method for the characterization of protein patterns in membranes (31) and for the separation and detection (by staining) of enzymes. Peripheral membrane and SM mesosomal membrane fractions were dissolved in TG buffer containing 0.5% sodium deoxycholate and subjected to gel electrophoresis as described. (0.1% Sodium dodecyl sulfate was an equally good membrane solvent, but gels did not stain as well for enzymes, suggesting that partial denaturation occurred.) It was observed that about 25% of the protein did not migrate far into the gel, possibly because it consisted of particles of large size. Parallel gel tubes from a given run were stained, respectively, for protein and for various enzymes.

Densitometric tracings of electrophoreograms of the two membrane preparations stained for protein are shown in Fig. 6. In these gels it is possible to identify approximately 14 proteins. The relative mobilities of these are indicated in the abscissa of Fig. 6. The great similarity of the two protein patterns is clearly evident. Still, in some areas, notably peaks 2, 3, 4, 8, 12, and 13, there appear to be differences between the two tracings. These differences are apparently not significant, however, since the same peaks are mutually comparable in electrophoreograms made with different membrane preparations. (Repeat electrophoreograms were run at approximately fourfold higher protein concentrations.)

Following the guidance of earlier research (25, 44, 47), electrophoreograms were surveyed for the
FIG. 3. Membranes from Bacillus subtilis 168. All preparations were banded twice in sucrose gradients and concentrated before negative staining. A, Peripheral membrane. B, Mesosome vesicles collected from protoplast supernatant—rapidly sedimenting vesicles (RM). C, Mesosome vesicles from protoplast supernatant—slowly sedimenting vesicles (SM). D, Mesosome material released during protoplast lysis—SM fraction. Note that all mesosome preparations are contaminated with flagellae. Scale indicates 0.2 μm in all micrographs.
Fig. 4. Membranes from Bacillus subtilis SB 108. All preparations were banded twice in sucrose gradients and concentrated before negative staining. A, Peripheral membrane. B, Mesosome material released during protoplast lysis: RM fraction. C, SM fraction. Scale indicates 0.2 μm in all cases.

presence of several enzymes (Table 2). It is evident from the last column of Table 2 that there are quantitative differences between peripheral and mesosomal membrane for each one of the five enzymes assayed. Substantial differences were reproduced consistently in repeat experiments. For example, a malate dehydrogenase tracing made with a different set of membrane
preparations shows a single peak of specific activity 16.0 for the mesosome preparation and three isozyme peaks in the peripheral membrane preparation with specific activities of 1.8 (mobility 0.66), 0.58 (mobility 0.59), and 0.98 (mobility 0.33). In the case of malate dehydrogenase, the enzyme differences between mesosomal and peripheral membranes may be considered qualitative. They are strikingly displayed in the Analytrol tracings (Fig. 7).

Relationship of mesosomal membrane to peripheral membrane. The apparent involvement of mesosomes with septum formation (5, 7, 29) and their postulated function in the segregation of chromosomes (16, 33, 35) suggest that mesosomal membrane might grow more rapidly than peripheral membrane. Further, the possibility has been raised that membranes might be formed only at the mesosomes and might be extended from there out over the peripheral cell surface. To explore these questions, we compared the incorporation of tracers into peripheral and mesosomal membrane fractions in pulse and pulse-chase experiments. \(^3\)H-acetate was used principally for following lipid synthesis (22), and \(^35\)S-sulfate was used for following protein synthesis.

Strain SB108 was grown in 1 liter of Spizizen medium with acetate substituted for glucose. When the cells were in late log phase they were pelleted and resuspended to one-tenth volume in the same medium, but lacking acetate, to which 10 mCi of \(^3\)H-acetate was added. Samples were taken after 5, 10, and 20 min of incubation. Incorporation was stopped by making the samples 0.5 M in nonradioactive acetate and 0.025 M in cyanide. Each sample was proteolyzed, lysed, and fractionated in sucrose density gradients, and the membranes were analyzed for protein, for radioactivity, and for lipid-extractable radioactivity (Table 3). The most important result is, of course, that there is no difference in specific radioactivity between peripheral membrane and the mesosomal fractions. This holds true after 5, 10, and 20 min of incubation, a period...
VOL. 107, 1971  MESOSOMAL AND PERIPHERAL MEMBRANES IN B. SUBTILIS  353

**Table 2. Mobilities and activities of membrane enzymes from Bacillus subtilis SB108 in acrylamide gels**

<table>
<thead>
<tr>
<th>Enzyme†</th>
<th>Stain band</th>
<th>Peripheral membrane</th>
<th>SM mesosomal membrane</th>
<th>Ratio‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein per gel (µg)</td>
<td>Specific activity</td>
<td>Mobility</td>
<td>Protein per gel (µg)</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>252</td>
<td>0.42</td>
<td>2.78</td>
<td>250</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>225</td>
<td>0.19</td>
<td>7.12</td>
<td>232</td>
</tr>
<tr>
<td>ATPase</td>
<td>225</td>
<td>0.96</td>
<td>4.90</td>
<td>232</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>1</td>
<td>250</td>
<td>0.65</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.57</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.53</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>0.34</td>
<td>1.80</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>249</td>
<td>—</td>
<td>—</td>
<td>120 ‡</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0.87</td>
<td>7.10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>0.58</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>0.55</td>
<td>5.62</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>0.37</td>
<td>3.21</td>
</tr>
</tbody>
</table>

† Attempts to stain electrophoregrams for nicotinamide adenine dinucleotide-dependent citrate dehydrogenase indicated absence of this enzyme. Abbreviations: ATPase, adenosine triphosphatase; NADH, reduced nicotinamide adenine dinucleotide.

‡ Specific activities are given in arbitrary units calculated by dividing units of density (of formazan deposits) measured with a Beckman Analysys instrument by micrograms of protein per gel.

§ Specific activity of peripheral membrane/specific activity of mesosomal membrane.

¶ The ratio becomes 0.3 when data from all peripheral bands are summed.

†† Considerable activity was present but too near the lipid band for resolution.

 Resolution was improved at the lower protein concentration.

The results of a pulse-chase experiments with $^{35}$SO₄²⁻ label are shown in Table 4. For this experiment, B. subtilis SB108 was grown to late-log phase in 2 liters of Spizizen medium lacking casein hydrolysate but containing all nonsulfur amino acids at a concentration of 20 µg/ml each. In this medium, the doubling time for B. subtilis is about 120 min. The cells were pelleted, washed once, and resuspended in the same medium, but lacking sulfate, to which 10 mCi of $^{35}$SO₄²⁻ was added. The suspension was incubated for an additional 30 min at 37 C with shaking. The culture was then divided equally and pelleted immediately. The pulse culture was suspended in MPS buffer with 0.01 M KCN, chilled, and set aside. The chase culture was suspended in Spizizen
medium with cold sulfate and casein hydrolysate and returned to the incubator. The chase was stopped at 90 min from the beginning of the pulse. Both cell preparations were now washed four times with 200 ml of MPS, protoplasted, and fractionated as usual; the membranes were then assayed for protein and radioactivity.

The $^{35}$SO$_4$" experiment showed (Table 4) that membrane protein synthesis followed the same pattern as membrane lipid synthesis. Again label incorporation was about the same in peripheral membrane as in the mesosomal fractions and, further, there was no evidence of movement of label from mesosomal into peripheral membrane (or vice versa) during the "chase" incubation. Peripheral and mesosomal membrane labeling appeared, as before, to be mutually independent and fairly similar. The specific radioactivity of peripheral membrane was slightly higher; however, this might simply be due to a higher content of sulfur-rich proteins in these membranes.

**DISCUSSION**

The aim of this research was to explore the similarities and differences between mesosomal and peripheral membrane and, if possible, to make an improved assessment of the role of mesosomes on the basis of our findings.

In the light of these objectives, the separation and clear identification of the two types of membrane is a matter of primary importance. Our belief that we achieved a significant separation and identification is based on three considerations. (i) Our electron microscopic examination of both peripheral membrane and mesosome vesicle fractions indicated that both fractions were substantially homogeneous. (ii) After water-shocking of the protoplasts, the vesicle fractions were separated from the ghost fractions after only a brief delay and in a low Mg$^{2+}$ environment. Under these circumstances there was little chance for self-assembly of large membranes from small fragments (17, 32). On the other hand, it must be assumed that some peripheral membrane fragments of mesosome vesicle size contaminate our mesosome vesicle fractions. (iii) Perhaps the best evidence of a significant separation of membrane fractions is the internal

### Table 3. Specific radioactivity of peripheral and mesosomal membrane fractions after pulses of $^{3}$H-acetate

<table>
<thead>
<tr>
<th>Incubation in $^{3}$H-acetate (min)</th>
<th>Membrane fraction</th>
<th>Total protein (mg)</th>
<th>Total radio-activity$^a$</th>
<th>Specific activity$^a$</th>
<th>Lipid-soluble radioactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Peripheral</td>
<td>1.28</td>
<td>18.5</td>
<td>14.4</td>
<td>87.4</td>
</tr>
<tr>
<td></td>
<td>RM-mesosomal</td>
<td>0.22</td>
<td>3.1</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SM-mesosomal</td>
<td>0.64</td>
<td>8.7</td>
<td>13.6</td>
<td>97.8</td>
</tr>
<tr>
<td>10</td>
<td>Peripheral</td>
<td>1.22</td>
<td>39.9</td>
<td>32.7</td>
<td>81.9</td>
</tr>
<tr>
<td></td>
<td>RM-mesosomal</td>
<td>0.42</td>
<td>11.1</td>
<td>26.4</td>
<td>93.7</td>
</tr>
<tr>
<td></td>
<td>SM-mesosomal</td>
<td>0.51</td>
<td>20.2</td>
<td>39.6</td>
<td>97.0</td>
</tr>
<tr>
<td>20</td>
<td>Peripheral</td>
<td>1.09</td>
<td>131.3</td>
<td>120</td>
<td>77.8</td>
</tr>
<tr>
<td></td>
<td>RM-mesosomal</td>
<td>0.22</td>
<td>22.2</td>
<td>102</td>
<td>90.7</td>
</tr>
<tr>
<td></td>
<td>SM-mesosomal</td>
<td>0.72</td>
<td>72.2</td>
<td>100</td>
<td>87.4</td>
</tr>
</tbody>
</table>

$^a$ Expressed as counts per minute when figures shown are multiplied times 10$^4$.

### Table 4. Specific radioactivity of peripheral and mesosomal membrane fractions after a pulse and a pulse-and-chase with $^{35}$SO$_4$" label

<table>
<thead>
<tr>
<th>Incubation in $^{35}$SO$_4$&quot; and cold media</th>
<th>Membrane fraction</th>
<th>Total protein (mg)</th>
<th>Total radio-activity$^a$</th>
<th>Specific activity$^a$</th>
<th>Relative specific activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse for 0.25 generation</td>
<td>Peripheral</td>
<td>4.75</td>
<td>34.80</td>
<td>73.5</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>RM mesosomal</td>
<td>1.02</td>
<td>6.14</td>
<td>60.2</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>SM mesosomal</td>
<td>1.20</td>
<td>7.66</td>
<td>63.8</td>
<td>0.87</td>
</tr>
<tr>
<td>Pulse followed by a chase for approximately 0.5 generation</td>
<td>Peripheral</td>
<td>6.28</td>
<td>35.00</td>
<td>55.6</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>RM mesosomal</td>
<td>2.88</td>
<td>13.20</td>
<td>45.8</td>
<td>0.82</td>
</tr>
</tbody>
</table>

$^a$ Expressed as counts per minute when figures shown are multiplied times 10$^4$.

$^a$ Peripheral membrane = 1.00.
evidence showing that our fractions had divergent properties—especially that they differed in their isozyme patterns (Table 2).

Although the foregoing considerations provide reasonable assurance that a separation of *B. subtilis* membranes into two fractions was achieved by our methods, the present development of fractionation procedures is probably only partly effective in discriminating between the peripheral membrane and mesosomes. In particular, present methods yield only two membrane fractions where three fractions (mesosome vesicles, peripheral membrane, and mesosome sacs) are probably required for clear discrimination between mesosomal functions and peripheral membrane functions. The mesosome sacs presumably constitute a systemic contaminant of all available ghost preparations because, so far as is known, the mesosome sacs evaginate during protoplasting and become part of the peripheral membrane (33). Such interesting postulated mesosome features as the chromosome attachment site are thus expected to remain with the ghost fraction. Other possible mesosome-localized functions, such as the respiratory function, might well be partitioned between mesosome vesicles and mesosome sacs (41) and, if so, our fractionation procedure would not effect an informative separation.

Conceding the limitations of the existing fractionation procedures, our comparison of peripheral and mesosomal membranes, in conjunction with other such comparisons (8, 11, 12, 15, 27–29), nevertheless permits several tentative conclusions. (i) In gross chemical composition and in regard to principal protein constituents, mesosome vesicle membrane and peripheral membrane are quite similar. (ii) However, comparative analysis for individual enzyme activities often reveals quantitative differences and, occasionally, qualitative differences. (iii) With respect to the mode of formation of the two membrane types, most data indicate that they are of mutually independent origin, i.e., that mesosome vesicle membrane is not a precursor of peripheral membrane.

There is no clearcut agreement between different laboratories concerning gross chemical composition (item i). The results of Reavely (27) and of Ghosh and Murray (15) are in essential agreement with ours in showing that the ratio of lipids to protein is about the same in the two membrane types. By contrast, Fitz-James (11) and Ellar (J. Gen. Microbiol. 1969. 57:6–8; 58:7) report that their mesosome fractions contain relatively higher amounts of lipid. It is not profitable to dwell on these differences, since the analytical data vary quite markedly, depending on the species of bacteria used (39), the growth medium (39), the presence of nucleases, the Mg++ concentration (28), the use of detergents or other reagents during membrane isolation (12), and even on the method of protein analysis (15, 38).

A comparison by gel electrophoresis of the protein patterns of mesosomal and peripheral membrane fractions comparable to ours was made by Reavely (27). His findings were essentially the same: considerable overall similarity in the electrophoregrams with possible differences in some regions.

Differences between mesosomal and peripheral membranes in specific activity of different enzymes have been found in all cases where pertinent experiments were done (8, 15, 28, 29). A survey of such published data for different bacterial species reveals a seemingly chaotic picture, however. For example, the ratios of specific activity of mesosome membrane to specific activity of peripheral membrane for "succinic dehydrogenase" (reduction of various reagents in presence of succinate) in different organisms were reported as follows: *B. licheniformis*, 0.056 (29); *Micrococcus lysodeikticus*, 0.031 (29); *B. megaterium*, 0.06 (29); *Listeria monocytogenes*, 1.80 (15); *B. subtilis*, 0.47 (Table 2). Similarly, the ratios for reduced nicotinamide adenine dinucleotide dehydrogenase ranged from 0.48 (29) to 3.50 (15), and those for "malate dehydrogenase" ranged from 0.44 (29) to 3.30 (Table 2). Undoubtedly these results can be reconciled by attributing the difference to isozymes (Table 2), variations in membrane fractionation and enzyme assay procedures, and differences between strains. Nevertheless, it seems dubious that these inchoate data conceal an orderly distribution of enzymatic roles between mesosome vesicles and peripheral membrane. We have been skeptical of the proposition that mesosomes perform a definite, consistent enzymatic role in *B. subtilis* ever since we noted that cultures of this bacterium grow at normal exponential rates on gelatin media where three-fourths of the cells are devoid of mesosomes (21). The same conclusion—that mesosomes are metabolically not important—is suggested by the observation that deoxyribonucleic acid, ribonucleic acid, and protein synthesis as well as respiration continue undisturbed both during cell wall removal (with its attendant erosion and partial loss of mesosomes) and for several hours thereafter (25; C. Patch, Ph.D. Thesis, Georgetown University, 1968; Anraku and Landman, *unpublished data*; and Landman and Ryter, submitted for publication).

Regardless of whether mesosome vesicles contain metabolically functioning or nonfunctional
enzyme aggregates, the question remains whether they serve as precursor for peripheral membrane. A precursor-product relationship was postulated by Fitz-James (12) when, in short-term incubations of \( B. \) megaterium with \(^{14}C\)-acetate, he found a higher specific activity in the lipids of his slower-sedimenting fraction (mesosomes) than in the lipids of the denser fraction (ghosts?). Our experiments do not support such a postulate for \( B. \) subtilis. In both lipid-labeling experiments with \(^{3}H\)-acetate pulse and \(^{35}S\)-protein-labeling pulse and pulse-chase experiments, the specific activities of mesosome vesicle and peripheral membrane fractions were quite similar (Tables 3 and 4). Evidently, both lipids and proteins of the two membrane fractions were labeling at the same rate and independently of one another. Experiments of a similar nature were also performed by Mindich (personal communication) and by Morrison and Morowitz (26). Neither laboratory found evidence for the movement of label from mesosomes to peripheral membrane. Using an amino acid label, Fitz-James also was unable to document movement of labeled protein from the mesosomal membrane fraction to the denser fraction (11).

We conclude that the presently available data on the enzymatic activities or the labeling of mesosome vesicle proteins and lipids do not provide a substantial clue to the role of mesosomes. Since evolution has preserved these organelles in several bacterial genera, one may assume that further research will ultimately disclose their true role.

ACKNOWLEDGMENTS

We thank Roger Cole, NIH, for making the electron micrographs.

This investigation was supported by Public Health Service grant AI 05972 from the National Institute of Allergy and Infectious Diseases and by grant GB 7204 from the National Science Foundation.

LITERATURE CITED