The Occurrence of Bactoprenol in the Mesosome and Plasma Membranes of Lactobacillus casei and Lactobacillus plantarum

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(Accepted for publication 1 October 1971)

SUMMARY

About one-third of the C₅₅ isoprenoid alcohol, bactoprenol, in Lactobacillus casei and L. plantarum cells was in the mesosomes and two-thirds in the plasma membrane. After removal of bound lysozyme from the plasma membrane, the concentration of bactoprenol per mg protein was the same in the mesosomes and in the plasma membrane. Pulse-labelling experiments and chasing with unlabelled precursor showed that bactoprenol is synthesized in both the plasma and mesosome membranes and that mesosomal bactoprenol is not a precursor of plasma membrane bactoprenol. These findings are discussed in relation to the involvement of bactoprenol in cell-wall synthesis.

INTRODUCTION

Bactoprenol, a C₅₅ isoprenoid alcohol synthesized from mevalonic acid (Thorne & Kodicek, 1966), is located in the mesosomes (Thorne & Barker, 1969) and the plasma membrane (Barker & Thorne, 1970) of lactobacilli. Reappraisal of its mass spectrum shows that it does not, as originally believed, contain any saturated residues. It is therefore identical with the C₅₅ isoprenoid alcohol which has been implicated in cell envelope biosynthesis (Higashi, Strominger & Sweeley, 1967; Wright, Dankert, Fennessey & Robbins, 1967; Scher, Lennarz & Sweeley, 1968; Troy, Frerman & Heath, 1971; Watkinson, Hussey & Baddiley, 1971). If it functions as a carrier of cell envelope material through the membrane its location in the cell is important. For instance, if a greater concentration were present in the mesosomes than in the plasma membrane this would be evidence to support the contention (Chapman & Hillier, 1953; Ellar, Lundgren & Slepecky, 1967; Kakefuda, Holden & Utech, 1967; Higgins & Shockman, 1970a) that mesosomes are involved in cell-wall synthesis.

Testing such ideas with regard to lactobacilli is now possible as we have devised a method for preparing protoplasts under conditions which permits mesosomes to be separately and simultaneously prepared. It involves the sequential treatment of lactobacillus cells with ethylenediaminetetraacetate (EDTA) and then with lysozyme in the presence of Mg²⁺. Controlled release of the mesosomes is achieved by changing the Mg²⁺ concentration (Reaveley & Rogers, 1969). Bactoprenol content and rates of biosynthesis in mesosomes and plasma membrane have been assayed and the results are discussed. A preliminary account of this work has appeared elsewhere (Thorne & Barker, 1971).

METHODS

Growth of bacteria. Lactobacillus casei (ATCC 7469) and L. plantarum (ATCC 8014) were grown for 16 h at 37°, to a density of 0.3 to 0.8 mg dry wt/ml, in 100 ml of acetate-free medium (Thorne & Kodicek, 1962a) to which 1 μCi, 0.165 μmole of [2-¹⁴C]mevalonic acid
(Radiochemical Centre, Amersham, Buckinghamshire) was added, unless otherwise stated. Organisms were harvested by centrifuging at 1500g for 15 min and washed once with 0.9% (w/v) saline.

Preparation of protoplasts and mesosomes. Washed organisms were incubated with 25 ml 0.2 mM-EDTA in 0.12 M-tris-HCl buffer pH 7.6 for 10 min at 37°, harvested and resuspended in 25 ml 5 mM-sodium phosphate buffer, pH 7.0, containing 5 mg lysozyme (Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex) and 0.6 M-sucrose. The suspension was incubated at 37° for about 45 to 90 min until protoplast formation, as observed by phase-contrast microscopy, was complete. Protoplasts were collected by centrifuging at 20,000g for 30 min. The supernatant fraction was centrifuged for a further 2 h at 100,000g for preparation of a mesosome pellet. Plasma membrane was prepared from the protoplasts after the addition of 25 ml water. It was collected by centrifuging at 38,000g for 30 min and was washed once with 25 ml water. It was then either dissolved in 4 ml 0.2% sodium lauryl sulphate for assay of its bactoprenol and protein content, or further purified by gel filtration through Sepharose 2B (2.8 x 23 cm.) in water (Thorne & Barker, 1969).

Bactoprenol determination. Bactoprenol is synthesized by lactobacilli from [14C]mevalonic acid (Thorne & Kodicek, 1962b; 1966). For its determination bacterial fractions were saponified, and the unsaponifiable lipids were extracted and chromatographed on thin layers of silica gel G with 10% (v/v) ethyl acetate in n-heptane. The amount of bactoprenol present, RF 0.34, was determined from its radioactivity.

Chemical analyses. Protein was measured by the Folin–Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine plasma albumin as standard. Hexose was measured with anthrone and sulphuric acid by the method of Loewus (1952) using glucose as standard. Methyl pentoses were assayed by the method of Gibbons (1955) using rhamnose as a standard. Organic phosphate was determined by the method of Fiske & Subba Row (1925) after digestion with perchloric acid.

ATPase assay. ATPase was assayed by measuring the amount of inorganic phosphate produced after incubation for 15 min at 37° with 5 μmoles Na₄ATP and 2.5 μmoles MgCl₂ in 0.8 ml 0.05 M-imidazole-HCl buffer, pH 6.6.

Electron microscopy of thin-sectioned material. Samples of cells, protoplasts and membranes were fixed by one of three methods.

(i) Pelleted samples were suspended in 6-25% (w/v) glutaraldehyde in Kellenberger’s buffer (Kellenberger, Ryter & Sëchaud, 1958) for at least 1.5 h before being washed in Kellenberger’s buffer pH 6 for 1 to 20 h. Postfixation was by 1% (w/v) OsO₄ in Kellenberger’s buffer pH 6 for 12 to 16 h at 4°. As sodium veronal reacts with some aldehydes to produce a substance with no buffering capacity within the physiological range of pH values, this method is theoretically undesirable due to this buffer/fixed incompatibility. Practically, this method proved satisfactory for EDTA/lysozyme treated bacteria and protoplasts but was not satisfactory for untreated bacteria.

(ii) Kellenberger standard fixation modified: 1% (w/v) OsO₄ in Kellenberger’s buffer pH 6 was added to the bacterial culture or sample, pH 5, 0.1 ml/ml culture. The cells were centrifuged at 1800g for 5 min. The pellet was resuspended in 1.0 ml Kellenberger’s fixative pH 6 and 0.1 ml peptone (1 g Bactopeptone, 0.5 NaCl in 100 ml distilled water) for 16 h. at 20 ± 2°. Kellenberger’s fixative pH 6 (8 ml) was added and the cells centrifuged at 1800g for 5 min. The pellet was resuspended in 0.2 ml agar (3% w/v) at 45° and transferred to a microscope slide and allowed to cool before being cut into 1 mm³ cubes. Slight variations of this procedure, particularly in temperature, were used depending on the conditions prevailing in the samples and their ease of handling.
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(iii) Osmium tetroxide, 0.1 g, dissolved in the medium at pH 5 or suspending solution was added to preparations of bacteria or sphaeroplasts at 37° to a final concentration of 1% (w/v) and allowed to stand for 30 min at room temperature. The sample was centrifuged at 1800g for 5 min or faster at 20,000g. The pellet was resuspended in 1% (w/v) osmium tetroxide in Kellenberger's buffer pH 6 for 16 h at room temperature, 20 ± 2°, and centrifuged at 1800g for 5 min. The pellet was cut into 1 mm.9

No detectable difference in fixation was observed between methods (ii) and (iii) and therefore the simpler method (iii) was most frequently used.

After fixation all the samples were washed in Kellenberger's buffer for 1 h with three changes. The block was stained in 0.5% uranyl acetate in Kellenberger's buffer for 30 min. It was dehydrated through a graded series of ethanol or acetone and given two soaks in propylene oxide for 30 min. Depending on the sample, the specimens were passed through a series of immersions in 25/75, 50/50, 75/25 and 100% propylene oxide/Araldite mixtures without accelerator for three days. Finally the material was blocked and embedded in Araldite (CY 212 13.5 ml, HY 964 11.5 ml) (Ciba Limited, Cambridge) dibutyl phthalate 1.0 ml, and DMP-30 or BDMA 0.5 ml (Shell Chemical Co. Ltd, Sittingbourne, Kent). Sections were cut on Reichert OMU-2 or Cambridge Huxley ultramicrotomes. Silver and gold sections were mounted on coated grids, and stained with uranyl acetate and lead (Millonig, 1961).

Electron microscopy of negative-stained specimens. Specimens were examined at various dilutions from 1/1 to 1/20 using 2% ammonium acetate in distilled water. Two methods of staining and spreading were used.

(i) Samples were mixed with an equal volume of negative stain, drop by drop on a microscope slide; 483 grids (Smethurst, Highlight Limited, Bolton, Lancashire) coated with collodion plus carbon or formvar plus carbon were touched on to the surface of the mixed drop. Excess fluid was immediately removed by touching the side of the adhering drop with a filter paper. The grid was allowed to dry under a naked bulb light.

(ii) Samples were dropped on to the surface of a collodion or formvar/carbon-coated 483 grid. Excess fluid was pulled off by touching the side of the drop with a filter paper. The negative stain was then dropped on to the grid and allowed to remain for several seconds before excess was removed in the same way. The grid was then allowed to dry. We experimented with the nine commonly used negative stains before selecting three for routine use. In this study all samples were subjected to three negative stains: 2% (w/v) potassium phosphotungstate (KPT) at pH 6.5 to 8.0, 0.5% (w/v) uranyl acetate (UA), pH 6.5 to 3.5, and 2% (w/v) ammonium molybdate, pH 5.5 to 7.5. Lithium tungstate 1% (w/v) at pH 6.0 to 8.0 was also used occasionally.

As membranes with a high lipid content are subject to various artifacts due to interaction with negative stains, it is advisable that at least three negative stains should be used for each sample and that proper controls should be used wherever possible when membranes subjected to negative stain are fixed and sectioned. Ammonium molybdate is known to have a similar osmotic effect to 0.32 M-sucrose. Two per cent (w/v) KPT tended to cause extensive disruption of membrane in some preparations. Ammonium molybdate usually gave the most satisfactory results judged by the close correlation between the sectioned and negative-stained material. Preparations fixed with 6.25% (w/v) glutaraldehyde were also examined by negative staining. The material was examined in an AEI EM6B electron microscope with decontaminator, at instrumental magnifications of × 5000 to 100,000.
Fig. 1. Thin section of two cells of *Lactobacillus casei* showing the thick outer cell wall (ow) and middle cell wall (mw) extending into the area cross wall formation (cw). The darkly staining inner cell wall (iw) is seen in the peripheral region but not in the septum. The cell membrane (mpm) is closely applied to the wall and cross-wall, but also extends into and surrounds the mesosome (m) boundary. The tubular-vesicular (tv) elements of the mesosome also display a prominent darkly stained membrane. Fixation method (iii).

Fig. 2. After treatment with EDTA followed by treatment with lysozyme in the presence of sucrose and 10 mM-MgCl₂ protoplasts (p) with attached tubular-vesicular (tv) elements of mesosomes are formed. The tubular-vesicular elements also attach to the cell wall ghosts (cwg). Fixation method (i).
RESULTS AND DISCUSSION

Preparation of protoplasts. Cells of Lactobacillus casei and L. plantarum, treated with 0.2 mM-EDTA in 0.12 M-tris-HCl, were completely converted to protoplasts by lysozyme in 0.6 M-sucrose after 45 to 90 min. Protoplasts were only formed from organisms taken from cultures which had reached a density of at least 0.3 mg dry weight/ml, and were growing linearly.

Organisms treated with EDTA released some protein (2% of the cell dry weight) and traces of hexose (0.1%), methyl pentose (0.1%) and organic phosphorus (0.1%). Disc electrophoresis on polyacrylamide gels in sodium lauryl sulphate (Weber & Osborn, 1969) showed that the protein was heterogeneous with the main component having a molecular weight of about 30,000. Two of the minor components, one of molecular weight about 15,000 and the other only just entering the gel, stained for glycoprotein (Zacharius, Zell, Morrison & Woodlock, 1969). Proteins have been described on the surface of Staphylococcus aureus (James & Brewer, 1968) and Bacillus polymyxa (Nermut & Murray, 1967). No release of small molecular weight dialysable material with extinction at 260 nm was detected, although this was observed in Gram-negative bacteria (Wilkinson, 1967).

Preparation of mesosomes. The observation that mesosomal vesicles are detached from newly formed protoplasts when the Mg\(^{2+}\) concentration is below a certain critical level was used by Reaveley & Rogers (1969) in the preparation of mesosomes from Bacillus subtilis and B. licheniformis. When protoplasts of Lactobacillus casei and L. plantarum were made in the absence of MgCl\(_2\), the amount of mesosome released, measured as protein, was 4-5 mg protein per 100 mg dry weight of cell, while in the presence of 10 mM-MgCl\(_2\) it was only 1-5 mg protein per 100 mg dry weight of cell. Lactobacilli therefore resemble B. subtilis strain MARBURG where mesosomal vesicles remain attached to protoplasts in 10 mM-Mg\(^{2+}\) (van Iterson & op den Kamp, 1969) and not B. licheniformis 6346 where concentrations greater than 20 mM-Mg\(^{2+}\) are necessary to retain mesosomal adherence (Reaveley & Rogers, 1969).

The mesosomal identity of the material released from protoplasts, made in the absence of MgCl\(_2\), was demonstrated by electron microscopy. Fig. 1 shows a thin section of a cell of Lactobacillus casei with prominent mesosomes located at the site of cross-wall formation. Fig. 2 shows a protoplast of L. casei made in the presence of 10 mM-MgCl\(_2\), with mesosomal tubular-vesicular elements attached to the protoplast and to the cell-wall ghosts which persist in protoplast preparations from L. casei (Barker & Thorne, 1970). Fig. 3 shows a protoplast made in the absence of MgCl\(_2\), with the mesosomal tubular-vesicular elements detached. The liberated mesosomes were collected as a pellet by centrifuging at 100,000g for 2 h. They had the appearance shown in Fig. 4a when negatively stained and in Fig. 4b when sectioned. Sometimes tubules were also seen in the sectioned and negatively stained material (Fig. 4a, b). In all preparations the diameter of the tubules is 15 to 20 nm. It is possible that these tubules originate from the other tubules, which are also 15 to 20 nm in diameter, sometimes seen connecting vesicles to the protoplast membrane (Fig. 5). In agreement with the observations of Nanninga (1968) and Burdett & Rogers (1970) negatively stained mesosomal membrane had a smooth surface, while plasma membrane had surface protrusions (Fig. 6), possibly ATPases (Munoz, Freer, Ellar & Salton, 1968). The ATPase activity (μmoles ATP hydrolysed/min) was 0.1 in the mesosomes and 2.7 in the plasma membrane of L. casei and 0.1 in the mesosomes and 2.3 in the plasma membrane of Lactobacillus plantarum per 100 mg dry weight of cells, in agreement with the results of Ellar, Thomas & Postgate (1971). One explanation of the absence of ATPase from
Fig. 3. After treatment with EDTA followed by treatment with lysozyme in absence of MgCl₂, the tubular-vesicular elements (tv) of the mesosome are detached from the emerging protoplasts (p). The outer cell-wall layer (ow) becomes split off from the middle dense layer (mw). Cross-walls (cw) also become detached. Fixation method (i).

Fig. 4. Liberated mesosome tubular-vesicular elements from protoplasts made in the absence of MgCl₂. (a) Negatively stained with ammonium molybdate, pH 6.8, ×60,000. The width of the tubules (15 to 20 nm) is identical to those in Fig. 3 and (b). (b) Thin sectioned tubular-vesicular elements of the mesosomes. Fixation method (i).
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Fig. 5. Emerging protoplast with vesicles (v) attached to the plasma membrane (mpm) by a tubular element (t) both presumably of mesosome origin. The size of vesicle and width of tubule (15 to 20 nm) is consistent with the sizes recorded in pelleted liberated mesosomes tubular-vesicular elements Fig. 4a and b. Fixation method (iii).

Fig. 6. Protoplast plasma membrane (mpm) with stalked particles and surface protrusions similar to those found in inner mitochondrial membranes of eukaryotic cells. These particles have been correlated with ATPase activity. Negatively stained with ammonium molybdate, pH 6.8.
mesosomes may be that the concentration of Mg\(^{2+}\) in the mesosomal region of the cell is too low for ATPase to be bound to the membrane (Abrams, 1965). Hughes, Stow, Hancock & Baddiley (1971) have suggested that a locally high Mg\(^{2+}\) concentration may be maintained in the region of the plasma membrane by the cell wall–membrane system of teichoic acids.

Table 1 shows that 33% of the bactoprenol content was released from *Lactobacillus casei* and 32% from *L. plantarum* in the mesosomal fraction when protoplasts were made in the absence of MgCl\(_2\). When the MgCl\(_2\) concentration was increased to bind the mesosomes to the protoplasts the bactoprenol also remained bound.

**Bactoprenol content of mesosome and plasma membrane.** The bactoprenol contents of the plasma and mesosome membrane of *Lactobacillus casei* and *L. plantarum* are shown in Table 2. About one-third of the bactoprenol was found in the mesosomes and two-thirds in the mesosome-depleted protoplasts. The concentration of bactoprenol per mg protein in the mesosomes was measured using a pellet obtained by centrifuging at 100,000 *g* for 2 h. Only about half the mesosomal membrane can be recovered by this procedure (Thorne & Barker, 1969) even if the sucrose is first removed by dialysis against 5 mM-tris-HCl and 1 mM-MgCl\(_2\). The concentration of bactoprenol was measured in bacteria grown with saturating levels of mevalonic acid of 5 mg/100 ml (Thorne & Kocikek, 1962b). It was higher in the mesosomes of *L. casei* than of *L. plantarum*. This could be only an apparent difference explainable by endogenous synthesis of mevalonic acid in *L. plantarum*.

### Table 1. Release of bactoprenol during protoplast formation

<table>
<thead>
<tr>
<th>MgCl(_2) in protoplasting medium (mM)</th>
<th>Bactoprenol released from</th>
<th>Lactobacillus casei</th>
<th>Lactobacillus plantarum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>0</td>
<td>33</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.8</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.8</td>
<td>6.4</td>
<td></td>
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</table>

### Table 2. The bactoprenol content of plasma and mesosome membranes

<table>
<thead>
<tr>
<th></th>
<th>Bactoprenol distribution (%)</th>
<th>Protein (mg/100 mg cell dry wt)</th>
<th>Bactoprenol concentration (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus casei</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Released mesosomes</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100,000g pellet</td>
<td>17</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed membrane</td>
<td>57</td>
<td>11.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Membrane purified by gel filtration</td>
<td>18</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Released mesosomes</td>
<td>33.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100,000g pellet</td>
<td>19</td>
<td>2.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Protoplasts</td>
<td>62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed membrane</td>
<td>50</td>
<td>11.8</td>
<td>0.55</td>
</tr>
<tr>
<td>Membrane purified by gel filtration</td>
<td>25</td>
<td>2.8</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Plasma membrane was prepared from mesosome-depleted protoplasts disrupted in water. The membrane was washed once with water. The concentration of bactoprenol in plasma membrane seemed to be only half that of the concentration in mesosomes. It was discovered that lysozyme was binding to the plasma membrane or cell wall ghosts present in the preparation. The yield of protein in the plasma membrane fraction, but not in the mesosome fraction, was proportional to the amount of lysozyme added. Gel filtration through Sepharose 2B to remove the lysozyme (Thorne & Barker, 1969) while giving a poor yield, produced a membrane with as high a concentration of bactoprenol as that found in mesosomes.

Table 3. Effect of hypotonic medium used for disrupting protoplasts of Lactobacillus casei on bactoprenol content of plasma membrane

Protoplasts were prepared with and without MgCl₂ as described in Methods and were collected by centrifuging at 20,000 g for 30 min. The protoplasts were disrupted with 25 ml water or 0·1 M-tris-HCl pH 7·4 and centrifuged at 38,000 g for 30 min. The membrane pellet was washed with 25 ml water or 0·1 M-tris-HCl pH 7·4 and centrifuged again at 38,000 g for 30 min. The bactoprenol content was measured in the supernatant fractions from whole protoplasts, disrupted protoplasts and washed membrane and in the final membrane pellet.

<table>
<thead>
<tr>
<th>Percentage of cellular bactoprenol</th>
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<tr>
<td>Released from protoplasts</td>
</tr>
<tr>
<td>Protoplasts prepared without MgCl₂</td>
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<tr>
<td>Protoplasts prepared with 10 mM-MgCl₂</td>
</tr>
<tr>
<td>Protoplasts prepared with 10 mM-MgCl₂</td>
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</table>

Table 4. Biosynthesis of bactoprenol in plasma and mesosomal membrane

<table>
<thead>
<tr>
<th>Final cell concentration (mg dry wt/ml)</th>
<th>Total bactoprenol (d.p.m.)</th>
<th>Bactoprenol concentration (d.p.m./mg protein*)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mesosome membrane (d.p.m.)</td>
<td>Plasma membrane (d.p.m.)</td>
</tr>
<tr>
<td>Lactobacillus casei grown with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[¹⁴C]MVA for 10 min (5 μCi in 100 ml)</td>
<td>0·38</td>
<td>368</td>
</tr>
<tr>
<td>[¹⁴C]MVA overnight (0·4 μCi in 40 ml)</td>
<td>0·52</td>
<td>18,500</td>
</tr>
<tr>
<td>[¹⁴C]MVA overnight, then [¹³C]MVA for 1 h (10 mg in 40 ml)</td>
<td>0·74</td>
<td>15,300</td>
</tr>
<tr>
<td>L. plantarum grown with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[¹⁴C]MVA for 10 min (5 μCi in 100 ml)</td>
<td>0·50</td>
<td>501</td>
</tr>
<tr>
<td>[¹⁴C]MVA overnight (0·4 μCi in 40 ml)</td>
<td>0·35</td>
<td>2,450</td>
</tr>
<tr>
<td>[¹⁴C]MVA overnight, then [¹³C]MVA for 1 h (20 mg in 40 ml)</td>
<td>0·44</td>
<td>2,640</td>
</tr>
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</table>

* Protein corrected for bound lysozyme. MVA, mevalonic acid.
Table 3 shows the effect of disrupting protoplasts with water and with 0.1 M-Tris-HCl. Little bactoprenol was lost from protoplasts made in the absence of MgCl₂, either in 0.1 M-Tris-HCl or water. When protoplasts, made in 10 mM-MgCl₂ to retain the mesosomes, were disrupted and washed with 0.1 M-Tris-HCl they lost nearly half their bactoprenol, but with water they lost little. We have previously observed the opposite effect in protoplasts made in the simultaneous presence of lysozyme, trypsin and EDTA (Barker & Thorne, 1970). Half the bactoprenol was lost when these protoplasts were disrupted with water and none when they were disrupted with 0.1 M-Tris-HCl. It is evident that protoplasts of the same organism when prepared by different methods respond differently to hypotonic medium.

**Bactoprenol and cell-wall biosynthesis.** The possibility that only the mesosomal bactoprenol was functioning in cell-wall biosynthesis was investigated. The lipid intermediate in peptidoglycan synthesis was assayed in membranes from *Lactobacillus plantarum* grown with [³H]2,6-diaminopimelic acid (1 μCi/ml) and [²⁵⁴C]mevalonic acid (10 nCi/ml). Samples of the membranes were chromatographed on thin layers of silica gel G in isobutyric acid-1.0 N ammonia (5:3, v/v). The lipid intermediate of peptidoglycan synthesis, containing both [³H] and [¹⁴C], had an *Rf* of 0.6 to 0.7 (Dietrich, Colucci & Strominger, 1967). It represented 8.4% of the total bactoprenol content of the mesosome membrane and 8.7% of the bactoprenol content of the plasma membrane. It was evident therefore, that both mesosome and plasma membrane were equally active in lipid intermediate formation.

In Gram-positive bacteria new cell wall is synthesized equatorially at the site of septum formation in cocci (Cole & Hahn, 1962; Briles & Tomasz, 1970; Higgins & Shockman, 1970a). Hughes & Stokes (1971) observed several sites of new cell-wall synthesis in bacilli. The uniform distribution of bactoprenol in both plasma and mesosome membranes which we have found may be explained in three ways. (i) In cocci the process of cross-wall formation leads to peripheral wall synthesis and cell elongation. In rods of *Lactobacillus* the synthesis of cross-walls and peripheral walls may be separate processes. Peripheral walls are of uniform thickness and are thicker and denser than cross-walls and polar walls (Fig. 1). Separation of the daughter cells only starts when septum formation is complete. (ii) If cell-wall synthesizing activity is in the equatorial region, where large mesosomes are found, it may simply reflect the high amount of membrane which occurs here (Rogers, 1970). (iii) The potential for cell-wall synthesis may occur over the whole surface. Wall thickening occurs in cocci over a large area of the cell both during normal cell growth and when growth is inhibited (Giesbrecht & Ruska, 1968; Higgins & Shockman, 1970b).

**Biosynthesis of bactoprenol in plasma and mesosome membranes.** It was suggested by FitzJames (1968) that mesosomes are the site of membrane lipid synthesis and that mesosome membrane lipid is the precursor of plasma membrane lipid. If this were true for bactoprenol it would be synthesized in the mesosomes and would occur in the plasma membrane only fortuitously as a result of being transferred together with the rest of the membrane lipid. This was tested by studying the synthesis of bactoprenol from [²³⁴C]mevalonic acid (Table 4). If the site of synthesis of bactoprenol is mesosomal it would be expected that [¹⁴C]mevalonic acid would go initially into the mesosome fractions. However, after a 10 min incubation with [¹⁴C]mevalonic acid the pattern of labelling of the mesosome and plasma membrane bactoprenol was the same as after overnight incubation, showing that, within the limits of the present procedure, no preferential incorporation into the mesosomes was detected. It also proved impossible to chase radioactive bactoprenol, labelled by growing the bacteria overnight with [¹⁴C]mevalonic acid, from the mesosomes into the plasma membrane with unlabelled mevalonic acid. We conclude that the synthesis of bactoprenol occurs in both plasma and mesosome membranes. Ellar et al. (1971) showed that
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phospholipid synthesis from $^{32}$Pphosphate and $^{14}$Cacetate did not occur preferentially in the mesosomes of Micrococcus lysodeikticus and Bacillus megaterium. Daniels (1971) found no difference between mesosome and plasma membrane lipids of B. megaterium pulse labelled and steady state labelled from $^{3}$Hglycerol.

We are grateful to Dr E. Kodicek for his interest and advice. We thank Miss L. S. Swales, Mr K. R. Symonds and Mr K. C. Day for their skilful technical assistance.

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