The extracellular release of mycobacillin from Bacillus subtilis first occurred in the medium at the onset of stationary phase and continued at a high rate even after 6 days. Mycobacillin synthetase activity appeared earlier than late-exponential phase in the cytosol of producer cells and was not sedimentable even at 105000 g. The activity then quickly reached the maximum late in the stationary phase. With further increase in the age of the culture, the activity gradually disappeared from the cytosol to reappear concomitantly in the membrane in an insoluble particulate form, even in absence of protein synthesis. The membrane-bound synthetase activity was sedimentable at 10000 g and was fairly active even after 5 days.
INTRODUCTION

The biosynthesis of the antibiotic peptides gramicidin S and tyrocidin produced by different strains of *Bacillus brevis* has been elucidated by several groups (36, 172-174). It was observed that the synthesizing enzyme system, which happened to be present in the soluble supernatant in the early stage of growth, changed from a soluble to a membrane-bound form with the age of the culture in the case of tyrocidin (25), gramicidin S (22-24) and bacitracin (26), whereas in case of polymyxin (27, 41) the reverse phenomenon occurred. However, in case of edeine, the synthesizing polyenzyme system has been shown to be associated with a membrane-DNA complex (28) in the post exponential phase cells of *Bacillus brevis* Vm 4.

Mycobacillin synthesizing enzyme system has been shown to be present in the soluble supernatant of the producer *Bacillus subtilis* B3 during the early phase of growth (134). The synthesizing enzyme, mycobacillin synthetase, has been purified and appears to be a polyenzyme system that resolves itself into three fractions, which cannot carry out the synthesis unless added together (170). We therefore decided to study the localisation of the three-fraction enzyme in relation to the age of the culture.

MATERIALS AND METHODS

**Chemicals and radiochemicals**

Lysozyme from egg white, ATP and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). U$^{14}$C-labelled...
Chlorella protein hydrolysate (specific radioactivity 42 mCi/mg atom of C) was purchased from Bhabha Atomic Research Centre (Trombay, India). Other chemicals used were from commercial sources.

**Strain, media and growth condition**

*Bacillus subtilis* B3 producing mycobacillin was grown in a shaking incubator at pH 7.2 and at 30 ± 1°C in nutrient broth as reported in previous chapter.

**Measurement of growth**

The growth under agitation was monitored by measurement of $A_{660}$ in a photoelectric colorimeter.

**Microbial assay**

Extracellular and intracellular mycobacillin production under agitation was assayed by a cup-plate method against *Aspergillus niger* G3Br (143, 175). The concentration of the antibiotic was determined from the standard curve of mycobacillin.

**Preparation and fractionation of sonic extract**

A homogenized washed cell suspension of *B. subtilis* in 50 mM Tris/HCl buffer was sonicated (176). Portions of the sonicated extract were then separately centrifuged at 10000 g for 30 min in Sorval RC-5B refrigerated superspeed centrifuge and also at 105000 g for 90 min in ultracentrifuge below 5°C. The pellet and supernatant at each step were assayed for enzyme activity. For characterisation, the 10000 g pellet as obtained from 80 h
old cells was suspended in buffer and centrifuged first at 3000 g and then at 10000 g to give a heavy and a light pellet respectively.

**Purification of 10000 g light pellet by sucrose density gradient**

The 10000 g light pellet in Tris-HCl buffer was layered (5 ml) on top of a three-step sucrose-density-gradient column consisting of 7 ml of 60% (w/v) sucrose overlaid with 7 ml of 40%, and then with 13 ml of 20%, sucrose. The samples were then centrifuged for 30 min at 75000 g (Beckman model L-5-50 ultracentrifuge; swinging-bucket type SW 25.1 rotor) in the cold. Fractions (1 ml) were analysed for mycobacillin synthetase activity.

**Preparation of protoplasts**

Protoplasts were prepared by the method of Sengupta and Bose (134).

**Assay of mycobacillin synthetase activity**

The incubation mixture and the assay procedure was the same as described in previous chapter. The concentration of the enzyme and the period of incubation in the assay procedure were so adjusted as to maintain linear relationship during the process. In practice, the specific activity of the soluble enzyme preparations was 405 c.p.m./60 min per mg of protein and the enzyme synthesized mycobacillin at enzyme-protein concentrations of 3 mg/ml for at least 120 min, whereas at higher concentrations (4 and 5 mg/ml) a slight decline was observed after 90 min; the pellet preparations had a specific activity of
320 cpm/60 min per mg of protein and produced mycobacillin linearly for at least 120 min.

Protein determination

Protein was determined by the method of Lowry et al. (171) with bovine serum albumin as the standard.

RESULTS

Kinetics of cellular growth, antibiotic production and synthetase activity in subcellular fractions

The extracellular mycobacillin production started at the onset of stationary phase (Fig. 17) and continued for 6 days or more, the peak value being attained at about 136 h (stationary phase). The intracellular accumulation of mycobacillin occurred first at the lower limit of the assay, 40 µg/ml, at late stationary phase, and continued during the observed period. Fig. 17 also shows that mycobacillin synthetase activity first appeared in the 105000 g supernatant at the late exponential phase of growth, increased abruptly and reached the maximum at the onset of stationary phase (16 h). The activity then declined slowly to become almost zero at about 72 h. The synthetase activity in the 10000 g pellet appeared first at about 20 h, reached the maximum at about 80 h and then slowly declined to attain, at 100 h, 50% of its peak activity, which thereafter remained constant all through the observed period.
Distribution of synthetase activity under conditions of inhibition of protein synthesis

Studies on the pattern of distribution of mycobacillin synthetase activity between the 10000 g supernatant and the pellet over a short period, namely 40–46 h, when the activity was present in both the pellet and the supernatant (Table 8) and under conditions where protein synthesis was completely blocked by chloramphenicol, indicate that the total synthetase activity showed a slight downward trend over this period. However, whereas the activity in the pellet fraction increased continuously, that in the supernatant decreased during this period, even in absence of any protein synthesis.

Localisation of the 10000 g particulate synthetase activity of stationary-phase cells

Studies on the localisation of the particulate enzyme obtained in the 10000 g pellet of sonicated stationary-phase cells indicate that the activity was absent in the heavy pellet (3000 g) but present in the light one (10000 g). On further fractionation by sucrose-density gradient centrifugation the activity in the light pellet resolved itself into three fractions. The major synthesizing activity was found to be associated with fractions sedimented at 40% (w/v) sucrose (Fig. 18), whereas a minor one at 20% (w/v) sucrose. The heavy precipitate sedimented at the bottom of the 60% (w/v) sucrose was devoid of any synthesizing activity.
Characterisation of particulate mycobacillin synthetase system

a) Effect of lysozyme treatment: Mycobacillin synthetase activity of the unfractionated 10000 g pellet was decreased by 13% after lysozyme treatment (Table 9), whereas the fractions obtained from the sucrose density gradient possessed lysozyme-insensitive enzyme activity.

b) Presence of succinate dehydrogenase activity: Table 9 further indicates that succinate dehydrogenase activity, a membrane marker enzyme, was present in the 10000 g pellet and in the fractions sedimented at 40% (w/v) sucrose, but was completely absent in the fractions sedimented at 20% (w/v) sucrose.

c) Effect of detergents: The particulate enzyme activity of the 10000 g light pellet and that of the fractions sedimented at 40% (w/v) sucrose was destroyed almost totally (75%) by treatment with sodium deoxycholate and sodium dodecyl sulphate (Table 9), and this loss of activity was linearly related to detergent concentration. The enzyme activity in the 105000 g supernatant of exponential phase cells was unaffected by the treatment.

Site of membrane binding of particulate mycobacillin synthetase activity

The mycobacillin synthetase activity was determined not only in the supernatant from lysozyme treated cell suspension (40 h),
but also in the intact protoplast and in the protoplast lysate, and showed that neither the supernatant nor the intact protoplast contain mycobacillin synthetase activity, which was present only in the protoplast lysate.
Table 8: Effect of chloramphenicol on mycobacillin synthetase activity

Chloramphenicol (50 μg/ml) was added to the growing culture (40 h) at 30°C, which was further incubated with aeration. The cells of different age groups (200 ml) were harvested and the protein concentration as well as the synthesizing activity were determined separately for the 10000 g pellet and supernatant as described in the text.

<table>
<thead>
<tr>
<th>Age of the culture (h)</th>
<th>Incorporation of $^{14}$C-labelled amino acid to mycobacillin (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
</tr>
<tr>
<td>40</td>
<td>2890</td>
</tr>
<tr>
<td>42</td>
<td>2700</td>
</tr>
<tr>
<td>44</td>
<td>2545</td>
</tr>
<tr>
<td>46</td>
<td>2304</td>
</tr>
</tbody>
</table>
Table 9: Nature of the 10,000 g particulate enzyme synthesizing mycobacillin

Different fractions were treated with lysozyme (1 mg/ml) for 10 min at 30°C and centrifuged at 10,000 g in cold. The supernatant from each fraction was taken for sugar detection by the anthrone reaction (177). The synthetase activity of each fraction before and after lysozyme treatment, expressed as c.p.m., was determined as described in the text. The presence of the enzyme succinate dehydrogenase in different fractions was assayed by the method of Slatter and Bonner (178). The different fractions were treated with different concentrations of the detergents sodium deoxycholate (DOC) and sodium dodecyl sulphate (SDS) for 10 min in cold, washed twice in buffer and enzyme activity assayed (For brevity, only the results for 1% detergent are shown). The control was run with denatured protein.

<table>
<thead>
<tr>
<th>System</th>
<th>Lysozyme sensitivity</th>
<th>Succinate dehydrogenase activity</th>
<th>Effect of 1% (w/v) detergent (c.p.m.)</th>
<th>Incorporation ability (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DOC</td>
<td>SDS</td>
</tr>
<tr>
<td>10,000 pellet</td>
<td>+</td>
<td>+</td>
<td>505</td>
<td>480</td>
</tr>
<tr>
<td>Enzyme system at</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% (w/v) sucrose</td>
<td>-</td>
<td>-</td>
<td>1088</td>
<td>1050</td>
</tr>
<tr>
<td>40% (w/v) sucrose</td>
<td>-</td>
<td>+</td>
<td>450</td>
<td>430</td>
</tr>
</tbody>
</table>
Legend to the Figure 17: Correlation between cell growth, antibiotic production and mycobacillin synthetase activity

The enzyme activity in the supernatant as well as in pellet fraction was determined as described in the Materials and Methods section. The age of the culture was varied as required. Incubation temperature and period were respectively 30°C and 90 min. Key to symbols: , Cell growth, 0, extracellular and , intracellular, mycobacillin activity; , 105000 g supernatant and , 10000 g pellet mycobacillin synthetase activity; , change in pH of fermentation medium.
Legend to the Figure 18: Isolation of membrane-associated mycobacillin synthetase by sucrose-density gradient centrifugation

The 10000 g light pellet in Tris/HCl buffer was layered (5 ml) on top of a three-step sucrose-density gradient column consisting of 7 ml of 60% (w/v) sucrose overlaided with 7 ml of 40%, and then with 13 ml of 20%, sucrose. The samples were then centrifuged for 30 min at 75000 g (Beckman model L-5-50 ultracentrifuge; swinging-bucket type SW.25.1. rotor) in the cold. Fractions (1 ml) were analysed for mycobacillin synthetase activity.
Fig. 18

Sucrose Concentration

20%  40%  60%

$^{14}C$ amino acid incorporation (c.p.m. x 10$^{-3}$)

Top  Bottom

Fraction Number