The Formation of Functional Penicillin-binding Proteins*

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A method was developed which permitted determination of the $[^{14C}]$benzylpenicillin and $[^{14C}]$Cepha-pirin binding capacity of rapidly growing Bacillus subtilis cells in liquid culture. Over the concentration range of the binding plateau (0.1 to 0.8 µg/ml), $[^{14C}]$benzylpenicillin significantly inhibited formation of functional penicillin-binding proteins, but had comparatively little effect on total bacterial protein synthesis. The data suggest that penicillin covalently bound to the cells in a chemically stable manner alone is not sufficient to inhibit formation of functional binding proteins and that unbound penicillin in the growth medium is necessary. The concentration of unbound antibiotic in the culture medium, in turn, is a function of the cell-bound penicillinase activity whose significance increases with cell density.

$[^{14C}]$Cepha-pirin, a cephalosporin resistant to this cell-bound penicillinase almost completely inhibited the formation of functional Cepha-pirin-binding proteins, but had relatively little effect on total protein synthesis. At concentrations 250-fold higher than that required to inhibit formation of functional binding proteins, Cepha-pirin did not inhibit particulate $\beta$-alanine carboxypeptidase activity and presumably did not bind covalently to this penicillin-binding protein.

The specific interaction of penicillin with appropriate bacterial receptors can produce at least three major physiological consequences: the death of susceptible growing cells; the induction of penicillinase (penicillin amido-$\beta$-lactamase, EC 2.5.2.6) in specific resistant cultures; and the inhibition of bacterial sporulation. The role of covalently bound penicillin in the first two biological phenomena has been intensively studied. Several investigations established reasonable correlation between the concentration of antibiotic required to saturate the penicillin target proteins in Staphylococcus aureus cells and that required to kill the organisms (1-7). From these studies, it was concluded that the penicillin-binding site (or sites) of bacteria was probably the same as the killing site (cf. Ref. 8). The significance of bound penicillins in the induction of penicillinase has also been demonstrated (9, 10). Penicillin inhibits bacterial forespore seption at concentrations similar to that required to kill vegetative cells (11). The role of covalently bound antibiotic in preventing the sporulation process remains to be established but the capacity of sporulating bacteria to bind radioactive penicillins is elevated during the intervals when bacterial forespore peptidoglycan synthesis is maximal (12, 13). In vivo and in vitro studies indicate that penicillins kill bacteria by inhibiting the terminal steps of bacterial cell wall peptidoglycan synthesis (for a review see Ref. 14). Penicillins irreversibly inhibit a membrane-bound glycopeptide transpeptidase (14-22) and a $\beta$-alanine carboxypeptidase (23, 24) resulting in the synthesis of defective cell wall peptidoglycan. Both direct labeling experiments (8, 14, 24, 25) and the isolation of penicillin-binding components by covalent affinity chromatography (27) indicate that at least five penicillin-binding proteins are present in extracts from Bacillus subtilis membrane particles. Of these, only the $\beta$-alanine carboxypeptidase has been conclusively identified (8, 14, 24, 25, 27). The identity, catalytic capacity, and metabolic significance of the other proteins remains to be established. Since specific inactivation of the glycopeptide transpeptidase in some in vitro systems involves irreversible binding of the antibiotic (15) it presumably constitutes at least one of the other components.

The irreversible inhibition of some bacterial glycopeptide transpeptidases and the $\beta$-alanine carboxypeptidase contrasts with the apparent reversibility of the in vivo biological effects exerted by penicillin. If a rapidly growing bacterial culture is exposed to high concentrations of penicillins and the culture is treated with penicillinase or the unbound penicillin removed by washing or dilution, the bactericidal effect of the antibiotic is abolished (28). Similarly, penicillinase induction confers collective protection on a culture of resistant organisms. Nonetheless, the induction of penicillinase by penicillin required 6 to 12 min (29), whereas penicilloylation of bacterial target proteins is complete in a much shorter period of time (Ref. 30 and Table 1). Finally, penicillin inhibition of bacterial forespore septum formation in Bacillus megaterium cells (11) is completely reversible with penicillinase under conditions where little bound antibiotic is removed from cells (31).

One proposed explanation for this enigmatic behavior is the rapid replacement of penicilloylated target proteins with newly

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formed functional enzymes after removal of excess penicillin from the growth medium either by dilution or by hydrolysis of the antibiotic (2). Previous studies have attempted to determine the concentration of penicillin required to saturate specific penicillin binding sites of cells (1-7). Since highly concentrated cell suspensions were utilized, the procedures do not permit study of the formation of functional penicillin-binding proteins in cell culture. An in vivo method was therefore developed to determine directly the quantity of radioactive antibiotic bound to growing cells in liquid culture. This experimental approach permitted examination of the rate of formation of functional penicillin and cephalosporin-binding proteins in rapidly growing cells: (a) not exposed to the antibiotic during growth; (b) exposed continuously to various concentrations of $^{14}C$ benzylpenicillin or $^{14}C$ cephalosporin; (c) subjected only to the influence of covalently bound penicillin. It also provided information as to the significance of cell-bound penicillinase activity on the actual concentration of penicillin present in bacterial cultures under conditions of comparatively high cell density.

**MATERIALS AND METHODS**

**A. Organism and Growth Conditions**

The organism employed was a wild type strain, *Bacillus subtilis*-Marburg. Cells were grown in Difco Antibiotic Medium 3 at 37°C in a New Brunswick Gyrotory shaker. For reasons discussed below, it was necessary to utilize rapidly growing cells at a relatively early stage of logarithmic growth. An inoculum of growing cells was added to fresh media and culture allowed to grow to a cell density of $5 \times 10^8$ viable cells/ml of culture (optical density of 0.00 at 680 nm or 45 Klett units). This represents approximately 25% of the total logarithmic growth phase.

**B. Materials**

$^{14}C$ Benzylpenicillin (67 μCi/mg) was purchased from Amersham/Searle. $^{14}C$ Cephalosporin (1.6 μCi/mg) and unlabelled Cephalosporin were generous gifts from Bristol Laboratories. Unlabelled benzylpenicillin was obtained from Sigma.

**C. Antibiotic Binding Studies**

1. **Time Dependence of Reaction**—The principal goal of this study was to examine the rate of formation of functional binding proteins in rapidly growing cells. It was desirable to determine the specific binding capacity of the cells under conditions which prevented additional target protein formation while the binding reaction took place. Experiments were designed to determine, if, at specific concentrations of $^{14}C$ benzylpenicillin: (a) the time required to saturate the binding proteins was the same at 4°C as at 37°C; (b) if the quantity of radioactive antibiotic bound at 4°C reflected the specific binding capacity of the cells at 37°C. A culture at a cell density of $5 \times 10^8$ viable cells/ml was diluted with fresh medium and cell culture allowed to grow to a cell density of $5 \times 10^8$ viable cells/ml of culture. This represents approximately 25% of the total logarithmic growth phase.

2. **Saturation Binding**—A procedure was developed which permitted determination of the quantity of $^{14}C$ benzylpenicillin required to saturate the penicillin binding sites of cells under conditions of cell density identical with those employed in the *in vivo* binding studies (Section C, Parts 1, 3, 4, 6, and 7). A cell culture was grown to a cell density described above and immediately chilled at 4°C. Aliquots of 20 ml each, were added to cold flasks containing different quantities of $^{14}C$ benzylpenicillin. Control flasks contained both radioactive benzylpenicillin and excess unlabelled benzylpenicillin (final concentration, 20 mg/ml). Samples were incubated for 15 min at 4°C and the reaction was terminated by adding unlabelled antibiotic. The cells were washed, filtered, and counted as described above. Procedures for determining the specific penicillin binding capacity of cells in concentrated, nongrowing suspensions have been described elsewhere (12, 13).

3. **Formation of Functional Penicillin-binding Proteins under Growth Conditions**—The following procedure was employed to determine quantitatively the formation of functional penicillin-binding proteins in cells growing in liquid culture. A rapidly growing culture (cell density described above) was incubated with an equal volume of radioactive benzylpenicillin at 37°C. Then radioactive benzylpenicillin and excess unlabelled benzylpenicillin (0.8 μg/ml) and incubated for 15 min at 4°C. The cells were washed, filtered, and counted as described previously.

4. **Formation of Functional Penicillin-binding Proteins in Cells Grown in Continuous Presence of $^{14}C$ Benzylpenicillin**—To measure the appearance of functional penicillin-binding proteins in cells which were incubated under normal growth conditions in the continuous presence of radioactive penicillin, a growing cell culture (cell density described above and immediately chilled at 4°C) was added to an equal volume of fresh medium containing $^{14}C$ benzylpenicillin (0.8 μg/ml) and agitated at 37°C. Then 20 ml aliquots were withdrawn at various times, chilled at 4°C, and the reaction was stopped by adding excess amounts of unlabelled penicillin. Samples were centrifuged at 20,000 x g for 10 min and washed twice in Buffer A. Cells were filtered and counted as previously described. To compensate for nonspecific binding of radioactive penicillin, an identical control was added to a second volume of medium containing both $^{14}C$ benzylpenicillin and an excess amount of unlabelled penicillin. This control suspension was incubated and assayed as described above.

5. **Presence of Cell-bound Penicillinase Activity and Its Effect on Binding of $^{14}C$ Benzylpenicillin to Cells Grown in Continuous Presence of $^{14}C$ Benzylpenicillin**—The organism examined *B. subtilis* strains Porton and 168; *Bacillus megaterium* ATCC 19213; Staphylococcus aureus-Copenhagen) all exhibited a potent cell-bound enzyme activity on the actual concentration of penicillin. The enzymatic activity appears tightly bound to the cells and is not released into the medium under normal growth conditions. The effect is particularly significant when concentrated cell suspensions are incubated at relatively low concentrations of the antibiotic and is especially important when the concentration of antibiotic is required to kill a highly diluted suspension of cells. It is to be compared to that required to saturate a given penicillin binding component or inhibit a specific enzyme in a concentrated cell suspension 5

The following procedure was devised to detect cell-bound penicillinase activity in *B. subtilis*-Marburg cells. A rapidly dividing culture was grown to a cell density described above, centrifuged at 3800 x g in an International centrifuge, washed once with Buffer A and finally resuspended in the same buffer. A 25 ml aliquot of the cell suspension was incubated with 25 μl of $^{14}C$ benzylpenicillin (final concentration, 0.8 μg/ml) in 1 ml of medium containing 90% ethanol/1 butanol/27% (NH₄)₂SO₄, 9(1/5). The dried plates were exposed to Kodak Medical x-ray film to locate the reaction products. The appropriate portions of the cellulose plates were then scraped, mixed with the previously described scintillation

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1. P. J. Lawrence, unpublished observations.
2. T. E. Hamilton and P. J. Lawrence, manuscript in preparation.
mixture, and counted in a liquid scintillation counter.

To determine the effect of cell-bound penicillinase or other hydrolytic enzyme activity on the binding capacities obtained in Section C4 above, cells grown in the continuous presence of [14C]benzylpenicillin (0.8 &mu;g/ml) were removed from the growth flask at various intervals and added to sufficient additional radioactive antibiotic to raise the concentration of the antibiotic by 0.2 &mu;g/ml. The cells were incubated under these conditions for 15 min at 4° and bound radioactive antibiotic determined as described above. If such hydrolytic enzyme activity is absent, the cells would be expected to bind no more antibiotic than was found in Section C4 above. Conversely, the presence of penicillinase activity at 37° would hydrolyze the initial [14C]benzylpenicillin present (0.8 &mu;g/ml) and permit unlabelled penicillin-binding proteins to bind additional quantities of the antibiotic added during the second incubation.

6. Effect of Bound Penicillin on Formation of Functional Penicillin-binding Proteins—A third technique was utilized to determine whether bound penicillin affected the formation of functional penicillin-binding proteins. For these experiments it was necessary to have antibiotic concentrations below those employed in the methods described above, but not present in the medium in unbound form. A cell culture was grown to a cell density as described above and chilled at 4°. Unlabeled benzylpenicillin (0.8 &mu;g/ml) was then added to the culture and the suspension incubated for 10 min at 4°. Cells were centrifuged at 3500 × g in an International centrifuge, washed once with 10 mM Tris buffer, pH 7.2, to remove free penicillin, and resuspended in a volume of fresh medium (37°) equal to the original culture volume. This suspension was agitated at 37° and 20-ml aliquots were removed at appropriate intervals to determine the specific binding capacity of the cells at 4° as outlined above. As a control, aliquots were also added to flasks containing both radioactive penicillin and excess unlabelled penicillin. All determinations in the penicillin binding studies were performed in triplicate.

7. Formation of Functional Cephapirin-binding Proteins—A penicillinase-resistant cephalosporin, Cephapirin (Bristol), was employed to determine its effect on the formation of functional Cephapirin-binding proteins of cells grown in the continuous presence of [14C]Cephapirin. Since the specific activity of [14C]Cephapirin (1.6 &mu;Ci/mg) was 40-fold less than that of [14C]benzylpenicillin (67 &mu;Ci/mg), the above procedures (Sections C3 and C4) were modified so that the effect of radioactive Cephapirin on the formation of Cephapirin-binding proteins could be measured. Cells were grown under forced aeration to the same cell density described above; the culture was diluted with an equal volume of fresh medium (37°) containing [14C]Cephapirin (0.1 &mu;g/ml) and agitated at 37°. Samples, 500 ml were withdrawn at various intervals and chilled immediately at 4°. Samples were centrifuged at 3500 × g for 20 min, washed once in Buffer A, and filtered on Millipore filters as previously described. The filters were dried and burned in a Packard sample oxidizer to determine the amount of [14C]Cephapirin bound to the cells. Samples were then counted in a Packard Tri-Carb liquid scintillation counter. To compensate for nonspecific [14C]Cephapirin binding, cultures containing both excess unlabelled Cephapirin (1.0 mg/ml) and radioactive Cephapirin (0.1 &mu;g/ml) were utilized.

To determine the rate of formation of Cephapirin-binding proteins of cells grown in the absence of Cephapirin, cells were grown to the cell density described above, diluted 1:2 with fresh medium, and agitated at 37°. Then 500 ml samples were taken at various times and added to either [14C]Cephapirin (0.1 &mu;g/ml, final concentration) alone or to [14C]Cephapirin and excess unlabelled Cephapirin (1 mg/ml). The cells were collected and the bound radioactive Cephapirin was determined as above.

D. Protein Determinations
In each of the above penicillin binding assays, 20-ml aliquots were taken at appropriate intervals. The cells were centrifuged, washed in Buffer A, resuspended in water, and the total protein content determined by the Lowry method (26). Determinations were performed in triplicate.

E. D-Alanine Carboxypeptidase Activity in B. subtilis
Particulate Fraction
To determine the d-alanine carboxypeptidase activity of a B. subtilis particulate fraction, cells were grown to early logarithmic phase (cell density described above), harvested, and the particulate fraction prepared as previously reported (32). The d-alanine carboxypeptidase assay has already been described (23).

**RESULTS**

**Effects of Time and Temperature on Binding of [14C]Benzylpenicillin to Rapidly Growing Bacillus subtilis Cells**—At a concentration of 0.8 &mu;g/ml, [14C]benzylpenicillin reacted quickly with growing B. subtilis cells. When the cells were exposed to the antibiotic at 4° (Table I) the reaction was essentially completed within 1 min and little additional binding occurred over a 3-hour interval (data not presented) despite the capacity of concentrated cell suspensions to bind additional quantities of the antibiotic at higher penicillin concentrations (Fig. 1). Moreover, the quantity of [14C]benzylpenicillin bound to cells exposed to the antibiotic at 37° was essentially the same as that at 4° (Table I). These data indicate that exposure of cells to the radioactive antibiotic for 15 min at 4° was sufficient to saturate the penicillin-binding proteins which could react with this concentration of antibiotic at 37°. Unless indicated otherwise, subsequent binding assays were performed at 4° to prevent additional cell growth where such growth was undesirable.

Specific [14C]Benzylpenicillin Binding Capacity of Rapidly Growing B. subtilis Cells—When a rapidly growing culture of B. subtilis Marburg cells was exposed to various concentrations of [14C]benzylpenicillin at 4° and at the cell densities present during conditions of rapid growth, a definite binding plateau similar to that previously observed with concentrated bacterial cell suspensions (17) and cell membrane fragments (23) was observed (Fig. 1). The specific binding capacity of the cells under these conditions remained essentially constant at penicillin concentrations between 0.1 and 0.8 &mu;g/ml. Benzylpenicillin, at the concentration of this plateau and at cell densities equal to those employed in Fig. 1, was bactericidal. Thus, the target proteins saturated at a 0.1 to 0.8 &mu;g/ml of benzylpenicillin reflect those which react covalently with the antibiotic at bactericidal concentrations (33).

When the specific binding capacity of a concentrated cell suspension under nongrowing conditions was determined, a similar over-all binding pattern was observed (Fig. 1). Somewhat more penicillin was required to saturate the cells in concentrated suspension (0.6 to 1.1 &mu;g/ml), but essentially the same specific penicillin binding capacity was observed (approximately 11 pmol of [14C]benzylpenicillin bound/mg of protein). However, binding studies performed with concentrated cell suspensions does not permit examination of the rate of formation of functional antibiotic binding proteins.

The quantity of [14C]benzylpenicillin bound to the cells was determined as above.

| TABLE I
<p>| Amount of [14C]benzylpenicillin bound to vegetative Bacillus subtilis cells at 4° and 37° |
|----------------------|-----------------------------------------|</p>
<table>
<thead>
<tr>
<th>Incubation time</th>
<th>[14C]Benzylpenicillin bound (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°</td>
<td>10.9</td>
</tr>
<tr>
<td>7</td>
<td>10.8</td>
</tr>
<tr>
<td>15</td>
<td>10.9</td>
</tr>
<tr>
<td>37°</td>
<td>9.7</td>
</tr>
</tbody>
</table>
To insure uniformity in experimental procedure, all subse-

Thus appear to be a function of the cell density of the culture. Their relative rates of formation

Total cell protein synthesis. Under these conditions forma-

20 min of growth, formation of functional penicillin-binding proteins reproducibly proceeded at a rate approximately twice

content of the cells was then determined (Fig. 2). For the first

specific binding capacity occurs at a concentration shown to

high antibiotic concentrations does not appear to be related

studies with rapidly growing cultures were not examined at

over this concentration range does not appear to reflect binding

to the n-alanine carboxypeptidase since (a) the concentra-

tion of benzylpenicillin required to inhibit the activity of this

enzyme 50% in concentrated cell suspension is approximately 2

ng/ml (24); (b) Cephapirin, at a concentration of 0.1 ng/ml,

completely prevents the binding of [14C]benzylpenicillin to

concentrated cell suspensions over a benzylpenicillin concen-

tration range of 0.1 to 1.0 ng/ml. However, even at a concen-

tration of 25 ng/ml, Cephapirin does not inhibit the n-alanine

carboxypeptidase activity of particulate enzyme preparations

obtained from this organism (Table II). When the penicillin

concentration was elevated further in experiments utilizing

concentrated cell suspensions, the quantity of radioactive

antibiotic bound to the cells nearly doubled (Fig. 1). Binding

studies with rapidly growing cultures were not examined at

the higher benzylpenicillin concentrations. The biological sig-
nificance of the protein(s) which bind penicillin only at these

high antibiotic concentrations does not appear to be related to

the bactericidal effects of the antibiotic. This increase in

specific binding capacity occurs at a concentration shown to

inhibit the n-alanine carboxypeptidase activity of B. subtilis-

Porton cells. Inhibition of the latter enzyme does not appear to

be involved in the bactericidal effect of penicillin (24).

Formation of Functional Penicillin-binding Proteins by B.

subtilis Cells Growing in Liquid Culture—B. subtilis cells were

grown under conditions identical with those described in Fig. 1

(cells diluted 1:2 with fresh antibiotic medium 3). Aliquots,

removed after the desired intervals, were exposed to [14C]benzylpenicillin at a final concentration of 0.8 ng/ml at 4°C. The

specific penicillin-binding capacity and the total protein content of the cells was then determined (Fig. 2). For the first

20 min of growth, formation of functional penicillin-binding proteins reproducibly proceeded at a rate approximately twice

that of over-all cell protein synthesis. Thereafter, the rates of total cell protein synthesis and functional penicillin-binding

protein formation were approximately equal. The effect of

relative cell density on these phenomena was further illus-

trated when a culture was allowed to reach a cell density of 5 x

10^7 cells/ml and undiluted samples were exposed to the

radioactive antibiotic. Under these conditions formation

of the penicillin-binding proteins essentially paralleled total cell protein synthesis. Their relative rates of formation

thus appear to be a function of the cell density of the culture. To

insure uniformity in experimental procedure, all subse-

quent cultures were diluted 1:2 with fresh medium prior to their use in this study.

Formation of Functional Penicillin-binding Proteins of B.

subtilis Cells Growing in Liquid Culture While Continuously Exposed to [14C]Benzy1penicillin—Cells were grown under

conditions identical with those described in Fig. 1, When the cell density reached 5 x 10^7 cells/ml, the culture was diluted

1:2 with fresh medium containing sufficient [14C]benzy1penicillin to saturate the penicillin-binding sites of the first

plateau shown in Fig. 1 (0.8 ng/ml). The resulting cultures were

shaken at 37°C and 20-ml aliquots were removed at appropriate

intervals. Unlabeled penicillin was added to the aliquots to

stop binding of the radioactive antibiotic and the specific binding capacity and total protein content of the cells was

determined (Fig. 4). The rate of bacterial protein synthesis was

similar to that in an untreated culture over a 30-min period

(Table III). Despite the rapid rate of total bacterial protein

synthesis under these conditions, no additional formation of

functional penicillin-binding proteins could be detected over a

30-min period (Curve B, Fig. 4). The total binding capacity of

the cells actually appeared to decline slightly during the interval. Presumably this effect results at least partially from

the cell-bound penicillinase activity (see below). To determine

more precisely the effect of enzymatic hydrolysis of benzyl-

penicillin on the detection of functional penicillin-binding

protein formation in cells grown in the presence of a saturating

concentrating of radioactive antibiotic (0.8 ng/ml), the follow-

TABLE II

Effect of Cephapirin on n-alanine carboxypeptidase activity in

Bacillus subtilis particulate fraction

<table>
<thead>
<tr>
<th>Cephapirin concentration (μg/ml)</th>
<th>d-Alanine carboxypeptidase activity (dpm/nl [14C]alanine released/mg protein x 10^-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.18 (100%)</td>
</tr>
<tr>
<td>0.1</td>
<td>2.18 (100%)</td>
</tr>
<tr>
<td>1.0</td>
<td>2.01 (99%)</td>
</tr>
<tr>
<td>4.0</td>
<td>2.20 (108%)</td>
</tr>
<tr>
<td>25.0</td>
<td>2.18 (100%)</td>
</tr>
</tbody>
</table>
Fig. 4. The formation of functional penicillin-binding proteins in Bacillus subtilis cells. A, rapidly growing, untreated cells; B, cells grown in the continuous presence of 0.8 µg/ml of ['^14C'] benzylpenicillin; C, cells grown in the continuous presence of ['^14C'] benzylpenicillin (0.8 µg/ml) but at the indicated times, an aliquot of ['^14C'] benzylpenicillin sufficient to increase the antibiotic concentration by 0.2 µg/ml was added at 4°C. D, cells pretreated with unlabeled penicillin, 1.0 µg/ml, washed free of unbound antibiotic, and allowed to grow in fresh medium ("Materials and Methods," Section C parts 3, 4, and 6 and Section D).

**Table III**

**Comparative effect of benzylpenicillin or Cephapirin on formation of functional antibiotic binding proteins and on total bacterial protein synthesis**

The procedures employed to determine parameters A and B are discussed in detail ("Materials and Methods," Sections C, Parts 3, 4, 6, and 7 and Section D).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Effect on protein synthesis and on formation of functional antibiotic binding proteins (ABP)</th>
<th>A&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B&lt;sup&gt;+&lt;/sup&gt;</th>
<th>A/B&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylpenicillin</td>
<td></td>
<td>113%/55% = 2.13</td>
<td>51%/45% = 1.13</td>
<td>2.1/1.1 = 1.91</td>
</tr>
<tr>
<td>Cephapirin</td>
<td></td>
<td>74%/6% = 12.3</td>
<td>60%/39% = 1.54</td>
<td>12.3/1.54 = 7.98</td>
</tr>
</tbody>
</table>

<sup>a</sup>A = [% increase in the formation of ABP during initial 20-min growth]/untreated culture [% increase in the formation of ABP during initial 20-min growth] antibiotic present during growth.

<sup>b</sup>B = [% increase in total protein synthesis during initial 20-min growth]/untreated culture [% increase in total protein synthesis during initial 20-min growth] antibiotic present during growth.

The comparative effect of either Cephapirin or benzylpenicillin on formation of functional antibiotic binding proteins relative to total protein synthesis during the initial 20-min of growth.

The experiment was performed at appropriate intervals, samples were removed from a culture grown in the presence of ['^14C'] benzylpenicillin in the same manner and incubated at 4°C with sufficient ['^14C'] benzylpenicillin to increase the final concentration of antibiotic by 0.2 µg/ml. This concentration of antibiotic alone is sufficient to saturate the penicillin-binding proteins of the first plateau (Fig. 1). The additional antibiotic, however, did not raise the concentration of ['^14C'] benzylpenicillin sufficiently to saturate the proteins of the second binding plateau (Fig. 1) since the initial binding capacities of these cells at 1 and 10 min were identical with that observed in Figs. 1 to 4 (Curve D, Fig. 4). This procedure therefore permitted the accurate detection of the formation of penicillin-binding proteins despite the possible presence of the cell-bound penicillinase. The binding capacity of the cells remained constant over a 15-min interval, but then increased at a rate similar to that of untreated cells. Failure to detect formation of the penicillin-binding proteins after 15 min in the earlier experiments (Curve B, Fig. 4) may be attributed to the penicillinase activity of the cells (see below). Presumably the concentration of radioactive antibiotic under growth conditions decreased to a value below 0.1 µg/ml during this interval, since even at this concentration functional penicillin-binding proteins could be detected (Fig. 1). The effect of ['^14C'] benzylpenicillin on the formation of functional penicillin-binding proteins and on total protein synthesis over a 20-min interval is further illustrated in Fig. 5. In cultures grown as indicated above, the rate of formation of the penicillin-binding proteins was approximately twice that of total cellular protein synthesis. The addition of ['^14C'] benzylpenicillin at a concentration of 0.75 µg/ml drastically reduced the rate of formation of the penicillin-binding proteins, but had a comparatively minor effect on total cell protein synthesis.

The addition of higher concentrations of penicillin reduced the formation of both penicillin-binding proteins and total protein synthesis in a parallel fashion. The inhibition of penicillin-binding protein formation presumably does not reflect an effect on the formation of the D-alanine carboxypeptidase for the reasons described above. The effect of ['^14C'] Cephapirin on cephalosporin-binding protein formation (below) also indicates that the binding described in Figs. 1 to 6 and Tables I and III do not represent binding to this enzyme. The above data do not indicate whether the effect of benzylpenicillin on the formation of functional penicillin-binding proteins of B. subtilis cells is caused by penicillin covalently bound to the cells or by excess antibiotic present in the medium. To examine further this question the following experiment was performed.

**Effect of Penicillin Covalently Bound to Bacterial Membrane Proteins on Formation of Functional Penicillin-binding Proteins**—A rapidly growing culture was diluted (1:2) with fresh medium as described above and exposed to a saturating concentration of unlabeled benzylpenicillin (1.0 µg/ml) for 15 min at 4°C. The cells were washed to remove unbound antibiotic, resuspended in the same volume of fresh growth medium, and growth was continued at 37°C. At appropriate intervals, 20-ml aliquots were removed and exposed to radioactive benzylpenicillin for 15 min at 4°C. The capacity of the cells to bind radioactive antibiotic increased as growth commenced (Fig. 6). The rate of formation of the functional binding proteins was essentially the same as that of an untreated control culture. The absolute difference in ['^14C'] benzylpenicillin capacity remained constant and represents unavailable target proteins saturated with unlabeled antibiotic. The rate of reappearance of ['^14C'] benzylpenicillin-binding capacity of pretreated cells as a function of time is also presented in Curve D, Fig. 4. It resembled that of the control culture, but contrasted sharply with that of a culture continuously exposed to the antibiotic during growth (Curve C, Fig. 4). The data indicate that antibiotic bound to cellular proteins in a chemically stable manner is not sufficient to prevent the appearance of func-
penicilloylate newly synthesized target proteins or to repenicilloylate those proteins from which the antibiotic has been removed when cells were incubated with concentrations of [14C]benzylpenicillin, however, would have resulted in the labeling of penicillin-binding proteins even more dramatically than Cephapirin (0.1 μg/ml) (Table III). Over a 20-min interval, cells grown as described under "Materials and Methods," diluted 1:2 with fresh medium containing a bactericidal concentration of [14C]Cephapirin (0.1 μg/ml), and shaken at 37°C. The reaction was terminated by adding 80% ethanol and the quantity of enzymatically produced [14C]benzylpenicilloic acid (Rf = 0.59) was determined ("Materials and Methods," Section C5). Cells Exposed to [14C]Cephapirin—The transient and incomplete inhibition of the formation of functional penicillin-binding proteins by saturating concentrations of [14C]benzylpenicillin (Curve C, Figs. 4 and 5) may have resulted from enzyme-catalyzed hydrolysis of the antibiotic (Table IV). A critical concentration of free [14C]benzylpenicillin could have been maintained for a longer interval by adding much larger quantities of labeled antibiotic to the medium initially. Increased concentrations of [14C]benzylpenicillin, however, would have resulted in the labeling of penicillin-binding proteins apparently not involved in the lethal effect of the antibiotic (see above, Fig. 1) including the p-alanine carboxypeptidase (24). For these reasons, the effect of [14C]Cephapirin on the formation of the cephalosporin-binding proteins was examined.

Cephapirin is a cephalosporin with an antibacterial spectrum similar to that of cephalothin (34). Its bactericidal efficacy against growing B. subtilis cells is virtually identical to that of cephalothin; it is almost completely resistant to the B. subtilis cell-bound penicillinase, and at a concentration of 0.1 μg/ml completely prevents the binding of [14C]benzylpenicillin (0.8 μg/ml) to growing B. subtilis cells. However, when cells were incubated with concentrations of [14C]benzylpenicillin sufficient to saturate the second binding plateau, Cephapirin (0.1 μg/ml) did not prevent binding of penicillin to the proteins responsible for this additional binding. Cells were grown as described under "Materials and Methods," diluted 1:2 with fresh medium containing a bactericidal concentration of [14C]Cephapirin (0.1 μg/ml), and shaken at 37°C. Then 500-ml samples were removed at various intervals, and the amount of [14C]Cephapirin bound to the cells and their total protein content determined. Since Cephapirin kills more than 90% of the cells within 30 min under these conditions, the formation of the binding proteins could be examined only over a 20-min period. Cephapirin inhibited formation of the cephalosporin-binding proteins even more dramatically than penicillin (Fig. 7, Table III). Over a 20-min interval, cells...
grown in the absence of the antibiotic exhibited a 74% increase in the quantity of Cephapirin-binding proteins, whereas only a 6% increase was observed in cells grown in the presence of [14C]Cephapirin. Total protein synthesis, alternatively, increased 60% and 39%, respectively. Thus, [14C]Cephapirin inhibition of cephalosporin-binding protein formation was 8 times greater than its effect on total protein synthesis (Table III). [14C]Benzylenpicillin inhibited penicillin-binding protein formation twice as much as it inhibited total protein synthesis (Fig. 5 and Table III). Thus, both β-lactam antibiotics, especially Cephapirin, inhibited formation of functional β-lactam antibiotic binding proteins much more than they inhibited total bacterial protein synthesis.

Interaction of Cephapirin with Particulate D-Alanine Carboxypeptidase of B. subtilis Cells—The D-alanine carboxypeptidase has been identified as the major penicillin-binding protein of B. subtilis-Porton cells (at penicillin concentrations much higher than those utilized in the present study (8)), but is not considered the lethal penicillin target (24) and is inhibited by cephalothin only at high concentrations (8, 35) of the antibiotic. At the [14C]benzylenpiciillin or [14C]Cephapirin concentrations employed in the present study, the radioactive antibiotics would not be expected to react with the D-alanine carboxypeptidase (Ref. 24 and above). Nonetheless, it was conceivable that the total observed [14C]Cephapirin binding capacity of the cells also reflected partial binding to this protein. Thus, the effect of Cephapirin on a particulate D-alanine carboxypeptidase from B. subtilis cells was determined. Even at a concentration of 25 μg/ml, Cephapirin exerted no detectable inhibition of the D-alanine carboxypeptidase (Table II) and presumably did not react with the enzyme. In this respect, it resembles the cephalosporin, cephalothin, employed in earlier studies (8, 35). Thus, these data combined with those of Fig. 7 and Table III strongly suggest that (a) [14C]Cephapirin does not bind to the D-alanine carboxypeptidase at the Cephapirin concentrations employed in this study (0.1 μg/ml) and (b) the almost complete inhibition of formation of [14C]Cephapirin-binding proteins (greater than 90%) reflects failure of the cells to form cephalosporin-binding proteins other than the D-alanine carboxypeptidase.

**DISCUSSION**

The data presented indicate that the formation of functional penicillin-binding proteins in a rapidly growing culture proceeds at a rate approximately twice that of general protein synthesis (Figs. 2 and 5). After a culture density of 5 × 10⁶ cells/ml is reached, however, the penicillin-binding proteins are formed at a rate essentially the same as that of total protein synthesis (Figs. 2 and 3). In addition to the data presented, this observation is substantiated by the isolation of the penicillin-binding proteins by covalent affinity chromatography on derivatized Corning porous glass beads (36). In this case, if the cells are harvested at a higher cell density, the quantity of functional penicillin-binding proteins present in a given quantity of cells is less than half that present in cells harvested before the above cell density is reached. The identity of the specific penicillin-binding proteins formed in both types of cells is currently under investigation (36). Benzylenpiciillin at a concentration sufficient to saturate the binding sites of the first plateau (Fig. 1) does not profoundly influence total bacterial protein synthesis under the incubation conditions described, but sigificanty inhibits formation of functional penicillin-binding proteins in the exposed cells (Figs. 4 (Curve B), and 5 and Table III). The effect is apparently not exerted by penicillin covalently bound to the organism in a chemically stable fashion since cells pretreated with unlabeled antibiotic and washed free of excess penicillin form functional penicillin-binding proteins at a rate approximately that of untreated cells (Figs. 4 (Curve D) and 6). Hence, unbound penicillin present in the media appears necessary to prevent formation of functional target proteins. A number of investigations showed that the amount of penicillin bound to S. aureus cells increased when growth occurred in the presence of the antibiotic (8, 37–39). However, this was shown to be a result of the exposure of "reserve" penicillin-binding proteins not normally accessible to the antibiotic (37, 40). It did not result from an increased rate of synthesis of these proteins (2). Previous investigations have shown that vegetative B. subtilis cells have no inaccessible penicillin-binding proteins analogous to those observed in S. aureus cells (41). The observed effect of unbound penicillin in the medium offers one explanation for the reversibility of the biological effects of these antibiotics despite their irreversible inhibition of the enzymes used in the terminal stages of bacterial cell wall synthesis. At a concentration sufficient to cause cell death, 4 the antibiotic inhibits these enzymes in an irreversible fashion while simultaneously preventing their formation. When unbound antibiotic is removed from the growth medium, whether by induced or cell-bound β-lactamase-catalyzed hydrolysis, or by experimental dilution of the medium, the formation of functional penicillin-binding protein resumes at a rate equal to that present in an untreated culture (Curve D, Fig. 4). Irreversible inhibition of the target enzymes by itself is clearly insufficient to cause cell death (28) or inhibition of bacterial forespore septum formation (31). The striking bactericidal efficacy of the penicillins apparently requires not only the saturation of specific bacterial components, but also the presence of unbound antibiotic in the medium. The actual concentration of antibiotic present may be strongly influenced by the quantity of cell-bound penicillinase which, in turn, is a function of cell density. The unbound antibiotic may serve to penicilloylate newly formed penicillin target proteins, or those from which penicillin has been removed by hydrolysis of other mechanisms. This observation is particularly significant when attempts are made to correlate the concentration of antibiotic required to saturate given bacterial components, and that needed to inhibit either specific enzyme activities, or cause cell death. 5

4 Because of the low specific activity of the [14C]Cephapirin (1.6 μCi/mg) utilized, it was necessary to grow the cells on a much larger scale than that employed in the studies performed with [14C]benzylenpiciillin (67 μCi/mg). These differences in growth conditions resulted in inherently different rates of bacterial protein synthesis.

5 [14C]Cephapirin, a penicillinase-resistant cephalosporin, almost completely inhibits formation of functional antibiotic binding proteins while exerting a comparatively minor effect on total bacterial protein synthesis. Thus, under conditions where the β-lactam antibiotic is not hydrolyzed, Cephapirin inhibits formation of functional binding proteins to a greater extent than benzylenpiciillin (Fig. 7, Table III). Since Cephapirin does not inhibit B. subtilis, D-alanine carboxypeptidase activity even at a concentration of 25 μg/ml (Table II), it presumably does not bind to this protein at a concentration of 0.1 μg/ml. In this respect, Cephapirin is similar to cephalothin. The latter antibiotic does not react with the B. subtilis or B. stearothermophilus D-alanine carboxypeptidases at concentrations exceeding 20 μg/ml (35). Collectively, these data suggest...
that the observed [14C]Cephapirin binding to B. subtilis cells does not reflect binding to β-alanine carboxypeptidase. Furthermore, inhibition of the formation of the [14C]Cephapirin-binding proteins is almost complete; Cephapirin inhibition of formation of Cephapirin-binding proteins is inhibited by more than 90% (Fig. 7, Table III). Thus, even if [14C]Cephapirin did bind to the β-alanine carboxypeptidase of the cells, the formation of this enzyme is also inhibited. Finally, exposure to [14C]-Cephapirin at 0.1 μg/ml is lethal; more than 95% of the cells are killed in less than 30 min.

Several possible mechanisms may be postulated to explain the observed inability of growing cells to form functional penicillin-binding proteins in the presence of excess antibiotic: penicillin may prevent synthesis of the specific proteins at either the transcriptional or translational level; the insertion of the proteins into the membrane may be inhibited; the conversion of inactive penicillin-binding proteins to active forms (zymogen activation) may be inhibited. Conceivably the enzymes are synthesized in an inactive form and penicillin prevents their activation. Penicillins, at concentrations similar to those required to kill B. megaterium cells, prevent the synthesis of the forespore septum in sporulating cultures of the latter organisms (11). The forespore membrane is apparently devoid of cell wall peptidoglycan. Since penicillins inhibit the terminal enzymes utilized in the synthesis of cell wall peptidoglycan (14) these data suggest that inhibition of cell wall biosynthesis can also prevent normal membrane development. The inhibitory effect of penicillins on forespore septum development is completely abolished if penicillinase is subsequently added to the culture (31). Conceivably, penicillins could prevent the formation of functional penicillin-binding proteins located in the bacterial membranes (14) by an analogous mechanism.

Efforts to eliminate any of the multiple penicillin-binding proteins from consideration as the "lethal binding protein(s)" may not be valid if based primarily on comparisons of the concentrations of the antibiotic required to inhibit the enzymes and that required to kill the cells (2, 4). While data thus obtained may indicate a given enzyme is not inhibited at penicillin concentrations which cause cell death, the antibiotic may prevent formation of the functional component at a lower concentration, thereby rendering its inhibition unnecessary. The same may be said for the differential utilization of various semisynthetic penicillins in similar studies. Thus, penicillin at a given concentration may kill cells by irreversibly inhibiting the enzymatic activity of an undetermined number of penicillin target proteins, by simultaneously preventing functional formation of all of the penicillin-binding proteins, or by a combination of these effects. If the antibiotic can prevent formation of these enzymes at concentrations which cause cell death, conceivably it could kill the cells without inhibiting the enzymes. The relative significance of these mechanisms in the biological effects of the penicillins remains to be clarified.

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