The Peptide Antibiotics of Bacillus: Chemistry, Biogenesis, and Possible Functions

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INTRODUCTION

In a recent review, Berdy stated that the number of antibiotics produced by members of the genus Bacillus was 167 (8). Only a few of the total number elaborated are listed in Table 1. As is generally recognized, these antibiotics are mainly polypeptides. Of this total, 66 different peptide antibiotics are elaborated by strains of Bacillus subtilis and 23 are products of Bacillus brevis. Polymyxin and the closely related colistin, bacitracin, the tyrothricin complex (linear gramicidin plus tyrocidine), and gramicidin S have been used, to some extent, for antibacterial therapy. Most of the peptide antibiotics produced by bacilli are active against gram-positive bacteria; however, compounds such as polymyxin, colistin, and cirucin exhibit activity almost exclusively upon gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts. Although bacilli mainly synthesize peptides, one should not lose sight of the fact that antibiotics belonging to other chemical classes are also produced by these microorganisms (e.g., butirosin [2]), an aminoglycoside derived from Bacillus circularus and protacin [111], a phosphorus-containing triene elaborated by Bacillus licheniformis var. mesentericus).

PROPERTIES OF PEPTIDE ANTIBIOTICS

A number of reviews relating to peptide antibiotics has appeared in the literature (14, 15, 55, 71, 72, 80, 109, 117, 132). As a consequence, the properties of peptide antibiotics will be described only briefly below.

(i) Peptide antibiotics are generally much smaller than proteins. Their molecular weights range from 270 (bacilysin) to about 4,500 (licheniformin).

(ii) Generally, a family of closely related peptides rather than a single substance is produced by an organism. The members may differ from each other by one or, at most, a few amino acid residues. In the case of B. brevis, both the linear gramicidins (A, B, and C) and the cyclic tyrocidines (A, B, and C) are formed simultaneously. The linear gramicidins (pentadecapeptides) and the tyrocidines (decapetides) differ markedly in chemical composition, yet within each family the principal variation resides in the aromatic amino acid substitutions of the molecule (94, 73, 114, 131, 134, 135, 136, 137).

(iii) Most of the peptide antibiotics formed by these organisms are composed entirely of amino acids, whereas others may contain amino acids plus other constituents. For example, edeine A contains the base spermidine in addition
TABLE 1. Some antibiotics elaborated by species of the genus Bacillus

<table>
<thead>
<tr>
<th>Species</th>
<th>Antibiotic</th>
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<tbody>
<tr>
<td>Bacillus brevis</td>
<td>Gramicidin S</td>
</tr>
<tr>
<td></td>
<td>Tyrocidine</td>
</tr>
<tr>
<td></td>
<td>Linear gramicidin</td>
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<tr>
<td></td>
<td>Brevin</td>
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<tr>
<td></td>
<td>Edeine</td>
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<td></td>
<td>Eseine</td>
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<td></td>
<td>Bresseine</td>
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<tr>
<td></td>
<td>Brevistin</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Mycobacillin</td>
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<tr>
<td></td>
<td>Subtilin</td>
</tr>
<tr>
<td></td>
<td>Bacilsyn</td>
</tr>
<tr>
<td></td>
<td>Bacillomycin</td>
</tr>
<tr>
<td></td>
<td>Fungistatin</td>
</tr>
<tr>
<td></td>
<td>Bulbiformin</td>
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<tr>
<td></td>
<td>Bacillin</td>
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<tr>
<td></td>
<td>Subsporin</td>
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<tr>
<td></td>
<td>Bacillocin</td>
</tr>
<tr>
<td></td>
<td>Mycosubtilin</td>
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<tr>
<td></td>
<td>Fungocin</td>
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<tr>
<td></td>
<td>Iturin</td>
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<tr>
<td></td>
<td>Necidin</td>
</tr>
<tr>
<td></td>
<td>Eumycin</td>
</tr>
<tr>
<td>Bacillus pumilis</td>
<td>Micrococcin P</td>
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<tr>
<td></td>
<td>Pumilin</td>
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<tr>
<td></td>
<td>Tetain</td>
</tr>
<tr>
<td>Bacillus mesentericus</td>
<td>Esperin</td>
</tr>
<tr>
<td>Bacillus thiaminolyticus</td>
<td>Octopytin (Thianosine)</td>
</tr>
<tr>
<td></td>
<td>Baciphelacin</td>
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<tr>
<td>Bacillus licheniformis</td>
<td>Bacitracin</td>
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<tr>
<td></td>
<td>Licheniformin</td>
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<tr>
<td></td>
<td>Proticin</td>
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<tr>
<td>Bacillus polymyxa</td>
<td>Polymyxin</td>
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<tr>
<td></td>
<td>Colistin</td>
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<tr>
<td></td>
<td>Gatavalin</td>
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<tr>
<td></td>
<td>Jolipeptin</td>
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<tr>
<td>Bacillus circulans</td>
<td>Butirosin</td>
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<tr>
<td></td>
<td>Circulin</td>
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<tr>
<td></td>
<td>Polypeptin</td>
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<td></td>
<td>EM-49</td>
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<tr>
<td></td>
<td>Xylostatin</td>
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<tr>
<td>Bacillus laterosporus</td>
<td>Laterosporamine</td>
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<tr>
<td></td>
<td>Laterosporin</td>
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<tr>
<td>Bacillus cereus</td>
<td>Biocerin</td>
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<tr>
<td></td>
<td>Cerexin</td>
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<td></td>
<td>Thiocillin</td>
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</table>

to five amino acid constituents (48). Polymyxins possess 6-methyloctanoic acid or 6-methylheptanoic acid as a fatty acid residue along with a number of amino acid constituents (71, 163).

(iv) Frequently, peptide antibiotics contain amino acids which are unique and are not found in proteins (14, 15, 71). β-Amino acids, basic amino acids (ornithine, diaminobutyric acid), β-amino acids, dehydroamino acids (dehydroalanine), and sulfur-containing amino acids (lanthionine) are often present. In contrast to peptides synthesized by fungi and actinomycetes, peptides from bacilli do not contain N-methylamino acid residues.

(v) Most of the peptides are cyclic structures; however, a few are linear (e.g., edeine, linear gramicidins). Besides the cyclic nature of a molecule, there may be unusual linkages or arrangements of the amino acids in the antibiotic. Bacitracin provides an example, for it possesses a thiazoline ring (derived from the condensation of cysteine and isoleucine), a cyclic hexapeptide, as well as an amide bond between the β-carboxyl group of aspartic acid and the epsilon amino group of lysine (1, 167).

(vi) Peptide antibiotics are generally resistant to hydrolysis by peptidases and proteases of animal and plant origin (14, 15, 71, 72, 117), although a few are susceptible to enzymatic attack (polymyxin B by ficin and papain [116]; edeines A and B by carboxypeptidase [49]; bacilysin by leucine aminopeptidase and Pronase [124]; gramicidin S by subtilopeptidase A [174]).

FORMATION OF ANTIBIOTICS IN RELATION TO GROWTH

A number of investigations have shown that the synthesis of peptide antibiotics is initiated after the organism has passed the rapid growth phase. This was noted with gramicidin S (180), tyrocidine (36, 90), polymyxin (116), edeine (82), bacitracin (11), mycobacillin (5), and bacilysin (124, 126). Tomino et al. (160) demonstrated that gramicidin S and the enzymes necessary for its synthesis were produced by B. brevis during the late logarithmic phase of growth (107, 180). The enzymatic activity for antibiotic synthesis then disappeared within a few hours. Similar results were observed with tyrocidine synthetase (36). The reason for the loss of enzyme activity has not been established conclusively. Recently, Lee et al. (90) observed that the tyrocidine-forming enzymes disappeared from the soluble portion of the cell and became associated with the forespore membrane during stage IV of sporulation. However, even this membrane-bound activity disappeared soon thereafter. Demain et al. (25) found...
that the disappearance of gramicidin S synthetases in B. brevis is due to an oxygen-dependent inactivation.

Although antibiotic formation usually follows logarithmic growth (presumably due to some type of repression of antibiotic synthetases in the growth phase), this is not universally observed. It is clear that antibiotics are sometimes produced during growth and that both genetic and nutritional modifications can shift the time of antibiotic synthesis in relation to the growth phase (23). It is quite possible that peptide antibiotic formation is controlled by carbon and nitrogen catabolite repression or is under growth rate control (106); manipulations which affect these controls would be expected to modify the temporal relationship between antibiotic synthesis and growth. In this regard, production of bacitracin by B. licheniformis parallels growth in a chemically defined medium (47, 152). However, Haavik (41, 42) reported that when glucose was added to a defined medium, bacitracin synthesis was inhibited during the first few hours of growth. He initially concluded that this delay in antibiotic production was not due to carbon catabolite repression but to the low pH created by the organism’s metabolism. His conclusion was based on the reversal of the glucose effect by added CaCO₃ (41). However, he later (42) found that the depression of bacitracin synthesis was not due to pH per se but to the acetic and pyruvic acids produced from the glucose, i.e., these acids were only inhibitory when undissociated and thus neutralization eliminated their inhibition. Lowering pH with HCl or phthalic acid did not inhibit bacitracin formation. It should be emphasized that this work does not eliminate the possibility that repression of bacitracin synthetase is due to the undissociated organic acids produced by glucose catabolism.

In addition to bacitracin, the production of tyrocidine (79), linear gramicidins (79), and polymyxin E (colistin) (28) has also been observed to parallel active growth of the producing bacilli under certain conditions. Moreover, gramicidin S (13) and its synthetases (106) can be synthesized by exponentially growing cells in continuous culture. Thus, it is clear that peptide antibiotics can be synthesized during active growth. Whether they function in the producing cells’ metabolism at some stage during growth, as some investigators (43, 52, 70) have considered, will be discussed in a later section of this review.

CELL-FREE SYNTHESIS OF PEPTIDE ANTIBiotics

The cell-free synthesis of gramicidin S, tyrocidine, linear gramicidin, edeine, bacitracin, colistin, and mycobacillin has been reviewed (15, 55, 72, 80, 117). These biosynthetic systems are quite distinct from those synthesizing proteins. The requirements for antibiotic synthesis are fairly similar and generally include the requisite amino acids of the peptide antibiotic in question, adenosine 5'-triphosphate (ATP), Mg²⁺ ion, a reducing agent, and the particle-free supernatant fluid. Many of the synthetases have been extensively purified by conventional procedures, and considerable success has been achieved in resolving the polyenzyme complexes by column chromatography (diethylaminoethyl [DEAE]-cellulose, hydroxylapatite, Sephadex G-200). The peptide-synthesizing systems have proven to be insensitive to chloramphenicol, puromycin, deoxyribonuclease, and ribonuclease (RNase), and it is quite clear that transfer ribonucleic acid (tRNA), messenger RNA (mRNA), and ribosomes are not directly involved in the biosynthetic process. The contributions of several laboratories have led to our present understanding of the mechanism of biosynthesis of gramicidin S and tyrocidine (9, 12, 35–38, 65, 75, 81, 87, 88, 94, 95, 97, 113, 119, 133, 160, 162, 175). Far less is known, however, regarding the cell-free synthesis of other peptide antibiotics produced by bacilli.

ENZYMATIC SYNTHESIS OF GRAMICIDIN S

Gramicidin S (Fig. 1), formed by certain strains of B. brevis, is a cyclic decapeptide consisting of two identical pentapeptides, D-phenylalanyl-L-prolyl-L-valyl-L-ornithyl-L-leucine (15, 55, 71). The presence of D-phenylalanine and L-ornithine, constituents which are not found in proteins, is noteworthy.

In the formation of gramicidin S in vivo, the

![Diagram](http://mmbr.asm.org/Downloaded from Oct 24, 2017 by guest)
first amino acid added is phenylalanine; in vitro it can be replaced by tyrosine, but not by tryptophan (128). Phenylalanine (or the tyrosine replacing it) is incorporated as the D-amino acid. An enzyme (designated as fraction II or the light enzyme) with a molecular weight of 100,000 (Table 2) activates L-phenylalanine (or D-phenylalanine) and racemizes it (37, 38, 160, 171, 172). Activation (Fig. 2) is an ATP-dependent process and, as observed in protein synthesis, there is formation of a phenylalanyl adenylate-enzyme complex (37, 75, 160, 171, 172). The remaining four amino acids, L-proline, L-valine, L-ornithine, and L-leucine, are activated by a second protein designated fraction I or heavy enzyme (Table 2) which has a molecular weight of 280,000 (37, 75, 160). In addition to the amino acids, ATP and Mg$^{++}$ ion are requirements for the activation step. Each of the four amino acids is activated independently on separate sites of the polyenzyme, also being bound as aminoacyl adenylates (Fig. 2). The activation process, as measured by the ATP-inorganic pyrophosphate (PP$_i$) exchange reaction, appears to be relatively specific since only amino acids chemically related to those in gramicidin S are activated. In contrast to the ribosomal system, acylation of tRNA does not take place during activation with either the light or the heavy enzyme (80, 87, 94, 97).

Both enzymes have been shown to catalyze not only an ATP-PP$_i$ exchange reaction, but an ATP-adenosine 5' monophosphate (AMP) exchange as well with the appropriate amino acid substrates. This is due to the presence of secondary acceptors of the bound amino acids on the enzymes (37, 38). The aminoacyl moiety is transferred from the adenylate site to an SH group on the enzyme, yielding a covalently bound thioester-linked amino acid. Thus, the heavy enzyme contains four activating enzymes organized into a tightly associated multienzyme complex; the binding of the amino acid substrates to this complex exhibits a 1:1:1:1 stoichiometry (75). Both the adenylate and SH sites will be occupied under saturating conditions, because once the amino acid is transferred to a thiol group, the adenylate site is free to accept another molecule of activated amino acid (75, 94, 97).

Several experiments have provided support for the view that covalent thioester bonds are formed between the individual amino acids and the specific enzymes involved (32, 38, 88, 129).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fraction</th>
<th>Nomenclature</th>
<th>Amino acid activated</th>
<th>4'-Phosphopantetheine</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gramicidin S synthetase</td>
<td>II</td>
<td>Light enzyme</td>
<td>L- and D-phenylalanine</td>
<td>0</td>
<td>100,000</td>
</tr>
<tr>
<td>Tyrocidine synthetase</td>
<td>I</td>
<td>Heavy enzyme</td>
<td>L-Proline, L-valine, L-ornithine, L-leucine</td>
<td>1</td>
<td>280,000</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>Light enzyme</td>
<td>L- and D-phenylalanine</td>
<td>0</td>
<td>100,000</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Intermediate enzyme</td>
<td>L-Proline, L- and D-phenylalanine (tryptophan)</td>
<td>1</td>
<td>230,000</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Heavy enzyme</td>
<td>L-Asparagine, L-glutamine, L-phenylalanine (tryptophan, tyrosine)</td>
<td>1</td>
<td>460,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-valine, L-ornithine, L-leucine</td>
<td></td>
<td></td>
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</tbody>
</table>

**ACTIVATION**

(1)  \[ E^{SH} + aa + ATP \xrightarrow{\text{Mg}^{++}} E^{...aa ~ AMP + PP_i} \]

(2)  \[ E^{SH} \xrightarrow{\text{AMP}} E^{S\sim aa} \]

(3)  \[ E^{S\sim aa} + aa + ATP \xrightarrow{\text{Mg}^{++}} E^{S\sim aa} \xrightarrow{...aa ~ AMP + PP_i} \]

**FIG. 2. Proposed mechanism of activation of amino acids in gramicidin S.** (Reprinted with permission of F. Lipmann.)
(i) After an incubation, an activated amino acid-enzyme complex can be recovered by Sephadex G-50 chromatography; precipitation of such a complex with trichloroacetic acid releases all of the bound adenylate, but only one-half of the bound amino acid is liberated. (ii) Dilute alkali (at pH 9 to 10) or treatment with mercuric salts at neutral pH brings about a quantitative discharge of the remaining amino acid from the trichloroacetic acid precipitate. (iii) Hydroxylamine treatment of the precipitate will also release the amino acid as the hydroxamate. (iv) Treatment of the precipitate with sodium borohydride reductively cleaves the bond with release of the amino acid as the corresponding amino alcohol. (v) Oxidative cleavage with performic acid also will liberate the amino acid. Thus, the behavior shown by the covalently bound amino acid to acid, base, mercuric salts, etc., comprises all of the characteristic properties of a thioester linkage.

Initiation of peptide bond synthesis. It is now well established that free peptide intermediates are not participants and that an enzyme-bound, activated d-phenylalanine residue is the starting point for gramicidin S formation (32, 38, 53, 98). The direction of peptide chain growth is from the N-terminal to the C-terminal end as in protein synthesis, and only enzyme-bound intermediates are involved. These conclusions were based on in vivo (53, 159) and in vitro (16, 38, 75) experiments. During gramicidin S synthesis, the amino acids are added singly and in a fixed order until a pentapeptide is formed; omission of one amino acid interrupts the elongation process.

For peptide synthesis to occur, the charged or activated enzymes have to be combined (75). The first peptide bond involves thioester-bound d-phenylalanine on the light enzyme and thioester-linked proline on the heavy enzyme (74, 81, 87, 94, 95, 97). The carboxyl-activated d-phenylalanine is transferred to the imino group of proline on the heavy enzyme (Fig. 3). In turn, the thioester-linked dipeptide is transferred to the next thioester-linked amino acid, L-valine, to form a tripeptide. All peptides remain attached to the enzyme until leucine, the last amino acid in the sequence, is added (Fig. 4). Leucine addition brings about a rapid release of cyclic gramicidin S from the polyenzyme. The mechanism of the cyclization step is not well understood. Lipmann’s group proposed that the cyclic decapptide of gramicidin S may arise by an antiparallel doubling reaction between two carboxyl-activated pentapeptide units presumably attached to different heavy enzyme components (94, 97) (Fig. 4). Some evidence, however, suggests an intramolecular cyclization (87, 171). Stoll et al. (153) reported that, after the first pentapeptide chain is formed, it is transferred to a holding site (—SH group) on the heavy enzyme. When the second pentapeptideyl unit is synthesized subsequently, the two pentapeptide chains attached to the same heavy enzyme cyclize by head-to-tail condensation to yield a molecule of gramicidin S (Fig. 5).

4’-Phosphopantetheine, bound to the heavy enzyme of gramicidin S synthetase, mediates the biosynthesis of the tri-, tetra-, and pentapeptides in alternative transthiolation and transpeptidation reactions (38, 39, 76, 77, 87, 94, 95, 97, 98). 4’-Phosphopantetheine is conceived to function as a swinging arm in which it alternates as acceptor of the growing peptide chain and donor of the peptide to the next thioester-linked amino acid in sequence (87, 95, 119). These reactions continue until the tetrapeptide is linked to the terminal amino acid, leucine, to complete the nascent pentapeptide chain. Lipmann has postulated that the thioester-linked polymerization of polypeptides may have evolved from thioester-linked fatty acid synthesis (94, 96).

**ENZYMATIC FORMATION OF TYROCIDINE**

The mechanism of biosynthesis of tyrocidine is similar to that of gramicidin S (80, 94, 95, 97), and only a brief discussion will be presented here. Tyrocidines A, B, and C are cyclic decapptides (Fig. 6) (34, 71, 73, 114, 131) produced by the same strains of B. brevis that elaborate the linear gramicidins. This strain cannot produce the cyclic gramicidin S. Although they are cyclic decapptides like gramicidin S, tyrocidines possess a somewhat different sequence of amino acids. The biosynthesis of tyrocidine also begins with d-phenylalanine and continues with proline (33, 35, 36, 89, 91, 94, 95, 97, 119, 128). However, five different amino acids, L-phenylalanine, d-phenylalanine, L-asparagine, L-glutamine, and L-tyrosine, follow. After tyro-
Elongation and Cyclization

\[
\begin{align*}
-I - S & \sim \text{PRO-PHE} \\
-I - S & \sim \text{VAL-PRO-PHE} \\
-I - S & \sim \text{ORN-VAL-PRO-PHE} \\
-I - S & \sim \text{LEU-ORN-VAL-PRO-PHE} \\
\text{PHE-PRO-VAL-ORN-LEU} & \sim - S
\end{align*}
\]

Fig. 4. Proposed mechanism of elongation and cyclization for gramicidin S biosynthesis. (Reprinted with permission of F. Lipmann.)

Fig. 5. Model for the role of enzyme I in gramicidin S biosynthesis. Positions 1, 2, 3, and 4 are occupied by thioester-linked proline, valine, ornithine, and leucine, respectively. Position 5 is envisioned as the thiol "waiting" site for the pentapeptide. The zigzag line represents the pantetheine arm with its terminal SH group. (A) Growth of nascent peptide chain. (B) Head-to-tail cyclization reaction involving two identical pentapeptides. (Reprinted with permission of S. G. Laland.)

Sine, the sequence of gramicidin S is seen again with L-valine, L-ornithine, and then L-leucine being the last amino acid added.

Purification of the tyrocidine-synthesizing system yields three complementary enzymes: a light fraction (molecular weight 100,000), an intermediate one (molecular weight 230,000) and a heavy enzyme (molecular weight 440,000) (22, 36, 69, 91, 127). The amino acids activated by each of these fractions are shown in Table 2. The light enzyme activates and racemizes phenylalanine (tyrosine or tryptophan can replace it) (97, 127). The intermediate enzyme activates proline, phenylalanine, and phenylalanine in positions 2, 3, and 4, respectively. Phenylalanine at site 4 is also racemized on the enzyme. The heavy enzyme catalyzes the activation of the remaining six amino acids. If one divides the molecular weight of the heavy enzyme of the gramicidin S synthetase complex or the intermediate or heavy enzyme of tyrocidine synthetase by the number of amino acids activated, one obtains a value of 70,000 to 75,000 daltons per amino acid activated and thioesteri-
The intermediate and heavy enzymes contain 1 mol each of 4'-phosphopantetheine bound per mol of enzyme protein. In tyrocidine synthesis, 4'-phosphopantetheine also functions in the alternative transthiolation and transpeptidation reactions during nascent peptide elongation (76, 77, 89, 128). Figure 7 schematically depicts the mechanism of initiation, elongation, and cyclization of the decapeptide of tyrocidine. Initiation of chain growth is similar to that seen in gramicidin S biosynthesis. Addition of amino acids occurs singly,
with a tetrapeptide being formed on the intermediate enzyme. The tetrapeptide is then transferred to the heavy enzyme where the remaining six amino acids are added sequentially to complete the peptide chain (127, 128). Cyclization of the tyrocidine decapetide appears to be a relatively slow reaction, the linear decapetide having been isolated from the heavy enzyme and characterized. Cyclization involves the activated enzyme-bound thioester-linked carboxyl group of leucine and the free amino group of $\beta$-phenylalanine. As with gramicidin S synthesis in vitro, omission of one amino acid in the sequence halts further chain elongation.

Disaggregation of the intermediate and heavy polyenzymes into subunits (89, 91, 95). Each of the polyenzyme components of the tyrocidine-synthesizing system appears to consist of subunits and contains 1 mol of covalently bound 4'-phosphopantetheine per mol of polyenzyme. On the basis of dissociation experiments, Lee et al. (89, 91) confirmed that the intermediate enzyme comprises three, and the heavy enzyme comprises six, amino acid-activating subunits of the 70,000-dalton size. The subunits retained the ability to catalyze ATP-P$P$ exchanges and the thioesterification reactions with the appropriate amino acid substrates, but were unable to carry out the polymerization step. Upon dissociation, both polyenzymes also yielded a pantetheine-containing protein fraction with a molecular weight of 17,000 (77, 89, 91, 95). Evidence that 4'-phosphopantetheine participates in the polymerization reaction was provided also by the dissociation experiments. After incubation of lysates of $B$. brevis or purified polyenzymes with amino acid substrates, the polyenzymes (from the crude or purified enzyme system) were degraded into subunits and subsequently purified. In both cases, a product containing both pantothenic acid and a nascent tyrocidine peptide was recovered (89, 95). These studies demonstrate that the intermediate and heavy enzymes of tyrocidine synthetase consist of specific amino acid-activating subunits (molecular weight 70,000) and a pantetheine-bearing protein of 17,000 daltons which functions in the biosynthesis of the nascent tyrocidine peptide chain (77).

**LINEAR GRAMICIDINS**

The gramicidins are linear pentadecapeptides produced by the same strain of $B$. brevis that elaborates the tyrocidines (Fig. 8). Gramicidins A, B, and C are a mixture of valine and isoleucine gramicidins (134, 137). They differ only in the aromatic amino acid (tryptophan, tyrosine, or phenylalanine) incorporated at position 11 of the peptide. The amino terminal $L$-valine (or $L$-isoleucine) is formylated and the carboxyl terminal tryptophan is linked to the amino group of ethanolamine. It should be noted that the peptide molecule consists almost entirely of alternating $L$- and $D$-amino acid residues.

Bauer et al. (7) were able to achieve a partial biosynthesis of linear gramicidin, using a solu-

<table>
<thead>
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<th>COMPOSITION OF GRAMICIDIN</th>
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<tr>
<td><strong>Compd.</strong></td>
</tr>
<tr>
<td><strong>I</strong> Valine-gramicidin A:</td>
</tr>
<tr>
<td><strong>II</strong> Isoleucine-gramicidin A:</td>
</tr>
<tr>
<td><strong>III</strong> Valine-gramicidin B:</td>
</tr>
<tr>
<td><strong>IV</strong> Isoleucine-gramicidin B:</td>
</tr>
<tr>
<td><strong>V</strong> Valine-gramicidin C:</td>
</tr>
<tr>
<td><strong>VI</strong> Isoleucine-gramicidin C:</td>
</tr>
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**Fig. 8. Composition of linear gramicidins A, B, and C.**
ble enzyme system obtained from extracts of \textit{B. brevis} that had been purified through ammonium sulfate fractionation and Sephadex G-200 column chromatography. The location of the linear gramicidin-synthesizing enzymes was followed by their amino acid-dependent ATP-\textsuperscript{32}P\textsubscript{1} exchange activities. The fractions that activated the amino acids incorporated into linear gramicidin were devoid of amino acid-tRNA ligase activity. However, due to its instability, further resolution of the linear gramicidin synthetase into complementary enzyme fractions was not attempted. Despite these difficulties, the enzyme system catalyzed the synthesis of an enzyme-bound pentadecapeptide. Chemical ethanolaminolysis liberated the peptide from the enzyme, and subsequent chemical formylation of the N-terminal valine yielded a compound that appeared to be chromatographically identical to authentic linear gramicidin. Bauer et al. (7) noted that enzymatic formylation of the N-terminal valine could only be achieved with crude extracts derived from \textit{B. brevis}. Unfortunately, attempts to add ethanolamine to the carboxyl terminal tryptophan enzymatically to complete the biosynthesis of linear gramicidin were unsuccessful.

All the amino acid precursors of linear gramicidin can be incorporated into a protein-bound thioester-linked peptide which proved to be stable to acid, but liberated by alkali (pH 11) or peroxidation (7). The stoichiometry of the amino acids incorporated into the peptide as deduced from double-labeling experiments was 1 Gly: 2 Ala: 4 Leu: 4 Val: 4 Phe in agreement with the stoichiometry of the amino acids in linear gramicidin. Alanine was found only in the L-configuration, and valine was present equally as D and L, whereas leucine was only found in the D-configuration, which correlates rather well with the optical configuration of the amino acids in linear gramicidin. These results clearly suggest that a pentadecapeptide which remains thioester-linked to the enzyme is biosynthesized. The peptide may be released enzymatically by aminoethanolysis. The stage during biosynthesis at which formylation of N-terminal valine occurs remains uncertain. However, Bauer et al. (7) suggested that formylation (presumably requiring tetrahydrofolate) most likely is carried out after synthesis of the polypeptide is completed. The participation of 4′-phosphopantetheine as a cofactor in linear gramicidin formation is anticipated, but this point has not been established to date.

**BACITRACINS**

The bacitracins are synthesized by certain strains of \textit{B. licheniformis} (167). Although several bacitracins have been described in the literature, the most completely investigated is bacitracin A (Fig. 9), a dodecapeptide with four of the amino acids (glutamic, ornithine, phenylalanine, and asparagine) in the D-configuration (1, 22, 99, 100). As already noted, the molecule contains a cyclic hexapeptide and a thiazoline ring structure.

Several laboratories have reported the cell-free synthesis of bacitracin (30, 31, 56–58, 118, 148, 149). Purified bacitracin synthetase (molecular weight 800,000) has been resolved into three complementary fractions by means of Sepharose affinity column chromatography (30) or a combination of hydroxyapatite column chromatography and sucrose density gradient centrifugation (56). It appears that all three fractions are required for maximal bacitracin synthesis; a combination of any two compo-
ponents is less than 25% as active as the three fractions together. The enzyme in peak A (designated component I) has a molecular weight of 200,000 and activates L-isoleucine, L-cysteine, L-leucine, and L-glutamic acid. A second fraction, B (component II), has a similar molecular weight (210,000) but only activates L-lysine and L-ornithine, whereas the third fraction, C (component III), possesses a molecular weight of 380,000 and activates L-phenylalanine, L-histidine, L-aspartic acid, L-asparagine, and L-isoleucine (or L-valine). Amino acid activation requires ATP and Mg\(^{2+}\), and it has been noted that aminoacyl adenylates are formed; subsequently, the amino acids are thioester-bound to the enzyme before polymerization occurs on the polyenzyme. Froyshov and Laland (31) first presented evidence that pantothenic acid is present in bacitracin synthetase and concluded that the enzyme in peak A is a polyenzyme. However, only one component may contain one equivalent of covalently bound 4'-phosphopantetheine. These components are relatively impure (40 to 70%), so that the results should be considered as somewhat preliminary in nature.

Earlier studies with intact cells, protoplast preparations, and in vitro systems indicated that D-amino acids could not replace their respective L-enantiomorphs as precursors for bacitracin formation (11, 21, 56, 57, 118, 151). However, studies with the partially purified enzyme preparation revealed that the L-enantiomorph of glutamic acid, phenylalanine, and asparagine can be replaced, to a considerable extent, by the D-isomers (glutamic acid, 27%; phenylalanine, 79%; asparagine, 81%) for antibiotic synthesis (31). By contrast, D-ornithine could not substitute for its L-isomer. It will be of considerable interest to learn the detailed mechanism of bacitracin biosynthesis as there are obvious differences as well as similarities between the structure of this molecule and those of gramicidin S and tyrocidine.

**EDEINE**

The edeines (Fig. 10) are strongly basic, linear oligopeptide antibiotics produced by *B. brevis* strain Vm4 (48, 50, 82, 125). The amino acid composition is unusual; besides glycine, the molecule consists of isoserine, β-tyrosine, α,β-diaminopropionic acid, and 2,6-diamino-7-hydroxyazaleic acid. The glycine residue in edeine A is attached to the basic constituent, spermidine. In the case of edeine B, the base is guanylspermidine (N-guanyl-N'-(3-aminopropyl)-1,4-diaminobutane). The structure of edeine D is similar to edeine A with α-phenyl-β-alanine substituting for β-tyrosine (169).

Although a number of papers have appeared on the cell-free synthesis of edeine (82, 84-86), the mechanism of its biosynthesis is not fully understood. Polyenzyme fractions that are specific for the activation and polymerization of the constituent amino acids of edeine have been isolated from *B. brevis* Vm4 by Kurylo-Borowska (85). The size and number of fractions that are resolved by DEAE-cellulose chromatography and Sephadex G-200 sieving appear to vary with the age of the culture and method of extract preparation (e.g., lysozyme treatment, grinding). However, only two polypolyenzyme fractions (designated A and B) are needed for edeine formation in the presence of constituent amino acids, spermidine (or guanyspermidine), ATP, and Mg\(^{2+}\). Fraction C, which may be a disaggregation product of the more complex polypolyenzyme B fraction, enhances the formation of edeines A and B. The molecular weight of these fractions is reported to be 210,000 (fraction A), 180,000 (fraction B), and 100,000 (fraction C) (79). Fraction A activates only β-tyrosine; fraction B catalyzes the activation of the other four constituent amino acids of edeine (isoserine, 2,6-diamino-7-hydroxyazaleic acid, α,β-diaminopropionic acid, and glycine). Fraction C activates isoserine and diaminopropionic acid as well as certain nonconstituent amino acids such as L-α- and L-β-alanine and L-leucine. Fractions A and B also catalyze ATP-AMP exchanges, suggestive of secondary acceptor sites for the amino acids on the polypolyenzyme complexes. Fractions A and B were shown to contain covalently bound pantetheine. There is no information concerning the sequence of addition of amino acids during nascent peptide synthesis, nor are there any data regarding the mechanism of attachment of the base to glycine during edeine formation. The large size of frac-
tion A is surprising in light of the fact that it only activates \( \beta \)-tyrosine. A separate enzyme, tyrosine \( \alpha,\beta \)-amino mutase (molecular weight 75,000) catalyzes the conversion of \( L-\alpha \)-tyrosine to \( L-\beta \)-tyrosine (84).

**MYCOBACILLIN**

Mycobacillin (Fig. 11) is a cyclic peptide antibiotic that contains 13 residues of 7 different amino acids (6, 103, 144). The sequence and linkages of the amino acids in the molecule were recently elucidated (103, 144). Mycobacillin lacks free amino groups but contains two free \( \alpha \)-carboxyl groups. All of the aspartic acid residues of the antibiotic have been shown to be in alpha-peptide linkage; the glutamic acid residues (\( \delta \)-isomers), however, are gamma-linked to the adjacent amino acid. Sengupta et al. (144) demonstrated that both \( L \)-aspartic acid (position 5) and \( \delta \)-aspartic acid (positions 2, 8, 11, and 13) are present.

A 10,000 \( \times \) \( g \) supernatant fraction obtained from the protoplast lysate of \( B. \) subtilis B3 was reported to catalyze the synthesis of the antibiotic (145). The process required ATP and was insensitive to the action of RNase and inhibitors of protein synthesis such as streptomycin, chloramphenicol, and puromycin.

There appear to be certain features which distinguish mycobacillin formation from the biosynthesis of other peptide antibiotics (gramicidin S, tyrocidine, bacitracin, edeine). For example, the activation step may involve an ATP-inorganic orthophosphate (\( P_i \)) exchange reaction. Sengupta and Bose noted that such an exchange occurred with \( L \)-proline, a constituent amino acid of mycobacillin (146). This exchange was further stimulated by the other amino acid components of the antibiotic. Omission of certain of the constituent amino acids (\( \delta \)-aspartic acid, \( \delta \)-glutamic acid, \( L \)-tyrosine, and \( L \)-serine) diminished the stimulation observed. Pyrophosphate, an inhibitor of gramicidin S formation, stimulated mycobacillin synthesis in vitro. It was also reported that several peptides were synthesized as intermediates in the biosynthesis of the antibiotic in vitro (146). Peptides

---

**Fig. 11. Structure of mycobacillin.**
containing two, three, four, five, and six different amino acids were formed after incubation with proline plus one to six additional amino acid components of the mycobacillin molecule. No peptide containing all seven different mycobacillin amino acids was observed, nor was any straight-chain tridecapeptide obtained, suggesting that cyclization may occur immediately after such a peptide is synthesized. Characterization of the peptides revealed that each compound had proline as its N-terminal residue and, depending on the peptide in question, aspartic acid, glutamic acid, or tyrosine as its C-terminal amino acid. The peptides were di-, tri-, hexa-, octa-, and undecapeptides; however, the total sequence as well as the stereochemistry of the constituent amino acids in these peptides was not established. The participation of such compounds as intermediates in the biosynthesis of mycobacillin is certainly suggested by the data presented, yet very little is known concerning the detailed mechanism of synthesis of this antifungal antibiotic, particularly with regard to the substrate and cofactor requirements, the nature of the activation, initiation, elongation reactions, and the cyclization of the nascent linear mycobacillin peptide. It would be of interest to know whether, during antibiotic synthesis, the peptide intermediates are enzyme-bound or free. Also, the nature and properties of the polypeptide systems that catalyze the polymerization reactions are not well known. The answer to these as well as other questions pertaining to the biosynthesis of mycobacillin must await additional purification of the enzyme system(s) involved.

**POLYMIXIN AND COLISTIN**

Polymyxin is produced by strains of *Bacillus polymyx* and the related colistins are elaborated by *Bacillus colistinus* Koyama (115, 155–157, 163). Structurally similar to the polymyxins and colistins are the circulin (115). These antibiotics are branched, cyclic decapeptides linked to a fatty acid residue. For example, 6-methyloctanoic acid or isoocctanoic acid, present in colistins A and B, respectively, is present in α-amide linkage with the terminal α,γ-diaminobutyric acid residue (155, 157). The structure of colistin B is presented in Fig. 12. It is of interest to point out that 6-methyloctanoic acid and isoocctanoic acid are synthesized from isoleucine and valine, respectively (59). Two compounds, Nα-6-methyloctanoyl-α,γ-diaminobutyric acid and Nα-isoocctanoyl-α,γ-diaminobutyric acid, formed by colistin-producing cells, have been implicated as intermediates in the biosynthesis of colistins A and B, respectively (60). The cell-free synthesis of colistins A and B has been achieved using soluble extracts obtained from *B. colistinus* and an incubation mixture consisting of Nα-6-methyloctanoyl-α,γ-diaminobutyric acid (and Nα-isoocctanoyl-α,γ-diaminobutyric acid), leucine, α,γ-diaminobutyric acid, [14C]threonine, ATP, and Mg²⁺ (61, 62). The radioactive product formed was identified as colistin by paper chromatography. These studies suggest that the fatty acyl-diaminobutyrate intermediate may initiate colistin biosynthesis. As observed earlier in the case of polymyxin formation in vivo (115), synthesis of colistin in vitro was insensitive to chloramphenicol and puromycin (62). Enzyme activity for colistin synthesis was observed chiefly in the soluble fraction (100,000 × g supernatant fluid) of the cell. Additional evidence that the polymerization process is distinct from protein synthesis was the finding that antibiotic synthesis was not inhibited by RNase (62).
A activating enzyme which may function in polymyxin biosynthesis was partially purified from sonic extracts of the polymyxin B-producing organism, B. polymyxia 2459 (66). The enzyme catalyzes an L-α,γ-diaminobutyrate-dependent exchange of ATP with PP₁. The specific activity of this enzyme increased approximately 10-fold during vegetative growth, which correlated with the appearance of the antibiotic in the growth medium. L-α,γ-Diaminobutyrate activation was also noted in extracts of B. polymyxia ATCC 10401 and B. circulans 14040, producers of polymyxin D and circulin, respectively. By contrast, enzyme activity was absent from extracts prepared from several polymyxin-negative and asporogenous mutants derived from B. polymyxia. Although these data certainly suggest that the activating enzyme plays a role in polymyxin, circulin, and possibly colistin formation, its direct participation has not been established. No system for the cell-free synthesis of polymyxin has been described, nor is there any evidence that the diaminobutyrate-activating activity is present in extracts of the colistin producer, B. colistinus.

SPECIFICITY OF INCORPORATION

In vivo (102, 112, 130, 168) as well as in vitro (30, 33, 34, 37, 58, 63, 93, 97, 120, 128, 153) experiments have revealed that structurally similar amino acids and amino acid analogues can replace certain of the amino acid residues of the peptide chain during antibiotic synthesis. These studies have demonstrated that antibiotic formation is catalyzed by enzymes with rather broad specificities. Multiple forms of an antibiotic are generally produced simultaneously by an organism. These often differ only in the aromatic amino acid or branched-chain amino acid residue that is present at a given site in the peptide. Supplementation with one of these amino acids in vivo or in vitro can direct the biosynthetic process so that the antibiotic(s) formed preferentially will be that rich in the amino acid added exogenously. For example, tyrocidines A, B, and C (differing solely in the number of tyrosine, tryptophan and phenylalanine residues present) are produced in vivo in the ratio of 1:3:7 (102). When tryptophan is added to the medium, B. brevis synthesizes principally tyrocidine C and a new tyrocidine, designated D, both of which are rich in tryptophan; neither tyrocidine A or B (phenylalanine-rich) is produced in significant amounts under these conditions. If phenylalanine is supplied instead of tryptophan, there is synthesis of tyrocidines A and B without formation of the C and D components. Provision of both aromatic amino acids leads to the synthesis of tyrocidines A, B, C, and D simultaneously. Amino acid substitutions have also been noted in experiments with the purified gramicidin S and tyrocidine synthetases (33, 34, 37, 63, 93, 97, 120, 128, 153). Thus, tyrosine can replace phenylalanine (position 1) in both antibiotics, whereas tryptophan can substitute for phenylalanine or tyrosine only during formation of the tyrocidine peptides in vitro (34, 128). Additional examples are listed in Tables 3 and 4. In some instances there may be a replacement only at the activation step (i.e., in the formation of the

<table>
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<th>Table 3. Amino acid and analogue substitutions observed with growing cells</th>
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<td><strong>Antibiotic</strong></td>
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<td>Aromatic amino acids&lt;sup&gt;d&lt;/sup&gt;</td>
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Table 4—Continued

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<tr>
<th>Antibiotic</th>
<th>Amino acid replaced</th>
<th>Analogue</th>
<th>ATP-PP$_1$ exchange* (%)</th>
<th>Antibiotic synthesis* (%)</th>
<th>Reference</th>
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<td>Isoleucine</td>
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<td>100</td>
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<td>DL-Norleucine</td>
<td>73</td>
<td>11</td>
<td>34</td>
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<td>L-Ornithine</td>
<td>Lysine</td>
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<td>100</td>
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<td>L-Diaminopimelic acid</td>
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<td>0</td>
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<td>Hydroxy-L-proline</td>
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<td>100</td>
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<td>Tyrocidine</td>
<td>L-Valine</td>
<td>DL-Norvaline</td>
<td>90</td>
<td>5</td>
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</tbody>
</table>

* 100% is the enzyme activity or antibiotic synthesis obtained with the natural amino acid.

1 Trans-3-methylproline inhibits L-proline-dependent ATP-PP$_1$ exchange by 29%, whereas under similar conditions trans-4-fluoroproline inhibits the reaction by 93% (93).

2 The ATP-PP$_1$ exchange reaction with the L-amino acid was inhibited by the corresponding D-enantiomorph as follows: D-valine (80%), D-ornithine (82%), D-leucine (86%), and D-proline (83%) (93).

But see Rao and Hall (150) for strain differences (B. brevis ATCC 8185 versus 10068) with respect to effect of aromatic amino acids on cell-free synthesis of tyrocidines A, B, C, and D.

**Aminoacyl adenylate**, suggesting that a greater degree of specificity exists at the point of interaction between the aminoacyl adenylate and the thiol group on the enzyme (97).

**RACEMIZATION AND THE SYNTHESIS OF D-AMINO ACIDS**

D-Amino acids have been found as constituents of microbial cell walls (142), peptidolipids (3), capsules (161), toxins (46), and antibiotics (14, 20). Racemases for various amino acids have been observed in microorganisms (110); however, the biosynthetic mechanism for the D-amino acid residues in most of these peptide metabolites remains unclear. In some cases, both the L- and D-enantiomorph of an amino acid can be utilized for synthesis of the peptide-bound D-amino acid (71, 160). In other instances, there is evidence to suggest that only the L-isomer is used for the biogenesis of the amino acid in the peptide structure (11, 26, 151). Troy recently reported that a particulate fraction from an encapsulated strain of *B. licheniformis* catalyzed the polymerization of L-glutamic acid (but not the D-isomer) to form a high-molecular-weight poly(α)-D-glutamic acid (161). It has also been observed that the D-amino acid may actually inhibit peptide synthesis, such inhibition being reversed by the L-enantiomorph (26).

Several hypotheses have been advanced to explain the biogenetic origin of D-amino acids in microbial peptides. Mäuger (108) has postulated that D-amino acids in antibiotics are formed from L-amino acids after incorporation of the latter into stereochemically labile inter-

mediates such as cyclic dipeptides (e.g., diketopiperazines). Bycroft (20) proposed that a combined (presumably enzyme-bound) form of a dehydroamino acid derived from the corresponding L-amino acid might be reduced stereospecifically in vivo to the D-isomer during antibiotic formation. These hypotheses have not yet been substantiated experimentally. In fact, studies by Huang et al. (54) and Mason et al. (105) indicate that a dehydroamino acid does not participate as an intermediate in the inversion of L-valine to its D-enantiomorph during the respective syntheses of penicillin and actinomycin. By contrast, it now seems certain with respect to tyrocidine and gramicidin S that, after the activation step, racemization of phenylalanine takes place on an enzyme-bound, thioester-linked intermediate (38, 81, 97, 128). Purified light enzyme obtained from either the gramicidin S or tyrocidine synthetase and the intermediate enzyme (tyrocidine synthetase) catalyze the racemization reaction with ATP and L- or D-phenylalanine as substrate (38, 128, 160). The phenylalanine residue at position 1 of the gramicidin S or tyrocidine molecule, as well as the phenylalanine at position 1 of the tyrocidine peptide, is incorporated as the D-amino acid. Recently, Lipmann (95) observed that racemization of phenylalanine labeled with tritium at the α-carbon takes place with complete loss of hydrogen. Details of this experiment have not been published, so the mechanism of the reaction remains somewhat obscure. Likewise, the cell-free synthesis of D-amino acids present in other peptide antibiotics produced by bacilli is not well understood. Ra-
cemization of amino acids may proceed via an analogous mechanism (38, 95).

**MUTANT STUDIES**

Mutants have frequently provided a useful means to investigate biosynthetic mechanisms and to determine the genetic control of metabolite formation. In this regard, mutants have been obtained that are unable to synthesize a number of peptide antibiotics (e.g., gramicidin S [B. brevis] [64, 68, 81, 147], bacitracin [B. licheniformis] [43, 44], polymyxin [B. polymyxa] [66], and mycobacillin [B. subtilis] [121]). Certain of these mutants appear to have defects in structural genes, whereas others may be affected in regulatory sites of the genome which control antibiotic synthetase formation.

Kurahashi et al. (68, 81) and Saito and co-workers (64, 147) characterized several classes of mutants of B. brevis which are unable to synthesize gramicidin S. A summary of certain of these mutants is presented in Table 5. Mutants of group I were shown to lack the light enzyme, but possessed a normal heavy enzyme. Group II mutants had a normal light enzyme, but did not contain an L-proline-, L-valine-, L-ornithine-, and L-leucine-activating enzyme activity. Group III mutants lacked both the heavy and light enzyme fractions. As might be expected, cell-free extracts of these mutants were unable to synthesize gramicidin S and could not form D-phenylalanyl-L-prolyl diketopiperazine (i.e., could not initiate peptide synthesis on the heavy enzyme). On the basis of these results, Kambe et al. (68) suggested that different types of mutations may occur, i.e., mutations affecting the regulatory mechanism controlling both light and heavy enzyme synthesis as well as those mutations affecting the regulatory or structural genes of the light or heavy enzyme, individually.

Other types of gramicidin S-less mutants have been described (68, 147). A fourth group appears to have a normal phenylalanyl-activating enzyme, but an incomplete or defective enzyme I complex. Characterization of mutants in the fourth group has revealed that the heavy enzyme complex is missing one specific activating enzyme (e.g., for L-proline, L-valine, or L-leucine), but is still able to activate the remaining three amino acids. The fifth group of mutants retained all of the amino acid-activating enzymes needed for gramicidin S formation, but still cannot synthesize gramicidin S. The defect in mutants of groups IV and V may be due to (i) the absence of a subunit that catalyzes the activation of a specific amino acid, (ii) a defect only in the secondary amino acid acceptor site, i.e., the thioester-binding site, or (iii) a conformational change [possibly due to (ii)] in the heavy or light enzyme that results in reduced complex formation between the two polypeptide components. It has also been proposed that other mutational events may influence the interaction and association of the subunits of the heavy enzyme or the formation of the phosphopantetheine arm (68).

Haavik and Freyshov (43) recently isolated a mutant, obtained from B. licheniformis AL, that was unable to produce bacitracin as a result of a defective enzyme complex. As described earlier (30, 56), the parental strain produces a bacitracin synthetase that can be separated into three complementary fractions, A, B, and C; all three components are needed for bacitracin synthesis. Only two fractions (AC₈ and B₆) were obtained from the mutant, B. licheniformis SB319 (43). Component AC₈ activated the same amino acids as components A and C from the parent. B₆ activated the same amino acids as the parental B fraction. However, there were marked differences seen in the

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<th>Mutant class</th>
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<th>Ability to form:</th>
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<td>None, wild type</td>
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<td>+</td>
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<tr>
<td>I</td>
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<td>V</td>
<td>+</td>
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* DKP, D-Phenylalanyl-L-prolyl diketopiperazine.
degree of the ATP-PP\textsubscript{1} exchange reaction with
the enzyme fractions derived from the producer
and nonproducer cells. Mixing experiments re-
vealed that enzyme component B\textsubscript{0} could cata-
lyze the synthesis of bacitracin with com-
ponents A and C; by contrast, component AC\textsubscript{0},
when used with component B, was unable to
synthesize the antibiotic. The evidence, there-
fore, suggests that the bacitracin synthetase
obtained from the nonproducer was defective
and that the lesion may involve the activation
sites for the amino acids. The affinity between
the enzyme components may also be affected.

POSSIBLE FUNCTIONS OF PEPTIDE
ANTIBIOTICS IN THE PRODUCING
ORGANISM

The function of antibiotics in the metabolism
of the producing organism has been the subject
of considerable speculation and discussion (17,
18, 164, 166, 170). However, there has been
little understanding with respect to the role, if
any, that they actually do play. Several pro-
posed functions for antibiotics are no longer
accepted as likely candidates, whereas others
are still under consideration in several labora-
tories. Those that have been discarded involve
antibiotics as evolutionary relics, waste prod-
ucts of cellular metabolism, reserve food mate-
rials, spore coat components, or breakdown
products derived from cellular macromolecules
(166). Still under current consideration (40) is
the possibility that antibiotics function to kill
or inhibit the growth of other organisms in
nature, thereby providing a competitive advan-
tage to the producing species (17). Although one
usually thinks of this competition as between
bacterial species A versus bacterial species B or
bacterium versus fungus, it could involve bacte-
ria versus amoebae (S. H. Hutner, per-
sonal communication) since these protozoa use
bacteria as sources of food (150). Another pos-
ibility is that the competition exists between
strains of the antibiotic-producing species itself
(101; E. Rosenberg, personal communication).
A further variation of the competition hypothe-
sis involves the excretion of the antibiotic dur-
ing spore germination in order to eliminate
competitors in the immediate environment of
the germinating spore. A completely different
concept, proposed by Bu'Lock (18), is that anti-
biotic synthesis is a means of keeping the cellu-
lar machinery in working order during the time
when cell growth is not possible due to unfavor-
able conditions. Although this is an interesting
speculation, no data exist in support of the
maintenance hypothesis. Additional hy-
potheses currently being debated state that
synthesis of an antibiotic (or other secondary
metabolites) is a method of avoiding cell death
due to unbalanced growth (166), or that it pro-
vides a mechanism of detoxification (170). With
respect to the unbalanced growth hypothesis, it
is assumed that certain primary metabolites
(e.g., amino acids or nucleotides) are overpro-
duced, when conditions for exponential growth
are no longer favorable, due to ineffective con-
trol systems in the producing organism. These
metabolites are thought to be converted to anti-
biotics which are released from the cell. The
detoxification hypothesis is slightly different,
proposing that certain toxic antimetabolites are
synthesized when exponential growth termi-
nates, e.g., analogues of nucleosides, amino
acids, or amino sugars. Conversion to the anti-
biotic is thought to provide a method of detoxifi-
cation. Inherent in both the unbalanced growth
and detoxification proposals is the assumption
that the antibiotic is not toxic to the producing
organism (170). There exists no evidence in
support of these two hypotheses. Indeed, most
antibiotics are more toxic to the producing or-
ganism than are their precursors (24). Other
possibilities for antibiotic function include (i)
facilitation of metal transport into cells (43) and
(ii) autoinhibition of germination (29). The lat-
ter is suggested by the following observations:
many microorganisms produce self-inhibitors
of germination (154); various antibiotic producers
contain the antibiotic in their spores (27, 29,
67).

Much of the criticism against the competition
hypothesis is based on the inability of certain
investigators to detect antibiotic production in
unsterilized soil which has not been supple-
mented with organic nutrients (164). The same
type of criticism has also been used to support
the viewpoint that antibiotics are biological ac-
cidents which are of no direct benefit to the cell.
However, it is known that soil which contains
seeds and decaying vegetable matter and the
rhizosphere support antibiotic producion (17,
19, 40, 78).

Of the various functions postulated for anti-
biotics, the one which has received the most
attention in recent years is the view that anti-
biotics are important compounds in cellular dif-
erentiation, i.e., the transition from vegetative
cells to spores (52, 55, 132, 139). Numerous anti-
biotics are elaborated by microorganisms that
undergo sporulation. Due to the fact that the
vast majority produced by bacilli are peptidic in
nature, these peptides have been singled out as
playing a key role in the termination of vegeta-
tive growth, allowing sporulation to take place.
The following factors make this hypothesis at-
tractive (24, 45). (i) Practically all sporulating
microorganisms produce antibiotics. (ii) Although it was once believed that the antibiotics elaborated by an organism have no effect on the producing cell, it is now well established that they are inhibitory to vegetative growth at concentrations at which they are generally produced by sporulating cultures (65, 123, 138, 152). Peptide antibiotics produced by bacilli specifically inhibit important cellular processes, e.g., deoxyribonucleic acid (DNA) synthesis, cell wall synthesis, membrane function, and structure (52, 132). (iii) Production of peptide antibiotics usually begins at the late-logarithmic phase of growth and continues during the early stages of the sporulation process in bacilli (5, 10, 11, 36, 82, 115, 138, 160). (iv) Sporulation and antibiotic synthesis are induced by depletion of some essential nutrient. (v) There are genetic links between the synthesis of antibiotics and the formation of spores (24, 45, 139). For example, antibiotic formation by certain bacilli is an early event beginning at stage I of sporulation. Mutants that are asporogenous (and map at different loci) are almost always antibiotic negative and, when so, are blocked very early (66, 68, 143). Revertants, transductants, and transformants of stage 0 asporogenous mutants, restored in their ability to synthesize antibiotic, also regain the ability to sporulate (4, 51, 140). Also, conditional asporogenous mutants fail to produce antibiotic at the nonpermissive temperature (92, 158). (vi) Physiological correlations also favor a relationship between the production of an antibiotic and spore formation. As an example, inhibitors of sporulation also inhibit antibiotic synthesis (10, 65, 115). Furthermore, both processes are repressed by glucose (10, 139), and concentrations of manganese ion of at least two orders of magnitude higher than that required for normal cellular growth are needed for sporulation and antibiotic synthesis by certain species of Bacillus (165).

Because of the above observations, several investigators have concluded that antibiotic production is required for sporulation. Hodgson (52) speculated that peptide antibiotics might be used in several ways by an organism during the process of sporulation as modifiers of the cell membrane, e.g., as detergents disrupting structural components, or as ion carriers, modifying permeability properties. Hodgson argued that, by selectively functioning at certain stages, the sporulation process would be able to proceed normally. Sadoff (132) pointed out that the peptide antibiotics produced by bacilli exhibit effects upon membrane synthesis and function, cell wall synthesis, and nucleic acid synthesis, which are important processes for sporulation. He contended that the antibiotics, when produced by an organism, may act as selective modifiers of cell function — i.e., repressing or inhibiting vegetative cell macromolecular synthesis, but permitting endospores to be formed.

Although the above observations and conclusions point to an intimate relationship between antibiotic formation and sporulation, by no means do they prove that the antibiotic is necessary for sporulation. Furthermore, there are data which fail to support this hypothesis. For example, there are conditions in which increased sporulation is accompanied by a decrease in antibiotic production (143) and vice versa (11). Recent nutritional studies on gramicidin S formation by B. brevis revealed a dissociation between sporulation and antibiotic formation (25). Media used at the beginning of the investigation supported sporulation but yielded little to no gramicidin S. As more suitable media were devised for antibiotic production, sporulation became markedly poorer. Another indication of a physiological dissociation between gramicidin S formation and sporulation arose from chemostat experiments in which nutrient limitation was used to study formation of the light and heavy gramicidin S synthetases (106). The enzymes were produced at intermediate growth rates under carbon, nitrogen, sulfur, or phosphorus limitation. Although carbon limitation yielded the poorest synthetase activity, it was the only condition under which significant amounts of spores were formed. Also, the growth rate favoring sporulation was considerably lower than that which was optimal for gramicidin S synthetase formation.

The most damaging evidence to the hypothesis involving antibiotics in sporulation is the existence of mutants which are antibiotic negative but can still sporulate. For example, Kambe et al. isolated gramicidin S-negative mutants of B. brevis with genetic blocks in the formation of the heavy synthetase, the light synthetase, or both (68). Upon examining the sporulating abilities of the gramicidin S-negative mutants, Kambe et al. (68) found them all to be oligosporogenous. However, recent studies on mutant hh, reportedly containing a specific defect in the leucine-activating function of the heavy synthetase (68), revealed that this gramicidin S-negative mutant sporulates normally (25).

The ability of peptide antibiotic-negative mutants to sporulate is also seen with other bacilli. Adenine-requiring mutants of B. subtilis B2, which fail to produce intra- or extracellular mycocabillin, sporulate and germinate very well, the spores containing normal concentrations of dipicolinate (121). Similarly, B. licheni-
formis mutant SB 319 produces no detectable intra- or extracellular bacitracin, yet it sporulates normally (44). Both parent and mutant have the same growth rate, morphology, and biochemical characteristics, but the mutant has a defective bacitracin synthetase complex (43).

Much of the enthusiasm in favor of a function of antibiotics in sporulation derives from the work of Sarkar and Paulus (138). They noted a specific inhibition of RNA synthesis by the tyrothricin complex during growth of the producing culture, B. brevis ATCC 8185. Moreover, purified B. brevis RNA polymerase was also inhibited by the complex. Both components of the tyrothricin complex (linear gramicidin and tyrocidine) but not gramicidin S, produced by another strain of B. brevis, were shown to exhibit similar inhibitory activity, indicating the specificity of the effect. They also observed that the cessation of exponential vegetative growth \((t_a)\) was accompanied by a sharp decline of net RNA synthesis. Tyrothricin synthesis began at \(t_a\); the antibiotic complex remained largely associated with the cell material and reached a maximum level 2 h after exponential growth \((t_e)\). Sarkar and Paulus (138) advanced the view that peptide antibiotics regulate gene transcription during the transition from vegetative growth to sporulation by selectively terminating the expression of vegetative genes. Unfortunately, they erroneously claimed that all peptide antibiotics were produced by sporulating microorganisms, a statement that ignores the production of comirin by Pseudomonas antimyctica, micrococin P by Micrococcus sp., and nisin by Streptococcus lactis and Streptococcus cremoris. It is even more disconcerting to read (65, 141) that all antibiotics are made by sporulating cultures!

Kleinkauf and co-workers (122, 123, 141) also proposed a role for tyrocidine and linear gramicidin in B. brevis sporulation. Their data suggest opposing effects of the two peptides in vivo. In vitro, tyrocidine inhibited transcription by forming a complex with DNA. Although linear gramicidin also inhibited transcription, it did not complex with DNA. Of particular interest was the ability of linear gramicidin to abolish the action of tyrocidine, allowing RNA synthesis to proceed. Tyrocidine also inhibited in vivo transcription in B. brevis cells; in the presence of linear gramicidin, however, a higher concentration of tyrocidine was required to attain complete inhibition. To explain these data, Ristow et al. (122) proposed that in vivo a complex may be formed between linear gramicidin and tyrocidine which would have less affinity for DNA than tyrocidine alone. They argued that perhaps both peptides are required to ensure that not all of the genes are turned off at the end of vegetative growth so that some can be transcribed during sporulation. What is disturbing about this hypothesis are their data showing that, although linear gramicidin reversed the effect of tyrocidine on growth and in vivo RNA synthesis, it did not reverse tyrocidine's inhibition of sporulation; in fact, in the presence of the antibiotic mixture (i.e., in the proportion in which they are made as the tyrothricin complex), sporulation was completely inhibited. To us, this means that the inhibition of RNA synthesis has nothing to do with sporulation. The main conclusions that one can derive from these studies is that both tyrocidine and linear gramicidin inhibit B. brevis RNA polymerase. However, this is not specific nor surprising since this group (141) has shown that tyrocidine also inhibits E. coli RNA polymerase, and the effect of B. brevis polymerase is also exerted when calf thymus DNA is substituted for B. brevis DNA as template.

All of the experimental data described to date could fit in well with the hypothesis that sporulation and antibiotic formation, although independent phenomena, are regulated by a common (or a similar) regulatory mechanism. Many of the mutants used in obtaining the data suggesting a role of antibiotics in sporulation were pleiotropic and probably blocked in regulatory genes. An attempt to demonstrate a function for the antibiotic in sporogenesis by adding crude antibiotic to pleiotropically asporogenous and antibiotic-negative Bacillus mutants failed to induce sporulation (139). As suggested by Schmitt and Freese (143), the critical experiment involving the isolation of an asporogenous mutant that can sporulate only upon addition of pure antibiotic has never been reported. The closest to such a finding was the claim of Jayaraman and Kannan (65) that addition of tyrothricin to the sporogenic B. brevis ATCC 8185 culture inhibited growth but enhanced sporulation. However, Ristow et al. (123) failed to confirm this result. Jayaraman and Kannan (65) also reported that addition of polymyxin to its producer, B. polymyxa, inhibited growth and stimulated sporulation. However, the significance of this observation is obscured by their finding that the antibiotic also inhibited glucose uptake. Thus, sporulation could have been enhanced merely by release of sporulation from catabolite repression. When D-leucine, an inhibitor of polymyxin synthesis (but not growth), was supplied to B. polymyxa, sporulation was strikingly inhibited. L-Leucine reversed the effect of D-leucine on both antibiotic production and sporulation. However, since L-leucine is needed for both polymyxin synthesis and for protein synthesis during sporulation, the effect of D-leucine
can be explained by an inhibitory effect on two independent phenomena. An oft-quoted and misquoted (65, 122, 123, 141) finding in support of a sporulation function for antibiotics are the data of Yoshida et al. (173), which revealed that addition of actinomycin to young cultures of the producer organism \textit{(Streptomycetes antibioticus)} markedly inhibited protein synthesis, whereas later addition during actinomycin production had no effect. It is claimed by these investigators (65, 122, 123, 141) that, since the antibiotic exerts its effect during growth but not during the stationary phase, it plays an important role in the transition between a growing and a differentiating cell. However, the subsequent finding (104) that \textit{S. antibioticus} becomes impermeable to exogenous actinomycin when the organism enters the stationary phase has apparently been missed by those who quote the original observation and surely invalidates their claim. Some recent experiments by Lee et al. (90) have provided an additional, although as-yet-unexplained, facet to the present controversy. It has been known that the enzymes for tyrocidine synthesis suddenly appear at the end of the exponential growth phase; only during a short period at the onset of the stationary phase has it been possible to obtain active soluble enzyme extracts. Later in the stationary phase, only particulate enzymes are recovered. Biochemical investigations carried out concomitantly with electron microscopy have shown most of the tyrocidine-forming activity becomes associated with forespores in \textit{B. brevis}. The significance of the migration of antibiotic-synthesizing activity from the soluble portion of the cell into the forespores is not clear. Perhaps it merely represents a nonspecific trapping of vegetative cell components that are truly unessential to the sporulation process or to the spore itself. The authors (90) are properly cautious in their interpretation of these results, stating that their data are “consistent with the assumption of an effect of the antibiotic facilitating the transition from the vegetative phase into sporulation. However, this laboratory has not yet probed into the causative connection between antibiotic and spore formation.”

It should be clear to the reader that the mystery of antibiotic function has not been solved. We feel, however, that peptide antibiotics must have some role in the survival of the producing organism. It is inconceivable to us that the intricate reaction sequences of antibiotic biosynthesis would have been retained in nature without benefit to the organism. It is often argued that the strain degeneration problem in the antibiotic industry speaks against a survival function. However, this argument is irrel-
evant since the amounts produced in commercial antibiotic fermentations are many orders of magnitude greater than that needed for an organism’s survival. In fact, the abnormal degree of synthesis observed in industrial fermentations is probably detrimental to the well-being of the organism and it is logical to assume that the culture would revert to a lower-producing, more rapidly growing form which would be capable of more balanced metabolic activity. Whatever the true function of antibiotics, many mechanisms exist whereby organisms can protect themselves from the antibiotics they elaborate (24). Permeability changes, compartmentalization, and the presence of an inactive form of the antibiotic intracellularly may all play a role in preventing self-annihilation. In support of the idea of “a built-in survival kit,” Kurylo-Borowska (83) demonstrated in \textit{B. brevis} Vm4 that edeine B synthesized in vivo is present in an inactive form covalently bound to the edeine-synthesizing polyenzymes and associated with a complex of cytoplasmic membrane and DNA. Free edeine is not found in the cells and the polyenzyme-membrane-DNA-bound edeine neither exhibits antimicrobial activity nor influences DNA synthesis. Biological activity is seen only when the antibiotic is released from the cell or from the complex in vitro.

**CONCLUDING REMARKS**

It is now evident that the biosynthesis of peptide antibiotics by members of the genus \textit{Bacillus} is directed by multienzyme thiotemplates. Amino acids employed as substrates for antibiotic synthesis are activated by ATP to form enzyme-bound aminoacyl adenylates. The aminoacyl moiety is then transferred to secondary acceptors, i.e., to specific SH groups, to form covalent thioester bonds. Antibiotic synthesis involves polyenzyme complexes that exhibit a broader specificity than that shown by ribosomal protein-synthesizing systems. tRNA, mRNA, and ribosomes are not directly involved. In antibiotic synthesis, the polyenzyme complex serves as template for the proper sequencing of the amino acids in the peptide. The processes of initiation, elongation, and termination are inherent properties of the polyenzyme system. The energy derived from the thioester bonds provides the driving force for the peptidation reactions. As observed in the growth of ribosomal polypeptide chains, antibiotic formation is initiated from the N-terminal position and ends at the carboxyl terminus. In the case of antibiotics such as gramicidin S and tyrocidine, cyclization is the final step before release of the antibiotic from the enzyme. This reaction, as well as the mechanism of amino acid racemization, is not well under-
stood. 4'-Phosphopantetheine plays an important role as coenzyme in the process of peptide chain elongation, mediating alternative trans-thiolation and transpeptidation reactions.

Little progress has been made in elucidating the function(s) of antibiotics in the producing organism. We believe that they indeed have a function(s) and that they are made in nature, especially in microenvironments containing organic matter. It is doubtful that all antibiotics have the same general function; we certainly accept the fact that different classes of proteins have different functions.

The view that peptide antibiotics function in cellular differentiation (i.e., sporulation) is not supported by data obtained with producers of gramicidin S, mycobacillin, and bacitracin. In these cases, nonproducing mutants are capable of sporulation. Of course, not enough work has been done to eliminate the possibility that other peptide antibiotics are involved in sporulation.

If the antibiotic is not an obligatory part of the sporulation process, then why are antibiotic production and sporulation so often related to one another? We propose that they are regulated by the same or similar control mechanisms and, furthermore, that there are reasons why it is beneficial for the sporulating cell to produce an antibiotic. For example, (i) the antibiotic could be packaged in the Bacillus spore and excreted during germination to inhibit or kill competitors, thereby providing an environment favorable for the development of the germinating spore; or (ii) the antibiotic could be packaged in the spore and inhibit germination until environmental conditions become more favorable. It is hoped that the availability of nonproducing Bacillus mutants will now allow us to approach the problem of antibiotic function in a more conclusive manner.

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ADDENDUM IN PROOF

Akers et al. (1b) recently reported that two enzyme fractions (derived from B. brevis) catalyzed the synthesis of gramicidin A to the heptapeptide stage. By contrast, Akashi et al. (1a) noted that the synthesis of formylvaline and formylvalylglycine could be achieved employing a partially purified enzyme system from B. brevis ATCC 8185. The enzyme preparation (component I) catalyzed an ATP-PP, exchange reaction that was dependent solely on valine and glycine. The molar ratio of valine to glycine bound by component I was approximately 1. Evidence was also obtained that the amino acids were bound to component I by thio-ester linkage and that formylvaline and formylvalylglycine were synthesized (in the presence of a formyltetrahydrofolate-synthesizing system, ATP, valine, glycine, and an "AS45" fraction from B. brevis) as enzyme-bound intermediates. The authors (1a) proposed the following biosynthetic sequence for gramicidin A formation: (i) initiation via the formylation of valine bound to component I, followed by the synthesis of formylvalylglycine; (ii) peptide elongation through transfer of formylvalylglycine to a second enzyme component that activates and binds alanine and leucine; (iii) further elongation of the nascent peptide, which may require additional enzyme components. The biochemical mechanism for linking ethanolamine to the C-terminal tryptophan residue of gramicidin A still remains undetermined.

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