BIOSYNTHESIS OF POLYPEPTIDE ANTIBIOTICS

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ABSTRACT

Peptide antibiotics possess a variety of chemical structures, e.g. cyclic di- and oligopeptides, depsipeptides, linear peptides with repeating sequences of L- and D-amino acids, substituted peptides containing non-peptide components. These antibiotics frequently contain amino acids which are not constituents of proteins.

In general, biosynthesis of peptide antibiotics follows active growth and macromolecular synthesis by microorganisms. Studies to date indicate that the mechanism of formation of peptide antibiotics differs markedly from that of protein synthesis. *In vivo* experiments reveal that antibiotic synthesis is insensitive to inhibitors of protein and nucleic acid synthesis. Certain amino acid analogues can block either antibiotic or protein synthesis with minimal inhibition of the other process. Protein and peptide antibiotic synthesis probably compete for the intramolecular amino acid pool; when one process is inhibited there may be marked stimulation of the alternate system. *In vitro* experiments reveal that antibiotic synthesis is RNAse insensitive and there is no requirement for ribosomes, tRNA, or mRNA. Enzymatic synthesis generally requires ATP, Mg^{2+}, a reducing agent, and the requisite amino acids. The process (gramicidin S, tyrocidine) is catalysed by multi-enzyme complexes. The activation of amino acids is mediated by aminoacyl synthetases (in the protein complex) distinct from the aminoacyl-tRNA synthetases that activate amino acids for protein synthesis. The activated amino acids are transferred to thiol groups in the complex, and peptide synthesis occurs subsequently. The sequence of amino acids in the antibiotic is determined by the unique arrangement of specific enzymes in the multi-enzyme complex.

Studies with cell-free systems as well as with intact organisms have revealed that chemically-related amino acids may substitute for normal constituents in antibiotic peptides. These data support the view that antibiotic peptide synthesis is catalysed by enzymes with relatively broad or low specificities.

D-Amino acid biogenesis appears to involve an ATP-dependent racemization catalysed by a component of the multi-enzyme complex.

Since the discovery of penicillin a large number of peptide antibiotics have been isolated and described in the literature[1–6]. These compounds, elaborated by a variety of microorganisms, contain one or more amino acids or moieties derived from amino acids. Studies have been carried out on the chemistry, biological activity, mode of action, and biosynthesis of these compounds. The evidence to date concerning the mechanism of formation of

peptide antibiotics has revealed that a different type of mechanism is involved in their biogenesis in contrast to that described for cellular proteins[7]. Today I would like to discuss certain aspects of their biosynthesis.

First I wish to consider some of the properties of peptide antibiotics[8,9] which distinguish them from proteins (*Table 1*):

Table 1. Properties of peptide antibiotics

1. Molecular weight—350 to 3000.
2. Family of closely related substances produced by a microorganism.
3. Some composed solely of amino acids; other contain amino acids plus other constituents.
4. Unusual amino acids: D-amino acids, methylated amino acids, β-amino acids, imino acids, basic amino acids, S-containing amino acids.
5. Cyclic structures.
6. Unusual linkages or arrangements of amino acids.
7. Generally resistant to hydrolysis by peptidases and proteases (animal, plant).

(1) To begin with, peptide antibiotics are generally much smaller in size than proteins. The molecular weights of the antibiotics range from about 350 to 3000. However, not all peptide antibiotics are small. For example, neocarzinostatin, an acidic protein, possesses a molecular weight of 9000.

(2) Microorganisms generally synthesize a family of closely related substances rather than a single entity. The members of a group generally differ from each other by one or, at most, a few amino acids, the rest of the molecule being the same.

(3) Some peptide antibiotics are composed entirely of amino acids, whereas others contain fatty acids, pyrimidines, amino sugars, hydroxy acids, amines, and other constituents besides the peptide portion of the molecule.

(4) Frequently, peptide antibiotics contain amino acids which are unique and are not found in proteins. For example, D-amino acids, methylated amino acids, β-amino acids, imino acids, basic amino acids (e.g. ornithine) and S-containing amino acids.

(5) Many antibiotics possess cyclic structures with no free α-amino or carboxyl groups. Besides the cyclic nature of a molecule, there may be unusual linkages or arrangements of the amino acids.

(6) Peptide antibiotics are generally resistant to hydrolysis by peptidases and proteases of animal and plant origin.

IN VIVO STUDIES

A. Formation of peptide antibiotics in relation to growth and protein synthesis

A number of studies have shown that the synthesis of peptide antibiotics is initiated after the organism has passed the rapid growth phase. This has been noted in the case of actinomycin, triostin, gramicidin S, tyrocidine, polymyxin, and many other antibiotics[10–25]. In the case of actinomycin[10], it has been reported that approximately 70 per cent of the growth of the organism is completed before antibiotic production is detected in the medium, and that very little growth occurs after the actinomycin titre reaches 15 to 20 μg/ml of medium. Kurahashi *et al.*[12,13] showed that gramicidin S and the enzyme systems necessary for its synthesis were produced in cells of *B.*

brevis during the late log phase of growth. The specific as well as total gramicidin S-forming activity was low at the mid log phase of growth, but they both increased abruptly and reached a maximum at the late log phase. The enzymatic activity for antibiotic synthesis then fell within a few hours to the level of the middle log phase. The activity of an ATP-dependent phenylalanine racemase, involved in gramicidin S synthesis, followed the same pattern[13].

The synthesis of cellular proteins and of a peptide antibiotic by an organism also varies considerably during the course of growth and antibiotic formation[26,27]. During the period of rapid cell synthesis there is a maximal incorporation of a ^{14}C-labelled amino acid into cell proteins, but negligible incorporation of ^{14}C-label into antibiotic, as synthesis of the latter is generally not detected[26]. After the organism has entered the late log or stationary phase, there is considerably less incorporation of the amino acid into protein ($\frac{1}{3}$ to $\frac{1}{6}$). The synthesis of antibiotic is initiated during this period and incorporation of an amino acid precursor into an antibiotic increases appreciably with the length of incubation until maximum synthesis of antibiotic occurred.

B. Inhibitors of protein and nucleic acid synthesis—antibiotics

Not only does antibiotic production follow maximal protein synthesis and cell growth, but it is now clear from studies with certain inhibitors of protein and nucleic acid synthesis that antibiotic synthesis is generally not blocked by these inhibitors. For example, chloramphenicol and puromycin markedly inhibited (>90 per cent) the incorporation of L-valine-^{14}C into cellular protein of *S. antibioticus*. By contrast, there was no inhibition of actinomycin synthesis and, in fact, a two- to three-fold stimulation of the incorporation of the labelled amino acid into the antibiotic was observed[10,26,28]. Streptomycin, neomycin, tetracycline and erythromycin, inhibitors of protein synthesis, also were found to stimulate actinomycin formation (*Table 2*). By contrast, vancomycin, penicillin, actinomycin and mitomycin C, inhibitors of cell wall or nucleic acid synthesis had no influence on actinomycin formation. The stimulation of incorporation of amino acids into actinomycin in the presence of chloramphenicol and the other antibiotics can be explained by the availability of the intracellular amino acid pool for antibiotic synthesis in the absence of protein synthesis[10,11]. Abraham *et al.*[1] suggested that a rate limiting factor for the biosynthesis of peptide antibiotics is the availability of precursors in the amino acid pool which could increase when the demands of protein synthesis fall off. This appears to be the case, as our data suggest that appreciable competition for amino acids exists between the processes of antibiotic and protein biosynthesis.

Observations similar to those found for actinomycin have now been reported for a number of peptide antibiotics, e.g. polymyxin B[17,18], edeine[29], and triostin[11]. The formation of bacitracin[22,30], tyrocidine[15], or gramicidin[31,32] was also unaffected, but not stimulated by antibiotic inhibitors of protein and nucleic acid synthesis. However, Eikhom and Laland[33] have reported that there was enhanced incorporation of ornithine-^{14}C into gramicidin S in the presence of actinomycin D.

Table 2. Effect of antibiotics on incorporation of ^{14}C-labelled amino acid precursors into actinomycin

^{14}C-Amino acid precursor	Antibiotic	^{14}C-incorporation into actinomycin cpm/ml	Increase per cent
L-Valine-1-^{14}C	—	1910	0
	penicillin	1780	0
	chloramphenicol	5200	172
	puromycin	4270	124
	mitomycin	1960	0
L-Threonine-^{14}C	—	1420	0
	chloramphenicol	3165	123
Glycine-1-^{14}C	—	220	0
	chloramphenicol	625	184
L-Methionine-$^{14}CH_3$	—	1340	0
	chloramphenicol	2225	66
DL-Tryptophan-^{14}C	—	1900	0
	chloramphenicol	3230	70
L-Proline-^{14}C	—	1700	0
	chloramphenicol	3700	118
	tetracycline	4000	135
	neomycin	4200	147
	streptomycin	2900	71
	erythromycin	2800	65
	vancomycin	1700	0

Nisin, on the other hand, may be synthesized on a ribosomal template. Hurst[34], and more recently, Ingram[35], have reported that chloramphenicol, puromycin and terramycin were more effective inhibitors of nisin formation than of protein synthesis. Mitomycin C and actinomycin D affected nisin formation to a very limited extent, however.

C. Amino acid and nucleic acid analogues

Amino acid and nucleic acid analogues have also been employed to differentiate between antibiotic peptide and protein synthesis. Winnick *et al.*[16] observed that 5-fluorouracil, 5-bromouridine and 5-fluorotryptophan were able to inhibit *B. brevis* growth and protein synthesis significantly with only slight or partial effects on gramicidin S synthesis. On the other hand, a number of amino acid analogues, e.g. norleucine, norvaline, fluorophenylalanine and hydroxyproline were highly inhibitory to gramicidin S formation without affecting protein synthesis appreciably. Certain analogues of amino acids present in tyrocidine were also shown by Winnick *et al.*[36] to inhibit tyrocidine synthesis by *B. brevis*; at the same time, gramicidin formation was stimulated, and the incorporation of valine-^{14}C into gramicidin was enhanced when tyrocidine synthesis was partially inhibited. Mach *et al.*[15] also noted a selective effect of amino acid analogues on tyrocidine and protein synthesis. Some of their results are shown in *Figure 1*. With most compounds examined, tyrocidine synthesis was almost completely stopped with only a limited effect (20 per cent) on protein synthesis. By contrast, some of the analogues proved

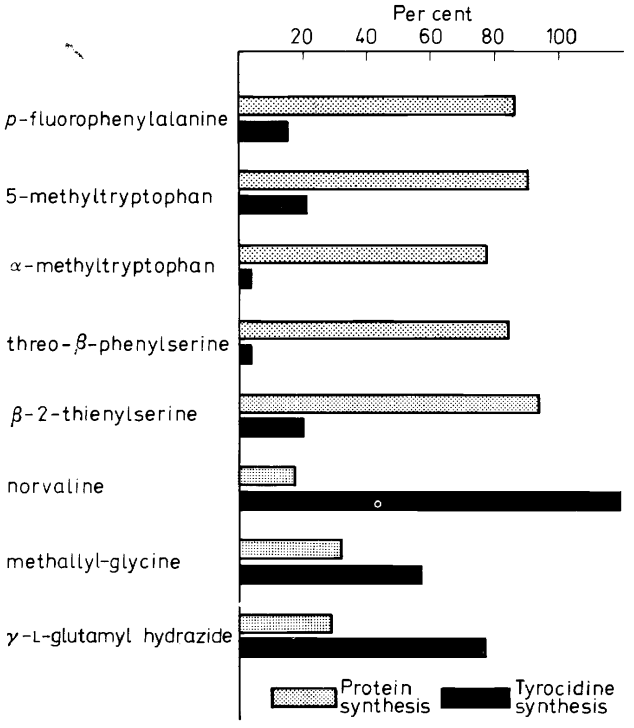

Figure 1. The effect of some amino acid analogues on the biosynthesis of tyrocidine and of protein by *B. brevis*.

to be more inhibitory for protein synthesis. Similar effects have been observed with analogues structurally related to amino acids in the actinomycins[37]. Thus, the results obtained with inhibitors of protein, RNA and DNA synthesis indicate that there is not a direct involvement of DNA or RNA in peptide antibiotic synthesis.

D. Controlled or directed biosynthesis

In vivo studies also have suggested that antibiotic formation is catalysed by enzymes with relatively broad specificities, as the mechanism for the recognition of amino acids incorporated at certain positions in a number of different peptide antibiotics appears to have a lower specificity than the corresponding one for protein synthesis. Amino acid substitutions in peptide antibiotics have been reported by several investigators[38–50]. This controlled or directed biosynthesis *in vivo* has been achieved by employing two nitrogen compounds in the culture medium. One compound serves as nitrogen source for protein and nucleic acid synthesis and hence growth. The second nitrogen compound serves to direct or influence antibiotic formation. The exogenously supplied compound (identical or chemically similar) competes with an endogenously synthesized amino acid for the latter's site in the peptide and either (i) is incorporated into the antibiotic peptides with increased formation

Table 3. Influence of exogenous compounds on antibiotic synthesis

Antibiotic family	Site in peptide affected	Exogenous compound	Result
Actinomycin	Proline	Hydroxyproline	Actinomycin I, 7% → 35%
		Sarcosine	Actinomycins II + III, 5% → 65%
		Pipecolic acid	Six new components:

	Proline	Pip acid	4-Oxo pip acid	4-Hydroxy-pip acid
Pip 2	0	2	0	0
Pip 1β	1	1	0	0
Pip 1α	0	1	1	0
Pip 1γ	0	1	0	1
Pip 1Δ	1	0	1	0
Pip 1ε	1	0	0	1

	Azetidine-2-carboxylic acid 3-, 4- or 5-methyl proline 4-F-, Br-, Cl- or thioproline	New components
	D-Valine-D alloisoleucine	L-Valine L-isoleucine D-isoleucine L-alloisoleucine
	N-methyl-L-valine	Actinomycin IV, 10% → 85% New components containing *N*-methyl-L-alloisoleucine
Tyrocidine		
A	Phenylalanine (3) tyrosine (1)	
B	Phenylalanine (2) tyrosine (1) tryptophan (1)	
C	Phenylalanine (1) tyrosine (1) tryptophan (2)	
		L-tryptophan
		Tyrocidine D { 1 phenylalanine, 0 tyrosine, 3 tryptophan }
		Tyrocidine A, B, inhibited
		L-tryptophan plus L-phenylalanine
		Tyrocidines A, B, C, D

of certain trace components or the formation of new antibiotics, or (ii) is incorporated after enzymatic modification. In some instances the exogenously supplied compound may selectively inhibit synthesis of only certain of the antibiotics formed by an organism. Some examples of amino acid substitutions or replacements are presented in *Table 3*.

The specificity of the amino acid sequence in a protein is determined genetically, and the translation of a nucleotide sequence into a sequence of amino acids generally occurs with virtually absolute specificity[51–58]. Single amino acid substitutions observed in proteins, e.g. tryptophan synthetase, phage coat proteins, haemoglobin or tobacco mosaic virus, are due to changes in the nucleotide sequence of structural genes rather than due to variations in environmental or metabolic conditions[59, 60]. Although the incorporation of certain amino acid analogues into protein has been frequently shown, there is no competition between the naturally occurring amino acids of proteins. Supplying large excesses of certain amino acids does not result in a single amino acid substitution involving these amino acids, e.g. isoleucine for valine or tryptophan for phenylalanine. The formation of an aminoacyl-tRNA *in vitro* is characterized by a rigorous specificity. Isoleucyl-tRNA synthetase catalyses the formation of an enzyme-bound aminoacyl adenylate with either L-isoleucine or L-valine. The enzyme, however, transfers isoleucine but not valine to the homologous isoleucyl tRNA[52, 54–56]. Valine is released from the enzyme as the free amino acid in the presence of isoleucyl tRNA.

The tyrocidines, actinomycins, quinomycins, and many other peptide antibiotics synthesized by an organism normally differ, respectively, by single amino acid substitutions. If antibiotic synthesis were under direct genetic control, then, *a priori*, it would be necessary to assume that different strains within the culture would be involved in the synthesis of the individual components of an antibiotic family. It has been shown, however, that a single clone isolated from a culture produces all of the various forms of actinomycin or tyrocidine. Another less likely possibility would be the existence, in the DNA of each cell, of several templates differing from one another by single nucleotide substitutions and directing the simultaneous synthesis of almost identical polypeptides. Although this possibility exists, it appears more likely that amino acid substitutions in antibiotic peptides are probably not the result of different templates arising through gene mutations, but rather depend on physiological and environmental factors. The synthesis of different forms of an antibiotic complex by a microorganism is, of course, genetically determined in so far as the elaboration of the enzyme systems for antibiotic synthesis is concerned. However, due to the relatively broad specificity of the specific enzyme systems, environmental and physiological conditions may influence quantitatively and qualitatively what is actually produced.

CELL-FREE STUDIES

A. General properties

The most conclusive evidence for the nonribosomal synthesis of peptide antibiotics has been obtained from cell-free systems. The cell-free synthesis of gramicidin S[12, 27, 61–63], tyrocidine [14, 64–67], edeine[20], malformin[68, 69],

bacitracin[70, 71] and colistin[72, 73] has been reported and it is evident that these systems are distinct from those synthesizing proteins. The requirements for synthesis of the antibiotics are fairly similar and generally include the requisite amino acids of the peptide antibiotic in question, ATP as energy source, occasionally an ATP generating system is required, Mg^{2+} ion, a reducing agent and the particle-free supernatant. Some laboratories have purified the enzymatic system by differential centrifugation, $(NH_4)_2SO_4$ fractionation, and column chromatography with DEAE cellulose, Sephadex G-200 or hydroxylapatite. The peptide synthesizing systems are reported to be insensitive to chloramphenicol, puromycin, DNAse and RNAse.

B. Amino acid activation

Until recently, investigations concerning the activation of amino acids for antibiotic synthesis did not shed much light on the specific nature of the process for antibiotic synthesis since components of the protein synthesizing system were still present in cell-free extracts. Activation of an amino acid for protein synthesis requires ATP, Mg^{2+} and a specific aminoacyl-tRNA synthetase. The enzyme bound aminoacyl adenylate is then transferred to a specific tRNA molecule. How then does one distinguish between the two kinds of activating enzymes in cell-free extracts of an organism? One approach would be to employ amino acids which are unique to antibiotic peptides and are not present in proteins. A second approach, since the *in vivo* studies have indicated that antibiotic synthesis does not involve RNA directly, would be to demonstrate the activation of an amino acid without its subsequent transfer to a tRNA specific for that amino acid as would be the case in protein synthesis. If the activated amino acid was then incorporated directly into the antibiotic peptide, this would clearly constitute a separate and distinct system for activation of amino acids for antibiotic synthesis.

Earlier studies had shown that a number of the L-amino acids in peptide antibiotics were activated by cell-free extracts; on the other hand, activation of the D-amino acids has been described as negligible to good. In these early attempts it was not demonstrated that the activated amino acid was employed specifically for synthesis of the antibiotic. In some cases, an ATP-pyrophosphate exchange reaction has been observed with amino acids unique to antibiotics. Brenner, Gray and Paulus[74] found an L-α, γ-diaminobutyrate dependent exchange in extracts of a strain of *B. polymyxa*. The enzyme was partially purified and was shown to be highly specific for the amino acid. Evidence that the enzyme is involved in polymyxin synthesis was deduced from the fact that mutants unable to produce the antiobiotic lacked the ability to activate L-α, γ-diaminobutyrate. Ciferri *et al.*[75], however, noted that the ATP-PP_i exchange reaction with this amino acid occurred with extracts obtained from only one of two polymyxin-producing strains tested. Otani *et al.*[76] have reported achieving a 30-fold purification of the ornithine-activating enzyme from extracts of *B. brevis*, producer of gramicidin S. The specific activity of the ornithine-activating enzyme was found to almost parallel that of gramicidin S formation. No formation of ornithinyl-tRNA was observed. This enzyme is part of a multi-enzyme complex required for gramicidin S synthesis (see below).

A number of laboratories have examined gramicidin S (*Figure 2*) synthesis

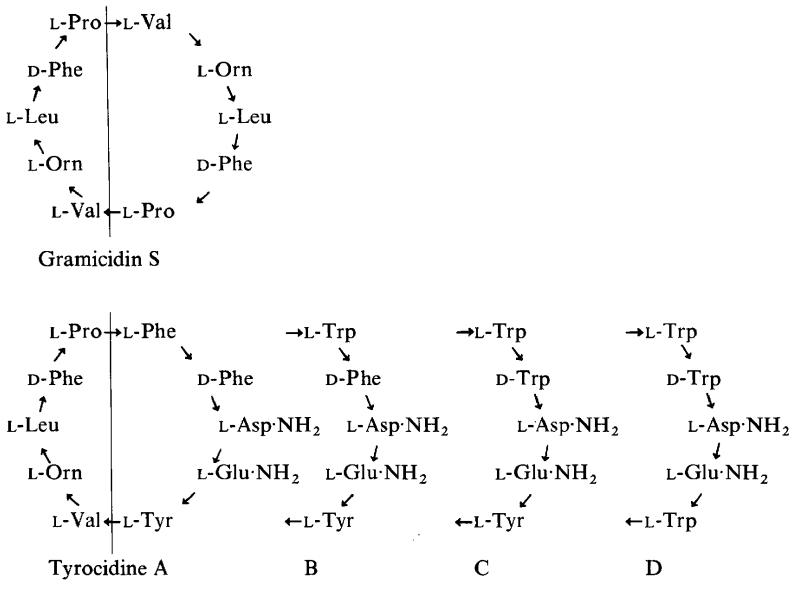

Figure 2. Structure of gramicidin S (above) and tyrocidines A, B, C, and D (below).

using cell-free systems derived from suitable strains of *B. brevis* [12,27,61–63,77–79] Kurahashi's group[14,65,78], Rao *et al.*[64] and, more recently, Roskoski *et al.*[66,67] have studied tyrocidine (*Figure 2*) formation using an *in vitro* system derived from a strain of *B. brevis*.

An extensive purification and resolution of the gramicidin S system has been achieved[12,63,76–81]. Two distinct protein fractions designated I and II were separated by Sephadex G-200 and DEAE cellulose chromatography. Gevers *et al.*[63,77,79], Kurahashi and his collaborators[12,78] and, more recently, Laland's group in Oslo[81] found that gramicidin S synthesizing ability with fraction I or II was very low when each fraction was tested separately; the two complementary fractions were necessary for gramicidin S synthesis (*Figure 3*). The amino acids L-proline, L-valine, L-ornithine, and L-leucine were activated by fraction I in the presence of ATP and Mg^{2+}; an ATP-dependent activation and racemization of L-phenylalanine $\rightleftharpoons$ D-phenylalanine occurred with fraction II. The activation process, measured by the ATP-PP_i exchange reaction, appears to be quite specific since relatively few amino acids other than the ones present in gramicidin S were activated. Aminoacyl-tRNA was not formed with either fraction I or II under conditions of ATP activation using *B. brevis* or *E. coli* tRNA and any one of the five gramicidin S amino acids. These data, along with the finding that gramicidin S synthesis is not inhibited by chloramphenicol, puromycin or RNAse, demonstrates that antibiotic synthesis occurs in the complete absence of

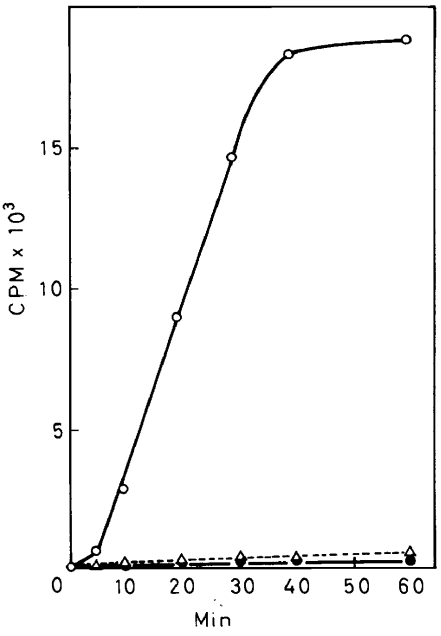

Figure 3. Rate of gramicidin S synthesis by the resolved fractions. Fraction I (- - - -), fraction II (●—●) and fraction I plus II (○—○).

polynucleotides. As pointed out by Roskoski *et al.*[66] the amino acid sequence in antibiotics such as gramicidin S or tyrocidine is determined by enzyme specificity and organization of the enzyme complex and not by RNA templates.

Kleinkauf *et al.*[63, 77, 79] have suggested that fraction I contains four activating enzymes (proline, valine, ornithine and leucine) organized into a tightly associated multi-enzyme complex. Sucrose density gradient centrifugation studies have indicated that its molecular weight is about 280000. Experiments carried out with fraction I reveal that there is a 1:1:1:1 stoichiometric binding of the amino acid substrates to the complex when the amino acids are added singly or in various combinations (with ATP + Mg^{2+}) (*Table 4*). When purified fraction I charged with all four amino acids was

Table 4. Binding of four amino acids to fraction I

Amino acids added	----- Amino acids bound (μμmoles) ----			
	L-Pro	L-Val	L-Orn	L-Leu
L-Pro	15.6	----	----	----
L-Val	----	13.6	----	----
L-Orn	----	----	15.0	----
L-Leu	----	----	----	14.6
L-Pro + L-Orn	13.6	----	16.6	----
L-Pro + L-Leu	13.6	----	----	16.6
L-Val + L-Orn	----	13.8	14.0	----

Kleinkauf *et al. Proc. Nat. Acad. Sci. U.S.*; **62**, 226 (1969)

heated to 90–100° for 5 minutes, the amino acids were obtained free in a yield of over 90 per cent, indicating that peptide bond formation does not occur following the initial activation step. From various studies employing labelled ATP (adenosine-3H, β-, γ-^{32}P) it was concluded that four aminoacyl adenylates are synthesized independently on separate active sites of the enzyme complex (fraction I).

Recent evidence has indicated that there are secondary acceptors of the bound amino acids on the enzymes and that these are sulphydryl groups[77, 82]. These —SH groups are reversibly esterified by transfer of the aminoacyl moieties from the aminoacyl adenylates. Evidence for the formation of covalent thioester bonds between the individual amino acids and the specific enzymes involved is as follows: (1) Acid stability—when a charged amino acid-enzyme complex I is precipitated with trichloroacetic acid, all of the bound adenylate is released, but only one half of the bound amino acid is liberated. (2) Dilute alkali (pH 9.0) or mercuric salts at neutral pH bring about a quantitative discharge of the amino acids from the trichloroacetic acid precipitates. (3) Hydroxylamine treatment of the precipitates releases the amino acids as hydroxamates. (4) Sodium borohydride reductively cleaves the bond with release of the corresponding amino alcohol.

Similarly, an aminoacyl adenylate complex is formed between fraction II (mol. wt. 100000) and phenylalanine in the presence of ATP and Mg^{2+}. The reversible formation of a thioester bond between phenylalanine and an SH group on fraction II follows the initial activation step (*Figure 4*).

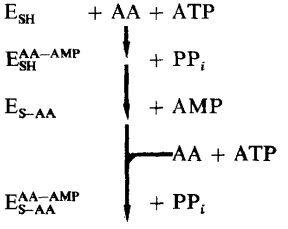

Figure 4. The mechanism of activation of each of the amino acids in gramicidin S and tyrocidine biosynthesis.

C. Initiation of peptide bond synthesis

Several groups have reported finding small amounts of free peptide derivatives after *in vitro* incubations[78, 83–86]. Compounds such as D-phenylalanyl-L-prolyl diketopiperazine as well as a tri- and tetrapeptide have been detected. While it is generally believed that free peptide intermediates are not involved in gramicidin S synthesis and that these compounds are probably artifacts of the cell-free incubation conditions, it is of interest to note that they were *all* found to contain D-phenylalanine in the N-terminal position next to L-proline. Based on this finding it was considered likely that the starting point for gramicidin S synthesis is at the D-phenylalanine residue

bound to fraction II and that the direction of peptide chain growth is from the N-terminal to the C-terminal position as in protein synthesis[78, 85].

It is now clear that peptide chains are formed with D-phenylalanine at the N-terminal position and that only enzyme-bound intermediates are involved in gramicidin S formation[79, 82, 87]. Recently, Ljones and co-workers[88] described *in vitro* pulse-chase experiments with labelled amino acids which indicated that protein-bound intermediates participate in gramicidin S synthesis. Gevers *et al.*[82] and Froshov *et al.*[87] have conclusively demonstrated that protein-bound covalently-linked peptide intermediates are formed during suitable incubations. These can be released from the protein (fraction I) by addition of hydroxylamine or performic acid oxidation. They found that D-phenylalanyl-L-proline, D-phenylalanyl-L-prolyl-L-valine, D-phenylalanyl-L-prolyl-L-valyl-L-ornithine, and even small amounts of the pentapeptide were present on the enzyme. Thus, during peptide synthesis the amino acids are added singly until a pentapeptide is formed. Elongation of the gramicidin S peptide chain takes place in a fixed order of amino acid additions. Omission of one amino acid was shown to interrupt the elongation process.

Lipmann *et al.*[77, 82] have proposed that the first peptide formed involves thioester bound D-phenylalanine on fraction II and thioester-linked L-proline bound on fraction I (*Figure 5*). Charged fraction II is thought to act

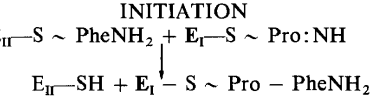

Figure 5. Initiation reaction for gramicidin S biosynthesis.

catalytically to initiate the formation of the first peptide on fraction I. The carboxyl activated phenylalanine forms a peptide bond with the imino group of proline. In turn, the thioester-bound dipeptide is believed to form a tripeptide with thioester-bound valine (*Figure 6*). The resulting tripeptide

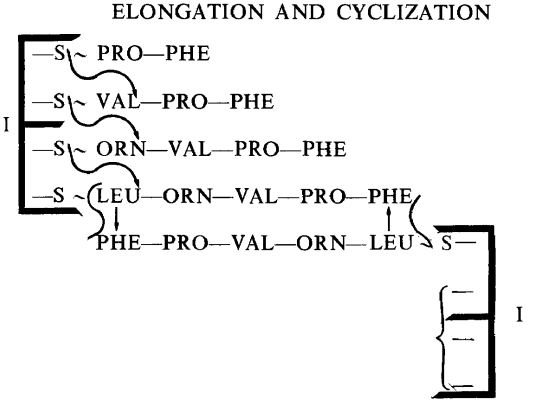

Figure 6. Proposed mechanism of elongation and hypothetical termination reactions for gramicidin S biosynthesis.

is joined to ornithine and, finally, the pentapeptide is synthesized by addition of leucine. The energy-rich thioester bonds provide the driving force for peptide-bond synthesis. The absence of peptide chains containing more than five amino acids suggests that these may never be formed. Lipmann's group has proposed that the cyclic decapeptide of gramicidin S may arise by an antiparallel doubling reaction between two carboxyl activated pentapeptidyl units, presumably attached to different fraction I components[82]. Stoll *et al.*[89], however, have very recently presented evidence suggesting that two pentapeptide chains attached to the same enzyme I molecule cyclize by head to tail condensations to yield a molecule of gramicidin S, i.e. that the cyclization is an intra rather than an inter molecular reaction.

D. Role of phosphopantotheine in peptide synthesis

Gevers *et al.*[82] advanced the interesting hypothesis that antibiotic peptide synthesis may be analogous to fatty acid elongation that occurs in a polyenzyme complex (fatty acid synthetase). On the basis of this similarity, it was suggested that pantotheine may play a role in peptide antibiotic synthesis. The first direct evidence, however, was provided by Gilhuus-Moë *et al.*[81] who demonstrated that pantothenic acid was present only in enzyme fraction I of the gramicidin S system. Based on microbiological assays, 0.8 mole of phosphopantothenic acid was found per mole of enzyme I. Kleinkauf *et al.*[90] confirmed that of the two complementary fractions, only the heavy one which carries 4 thioester-linked amino acids contains 1 mole of pantotheine/mole of enzyme. They also showed that ^{14}C-pantothenate was incorporated by *B. brevis* into the gramicidin S enzyme system. Subsequent separation by Sephadex column chromatography revealed that only fraction I contained bound ^{14}C-pantothenic acid. It was also reported[90] that pantotheine is present only in the heavy fraction of the enzymes that participate in tyrocidine synthesis (1 mole of pantotheine per mole of enzyme).

By analogy with its proposed function in the case of the fatty acid synthetase in fatty acid synthesis. Gilhuus-Moë *et al.*[81] have proposed that phosphopantotheine acts like an arm to accept the growing peptide chain after each succesive peptide bond is formed and to transfer the peptide to the next thioester-linked amino acid in sequence. Kleinkauf *et al.*[90] have considered a similar role for the compound; they have whimsically proposed that the coenzyme acts like a merry-go-round. Presumably then, one can think of the amino acids as the brass rings. More precisely, the phosphopantetheine is thought to function in vectorial peptidyl transfer reactions in which it alternates as acceptor of the growing peptide chain and donor to the free amino group of the next thioester-linked amino acid in sequence for peptide bond synthesis.

E. Tyrocidine formation

Kurahashi *et al.*[14,65] first obtained evidence for the cell-free synthesis of tyrocidine. They reported that two protein fractions were necessary for tyrocidine synthesis. One fraction activated and racemized phenylalanine, whereas the second fraction was believed to function for the activation of the other amino acids in tyrocidine.

Recently, however, Roskoski *et al.*[66, 67, 90] resolved the enzymatic system more extensively and found that each of three enzyme fractions are required for tyrocidine formation. The individual components catalyse ATP-$^{32}PP_i$ exchanges depending on the constituent amino acids. The molecular weights of the three components based on sedimentation coefficients by sucrose density centrifugation, as well as the amino acids activated by the individual components, are shown in *Table 5*. The light fraction displayed D- and L-phenylalanine dependent ATP-PP_i exchange activity and catalysed the racemization of phenylalanine. There was no demonstrable phenylalanyl-tRNA synthetase activity in the preparation. The intermediate fraction activated L-proline but was free of proline-tRNA synthetase activity. The heavy fraction contained exchange activities dependent on the amino acids which occur in positions 3–10 in the tyrocidine molecule and was free of the corresponding tRNA acylating enzymes. As in gramicidin S synthesis, each

Table5. Characteristics of complementary fractions in gramicidin S and tyrocidine biosynthesis.

Antibiotic	Amino acids bound	Fraction	Molecular weight
Gramicidin S	Phenylalanine, D- or L- L-Proline, L-Valine, L-Ornithine, L-Leucine	Light Heavy	100000 280000
Tyrocidine	Phenylalanine, D- or L- L-Proline L-Phenylalanine, D-Phenylalanine, L-Asparagine, L-Glutamine, L-Tryptophan (L-Phenylalanine), L-Valine, L-Ornithine, L-Leucine	Light Intermediate Heavy	100000 230000 460000

of the aminoacyl moieties of the aminoacyl adenylates was transferred to secondary acceptors (thiol groups). Evidence was also provided that chain growth in tyrocidine biosynthesis begins with the N-terminal amino acid, D-phenylalanine, and proceeds in order to the carboxyl terminus ending with leucine. It is believed that a linear decapeptide is formed on the heavy fractions are combined (*Figure 7*). Thus, the light component with bound nascent peptides are protein-bound.

The light, intermediate and heavy fractions can carry charged, i.e. activated, amino acids without polymerization. Polymerization occurs only when the fractions are combined (*Figure 7*). Thus, the light component with bound phenylalanine initiates peptide synthesis on the intermediate fraction by reacting with bound proline to form H_2N-phenylalanyl-prolyl-thioenzyme. This reacts with the third amino acid, i.e. with the amino group of the phenylalanyl thioester on the heavy component, forming a tripeptidyl thioenzyme and so on until a decapeptide is formed. Finally, the leucine carboxyl group is believed to react with the free amino group of D-phenylalanine at the N terminus of the peptide chain resulting in cyclization and release of a tyrocidine molecule. Chain elongation is halted when one of the amino acids (heavy fraction) is omitted from incubation mixtures. If phenylalanine (light

fraction) or proline (intermediate fraction) is omitted there is no peptide bond synthesis. Linear nascent peptides containing up to 10 amino acids have been isolated from the heavy fraction. The cyclization reaction to form the

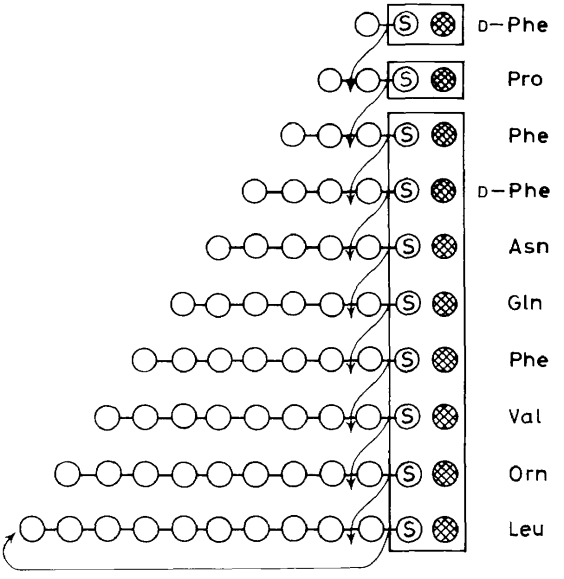

Figure 7. Summary of reactions involved in tyrocidine biosynthesis. The cross-hatched areas indicate individual sites in the three enzyme fractions for binding of the aminoacyl adenylates, each adjacent to sulphyhydryl groups responsible for thioester binding of amino acids and peptides as indicated.

tyrocidine molecule is thought to be a relatively slow reaction in contrast to the cyclization step in gramicidin S synthesis. As mentioned previously, phosphopantotheine is found only on the heavy fraction of the tyrocidine system, and its role in tyrocidine synthesis is considered to be analogous to that described earlier for gramicidin S formation[90].

F. Heterologous versus homologous systems

Roskoski *et al.*[66] have reported that when fraction II of the gramicidin S synthesizing system (activates and racemizes phenylalanine) is substituted for the tyrocidine light component in an otherwise complete tyrocidine synthesizing system, no antibiotic synthesis occurred. Similarly, the tyrocidine light fraction did not promote gramicidin S formation in the presence of fraction I (gramicidin S system). These results suggest a lack of heterologous complementarity between the gramicidin S and tyrocidine synthesizing systems. This observation differs, however, from that described earlier by Kurahashi *et al.*[75] who had reported that the purified phenylalanine racemase from their gramicidin S system could substitute for the tyrocidine light fraction (their fraction I). The reason for this discrepancy is not clear at the present time.

G. Enzyme specificity

We have already discussed that structurally related compounds can replace amino acids during the synthesis of the actinomycins, quinomycins, tyrocidines and other peptide antibiotics *in vivo*. Such data suggested that the enzyme systems had relatively broad specificities for particular amino acids. Cell-free studies with the tyrocidines and gramicidin S systems have provided additional proof for this hypothesis. For example, leucine can be replaced by isoleucine, ornithine by lysine, and tyrosine by phenylalanine in the enzymatic synthesis of tyrocidine[65]. Moreover, if tryptophan was omitted from incubation mixtures, tyrocidine A (2-phenylalanine, 1 tyrosine, 0 tryptophan) was synthesized preferentially. It has also been shown that azetidine-2-carboxylic acid can substitute for proline in the cell-free synthesis of gramicidin S[89]. In addition, certain amino acid analogues (5-methyltryptophan, *p*-fluorophenylalanine, thienylalanine, and *p*-fluorotryptophan) not only promote an ATP-$^{32}PP_i$ exchange reaction but also can be incorporated into tyrocidine *in vitro*.

H. D-Amino acids

Few studies have been reported on the biosynthesis of the amino acids which are unique to peptide antibiotics, e.g. the imino acids, *N*-methylamino acids, β-amino acids, and D-amino acids. Most attention has centred on the formation of D-amino acids.

The mechanism of biogenesis of D-amino acids, however, has still not been fully elucidated. In most cases, *in vivo* studies have revealed that the L-amino acid, but not the D-isomer, is the precursor of the D-amino acid in the antibiotic[22, 26, 92–94]. In a few instances the D-amino acid can be employed but not as effectively as the L-enantiomorph[69, 95]. Some investigators have reported that the D-amino acid inhibited antibiotic synthesis which was reversed by the L-amino acid[96–98].

Yukioka and Winnick[69] observed that both D- and L-leucine were utilized for malformin synthesis *in vitro*. However, an excess of L-leucine-^{12}C strongly depressed D-leucine-^{14}C incorporation whereas an excess of D-leucine-^{12}C had relatively little effect on the incorporation of L-leucine-^{14}C. Several groups have noted that both D- and L-phenylalanine can be utilized for the cell-free synthesis of the D-phenylalanine residue in gramicidin S[12–14, 27, 63, 66, 67, 77–79, 100–102]. It was noted generally that the L-isomer was preferentially employed. Bacitracin contains a residue each of D-asparagine, D-phenylalanine, D-ornithine and D-glutamic acid. Ishihara *et al.*[71] reported that addition of the D-isomers, singly or in combination, instead of the corresponding L-forms markedly depressed the incorporation of L-histidine-^{14}C into the antibiotic *in vitro*.

An ATP-dependent racemization of phenylalanine by cell-free extracts of *B. brevis* strains that produce gramicidin S was first described by Yamada *et al.*[102]. It has been well established since then that the enzyme is involved in the formation of the D-phenylalanine residue in gramicidin S[13, 63, 77, 79, 82, 100–102] and tyrocidine[66, 67, 78].

The enzyme was reported to require ATP, Mg^{2+} and PP_i for its action and the racemase activity could not be separated from the phenylalanine (D-

or L-) activating activity. It was proposed[13] that L-phenylalanine was activated by ATP forming an enzyme-bound L-phenylalanyl adenylate which was converted to its D-form on the enzyme (*Figure 8*). Release of the D-amino acid was pictured as a pyrophosphorylation of the D-aminoacyl adenylate

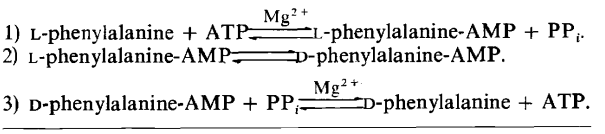

Figure 8. Postulated mechanism of D-phenylalanine synthesis *in vitro*.

to yield D-phenylalanine. The ratio of L-phenylalanine to D-phenylalanine at equilibrium was reported to be 3 to 7. When the enzyme was purified to near homogeneity, it was clear that a mechanism other than pyrophosphorylation had to be considered for the last step[101]. It was suggested that the final reaction might be a hydrolytic one, i.e. the hydrolysis of D-Phe-AMP-enzyme to yield D-Phe + AMP + Enz.

As we have already noted, Gevers *et al.*[68,82] reported that L-phenylalanine is activated by ATP with the formation of an L-phenylalanyl adenylate-enzyme complex (fraction II, gramicidin S; light fraction, tyrocidine). The aminoacyl moiety is then transferred to a thiol group on the enzyme to form a covalent thioester bond. Gevers *et al.*[82] have provided evidence that the racemization of phenylalanine occurs when the amino acid is present in the thioester form. The ratio of L-phenylalanine to D-phenylalanine at equilibrium was determined to be 1:1 and these results have been confirmed by Kurahashi *et al.*[78]. Generally amino acid racemases require pyridoxal phosphate or FAD or both for their activity. No evidence has been provided that either of these cofactors is involved in the reaction. The mechanism of the reaction, therefore, is still not entirely clear. Moreover, the nature of the racemization reaction involving phenylalanine or tryptophan at position 4 in a tyrocidine molecule has not been elucidated.

DISCUSSION

In an earlier review[8] of this subject I entitled my paper *Peptide Antibiotics—Proteins that Never Grew Up*? The title may have been misleading since it implied that these compounds are primitive proteins. At the time no attempt was made to consider them in that light. Little was known of the mechanism of peptide antibiotic synthesis; however, it was recognized that the first step in the biogenesis of these compounds was an activation of an amino acid by ATP in the presence of Mg^{2+} presumably with the formation of an aminoacyl adenylate. This reaction appeared to be the only one in common with protein synthesis and was the reason for selecting such a title. Although the suggestion has been made that peptide antibiotics may represent primitive

proteins[7], it is quite evident from the elegant studies carried out in a number of laboratories that the biosynthesis of peptide antibiotics is directed by multi-enzyme complexes and that these complexes consist of proteins. Hence, the notion that primordial soups begat peptide antibiotics seems quite unlikely.

As we have noted earlier amino acids for antibiotic synthesis are activated by ATP to form enzyme-bound aminoacyl adenylate complexes. The aminoacyl moiety is then transferred to a specific —SH group to form a covalent thioester bond. The broader or lower specificity of the antibiotic synthesizing systems is most likely due to the fact that there are no aminoacyl-tRNA synthetases, specific tRNAs or mRNA molecules to selectively direct the incorporation of a specific amino acid into its site in a protein. In antibiotic synthesis once an amino acid or a related compound is activated, it is generally transferred to the specific —SH group and ultimately into the antibiotic. There appears to be less selectivity in these systems.

In antibiotic synthesis, the enzyme 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 enzyme systems. As in ribosomal polypeptide synthesis, chain growth starts from the N-terminal position and ends at the carboxyl terminus end. However, the resemblance appears to end here. Phosphopantotheine has been shown to play an important role in the process of peptide chain elongation and hence there appears to be a similarity between antibiotic peptide synthesis and fatty acid elongation catalysed by polyenzyme systems (fatty acid synthetases)[103,104].

ACKNOWLEDGEMENTS

This review was prepared during a stay as a visiting scientist at the Roche Institute of Molecular Biology. Financial support was provided by the Roche Institute of Molecular Biology and by a research grant (CA06926) from the National Cancer Institute, N.I.H., United States Public Health Service. I wish to thank Mrs Betsy Connelly for her assistance in the preparation of the manuscript.

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Addendum in proof

Since this review was written, two additional papers concerning gramicidin S and tyrocidine formation haved appeared. These are:

R. Roskoski, G. Ryan, H. Kleinkauf, W. Gevers and F. Lipmann. *Arch. Biochem. Biophys*, **143**, 485 (1971).

F. Lipmann. *Science* **173**, 875 (1971).