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Nonribosomal Polypeptide Synthesis on Polyenzyme Templates

Fritz Lipmann

The Rockefeller University, New York, New York 10021

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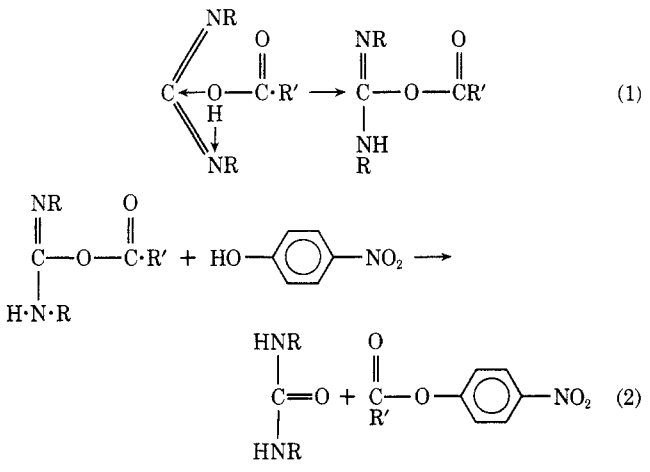
In the 1940's and 1950's, biochemical interest began to converge on protein biosynthesis. At that time one realized that, quite generally, growth and biosynthesis derive their energy supply from adenosine triphosphate (ATP). The historical development leading to the application of this concept to polypeptide synthesis has been discussed in detail elsewhere.[1]

To summarize, the mere addition of ATP to crude cell homogenates and extracts was found to promote the synthesis of peptidic bonds such as the acetylation of arylamines[2] and synthesis of benzoylglycine (hippuric acid),[3] as well as amino acid incorporation into proteins.[4] Thus, in biological reactions, ATP quite generally fulfills the function of a condensing reagent in a manner analogous, for example, to a carbodiimide in organic synthesis, as illustrated by eq 1-4. There the diimide may be formulated[5] to

Two-step Carboxyl Activation by Carbodiimide



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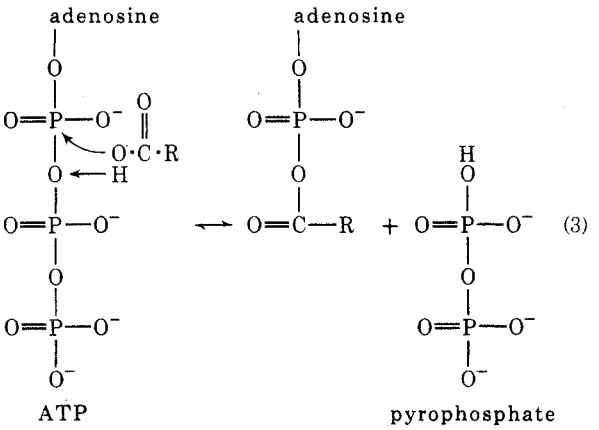
Two-step Amino Acid Activation by ATP



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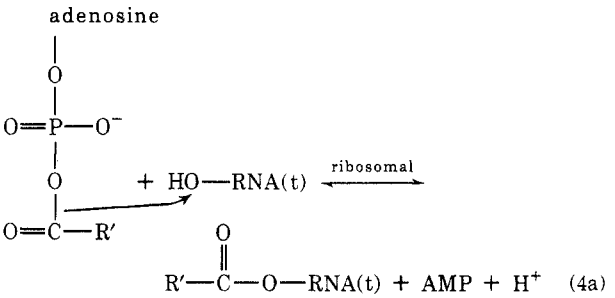
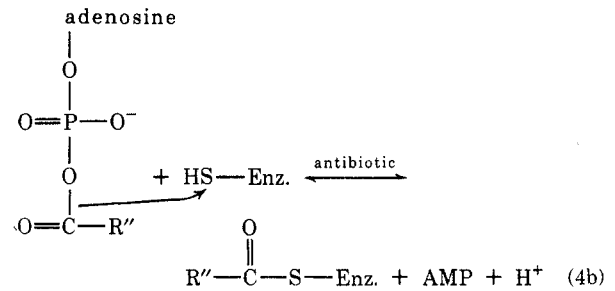


(4a)



(4b)

Fritz Lipmann was born in Koenigsberg, Germany, in 1899. From 1917 to 1922 he studied medicine at the Universities of Koenigsberg, Berlin, and Munich, and from 1923 to 1927, chemistry at Koenigsberg and Berlin. He received both his M.D. and Ph.D. from the University of Berlin. Professor Lipmann subsequently held posts in the Kaiser Wilhelm Institute, Berlin and Heidelberg, in the Biological Institute of Carlsberg Foundation, at Cornell Medical School, at Massachusetts General Hospital, and at Rockefeller University, where he has been since 1957. In 1953 he was awarded the Nobel Prize in Medicine for his discovery of coenzyme A and of its significance for intermediate metabolism.

(1) F. Lipmann, "Wanderings of a Biochemist," John Wiley, New York, N. Y., 1971, pp 80-91.
(2) F. Lipmann, *J. Biol Chem.*, **160**, 173 (1945).
(3) H. Chantrenne, *J. Biol. Chem.*, **189**, 227 (1951).
(4) P. C. Zamecnik and E. B. Keller, *J. Biol. Chem.*, **209**, 337 (1954).
(5) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley, New York, N. Y., 1961.

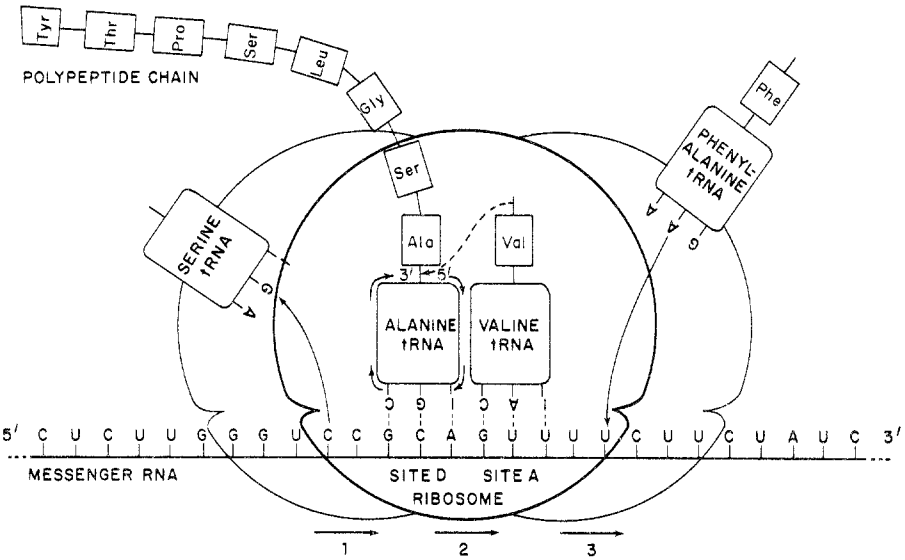


Figure 1. Survey of sequential addition of amino acids in ribosomal protein synthesis.[12] The ribosome is shown as gliding along on messenger RNA (mRNA); the peptidyl and aminoacyl terminals of the two interacting tRNAs are bound to the upper part and, through anticodon–codon interaction, are hydrogen bonding to mRNA below. On the left side of the figure, after peptidyl transfer, the tRNA, freed of its charged serine, is shown leaving. In the middle, transpeptidation to the newly adding valyl-tRNA is shown taking place and, on the right, elongation continues with phenylalanyl-tRNA aiming to connect its anticodon with the succeeding codon triplet on mRNA. The 5′ → 3′ arrows around the alanine tRNA emphasize the antiparallel nature of the binding between anticodon on tRNA and codon on mRNA. Site D means donor site and site A acceptor site. C, U, G, A, and I stand for the cytidylic, uridylic, guanylic, adenylic, and inosinic acids in mRNA or tRNA. Hydrogen bonding normally occurs between C-G and U-A. The I is a special nucleotide that appears in the third position of some anticodons.

react intermediately with a carboxyl group to form an acid anhydride like compound (eq 1), which may donate the carboxyl to a secondary acceptor to form a reactive ester, *e.g.*, with *p*-nitrophenol (eq 2). In this manner, carbodiimide activation of the carboxyl group of amino acids linked with esterification to *p*-nitrophenol has been applied in organic polypeptide synthesis.[6]

In principle, this is similar to the enzymatic two-step activation of amino acids initiated by ATP (eq 3) and continued in ribosomal protein synthesis (eq 4a) by esterification to the O-terminal of transfer RNA (tRNA).[7] Similarly, amino acid activation is initiated by ATP in antibiotic polypeptide synthesis (eq 3), but then it is continued by thioesterification to enzyme thiol (eq 4b).[8,9]

Since 1956, the development of the chemistry of genetic information transfer has forced attention to turn from amino acid activation to the dominant problem of how, in protein synthesis, the amino acids are added in a specific sequence to yield a functionally active product.[7] The guided addition of amino acids in sequence by the ribosomal system of protein synthesis is briefly outlined by the scheme in Figure 1, which should serve as background for comparison with the different mechanism of sequencing amino acids in antibiotic synthesis.

The very accurately working but rather complex amino acid sequencing machinery on the ribosome represents the final step in genetic information transfer. As such, it transmits from generation to generation information about the synthesis of specific proteins. Before this elaborate machinery had become known, a relatively simple scheme for amino acid sequencing had been proposed based on the amino acid selecting function of amino acid activating enzymes which had just then been recognized.[10] The proposition was to join together a number of amino acid specific activating enzymes into a polyenzyme, to use this assembly to fix amino acids in a predetermined sequence, and to polymerize them vectorially from such a polyenzyme to polypeptides.[11] Here, then, a polyenzyme was expected to act as template. This early scheme (Figure 2) is mentioned here since it proposes a mechanism similar to the one now found for the bacterial biosynthesis of antibiotic polypeptides to be described.

Gramicidin S and Tyrocidine Biosynthesis

After work in this laboratory had centered for a long time on the mechanism of ribosomal protein synthesis,[12] I became rather interested in exploring the nonribosomal and seemingly less complex mechanism of guided amino acid condensation that produces antibiotic polypeptides. Reports had appeared[13] that homogenates prepared from a *Bacillus brevis*, on addition of ATP, could synthesize the cyclic decapeptide antibiotic gramicidin S. This activity was found in particle-free supernatant fractions obtained from *B. brevis* (ATCC 9999) homogenates; it was resistant to known inhibitors of protein synthesis and to treatment with RNase,[14a,15] excluding the participation of an RNA. Subsequently, similar

(6) M. Bodansky and V. du Vigneaud, *J. Amer. Chem. Soc.*, 81, 5688 (1959).
(7) J. D. Watson, "Molecular Biology of the Gene," 2nd ed, W. A. Benjamin, New York, N. Y., 1970.
(8) W. Gevers, H. Kleinkauf, and F. Lipmann, *Proc. Nat. Acad. Sci. U. S.*, 63, 1335 (1969).
(9) F. Lipmann, *Science*, 173, 875 (1971).
(10) E. W. Davie, V. V. Koningsberger, and F. Lipmann, *Arch. Biochem. Biophys.*, 65, 21 (1956).
(11) F. Lipmann in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1954, p 599.
(12) F. Lipmann, *Science*, 164, 1024 (1969).
(13) T. L. Berg, L. O. Froholm, and S. G. Laland, *Biochem. J.*, 96, 43 (1965); S. Tomino, M. Yamada, H. Itoh, and K. Kurahashi, *Biochemistry*, 6, 2552 (1967).

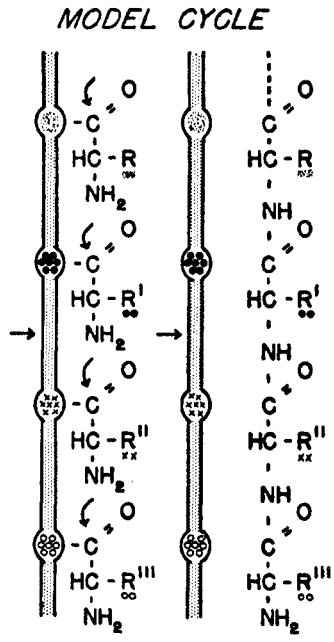


Figure 2. Early model of a template for polypeptide synthesis. The differently marked bulbous expansions indicate sites of activation and fixation of different amino acids that, as indicated by the arrows, are collected by progressive transpeptidation.

extracts of another *B. brevis* that produced the cyclic decapeptide tyrocidine[15] were prepared. This antibiotic, together with the linear gramicidins A, B, and C, were among the earliest antibiotics discovered;[16] they were produced by *B. brevis* (ATCC 8185). The mixture of these two antibiotics is often quoted in the literature under the name of tyrothricin.[17] We found that extracts of this *B. brevis* also synthesized in an analogous manner the linear pentadecapeptide gramicidin A; its partial biosynthesis was described,[18] but in view of its incompleteness it will not be included here. The mechanism of synthesis of the two rather similar cyclic antibiotics, however, was studied in great detail, and its discussion will be our main topic. Here we were able to analyze a mechanism for sequencing amino acids on templates composed of amino acid activating enzymes fused into polyenzymes of 230,000 to 440,000 molecular weight.

The structure of the decapeptide cycle of gramicidin S (Figure 3) indicates it to be made up of two identical pentapeptides. Its biosynthetic problem is least complicated since only the sequence of five amino acids, DPhe-Pro-Val-Orn-Leu, is produced; this cyclizes to the decapeptide as indicated by the dotted arrows in Figure 3.[14a-c] Active extracts are prepared by conventional methods of homogenization. They can produce quite large amounts of antibiotic when supplied with ATP. The ATP is split to AMP and pyrophosphate and, in somewhat purified extracts, a stoichiometry between disappearance of

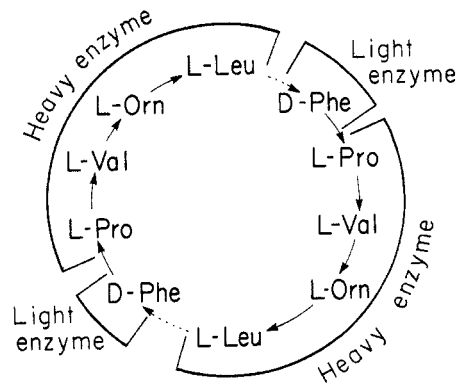


Figure 3. Sequence of amino acids in gramicidin S. The brackets indicate the enzymes activating the enclosed amino acids. The light enzyme has a molecular weight of 100,000 and the heavy enzyme of 280,000.

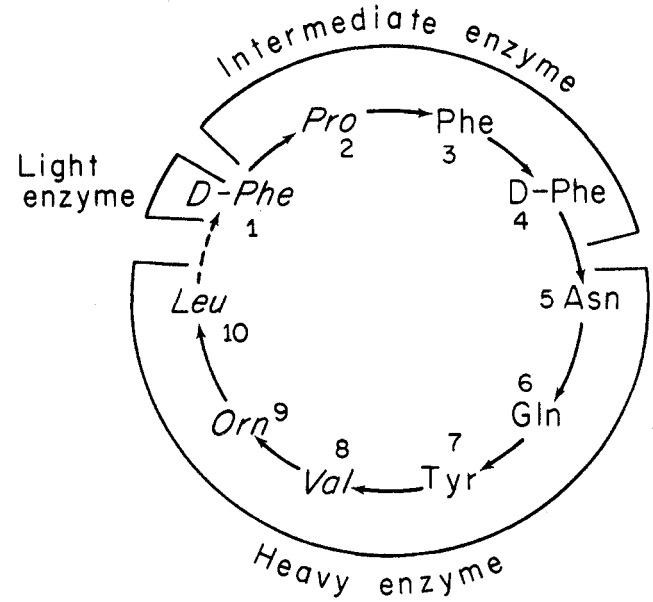


Figure 4. Amino acid sequence in tyrocidine. The brackets embrace the amino acids activated by the three complementary enzymes: light, 100,000, intermediate, 230,000, and heavy, 440,000 molecular weight.

ATP, the number of polymerized amino acids, and AMP produced can be approached. Thus, the initial step in the activation of amino acids resembles that in the ribosomal system (*cf.* eq 3).

The principles of biosynthesis for gramicidin S and tyrocidine are analogous and will be discussed jointly in order to avoid repetition. Although produced by a different strain of *B. brevis*, tyrocidine, as indicated in Figure 4, contains the five amino acids of gramicidin S in positions 1, 2, 8, 9, and 10. With synthesis of a ten amino acid sequence, it is more complex;[19,20] the extracts that produce it are not as easily obtained, and yields from the *in vitro* systems are less abundant. However, since its synthesis presents a more elaborate example of this mechanism, it was more thoroughly explored.

Interchangeability of Aromatic Amino Acids.[21] In gramicidin S, the first amino acid in the penta-

(14) (a) W. Gevers, H. Kleinkauf, and F. Lipmann, *Proc. Nat. Acad. Sci. U. S.*, **60**, 269 (1968); (b) H. Kleinkauf, W. Gevers, and F. Lipmann, *ibid.*, **62**, 226 (1969); H. Kleinkauf and W. Gevers, *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 805 (1969).

(15) K. Fujikawa, T. Suzuki, and K. Kurahashi, *Biochim. Biophys. Acta*, **161**, 232 (1968).

(16) R. J. Dubos, *J. Exp. Med.*, **70**, 1 (1939).

(17) "The Merck Index," 8th ed, Merck & Co., Inc., Rahway, N. J., 1968, p 1091.

(18) K. Bauer, R. Roskoski, Jr., H. Kleinkauf, and F. Lipmann, *Biochemistry*, **11**, 3266 (1972).

(19) R. Roskoski, Jr., W. Gevers, H. Kleinkauf, and F. Lipmann, *Biochemistry*, **9**, 4839 (1970).

(20) S. G. Lee, R. Roskoski, Jr., K. Bauer, and F. Lipmann, *Biochemistry*, **12**, 398 (1973).

(21) R. Roskoski, Jr., H. Kleinkauf, W. Gevers, and F. Lipmann, *Biochemistry*, **9**, 4846 (1970).

peptide chain is the only aromatic one and is commonly phenylalanine; in the *in vitro* system it was found to be replaceable by tyrosine but not by tryptophan.[21] Tyrocidine contains aromatic amino acids in positions 1, 3, 4, and 7. In the *in vitro* synthesis, phenylalanine and tyrosine were found to be easily interchangeable, whereas tryptophan when used as the only aromatic also replaced, but rather poorly so;[21] in practically all *in vitro* experiments, only phenylalanine was added to avoid complications. In the *in vivo* synthesis of tyrocidine, tyrosine is preferentially incorporated in position 7 and tryptophan in 3 and 4, while position 1 is commonly occupied by phenylalanine (for other replacements see ref 14c).

Racemization. As many antibiotics do, gramicidin S and tyrocidine contain D-amino acids. In both cases, only phenylalanine, or the tyrosine and tryptophan replacing it, are incorporated as D-amino acids. In both cases, the 100,000 molecular weight enzyme initiates biosynthesis and racemizes the aromatic amino acid; in tyrocidine a second D-phenylalanine appears in position 4 and is there activated and racemized by the intermediate enzyme.[20] Using the light gramicidin S enzyme (*cf.* Figure 3) charged with either L- or D-phenylalanine as substrate, racemization was practically complete in either direction after brief incubation.[8] Since no pyridoxal phosphate, a common coenzyme for racemization, could be detected in the racemizing enzymes, it was assumed that in this case racemization was thioester linked. As shown for the thioester of methylmalonyl-CoA,[22] this enzymatic racemization takes place with the loss of α-carbon–hydrogen. By use of α-carbon-tritiated phenylalanine, a rapid tritium loss during racemization has been found with the light tyrocidine enzyme and it is being studied in greater detail at present in this laboratory.[20,23]

Separation of Complementary Enzymes. *Amino Acid Activation.* Filtration through Sephadex G-200 separates from prepurified extracts of the two strains of *B. brevis* complementary fractions that, when combined, catalyze the synthesis of the two cyclic antibiotics. The extract that produces gramicidin S yields two fractions, a light and a heavy enzyme of 100×10^3 and 280×10^3 molecular weight, that activate the amino acids embraced by the brackets in Figure 3. The extracts that produce tyrocidine yield three fractions, a light, an intermediate, and a heavy enzyme of 100×10^3, 230×10^3, and 440×10^3 molecular weight, that activate the amino acids inside the brackets in Figure 4. The approximate molecular weights were obtained[14b,19] by sucrose gradient centrifugation using known markers.[24] The purification of the tyrocidine enzymes was more difficult, and the accurate assignment of the amino acids activated by the heavy, intermediate, and light enzymes required extensive purification.[20]

Transfer of ATP-Activated Amino Acids to SH Groups on the Enzymes. Indications for a secondary step in amino acid activation were obtained as follows. The similar reaction with ATP on amino acid ligases for protein synthesis (eq 3 and 4a) yields, in the absence of tRNA, a tightly but not covalently bound aminoacyl-AMP that filters with the enzyme through Sephadex G-50 but is released by denaturation with trichloroacetic acid. In our case, however, after trichloroacetic acid treatment,[14c,19] or precipitation with concentrated $(NH_4)_2SO_4$,[25] only half the enzyme-bound amino acid is released; the other half is stable to these treatments. For example, after charging ^{14}C-amino acids with [^{3}H]ATP to the three tyrocidine-producing enzymes, the amount of [^{3}H]AMP retained on filtration through Sephadex G-50 is only half that of the total ^{14}C-amino acids bound. This half presumably is bound as aminoacyl adenylate since it is released by trichloroacetic acid precipitation. The other half is stable to trichloroacetic acid; therefore it is bound differently, and presumably covalently, to the enzyme protein. The characteristics of the trichloroacetic acid stable bond are those of a thioester linkage.[8] From this, a two-step activation is deduced with secondary transfer of amino acids to an enzyme-bound thiol (Figure 5).

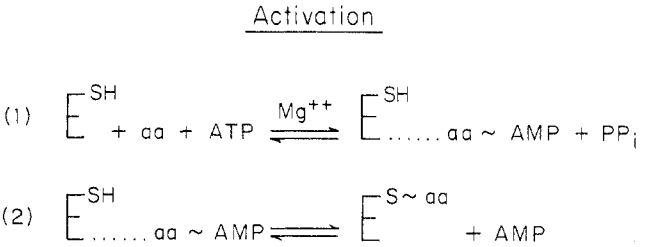


Figure 5. Two-step activation of amino acids. Reaction with ATP first yields enzyme-attached, but not covalently bound, aminoacyl-AMP; from there the aminoacyl residue transfers to an enzyme SH to which it becomes covalently linked.

The Mechanism of Polymerization[14b,21]

The data on activation, molecular weight, and pantetheine content of the complementary enzymes for the two antibiotics are summarized in Table I.[20] The function of the 1 mol of pantetheine present in those enzymes that activate several amino acids will be discussed later in connection with the mechanism of polymerization. It should be pointed out here that, by dividing the molecular weight by the number of amino acids activated, a quite constant fraction of 70,000–75,000 molecular weight obtains for every amino acid activated by the polyenzymes. This indicated early that they might be aggregates of subunits, each activating different amino acids.

Any of the polyenzymes that participate in gramicidin S or tyrocidine synthesis may be separately charged with ATP and the corresponding amino acids (Figures 3 and 4); however, these can be recovered by heating as single amino acids. For polymerization, the enzymes have to be combined. Furthermore, 1 mol of each amino acid is bound per mole of enzyme to one of three to six amino acid specific thiol acceptors, presumably of a cysteine.[14a,b]

Discussion of the process of polymerization is best begun with gramicidin S where only two enzymes take part in the synthesis. The condensation of amino acids is initiated when the light enzyme transfers D-phenylalanine to proline on the heavy en-

(22) P. Overath, G. M. Kellerman, F. Lynen, H. P. Fritz, and H. J. Keller, *Biochem. Z.*, 335, 500 (1962).
(23) S. G. Lee, unpublished results.
(24) R. G. Martin and B. N. Ames, *J. Biol. Chem.*, 236, 1372 (1961).
(25) R. Roskoski, Jr., G. Ryan, H. Kleinkauf, W. Gevers, and F. Lipmann, *Arch. Biochem. Biophys.*, 143, 485 (1971).

Table I
Enzyme Activities in the Biosynthesis of Gramicidin S and Tyrocidine

Decapeptide synthesized	No.	Mol wt of enzymes	Amino acid activated and fixed in sequence	Mol wt per amino acid $\times 10^3$	Pantetheine content, mol/enz
Gramicidin S[a]	1	100,000	DPhe	100	None
	2	280,000	Pro, Val, Orn, Leu	70	1
Tyrocidine	1	100,000	DPhe	100	None
	2	230,000	Pro, Phe, DPhe	76	1
	3	440,000	Asn, Gln, Phe, Val, Orn, Leu	74	1

[a] The decapeptide in this case is synthesized by antiparallel cyclization of two identical pentapeptides.[20]

zyme which is next in the sequence (Figure 3). During elongation, the polypeptide chain remains terminally thioesterified to the heavy enzyme.[8,26] When, in the presence of ATP, [^{14}C]phenylalanine and unmarked amino acids are added in sequence, and after each addition a sample is analyzed for enzyme-bound [^{14}C]phenylalanine, the proportional increase of [^{14}C]phenylalanine incorporation with amino acid addition indicates that all peptides stay attached until leucine, the last amino acid in the pentapeptide sequence, is reached. Leucine addition rather rapidly releases gramicidin S coincident with disappearance of protein-bound peptides. Since no peptides longer than a pentapeptide were formed, condensation of two pentapeptides with release of cyclic decapeptide must have taken place. These observations make one conclude that the active carboxyls of the two thioester-linked leucines at the end of two pentapeptides react head to tail with the free amino groups of two chain-terminal phenylalanines to form the decacycle.[14c,26]

In analogous experiments, it was found that if one amino acid in the sequence was omitted, further [^{14}C]phenylalanine incorporation stopped, although the amino acids following the one left out were present and presumably bound to the enzyme. This shows that polymerization cannot progress over an empty amino acid site. On the other hand, initiated by transfer of the N-terminal D-phenylalanine, the following amino acids added progressively in order until the pentapeptide chain length was reached. These results justify a preliminary formulation of gramicidin S synthesis as shown in Figure 6, including the ring closure which causes the release of the decapeptide. This scheme deliberately omits the complex participation of the single 4′-phosphopantetheine bound per mole of polyvalent enzyme (Table I), which preferably is left for later discussion.

Turning now to tyrocidine, its cycle consists of a continuous sequence of ten amino acids; it is synthesized through complementation by three enzymes (Figure 4). These are separated on Sephadex G-200[20]

(26) O. Froshov, T. L. Zimmer, and S. G. Laland, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, 7, 68 (1970).

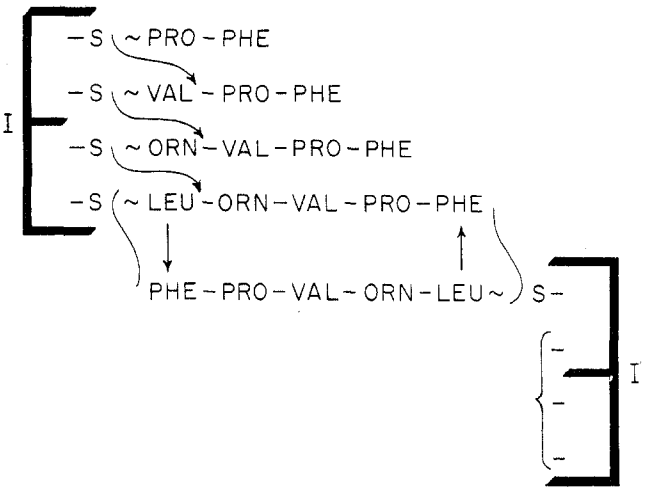


Figure 6. Scheme for polymerization of gramicidin S.

in a manner similar to that discussed for the gramicidin S enzymes. A light enzyme of 100,000 molecular weight, after activating and racemizing phenylalanine, initiates by transfer of D-phenylalanine to proline on a polyenzyme of intermediate molecular weight of 230,000. This intermediate enzyme then activates and further adds L-phenylalanine and D-phenylalanine in sequence to the D-phenylalanyl proline peptide. The second phenylalanine site on the intermediate enzyme is coincident with a racemase; from there, polymerization connects with the heavy enzyme of 440,000 molecular weight that activates the six amino acids, asparagine, glutamine, L-phenylalanine, valine, ornithine, and leucine, that complete the chain.

The *in vitro* synthesis of tyrocidine is slower than that of gramicidin S. Sequential addition of amino acids to the assembly of three enzymes in the presence of ATP results in binding of the growing peptide chains through the last added amino acid. The addition of leucine, the last in the ten amino acid sequence, yields a rather stable enzyme-bound decapeptide. The cyclization by reaction with the N-terminal amino group of initiating D-phenylalanine causes a relatively sluggish release of the tyrocidine decapeptide. Again, omission of amino acids causes polymerization to stop at the level reached before addition of the omitted amino acid. This confirms the interruption of vectorial amino acid polymerization by leaving out one in the sequence, as seen in gramicidin S synthesis. Figure 7 schematically presents the sequential addition of amino acids, simplified again by omission of the function of pantetheine in transpeptidation.

The Function of Enzyme-Bound 4′-Phosphopantetheine

In view of the similarity between the mechanism of this peptide synthesis and fatty acid synthesis,[27,28] indicated by the thioesterification of the building blocks before polymerization, here, as in fatty acid synthetase, a participation of phospho-

(27) F. Lynen, D. Oesterhelt, E. Schweizer, and K. Willecke in "Cellular Compartmentalization and Control of Fatty Acid Metabolism," F. C. Gran, Ed., Universitetsforlaget, Oslo, 1968, p 1.

(28) P. R. Vagelos, P. W. Majerus, A. W. Alberts, A. R. Larrabee, and G. P. Ailhaud, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 25, 1485 (1966).

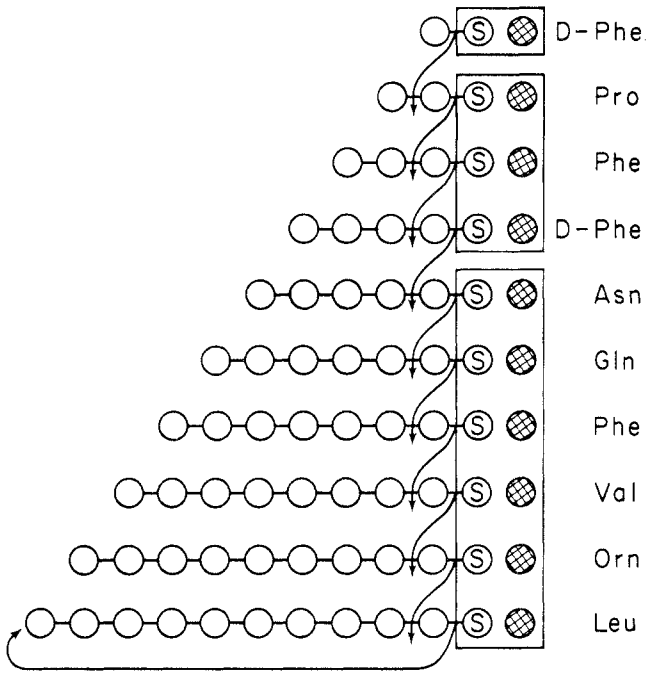


Figure 7. Scheme for elongation of enzyme-bound peptides on sequential addition of amino acids in tyrocidine biosynthesis. In the last line, formation of the decacycle by reaction between the thioester-linked chain-terminal leucine and the amino group of N-terminal phenylalanine is indicated by the arrow.

pantetheine was suspected.[8,29] After release from purified enzyme, the 4′-phosphopantetheine (Figure 8) was degraded[30] and determined as pantothenic acid by the highly sensitive bacterial assay. Alternatively, the organism was grown in the presence of [^{14}C]pantothenic acid and isolated enzymes were assayed for ^{14}C. The phosphopantetheine was found in those enzymes for gramicidin S[29,31] and for tyrocidine synthesis[20] which activated several amino acids. Its presence in the intermediate tyrocidine enzyme was overlooked at first when impure preparations were used.[31] The functioning of pantetheine may be reflected by the finding of a single mole of it in the various enzymes that activate, thioesterify, and polymerize three to six amino acids. Therefore, these amino acids must be bound to different SH groups in the polyenzyme proteins rather than to that in a single pantetheine. The latter might be assumed to collect the peripherally bound amino acids into polypeptides which may, at least temporarily, be bound to pantetheine. In confirmation, binding to pantetheine was found[32] when asparagine was incorporated into a peptide sequence, whereas singly bound asparagine was not attached to the enzyme through pantetheine. These results, amplified by recent isolation of a polypeptidylpantetheine carrier protein,[23] have been compounded into the preliminary scheme of Figure 9.

The drawing shows the enzyme-bound 20-Å-long pantetheine coming in as a swinging arm from above with a dipeptide linked to its thiol terminal in donor

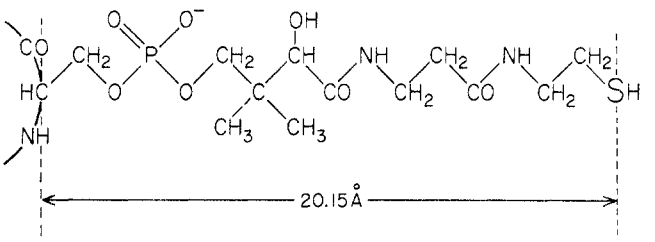


Figure 8. Enzyme-bound 4′-phosphopantetheine. On the left the phosphate bridge is shown between the hydroxyl group of protein-bound serine and the 4′-hydroxyl of the pantothenic acid part of pantetheine. 4′-Phosphopantetheine was first detected as part of coenzyme A, linked there to the phosphate group of adenylic acid.[12] Subsequently it was found to be enzyme bound in the acyl carrier protein of fatty acid synthetases.[27,28]

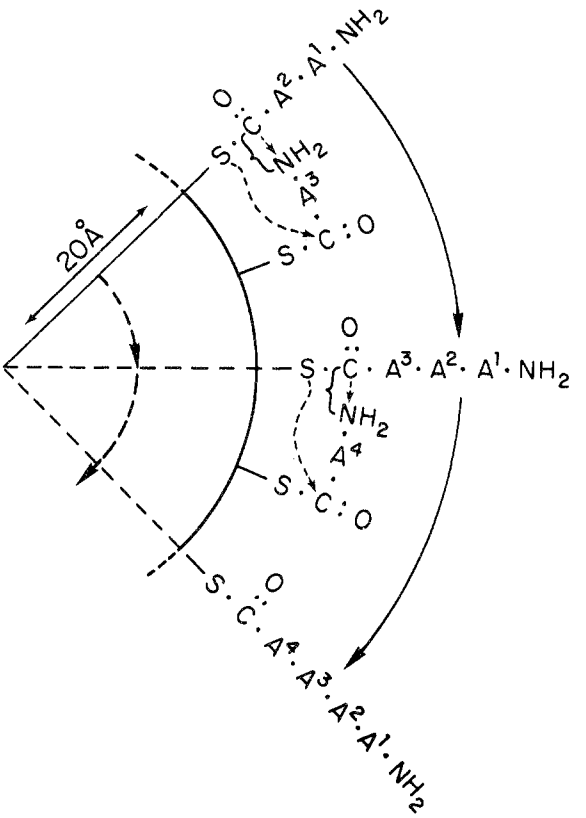


Figure 9. Scheme for participation of pantetheine in polymerization. The dotted lines and arrows indicate the movement of pantetheine collecting the peripherally bound amino acids.

position (compare with Figure 1); this brings the activated carboxyl in contact with the free NH_2 on the peripherally bound amino acid in acceptor position which is lined up for polymerization. After transpeptidation, the newly elongated tripeptide is linked peripherally, but for further elongation it should be returned to the donor position on the swinging arm, a reaction that corresponds to translocation in the ribosomal system.[12] Presumably it is transthiolated to the now free SH on pantetheine. The same sequence of transpeptidation and transthiolation should follow until the terminal amino acid is reached. On the one hand this sequence is analogous to a condensation to β-keto acid followed by reduction and transthiolation in the synthesis of fatty acids.[27] On the other hand it parallels transpeptidation followed by translocation in ribosomal protein synthesis (Figure 1). The part that pantetheine plays in polypeptide synthesis seems to be rather analogous to that played in the

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yeast polyenzyme for β-keto acid condensation and reduction in fatty acid synthesis,[27] from where we have adopted some of the terminology. I have tentatively proposed to look upon the thioester-linked polymerization of polypeptides as having evolved from thioester-linked fatty acid synthesis.[9] The participation of phosphopantetheine in both suggests a family relationship between these processes.

Disaggregation of the Polyenzymes for Tyrocidine Synthesis

In the concluding paragraphs it will be shown that it is possible to break up the large enzyme fractions into small subunits. By incubation of crude homogenates of *B. brevis* (ATCC 8185) at 37°, a degradation of the 230,000 and 440,000 molecular weight enzymes for tyrocidine synthesis can be observed to yield fractions of *ca.* 70,000 molecular weight.[20] The mixture of low molecular weight fragments activates and thioesterifies the amino acids normally activated by the large polyfunctional enzyme, but polymerizing activity is lost. Such decomposition is not obtained with purified enzymes, or it is very slow. It has the character of an enzyme-catalyzed reaction. The active principle is found in the low-speed sediment of the homogenate, from which it can be extracted with Triton.[23]

Degradation products of a similar type were obtained by sodium dodecyl sulfate gel electrophoresis, as shown in Figure 10. Using isolated enzymes of 230,000 and 440,000 molecular weight, by partial degradation the former yielded three closely spaced bands in the 68,000 to 70,000 molecular weight region, one for each of the three amino acids activated. The 440,000 molecular weight enzyme yielded five such bands, one of which was more heavily stained than the others, indicating two overlapping bands and therefore a total of six bands corresponding to the six amino acids activated by the 440,000 molecular weight polyenzyme. Furthermore, each enzyme yielded one band situated in both cases in the 17,000 molecular weight region. This has now been shown to correspond to a pantetheine-containing peptidyl carrier protein. A low molecular weight fraction containing pantetheine and polypeptide was obtained[25] after incubation with tritiated amino acids followed by disaggregation of bacterial homogenates containing [^{14}C]pantothenic acid.

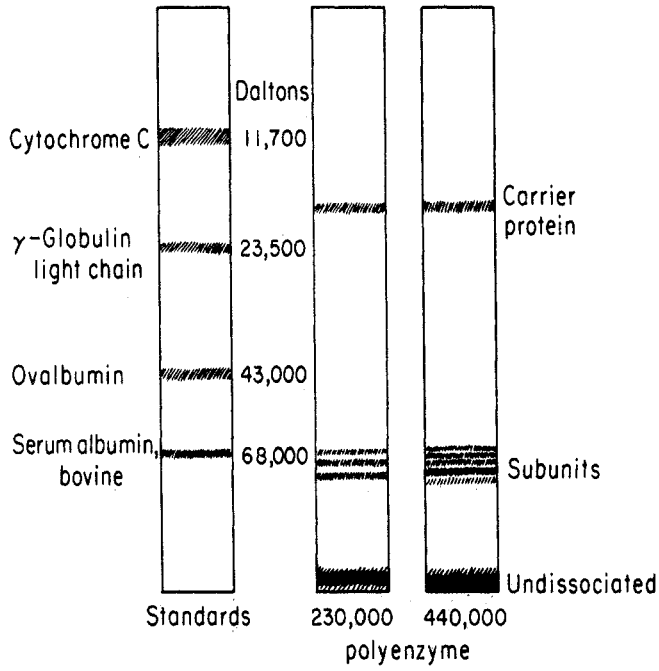

Figure 10. Separation of subunits by sodium dodecyl sulfate gel electrophoresis of purified intermediate and heavy enzymes for tyrocidine synthesis. In both cases a large part remained undissociated. (For experimental conditions see ref 20.)

We conclude that the large polyfunctional enzymes contain the activating enzymes arranged in sequence, that the thioester-linked terminal of the growing chain transpeptidates from pantetheine to the preactivated peripheral amino acids, and that amino acid addition proceeds in a prescribed order until termination by cyclization. The three-dimensional setup of the polypeptide synthesis on a polyenzyme template remains to be resolved. So far, reliable attempts to detect organized structures by electron microscopy have been unsuccessful, nor has enough of the purified enzyme fractions been available to aim at crystallization.

The work on gramicidin S synthesis was begun with Wieland Gevers and Horst Kleinkauf. The study of tyrocidine synthesis was initiated when Robert Roskoski, Jr., joined the group and has been most actively worked on recently by Sung G. Lee. Linear gramicidin biosynthesis was worked out largely by Karl Bauer. These studies were supported by Grant GM-13972 from the National Institutes of Health.