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Attempts to Map a Process Evolution of Peptide Biosynthesis

Synthesis of peptide antibiotics from thiol-linked amino acids parallels fatty acid synthesis.

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In trying to establish a continuity between prebiotic evolution and present-day biochemistry, I have attempted to find surviving metabolic relics from earlier stages (*1*). The retention of inorganic pyrophosphate as phosphoryl donor and inorganic phosphate as phosphoryl acceptor have been proposed as examples of metabolic fossils in the evolution of energy transfer (*1*, *2*).

In the domain of the transfer of genetic information I felt naïvely that, before translation into amino acid sequences could become the target of the genetic code, the extraordinary functional pliability of proteins would have to be established. In attempts to find a relatively simple process yielding peptides of defined composition, my attention was drawn to the recent preparation by Otani, Laland, and Kurahashi, and their co-workers, of ribonuclease-resistant systems for the synthesis in vitro of antibiotic polypeptides of reasonably long chain length (*3*, *4*). Our studies proved to be rewarding because, in this mechanism, activation was found to yield thioester-linked amino acids as precursors in polymerization (*5*). This finding presented a link in process evolution between the condensation of two-carbon fragments to β-keto acids in fatty acid synthesis, and of amino acids to polypeptides in ribosomal protein synthesis, between which striking parallels had long been obvious (*6*).

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Analogies between Fatty Acid Synthesis and Polypeptide Synthesis

During the isolation of a then new coenzyme for acetate activation, coenzyme A (CoA), a liver enzyme that catalyzed acetylation of aromatic amines, was used as a test system (*7*); Fig. 1 shows a thioester-linked reaction (*8*) yielding a peptide bond. Subsequently, Chantrenne (*9*) added hippuric acid synthesis, the benzoylation of glycine, to the family of CoA-thioester-linked reactions. In this way, carboxyl transfer from a thioester became more clearly connected with a peptide synthesis for which hippurate condensation had been considered a good model system. This peptide was among the first where the free energy change, ΔF, necessary to form a peptide bond was determined; it was found to be about +3 kilocalories (*10*). One of the more important results of these findings was that they strongly suggested that the carboxyl group would be the point of activation for amino acids in protein synthesis.

Although the initial energy donor for synthesis of acetyl-CoA was clearly adenosine triphosphate (ATP) (*7*), it took a long time to ascertain the details of the two-step reaction which yields acetyl-CoA by an energy transfer from ATP by pyrophosphate (PP_i) displacement (*11*). In eukaryote acetate activation, acetate-CoA ligase catalyzes the following two-step reaction:

$$\text{Acetate} + \text{ATP} \rightleftharpoons \text{acetyl}\sim\text{adenylate} + PP_i \quad (1a)$$

$$\text{Acetyl}\sim\text{adenylate} + \text{CoASH} \rightleftharpoons \text{acetyl}\sim\text{SCoA} + \text{adenylate} \quad (1b)$$

Acetate initially reacts with ATP to form an acetyl adenylate (*12*), followed by a second step (Eq. 1b) in which acetyl transfers from the acetyl-adenylate enzyme complex to the -SH group of CoA. As the result of a double transfer on the same enzyme, we find as end products acetyl-CoA, PP_i, and adenylate. A rather analogous reaction was eventually found to be responsible for the first step in amino acid activation. In prokaryotes and eukaryotes (*13*) the amino acid–transfer RNA (tRNA) ligase catalyzes the following

$$\text{Amino acid} + \text{ATP} \rightleftharpoons \text{aminoacyl}\sim\text{adenylate} + PP_i \quad (2a)$$

$$\text{Aminoacyl}\sim\text{adenylate} + \text{tRNA} \rightleftharpoons \text{aminoacyl}\sim\text{tRNA} + \text{AMP} \quad (2b)$$

Here, different enzymes were required for the activation of different amino acids. As in acetate activation, the PP_i displacement of ATP in amino acid activation turned out to be the first step in a two-step reaction. The second step, however, involved an esterification to a reactive hydroxyl at the O-terminal adenosine of amino-specific tRNA's rather than to a thiol (*14*).

These parallels between the group activations for fatty acid synthesis and protein synthesis prepared us for finding analogies between the mechanisms of the two polymerizations, the repeating condensation steps of which are compared in Fig. 2 (*15*). The analogy between condensation in β-keto acid synthesis of carboxyl to methyl groups to yield $\cdot CO\text{-}CH_2\cdot$, and of carboxyl to amino groups to yield ·CO-

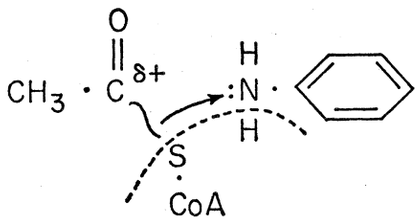


Fig. 1. Acetylation of arylamine forming a peptide bond.

NH· in peptide synthesis, was the topic of an interesting discussion at the 1959 Gatlinburg Conference, where I was very pleased that Professor Todd sided with my notion that the reactions that form these two linkages are quite similar (*16*). He went so far as to say that under certain conditions they are pretty much the same. Hydrolysis of β-keto acids by chymotrypsin may also be mentioned here (*17*).

Search for a Compact Mechanism of Polypeptide Synthesis

The studies on ribosomal protein synthesis (*18*) made us appreciate that the protein is a rather unusual chemical creation, composed as it is of various sequences of 20 different amino acids which have remained unchanged throughout the evolutionary process beginning with bacteria. Protein structure has the strange and useful property, depending on amino acid sequence, of giving rise to an inexhaustible variety of catalysts. We felt that this creation could not have been born, so to say, in one fell swoop. With the hope of finding remnants of evolutionary links, we looked in bacteria for a simpler mechanism of polypeptide synthesis and became interested in the biosynthesis of polypeptide antibiotics, particularly of gramicidin S (GS) and tyrocidine (Ty), the understanding of which had just begun to open up. After a brief period when it was thought that these polypeptides were manufactured on ribosomes (*19*), it appeared that their biosynthesis could be found in supernatant fractions of ribonuclease-treated bacterial homogenates centrifuged at high speed (*3, 20*). Extracts producing them can be obtained from certain strains of *Bacillus brevis*. Therefore, here seemed to exist a looked-for simpler biosynthetic mechanism. Indeed, since participation of nucleic acids was excluded, one had to expect that in this case a protein had to act as template. It was surprising, however, to discover here a process where initially thiol-linked amino acids polymerize in sequence on a polyenzyme system.

Table 1. Binding characteristics of complementary fractions in GS biosynthesis. Abbreviations: Phe, phenylalanine; Pro, proline; Val, valine; Orn, ornithine; Leu, leucine.

Amino acids bound	Sephadex G-200 fraction	Molecular weight	Remarks
Phe, D- or L-	Light	100,000	Contains racemase
Pro, Val, Orn, Leu	Heavy	280,000	Mol. wt./amino acid, 70,000

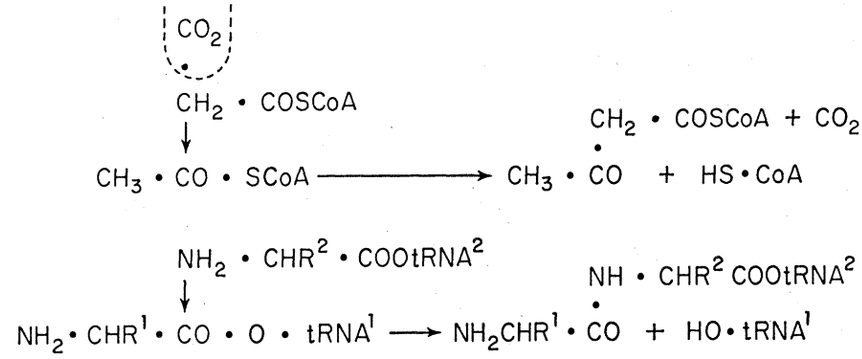


Fig. 2. Comparison of β-keto acid synthesis (upper equation) with peptide synthesis (lower equation).

Biosynthesis of Gramicidin S

The work of Saito, Otani, and Otani (*21*), of Kurahashi and his colleagues (*22*), and of Laland's group in Oslo (*23*) had laid a solid foundation on which to build. Both GS, the first to be studied, and Ty, an antibiotic similar to GS, are cyclized; their formulas appear in Figs. 3 and 4. The GS decapeptide is made up of two identical pentapeptides that cyclize head to tail (Fig. 3). In Ty the ten amino acids condense in a row and cyclize between starting D-phenylalanine and terminal leucine (Fig. 4). As general introduction, Fig. 5 illustrates the interrelation between formation of synthesizing enzyme and growth (*4*). Almost all spore-forming bacteria form antibiotics; these are produced only in the short period closely preceding sporulation, which is genetically linked to antibiotic production (*24*).

Before turning to details, I have to introduce the names of those responsible for all the work on GS and Ty in this laboratory. The early studies on GS were done by Wieland Gevers and Horst Kleinkauf, and Robert Roskoski, Jr., is largely responsible for the Ty analysis.

Tomino *et al.* (*4*) separated two complementary enzymes that function in the biosynthesis of GS; some basic facts are summarized in Table 1 (*25*). Figure 6 (*26*) is a Sephadex G-200 filtration profile for the GS system, showing the activation of amino acids by the respective peak fractions. The amino acid–dependent ATP↔PP_i exchange used for assay indicates that

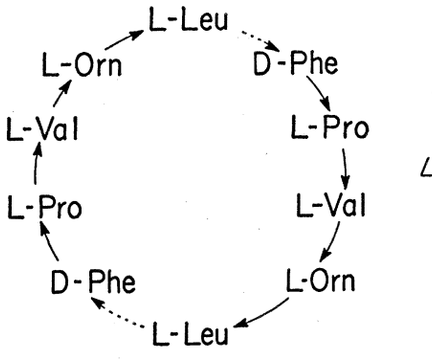


Fig. 3 (left). Gramicidin S. The stippled arrows indicate points of cyclization.

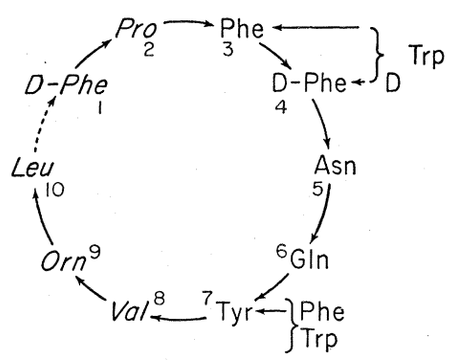


Fig. 4 (right). Tyrocidine. The stippled arrow indicates point of cyclization.

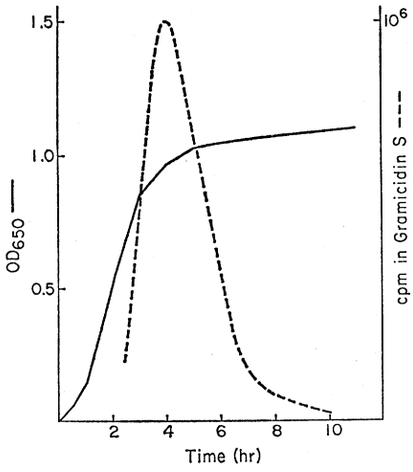


Fig. 5. Relationship between bacterial growth and enzyme production.

the primary activation in this system is of the type shown in Eq. 2a. When the enzyme that activates amino acids in the ribosomal system is filtered through Sephadex G-50, all protein-bound amino acid emerges as adenylate and links to added tRNA. In the antibiotic system, however, the filtration yields only half of the amino acid bound as adenylate (*25*); on precipitation with trichloroacetic acid (TCA), which destroys aminoacyl adenylates, the other half remains bound to the precipitate (*5, 27*).

The characteristics of this acid-stable protein-bonded amino acid are those of a thioester: it is acid stable but alkali labile, and is cleaved by mercuric acetate, a test for thioesters first used in biochemistry by Lynen *et al.* (*28*); it readily forms hydroxamate at slightly acid reaction (*29*); and it gives reductive cleavage with borohydride, which discharges the amino acid as the corresponding alcohol, and oxidative cleavage with performic acid. A reversible transfer from aminoacyl–adenosine monophosphate (AMP) had been postulated by our earlier finding (*5, 27*) that in addition to ATP↔PP_i exchange, all amino acids also catalyze an AMP↔ATP exchange. The secondary acceptor in the antibiotic system thus turned out to be an enzyme-bound thiol; from this the amino acid is fed into polymerization.

Mechanism of Peptide Synthesis

Charging all four amino acids to a preparation of the GS enzyme of 280,000 molecular weight (Table 2) causes them to become thioester-linked in equimolar proportions. However, so long as the complementary phenylalanine-charged enzyme, which has a molecular weight of 100,000, is not added, the four amino acids on the larger enzyme stay apart; polymerization is initiated by combination with the phenylalanine-charged enzyme (*25, 30*). Figure 7 presents a scheme of initiation found to be a transfer of thiol-linked D-phenylalanine from the smaller enzyme to proline linked to the larger enzyme, which yields the thiol-linked D-phenylalanylproline dipeptide with amino terminal phenylalanine (*5, 27*).

The further elongation of the enzyme-bound polypeptide chains with one-by-one addition of amino acids is shown in Table 3 (*5*). Up to the fourth, ornithine, the [^{14}C]phenylalanine incorporation is seen to increase proportionately with every step. Thus, up to then, the chain is lengthening, and all growing intermediate peptides are simultaneously enzyme-bound, which was shown by isolation of their hydroxamates from the TCA precipitates (*27*). After the fifth amino acid, leucine, is added, a large amount of GS is liberated from the enzyme (last column in Table 3), indicating rapid cyclization between two pentapeptides because no peptides longer than pentapeptides were determined (*5, 27*), as confirmed by the Oslo group (*31*). Of

Table 2. Stoichiometric binding of amino acids to 85 percent pure GS heavy fraction (*30*). The amino acid bound as thioester was measured in the TCA-precipitable material after Sephadex G-50 gel filtration as previously described (*39*).

Amino acid	Thioester bound (mole/mole enzyme)
[^{14}C]Proline	0.99
[^{14}C]Valine	1.05
[^{14}C]Ornithine	1.11
[^{3}H]Leucine	0.95
[^{14}C]Phenylalanine	0.00

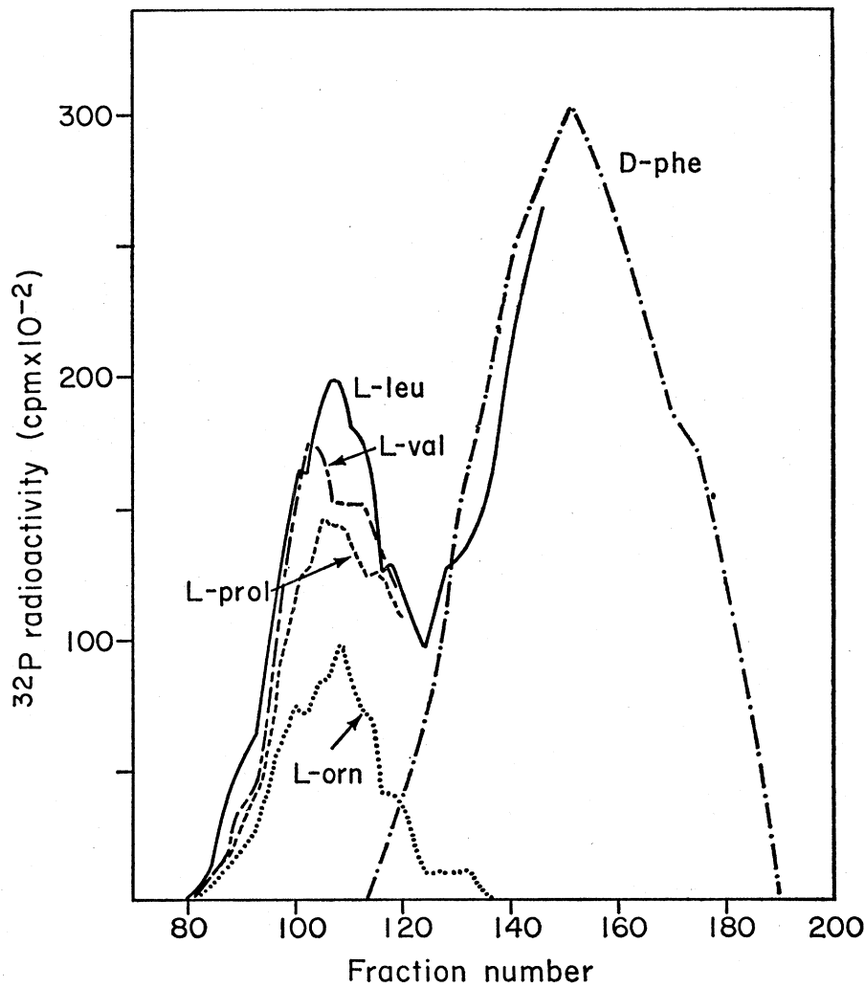


Fig. 6. Separation on Sephadex G-200 of fractions complementary in GS biosynthesis. The chromatography and amino acid–dependent ATP-$^{32}PP_i$ exchanges were carried out as described (*26*).

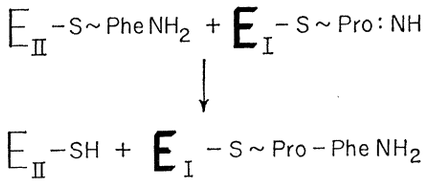


Fig. 7. Initiation reaction for biosynthesis of GS. For details, see (*27*).

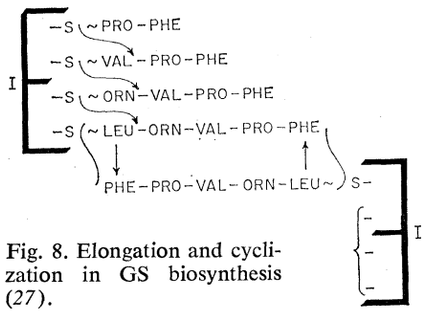


Fig. 8. Elongation and cyclization in GS biosynthesis (*27*).

great importance is the result of the last line in Table 3, namely, that when proline, the second amino acid, is omitted, but the following valine and ornithine are added, the enzyme-bound [^{14}C]phenylalanine incorporation is back to the stage when only phenylalanine alone was present. Thus, omission of one amino acid interrupts the progress of amino acid addition, similar to an amber mutation in ribosomal protein synthesis when an amino acid triplet in messenger RNA (mRNA) has undergone a mutation to a nonsense triplet (*32*). Although the template is a different one here, interruption of the sequence similarily leads to to premature termination. A preliminary scheme of elongation and cyclization is presented in Fig. 8 (*27*).

Table 3. Formation of protein-bound chains with increasing numbers of amino acids. For experimental details see Gevers *et al.* (*5*).

Peptide chains formed	Protein-bound thioesters (count/min)	GS (count/min)
[^{14}C]Phe	1,549	265
[^{14}C]Phe–Pro	6,001	375
[^{14}C]Phe–Pro–Val	10,005	1,531
[^{14}C]Phe–Pro–Val–Orn	14,325	1,005
[^{14}C]Phe–Pro–Val–Orn–Leu	2,029	25,409
[^{14}C]Phe–.... (Val, Orn)	1,610	376

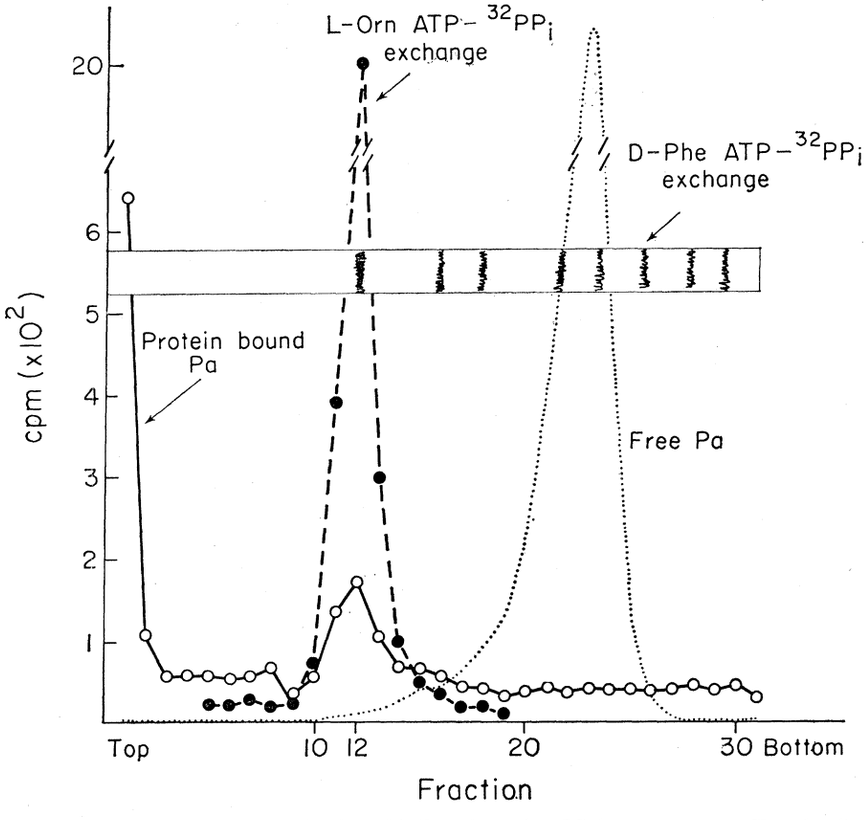


Fig. 9. Incorporation of pantothenic acid (*Pa*) into the GS heavy enzyme. For details see Kleinkauf *et al.* (*36*).

Table 4. Activity of the light fraction in GS biosynthesis after preliminary incubation with D-phenylalanine thiophenol. The D-phenylalanine has been bound (charged) to the light fraction from the synthetic thioester and, in combination with the heavy fraction—charged with ATP and the four other amino acids including [^{14}C]proline, [^{14}C]valine, and [^{14}C]-leucine of equal specific activity—has formed sizable amounts of GS, as measured by its content of ^{14}C.

Incubation	GS formed (pmole)
Light + D-phenylalanine	0
Light + D-phenylalanine thiophenol	5.8

Search for Pantothenic Acid

The discovery that enzyme-linked aminoacyl thioesters polymerize to enzyme-linked peptidyl thioesters introduced a closer resemblance to fatty acid synthesis. This led us to propose the possibility that 4′-phosphopantetheine, in analogy to its presence in the fatty acid–synthesizing polyenzyme system (*33, 34*), might take part in our polypeptide synthesis (*5, 27*). To test for the presence of pantothenic acid, we used a procedure analogous to that described by Vagelos *et al.* (*33*); they added radioactive pantothenic acid to wild-type *Escherichia coli* and found ample incorporation into acyl carrier protein. In our first experiments we failed to detect a sizable amount of pantothenic acid in our enzymes, presumably because of their late formation, at the end of the logarithmic growth period. In the meantime, the Oslo group, using the more sensitive microbiological assay, had found that pantetheine was present in the large enzyme fraction, 1 mole of pantetheine per mole of enzyme (*35*).

With a sample of *B. brevis* that grows somewhat better we have now detected (*36*) incorporation of pantothenic acid by the Vagelos method, but only in the larger enzyme (Fig. 9); there, with a rather pure preparation, the presence of phosphopantetheine was confirmed. As shown by Fig. 10, 1 mole of pantothenic acid per mole of enzyme was liberated for the micro-

biological assay by the method of Pugh and Wakil (*37*); this single pantetheine is to be compared with the thioester links to four amino acids in the large enzyme (Table 2). It was quite pleasant to meet here, unexpectedly, part of CoA, the composition of which is shown in Fig. 11.

Transfer of Amino Acid from Chemically Prepared Thioester

In the hope of confirming the polypeptide synthesis from thioester links, Gordon Ryan prepared in our laboratory D-phenylalanine thioesters of *N*-acetylcysteine and of thiophenol by the procedures devised by Wieland and Köppe (*38*). Both of these were active as precursors of the D-phenylalanine in GS, whereas the nitrophenol ester was inactive (*30*). A representative experiment is shown in Table 4. After incubation with the thioester, the amino acid–charged small enzyme, isolated by ammonium sulfate precipitation with retention of enzymatic activity (*30*), was combined with a similarily precipitated large enzyme that had been incubated with the four other amino acids, including [^{14}C]proline and ATP. The ammonium sulfate precipitate retained only the thioester-bound amino acids but not aminoacyl adenylates.

Mechanism of Tyrocidine Biosynthesis

Figure 5 shows the sequence in Ty, numbered in the order of incorporation, including the exchangeable aromatic amino acids. This interchangeability was shown to be competitive by Mach and Tatum (*39*), using the living organism; incorporation depends entirely on the concentration of the different amino acids present in the medium, which we have confirmed (*40*) for in vitro synthesis.

The Ty enzyme system is not as easily obtained as that for GS. Fujikawa *et al.* (*41*) were the first to report a reliable method for preparing the Ty system, and we have used it, with a little modification, to prepare our extracts from *B. brevis*, Dubos strain (ATCC 8185) (*40*). The component enzymes of the Ty system are described in Table 5. As indicated in Fig. 4, biosynthesis again begins with D-phenylalanine bound and racemized by a light enzyme having a molecular weight of

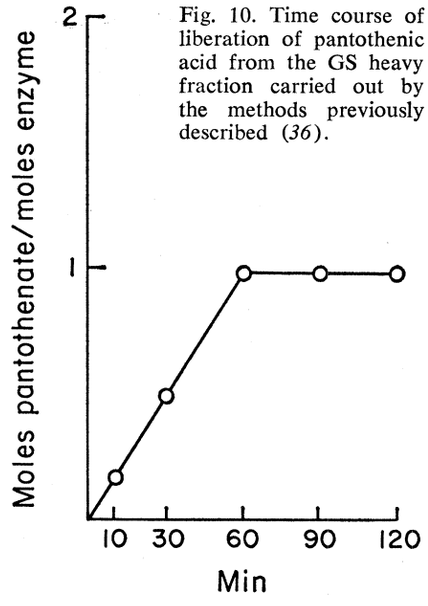


Fig. 10. Time course of liberation of pantothenic acid from the GS heavy fraction carried out by the methods previously described (*36*).

100,000. Proline, the next amino acid, is activated by a separate enzyme of intermediate size; the large enzyme activates and carries the eight other amino acids. The large enzyme also racemizes either phenylalanine or tryptophan in position 4. We can be brief with the description of the process because of the essential analogy to the GS system. The Sephadex G-200 filtration profile of a supernatant fraction is shown in Fig. 12 (*36*). The three fractions appear on this figure. The reason for the proline enzyme being so large (molecular weight 230,000) is not understood; it elutes after the heavy enzyme (molecular weight, 460,000) and often contaminates it. The light, phenylalanine-activating fraction elutes last. This diagram includes the assay for 4′-phosphopantetheine, which, we find, is present only in the large enzyme, in the ratio of 1 mole of pantetheine per mole of enzyme, and which, in this synthesis, carries eight thioester-linked amino acids (*36, 40*).

The mechanisms of activation and polymerization are the same in Ty and GS biosyntheses. All amino acids cause exchange of ATP↔PPi and ATP↔AMP (*40*), and ten ATP's are split to 10(AMP+PPi) with the formation of a decapeptide (*41*). Table 6

Table 5. Binding characteristics of complementary fractions in tyrocidine biosynthesis. Abbreviations as in Table 1; Asn, asparagine; Gln, glutamine; Tyr, tyrosine.

Amino acids bound	Fraction	Molecular weight	Mol. wt./ amino acid
Phe, D- or L-	Light	100,000	
Pro	Intermediate	230,000	
L-Phe, D-Phe, Asn, Gln Tyr(Phe)*, Val, Orn, Leu	Heavy	460,000	58,000

* When only Phe is present it will replace Tyr. Data from Roskoski *et al.* (*40*).

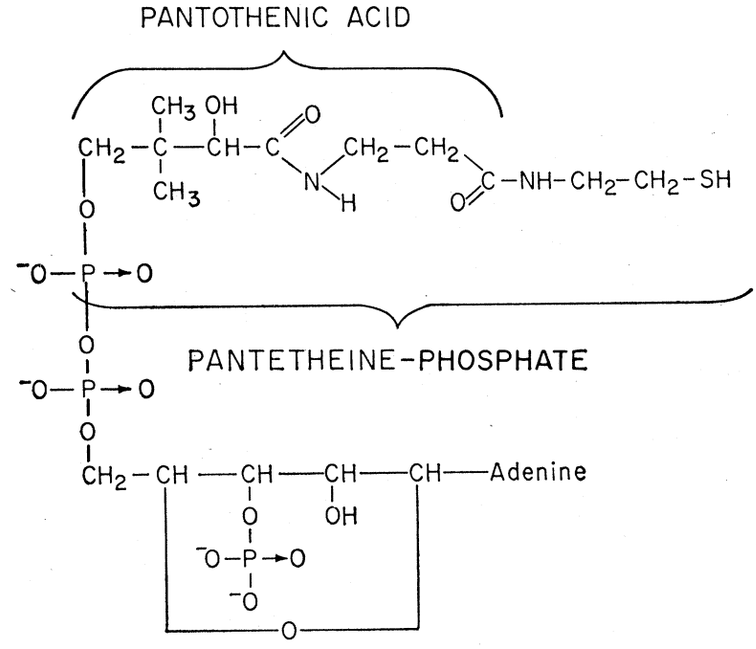


Fig. 11. Coenzyme A.

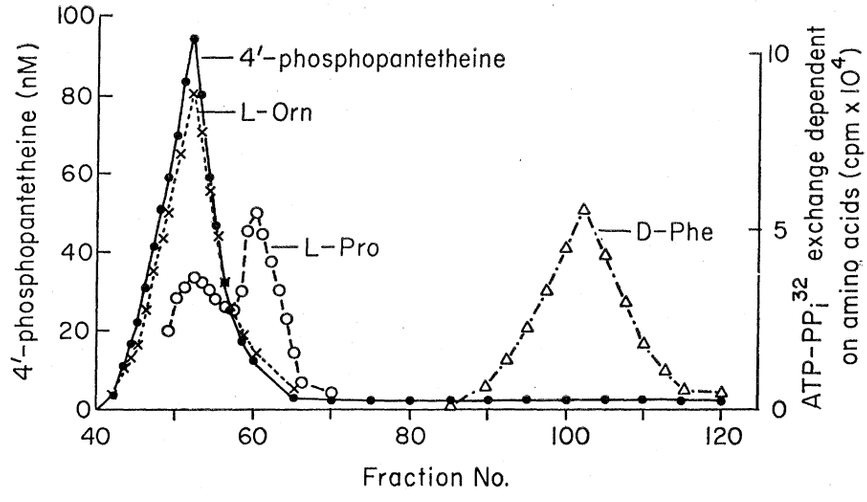


Fig 12. Resolution of the Ty-synthesizing system into three complementary fractions by Sephadex G-200 gel filtrations. The ATP-PP_i exchange activities correspond to L-ornithine for heavy, proline for intermediate, and D-phenylalanine for the light fraction appearing on the chromatogram in this order. Ornithine was chosen as the one of the eight amino acids activated and bound by the heavy fractions (see Table 5) because its activation is antibiotic-specific. Phosphopantetheine was determined as described (*36*).

Table 6. Amino acid binding of three tyrocidine complementary fractions. The total amino acid bound to protein in the Sephadex G-50 eluant is noncovalently linked aminoacyl-adenylate and covalently linked thioester. Precipitation by TCA discharges the aminoacyl-adenylate but not the thioester.

Labeled amino acid	Amino acid bound (pmole)					
	Light		Intermediate		Heavy	
	Total	TCA	Total	TCA	Total	TCA
L-[^{14}C]Phenylalanine	9.5	4.7			29.0	14.4
L-[^{14}C]Proline			10.0	5.0		
L-[^{3}H]Ornithine					10.1	5.0
L-[^{14}C]Asparagine					10.7	5.3

Table 7. Formation of protein-bound nascent peptide chains by sequential addition of amino acids with [^{14}C] marker in the proline (*40*). Addition of phenylalanine causes just short of a threefold normal increment in proline incorporation because it is incorporated simultaneously into the three peptides presented in items 2, 3, and 4. The addition of the other amino acids with single presence causes approximately equal increments, except for glutamine, which is followed by a phenylalanine already present on the enzyme where the increment is about doubled. The dashes indicate peptide links formed after each addition; the newly added amino acids are italicized. In items 11, 12, and 13, omission of an amino acid, indicated by dots, stops further incorporation of [^{14}C]proline posterior to omission.

Peptide chains formed	Bound radioactivity (pmole)	Increment
1. . . . [^{14}C]Pro	6	6
2. D-*Phe*–[^{14}C]Pro		
3. D-*Phe*–[^{14}C]Pro–*Phe*	20.5	14.5
4. D-*Phe*–[^{14}C]Pro–*Phe*–D-*Phe*		
5. D-Phe–[^{14}C]Pro–Phe–D-Phe-Asn	27.6	7.1
6. D-Phe–[^{14}C]Pro–Phe–D-Phe–Asn–*Gln*	39.0	11.4
7. D-Phe–[^{14}C]Pro–Phe–D-Phe–Asn–*Gln*–Phe*		
8. D-Phe–[^{14}C]Pro–Phe–D-Phe–Asn–Gln–Phe–*Val*	43.5	4.5
9. D-Phe–[^{14}C]Pro–Phe–D-Phe–Asn–Gln–Phe–Val–*Orn*	49.3	5.8
10. D-Phe–[^{14}C]Pro–Phe–D-Phe–Asn–Gln–Phe–Val–Orn–*Leu*	54.7	5.4
11. . . . –[^{14}C]Pro(Pro,Phe,Asn,Gln)	7.0	
12. D-Phe–[^{14}C]Pro–Phe–D-Phe– . . . (Gln,Phe,Val,Orn,Leu)	21	
13. D-Phe–[^{14}C]Pro–Phe–D-Phe–Asn–Gln–Phe–Val– . . . (Leu)	43.7	

* This Phe waiting in position 7 does not cause an increment before Asn, 5, and Gln, 6, are added, indicating that binding out of sequence does not cause elongation.

shows that the three enzyme fractions yield Sephadex G-50 filtrates that contain half the amino acids in acid-unstable form bound as adenylates and half as acid-stable thioesters; in tests with phenylalanine, 3 moles are bound to the large enzyme for every mole of ornithine and asparagine. The latter appear only once in the sequence, whereas phenylalanine incorporates in three places; when it is the only aromatic amino acid added, it replaces tyrosine. This shows equimolarity of binding on the large enzyme, every site being charged independently. Furthermore, of the three phenylalanines, only the one on site 4 is racemized. These data indicate a strictly topographical incorporation.

To obtain synthesis, the three enzymes are combined; the light, charged with phenylalanine, initiates the chain by condensing D-phenylalanine to proline on addition to the intermediate enzyme. The D-Phe-Pro dipeptide transfers to the large enzyme which carries all the other amino acids to complete the decapeptide. In Table 7 an experiment analogous to that for GS in Table 3 is presented. The amino acids with marker in [^{14}C]proline only are added in succession and proceed in the direction of synthesis; every addition causes an increase in [^{14}C]proline incorporation approximately proportional to the number of places of amino acid addition (for details see the legend of Table 7). Eventually, when leucine, the tenth amino acid, is added, the decapeptide is released, but cyclization is rather slow.

In contrast to GS synthesis, a fair amount of decapeptide can be found attached to the enzyme. When, in the overall synthesis, phenylalanine is added as the only aromatic amino acid, it is incorporated in four places, one of which derives from the light fraction. When phenylalanine is left out (line 11, Table 7), only proline is incorporated, as if the other amino acids were not there. On omission of asparagine (line 12, Table 7), incorporation proceeds only to the level of Phe-Pro-Phe-Phe, and, by omitting ornithine, the one next to the last (line 13, Table 7), it stops with the preceding valine at the level of enzyme-bound octapeptide. In other words, polymerization proceeds sequentially without jumping; the large enzyme functions as template. It has been possible to show by differential marking that during biosynthesis of Ty nine different peptides and the initiating

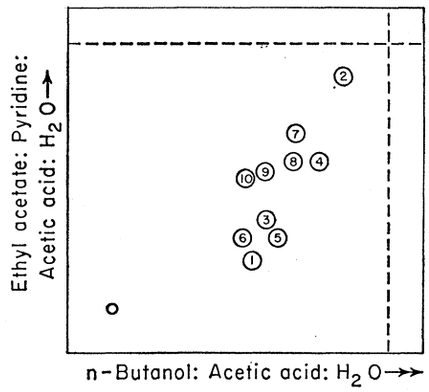


Fig. 13. Two-dimensional thin-layer chromatography of enzyme-bound peptides after alkaline liberation. The encircled residues indicate the number of amino acid residues in each peptide.

phenylalanine can be found bound to the enzyme. Figure 13 (*40*) shows their separation by two-dimensional thin-layer chromatography and their identification in ten different spots. This demonstration confirms the template-like activity of the large enzyme which takes over after initiation by transfer of D-Phe-Pro.

Preliminary Evidence for Gramicidin A Synthesis

The same organism that produces Ty also produces the gramicidins A, B, and C (*42*). Their constitutions are shown in Fig. 14. They have the same open-chain structure with a 15-amino acid sequence, and are distinguished only by amino acid replacements. We are in the process of exploring the synthesis of these gramicidins. Glycine and alanine, which are not present in Ty, have yielded TCA-stable activation products in our crude extracts, an indication that these gramicidin amino acid acids form thioesters with an enzyme protein (*40*). Karl Bauer, who recently joined us, is making further progress with this problem.

Summation

To complete the description of this new type of polypeptide synthesis, I present in Fig. 15 the structure of enzyme-bound 4′phosphopantetheine which takes part in fatty acid synthesis as well as in this peptide synthesis. In view of its alkali lability it appears to be esterified to a hydroxyamino acid in our enzyme proteins; it assays as in the acyl carrier protein, where its type of binding was proved more rigorously (*33*). The features of fatty acid synthesis which are repeated in our system are well brought out in Lynen's scheme in Fig. 16 (*34*). One sees the peripheral and central -SH groups; the latter is pantetheine-carried. When fatty acid synthesis begins, the peripheral -SH is charged with an acetyl residue which is the initiator, and the central -SH is charged by transfer of malonyl from CoA to pantetheine. There follows a condensation between the acetyl carboxyl and the CH_2 group of the malonyl, with liberation of CO_2, which yields an enzyme-bound acetoacetyl, written here as enol. Then, on the pantetheine, acetoacetyl swings around the polyenzyme through reduction and dehydration steps, until it reaches the butyric acid stage. A new round begins with the butyryl being translocated from pantetheine to peripheral thiol; a new malonyl is transferred to the liberated pantetheine-thiol, and condensation elongates to a six-carbon β-keto acid which continues the cycle and elongation. Translocation, to use the language of the ribosomal system, is from the acceptor to the donor site, where the peripheral thioester acyl is donated to a new malonyl on the acceptor site, and cycling goes on until a chain of 16 or 18 carbons is formed.

One realizes the close process similarity between fatty acid synthesis and polypeptide synthesis. However, in the former, one is dealing with a cyclic process where the same two-carbon unit is added in succession to the growing chain and the resulting β-keto acyl is reduced to fatty acid acyl until a certain length is reached. In antibiotic polypeptide synthesis one meets a synthetic machinery built on the same principle. However, the new combining units are different here, amino acids being added in a predetermined sequence; for this, a built-in template is required that dictates addition in the right order.

Antibiotic polypeptide synthesis, in terms of process evolution, is between fatty acid synthesis and the complex ribosomal polypeptide synthesis. It parallels features of the two reaction mechanisms; it runs in single steps through elongation to a product that grows

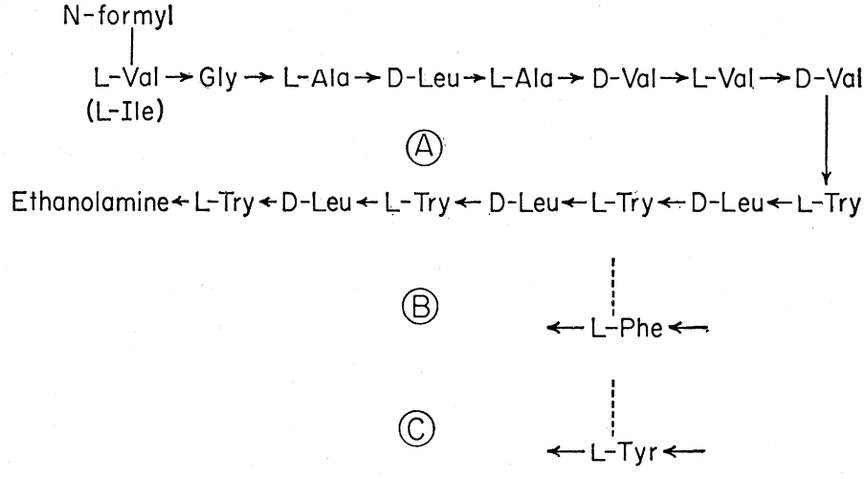


Fig. 14. Linear gramicidin (A, B, and C)

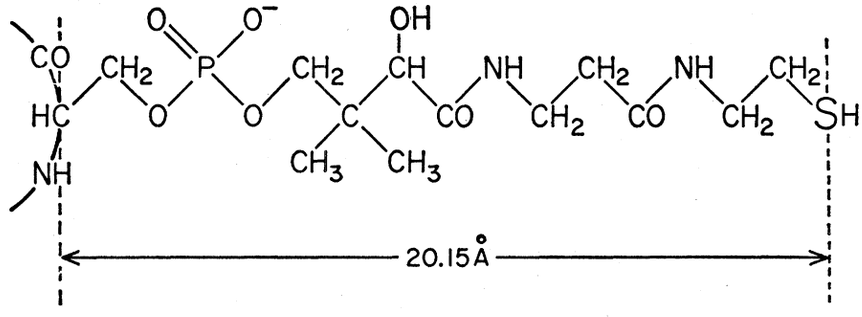


Fig. 15. Enzyme-bound 4′phosphopantetheine (*34*).

vectorially by transpeptidation of thiol-linked peptides to amino groups of thiol-linked amino acids. One has to assume, and we have preliminary evidence that this assumption is valid, that the pantetheine performs a switch or translocation by alternative condensation and transthiolation. As illustrated in Fig. 17, the pantetheine is drawn as a central thiol-carrying arm charged with a growing chain. As a result of the events after initiation of GS synthesis, which were shown in Fig. 7, it starts donating a dipeptidyl (*30*) from pantetheine to the amino group of the succeeding amino acid to form a tripeptide, which thereby temporarily comes to sit on a peripheral thiol. The

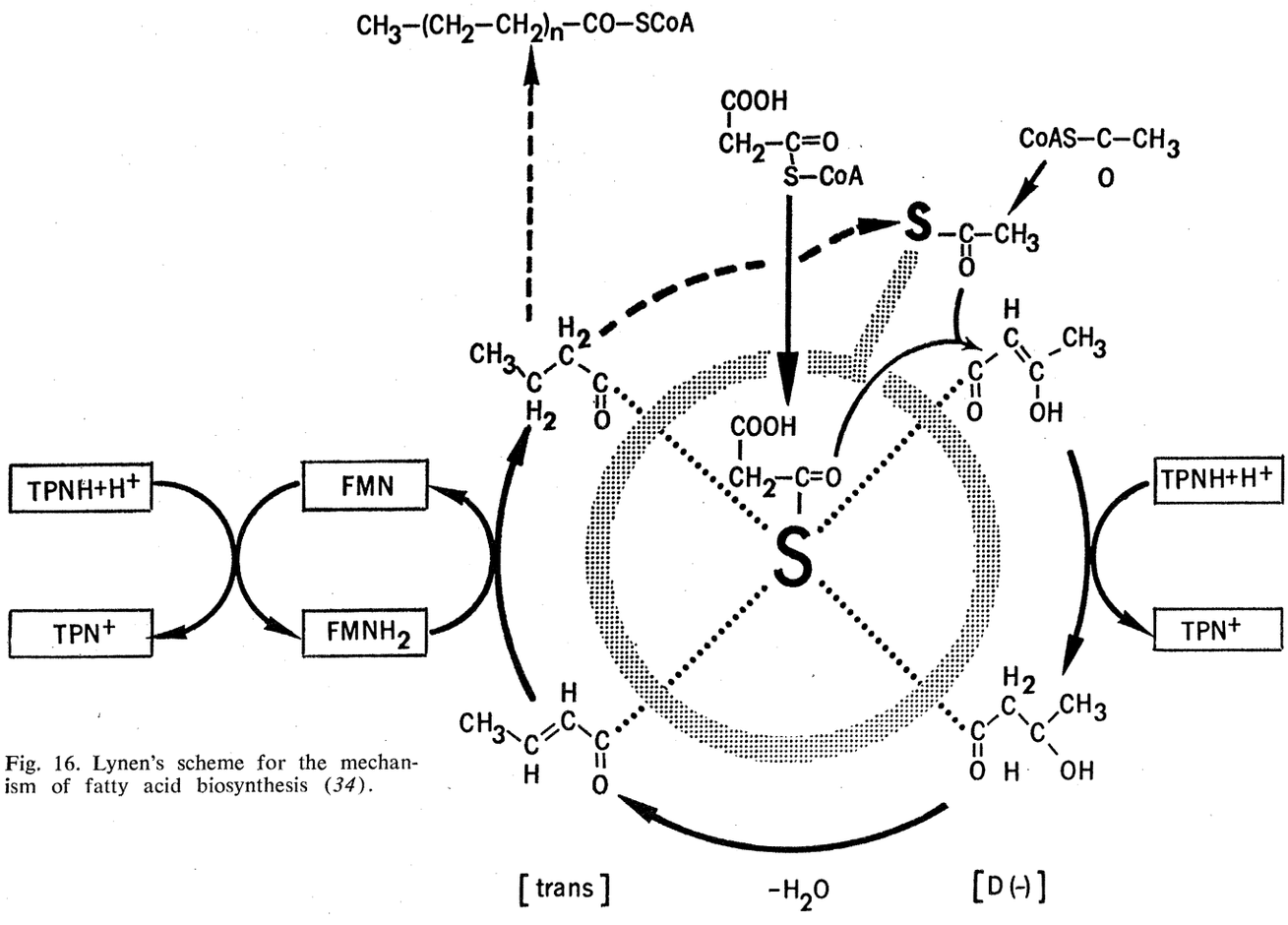
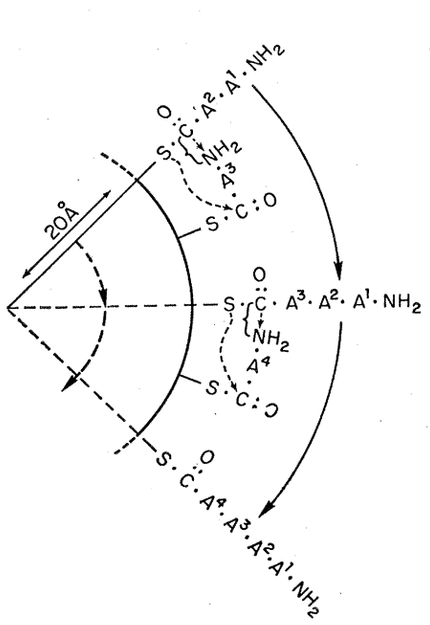


Fig. 16. Lynen's scheme for the mechanism of fatty acid biosynthesis (*34*).



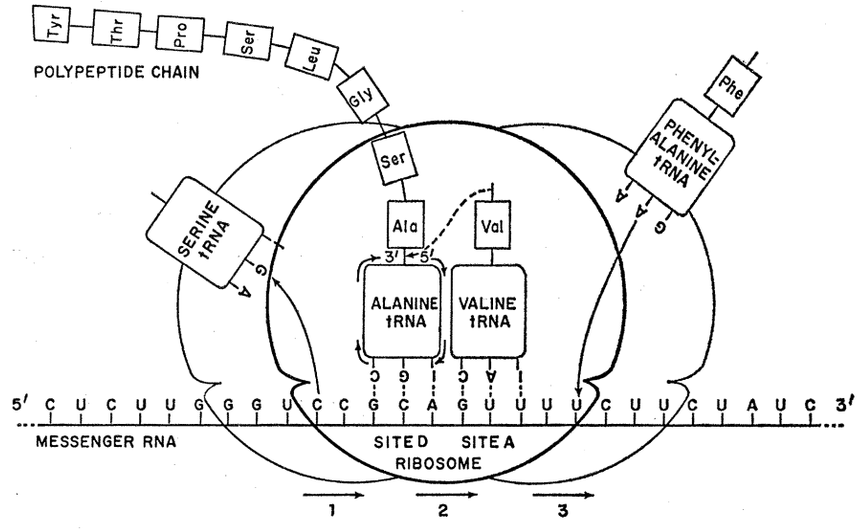


Fig. 17 (left). A schematic representation of transpeptidation and transthiolation, presumably mediated by enzyme-bound pantetheine in antibiotic peptide biosynthesis. Fig. 18 (right). Sequential addition of amino acids in ribosomal peptide synthesis, adapted from Crick (*43*) and discussed in (*18*).

liberated pantetheine-SH performs a translocation by transthiolation of the tripeptide from peripheral acceptor-SH to its own, the donor thiol. The next step is a repetition of transpeptidation from pantetheine thioester to neighboring groups of peripherally bound aminoacyl to form a tetrapeptide. This is again transthiolated, and elongation continues to the pentapeptide, two of which cyclize from the decapeptide to GS. The length of the pantetheine is 20 Å, and it seems that this is an appropriate length to go round.

For comparison, the ribosomal system is presented in action in Fig. 18. The alternation of transpeptidation and translocation is transacted here by some kind of linear propulsion rather than rotation. The ribosomal mechanism is different in detail but serves the same purpose: the growing peptide chain condenses from tRNA to the amino group of a newly adding aminoacyl-tRNA on the acceptor site, and then is translocated to the donor site on the ribosome for continuation.

In antibiotic biosynthesis the construction is very compact; activation and polymerization are transacted on the same protein, which makes the whole machinery much smaller, and this system impresses like a missing link in the transition in process evolution from fatty acid to ribosomal protein synthesis. The less complex system, a descendant of fatty acid synthesis, has gained the ability to assemble a line of different amino acids in prescribed order. In both Ty and GS synthesis, the sequence of addition is guided by a protein template laid out on a polyenzyme system. In ribosomal synthesis, genetic information is translated by codon-anticodon interaction on the mRNA template, which accurately transmits the information to produce sequences of long polypeptide chains. A protein template is not suited to do this; its limits are probably 15 to 20 amino acid sequences.

The three systems have a common mode of polymerization. They fall into a category that I like to call head-growth polymerization (*15*). As seen in Fig. 19, in this mode the group that joins the chain is preactivated, and activation of the previously added group heading the chain is used for the elongation step. This leaves the activation at the new head intact so that it is already primed to do the next step. The mode facilitates the binding of the chain terminal to the template, thereby prompting the adding unit to settle next to it.

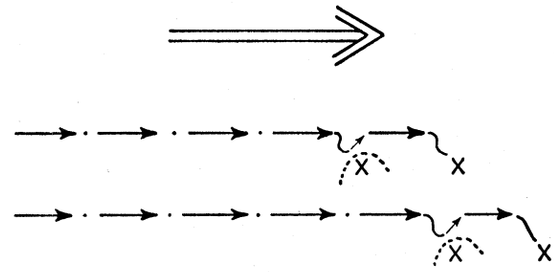


Fig. 19. Headward chain growth (*15*). The figure shows that the chain is headed by an activated annex, the displacement of which leads to addition of a new unit, whose preactivated head thereby becomes a new activated chain terminal.

Concluding Remarks

This study served two purposes, and it might be worthwhile to discuss them separately.

1) The exploration of this enzyme mechanism was initiated as an attempt to connect backward from the present to a prebiotic evolution. For this purpose we embarked on a study of a relatively simple enzymatic mechanism of amino acid sequentialization which produced polypeptides in an RNA- and particle-free solution. There can be no doubt that the *B. brevis* strains produce the enzyme proteins that act as templates through a DNA-derived message on ribosomes. This does not exclude the possibility that before the takeover by genetic information transfer there may have existed a mechanism similar to the one described here to connect amino acids. Such medium-sized polypeptides could have been early representatives of a class of compounds exhibiting the remarkable functional versatility that developed fully in the synthesis of proteins.

As was mentioned, an amino acid transfer from synthetic aminoacyl thioesters to one of the GS enzymes has recently been found. We hope to continue such synthetic approaches for exploring the use of -SH-charged proteins or other polymers in amino acid polymerization, as exemplified by antibiotic synthesis.

2) The unexpected finding of aminoacyl thioesters as donors in enzymatic polypeptide synthesis which, as in fatty acid synthesis, was also linked to pantetheine as carrier, encouraged me to formulate the evolution of a scheme of operation to activate and connect subunits into polymers of increasing complexity. Parallel modes of enzymatic activity were grouped together in a preceding essay on the relation between direction and mechanism of polymerization (*15*). It does not seem to me too absurd to think of the possibility that some genetic background may appear for the development of such parallel modes. However, calling this a process evolution should not be construed to imply use of the word evolution in a Darwinian manner.

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Bacterial Differentiation

The cell cycle of *Caulobacter* is used as a model system for studying the molecular basis of differentiation.

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Living cells, whether they are components of complex tissues and organs or function as unicellular organisms, are not static units but can change morphologically, adapt to environmental conditions by biochemical alteration, or express different functions at different times in the life cycle. A major task of modern biologists is to understand the control of these cell changes. Recent investigations of the genetic control mechanisms which operate in microbial cells indicate that not all the genes in a given cell are translated into protein at the same time (*1*), and that environmental factors can and do influence gene expression (*2*). In a differentiating cell, undergoing a series of biochemical and morphological changes within a regulated time sequence, controls must also exist to ensure the organization and sequential expression of these events during the life cycle of the cell. A persistent notion among developmental biologists has been that organizational control functions are a regular component of the genetic composition of each cell. Important advances in the understanding of cellular differentiation would seem to lie in exploring systems of interacting regulatory genes involved in the programmed expression of well-defined characteristics.

The control mechanisms that are ultimately shown to regulate the process of differentiation in one type of cell may not be applicable to all cells. In both prokaryotes and eukaryotes, however, the observable changes accompanying cell development do reflect variations in the patterns of protein synthesis. It is possible that the complex genetic machinery of the eukaryote demands different or additional controls of protein synthesis (*3*). A full understanding of the regulatory mechanisms of differentiating prokaryotic cells, however, might provide a valid basis for extrapolation to cells of higher organisms. Most importantly, prokaryotic cells that undergo cellular differentiation are accessible to study by the established techniques of bacterial genetics. One such area in which significant contributions to the control of cell regulation have come is sporulation and germination in the genus *Bacillus* (*4*). In these studies it has become apparent that control at the level of selective messenger RNA production (transcription) represents the basic mechanism whereby the cell organizes its development (*5*, *6*). Clues as to the universality of this type of control mechanism may come from the exploration of other readily studied bacterial systems. It is in this context that we have attempted to develop a simple system, using the dimorphic *Caulobacter* bacteria, to study the regulation of cellular differentiation.

Systems for Studying Bacterial Differentiation

Relatively few bacteria carry out a defined morphogenesis, other than cell division, during their normal life cycles. Still fewer bacterial species both differentiate and offer ease of genetic and biochemical manipulation. The criteria that make the use of bacteria reasonable as model systems for cell development include (i) a simple and well-defined differentiation pattern which can be studied in synchronized cell populations, (ii) the ability to grow the cells on defined media, permitting the correlation of biochemical events with morphological development, and (iii) the availability of mutants and a system for the exchange of genetic information. Differentiating bacteria that offer a

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