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Synthesis of a Linear Gramicidin by a Combination of Biosynthetic and Organic Methods†

Karl Bauer, Robert Roskoski, Jr., Horst Kleinkauf,‡ and Fritz Lipmann*

ABSTRACT: Linear gramicidin is a pentadecapeptide with the sequence *N*-formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. Ribosome-free extracts from *Bacillus brevis* (ATCC 8185), on Sephadex G-200 filtration, yielded fractions free of aminoacyl-tRNA ligases which catalyzed ATP–PP_i exchanges and covalent trichloroacetic acid stable binding of all amino acids incorporated in linear gramicidin. From these fractions Cl_3CCOOH-stable protein-bound peptides were prepared and, after alkali (pH 11) or peroxidative liberation, separated by thin-layer chromatography. Labeled valine, glycine, alanine, leucine, and tryptophan used alternatively with phenylalanine, *i.e.*, all the amino acids present in linear gramicidin, were incorporated into one of the peptides. The amino acid stoichiometry found in this peptide by double labeling as described in the text was Gly:Ala:Leu:Val:Phe, 1:2:4:4:4. Moreover, in agreement with the structure of linear gramicidin, alanine was found only in L configuration, valine in 50% D and 50% L, and leucine in the D configuration; all derived from L-amino acids used as biosynthetic precursors. Aminoethanolysis of this enzyme-bound intermediate, followed by organic formylation, yielded a product which comigrated with linear gramicidin in four solvent systems. These results indicate that biosynthetically a pentadecapeptide is formed which remains thioester linked to the enzyme and presumably is released enzymatically by aminoethanolysis. Crude extracts yielded a formylated peptide analogous to the one analyzed. The experiments suggest for the biosynthesis of linear gramicidin a mechanism analogous to that of gramicidin S and tyrocidine (Lipmann, F. (1971), *Science* 173, 875). However, a complete biosynthesis of linear gramicidin could not be achieved due to our inability to obtain extracts that linked the C-terminal aromatic amino acid to ethanolamine.

The linear gramicidins are antibiotics produced by the same strains of *Bacillus brevis* (ATCC 8185 or Dubos strain ATCC 10068) that produce tyrocidine (Hotchkiss, 1944). Figure 1 shows the structures of these pentadecapeptides, which differ only in the alternative incorporation of tryptophan, tyrosine, and phenylalanine as aromatic amino acids similar to the differences in the tyrocidines. The amino-terminal valine is formylated, and the carboxyl-terminal tryptophan is peptidically linked to ethanolamine. If glycine is considered an equivalent of a D-amino acid, the linear gramicidins consist throughout of alternating L- and D-amino acid residues, thereby placing constraints on the secondary structure (Urry *et al.*, 1971).

The development of cell-free systems for the synthesis of GS[1] and Ty made it possible to decipher the mechanism of biosynthesis of these cyclic decapeptides by nonribosomal systems (Saito *et al.*, 1970; Kurahashi *et al.*, 1969; Bredesen *et al.*, 1968; Lipmann *et al.*, 1971). These studies showed that the amino acids are activated by ATP on complementary enzymes of a molecular weight roughly proportional to the number of amino acids activated. From the resulting aminoacyladenylate the amino acid is then transferred to an enzymic sulfhydryl where it is bound covalently as thioester (Kleinkauf and Gevers, 1969). In both GS and Ty the N-terminal phenylalanine is activated and racemized by the smallest enzyme, mol wt 100,000. Reaction between it and the larger enzyme carrying the other amino acids initiates polymerization to peptide intermediates that remain thioester linked to enzymes until released by cyclization (Gevers *et al.*, 1969; Ljones *et al.*, 1968). Pantetheine, covalently bound to the larger enzyme proteins (Kleinkauf *et al.*, 1970; Gilhuus-Moe *et al.*, 1970), appears to mediate, by alternating transthiolation and transpeptidation, the elongation of successive peptidyl additions to enzyme–thioester-linked amino acids (Kleinkauf *et al.*, 1971).

The present experiments describe a soluble enzyme system that performs a partial LG biosynthesis. The mode of amino acid activation parallels that for GS and Ty biosynthesis. Thioester-linked glycine and alanine, exclusively present in LG, were already found in trichloroacetic acid precipitates during studies of Ty biosynthesis in the same extracts used here for studying LG biosynthesis (Roskoski *et al.*, 1970a). We have been able to identify an enzyme-bound pentadecapeptide intermediate of LG. After chemical ethanolaminolysis from its thioester link to denatured enzyme and chemical formylation, a compound was isolated that appeared to be identical with authentic LG. In crude extracts enzymatic N-terminal formylation was obtained; however, enzymatic conjugation of the carboxyl terminal with ethanolamine to complete the biosynthesis was not achieved.

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[1] Abbreviations used are: LG, linear gramicidin; GS, gramicidin S; Ty, tyrocidine.

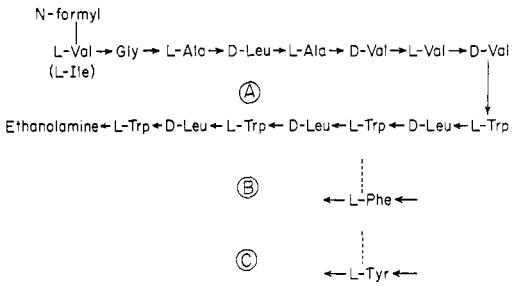

FIGURE 1: Linear gramicidins (A, B, and C).

Experimental Section

Growth of B. brevis. B. brevis (ATCC 8185) was cultured by the general method of Fujikawa *et al.* (1968). Spores from a single potato agar slant were suspended in sterile water, and equal portions were transferred to two 2-l. flasks containing 500 ml of milk–yeast extract medium. After incubation in a New Brunswick rotatory shaker (18 hr, 37°), about 300 ml of the culture was used to inoculate 10 l. of the meat extract–salt medium. The cells were grown at 37° with mechanical stirring (350 rpm) in a New Brunswick MMF-14 fermentation apparatus. The rate of sparging with air was 9 l./min until the A_{650} reached 0.5, when the rate was increased to 12 l./min. A decreased rate of stirring or aeration decreased the yield of cells and enzyme activity. The cells were harvested in the late log phase (A_{650} about 4), washed twice with 40 mM potassium phosphate (pH 7.0) buffer containing 2 mM $MgSO_4$, and then stored in liquid N_2.

Standard Solutions. The compositions of buffer A and medium M have been reported (Roskoski *et al.*, 1970a).

Preparation of Cell-Free Extracts. All operations were carried out at 0–4° unless otherwise specified; 100-g samples of cells were thawed and suspended in 1 l. of 25 mM triethanolamine-HCl (pH 7.4) containing 1 mM dithiothreitol, 2 mM $MgCl_2$, 200 mg of lysozyme, and 500 μg of DNase. After incubation for 15 min at 25°, the suspension was centrifuged at 10,000*g* for 30 min. The sediment was resuspended in another 500 ml of the same solution, and the incubation and centrifugation were repeated. The second step increased the enzyme yield about 25%. The two supernatants were combined and centrifuged at 100,000*g* for 1 hr. This supernatant, termed crude extract, was used immediately after preparation in the specified experiments. The crude extract was brought to 0.6% (w/v) with streptomycin sulfate and immediately centrifuged for 15 min at 10,000*g*. Solid $(NH_4)_2SO_4$ was added over a period of 15 min until 20% saturation. Thirty minutes after the $(NH_4)_2SO_4$ had dissolved, the suspension was centrifuged (10,000*g*, 15 min) and the precipitate was discarded. The supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and centrifuged 30 min later. The protein precipitate was dissolved in buffer A containing 10% sucrose (w/v) to give a protein concentration of 25 mg/ml, and was stored in liquid N_2.

Sephadex G-200 Gel Filtration. About 80 mg of the solution of 20–50% $(NH_4)_2SO_4$ fraction was applied to a Sephadex G-200 column (100 × 5 cm) equilibrated with buffer A, which was used for elution. Fractions were collected (5 ml) and the absorbancies at 278 nm were recorded. Amino acid dependent ATP-[^{32}P]PP_i exchanges were measured as previously described (Gevers *et al.*, 1968). Fractions 60–87 were pooled (about 1.1–1.6 exclusion volumes as indicated in Figure 2), and the protein was precipitated by $(NH_4)_2SO_4$ at 80% saturation for 1 hr prior to centrifugation at 10,000*g* for 15 min. The protein was taken up in buffer A containing 10% sucrose and stored in liquid N_2. Source of materials, determination of radioactivity, and methods for isolating enzyme-bound intermediates were identical with those described previously (Roskoski *et al.*, 1970a,b).

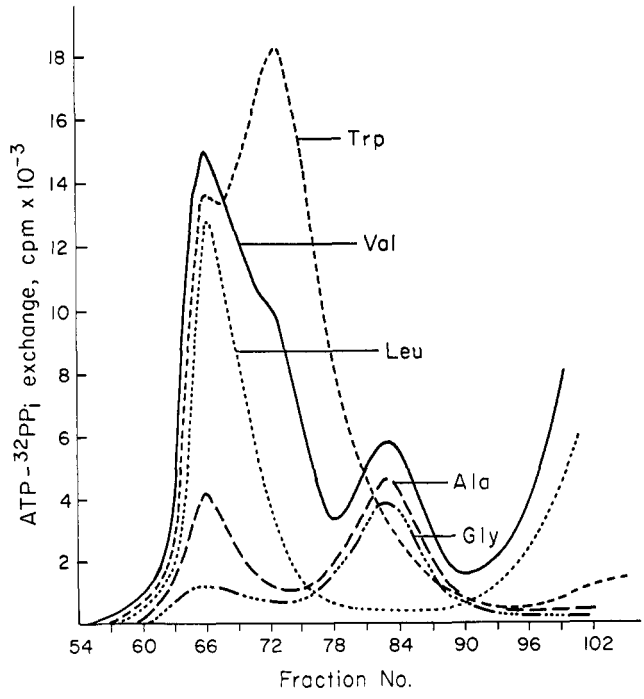

FIGURE 2: Separation of the linear gramicidin-synthesizing enzymes from amino acid–tRNA ligase activity by Sephadex G-200 gel filtration. ATP-[^{32}P]P_i exchanges dependent on valine (——), leucine (· · · ·), tryptophan (– – –), alanine (— — —) (all L configuration), and glycine (–· · ·–). The method for gel filtration is given in the Experimental Section. Fractions 60–87 (1.1–1.6 exclusion volumes) were used as the source of the "Sephadex enzyme." The amino acid dependent exchanges after fraction 90 are associated with amino acid–tRNA ligase activity measured as previously described (Roskoski *et al.*, 1970a).

Results

Amino-Acid Dependent ATP-[^{32}P]PP_i Exchanges of Sephadex G-200 Fractions. Previous studies in this laboratory indicated that exclusion chromatography is an effective way to resolve the enzyme fractions required for GS and Ty biosynthesis. The location of the biosynthetic enzymes was monitored by their amino acid dependent ATP-[^{32}P]PP_i-exchange activities. Figure 2 shows the Sephadex elution profile obtained by monitoring the exchange activities dependent upon the LG constituent amino acids. These exchanges eluted between 1.1 and 1.6 exclusion volumes. Moreover, these fractions were devoid of amino acid–tRNA ligase activity which eluted between 1.8 and 2.5 exclusion volumes (Roskoski *et al.*, 1970a).

The following experiments indicate that the early eluting fractions contain the enzymes that are activating the amino acids incorporated in LG. There were two peaks of activity for alanine, valine, and tryptophan, one main peak for glycine, and only one for leucine (Figure 2). The tryptophan and leucine peaks overlap with the ones seen in extracts synthesizing Ty since the Ty heavy enzyme (Roskoski *et al.*, 1970a),

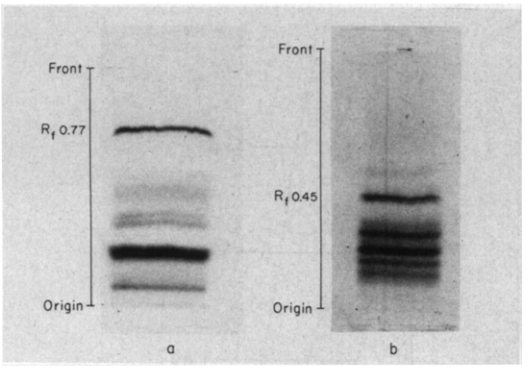

FIGURE 3: Isolation of the intermediate peptide (R_F 0.45) after liberation from enzyme by alkali. The "Sephadex enzyme" (2 mg of protein) was incubated with ATP, Mg^{2+}, [^{14}C]glycine, alanine, valine, leucine, and tryptophan for 30 min at 37°, and was passed through a Sephadex G-50 column to remove low molecular weight precursors as previously described (Roskoski *et al.*, 1970b). The protein in the fraction containing enzyme-bound intermediate was precipitated with 10% trichloroacetic acid and washed with the acid, then ethanol–ether (1:3), and then ether. The covalently linked intermediates were liberated by treatment with 500 μl of 0.01 N KOH for 1 hr at room temperature, neutralized to pH 6.5 with $HClO_4$, taken up in 3 ml of 90% methanol, and centrifuged for 10 min at 1000*g*. The supernatant solution was concentrated to dryness in a stream of air, 100 μl of 90% methanol was added to the residue, and the solution was applied to a silica gel thin-layer chromatogram which was developed in butanol–HOAc–H_2O (63:10:27) (chromatogram a). The major labeled product (R_F 0.77) was located by radioautography (72-hr exposure), eluted with 90% methanol, and chromatographed on a silica gel thin-layer chromatogram using the solvent ethyl acetate–pyridine–HOAc–H_2O (90:30:9:16). The main product, located by radioautography, had R_F 0.45 (chromatogram b).

which catalyzes valine, leucine, and tryptophan activity, is also found in this early eluting region. The glycine and alanine activation activities are exclusively related to LG biosynthesis (Roskoski *et al.*, 1971).

In view of the instability of the LG-synthesizing system, its resolution into complementary enzymes was postponed.

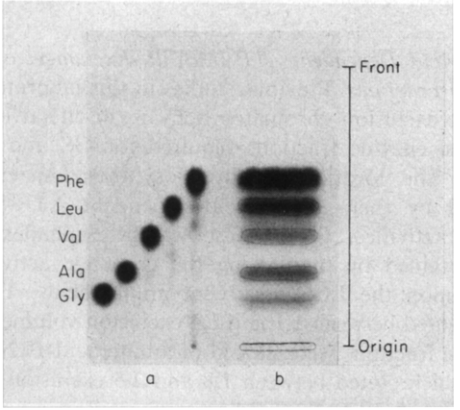

FIGURE 4: Recovery of the precursor amino acids from the 0.45 peptide after acid hydrolysis. The peptide, prepared as described in Figure 3 using labeled glycine, alanine, valine, leucine, and phenylalanine precursors, was treated with 6 N HCl for 84 hr at 110°, and the hydrolysate was chromatographed on EM silica gel plates (from Brinkmann) with phenol–H_2O (75:25, w/w) in a chamber atmosphere saturated with 3% NH_3. Since tryptophan is destroyed by acid hydrolysis, phenylalanine was used as the precursor amino acid.

TABLE I: Optical Configuration of Amino Acids Obtained after Hydrolysis of the R_F 0.45 Peptide.[a]

	L-Amino Acid (%)	D-Amino Acid (%)
Leucine	0	100
Valine	55	45
Alanine	100	0

[a] The R_F 0.45 peptide was prepared as described in Figure 3 using ^{14}C-labeled leucine, valine, and alanine, and was hydrolyzed as described in Figure 4. The optical configuration was determined with D- and L-amino acid oxidase as described previously (Gevers *et al.*, 1969).

The present study concentrated on the isolation of a large, enzyme-bound polypeptide that could be identified as the pentadecapeptide corresponding to LG and on its chemical conversion to the antibiotic.

Isolation of Enzyme-Bound Peptide Intermediates. Since peptide intermediates thioester linked to enzyme have been isolated in GS and Ty biosynthesis (*cf.* Lipmann, 1971), we tried to isolate similar intermediates in LG biosynthesis. The pooled Sephadex fractions were incubated with ATP, Mg^{2+}, and the LG amino acids (one or more of which was radiolabeled). After Sephadex G-50 gel filtration to remove low molecular weight precursors, the protein in the eluent was precipitated with trichloroacetic acid and washed to remove noncovalently bound material as previously described (Roskoski *et al.*, 1970b). The covalently bound products were liberated by alkali (pH 11.0) and resolved by two successive thin-layer chromatographic systems (Figure 3). It was surmised that the product with the highest R_F would be the most hydrophobic and consequently the longest intermediate. Therefore the R_F 0.45 peptide (Figure 3b) was further characterized.

All the LG constituent amino acids were incorporated into the R_F 0.45 peptide. With different LG amino acids labeled with ^{14}C and ^{3}H, radioactivity comigrated in a single zone. Moreover, after elution and rechromatography in a third solvent system (1-butanol–2-butanone–H_2O, 2:2:1), a single zone containing the double label was obtained with R_F 0.40. To show that the R_F 0.45 peptide product is an amino acid derivative, it was treated with 6 N HCl at 110° for 84 hr. In these experiments, as in the study of Ty biosynthesis (Roskoski *et al.*, 1970b), only phenylalanine was used as the aromatic amino acid. This was essential since tryptophan would have been decomposed by acid hydrolysis. The results show that, as found with Ty biosynthesis, phenylalanine substitutes well for tryptophan in the isolated enzyme system. The hydrolysate was chromatographed and the amino acids were recovered (Figure 4). This phenylalanine incorporation shows that analog substitution occurs to a greater extent *in vitro* than *in vivo*, which parallels the findings of the Ty–enzyme system (Roskoski *et al.*, 1970b).

To further substantiate the identity of the R_F 0.45 peptide as an intermediate in LG biosynthesis, the optical configuration of the amino acids in the hydrolysate was determined (Table I). In agreement with the primary structure (Figure 1), all the leucine from the R_F 0.45 product had the D configuration although L-leucine was used as the precursor;

TABLE II: Stoichiometry of Amino Acids in the R_F 0.45 Peptide.[a]

[^{14}C]Glycine	[^{3}H]Leucine	Ratio
1.45 pmoles	5.8 pmoles	1:4
[^{14}C]Alanine	[^{3}H]Leucine	Ratio
2.8 pmoles	5.4 pmoles	1:2
[^{14}C]Valine	[^{3}H]Leucine	Ratio
5.8 pmoles	5.4 pmoles	1:1
[^{14}C]Phenylalanine	[^{3}H]Leucine	Ratio
5.8 pmoles	5.4 pmoles	1:1

[a] The R_F 0.45 peptide for each experiment was prepared as described in Figure 3 using the specified labeled amino acids. After resolution, the peptides were eluted from the thin layers and radioactivity was determined by liquid scintillation spectrometry.

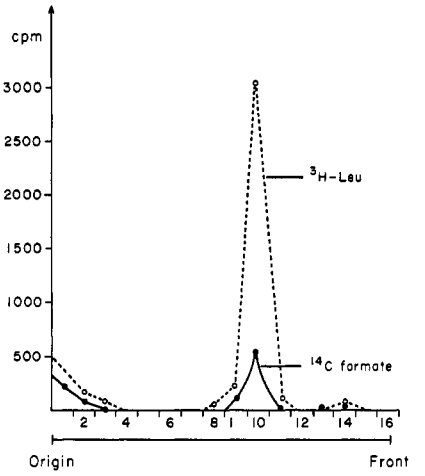

FIGURE 5: Isolation of the R_F 0.6 peptide after liberation from enzyme in the crude extract. The reaction was carried out as described in Figure 3 using 4 mg of protein from crude extract and [^{14}C]formate and [^{3}H]leucine markers. After chromatography on silica gel thin layer in ethyl acetate–pyridine–HOAc–H_2O (90:30:9:16) the plate was segmented and the radioactivity measured by liquid scintillation spectrometry.

valine was present in approximately equal amounts of D and L, and alanine was exclusively of the L configuration.

The incorporation of all the LG constituent amino acids into a single product does not specify the length of the peptide. To obtain a better estimate of chain length, the amino acid stoichiometry of the peptide was measured. Using [^{14}C]-glycine, [^{14}C]alanine, [^{14}C]valine, and [^{14}C]phenylalanine in successive experiments with [^{3}H]leucine, the ratio of Gly:Ala:Val:Leu:Phe was 1:2:4:4:4 (Table II). This is consistent with the stoichiometry of the complete pentadecapeptide (Figure 1), and appears to indicate that the entire peptide had been synthesized by the enzyme system.

Liberation of the Enzyme-Bound Peptide Chain by Performic Acid Oxidation. To further substantiate the notion that the intermediate peptide is bound as thioester, the complex was treated with performic acid, which cleaves the thioester but not the oxygen ester link (Harris *et al.*, 1963). The enzyme-bound intermediate was prepared as described in Figure 3 using [^{14}C]leucine as marker and a control with [^{3}H]leucine. The former was treated with performic acid (Roskoski *et al.*, 1970b) and the latter with alkali (Figure 3). The R_F 0.45 peptide was obtained in each case. The products were eluted after the second thin-layer chromatography as in Figure 3, and were shown to comigrate after re-chromatography in the second solvent by the coincidence of the ^{14}C and ^{3}H labels measured by liquid scintillation spectrometry.

Bioformation of a R_F 0.6 Peptide Intermediate. When crude extract was used as an enzyme source for preparation of enzyme-bound peptide intermediates, then the major product obtained after alkaline liberation chromatographed on silica gel in ethyl acetate–pyridine–HOAc–H_2O (90:30:9:16) with an R_F of 0.6 rather than 0.45. In addition to LG constituent amino acids, labeled formate was incorporated into the R_F 0.6 product (Figure 5). Through chemical deformylation, the R_F 0.6 product can be converted into the R_F 0.45 compound; treatment with methanol–HCl produces free formate and a product that comigrates with the R_F 0.45 peptide on thin-layer chromatography. Thus we conclude that the R_F 0.6 product is the formylated pentadecapeptide of R_F 0.45. The R_F 0.6 peptide is not formed by the Sephadex G-200 enzyme system but only with crude extract. Apparently components required for the formylation are removed or inactivated during purification. The conversion of the formylated compound into the biosynthetically obtained pentadecapeptide seems to indicate that formylation of the N-terminal valine may occur after polypeptide synthesis. This agrees with the experience that a formylvaline could not be detected after incubation of formylating extracts with valine and formate.

Combined Bio- and Organic Synthesis of LG. To prove more conclusively that the above characterized enzyme-bound peptide is identical with the LG pentadecapeptide, organic ethanolaminolysis and formylation were carried out on the biosynthetic product. For this purpose, the Sephadex G-200 enzyme fraction was incubated with ATP, Mg^{2+}, [^{14}C]leucine, and the four other LG components using trypophan as the aromatic amino acid. As described in detail in the legend of Table III, after Sephadex G-50 filtration, trichloroacetic acid precipitation, and washing, the enzyme–peptide complex was treated with ethanolamine in the expectation that the thioesterified peptide would undergo ethanolaminolysis. The product was formylated and then treated with alkali to saponify the *O*-formylethanolamine derivative (Sarges and Witkop, 1965). As shown in Table III, the final product cochromatographed with LG in four solvent systems.

Aminopterin Inhibition of LG Biosynthesis in Vivo. In an attempt to ascertain the requirements of the formylation reaction, antibiotic biosynthesis was studied in cultures in the presence of the formyltetrahydrofolate antagonist aminopterin. This drug inhibited LG biosynthesis completely, producing a twofold increase in Ty biosynthesis at high concentrations (1 mg/ml) (Table IV), and also a 40% decrease of amino acid incorporation into hot trichloroacetic acid precipitable material. These results indicate that formyltetrahydrofolate is required for LG as well as for bacterial protein synthesis.

Discussion

B. brevis (ATCC 8185) produces the pentadecapeptide linear gramicidins (A–C) and the cyclic decapeptide tyrocidine. However, the extract prepared from cells after lysis in hypotonic buffer, although active in Ty biosynthesis, did not

TABLE III: Comparison of the R_F Values of Standard and Biosynthetically Prepared Linear Gramicidin.[a]

Solvent	R_F[b]	R_F[c]
$CHCl_3$–CH_3OH (85:15)	0.60	0.60
Ethyl acetate–pyridine–HOAc–H_2O (120:30:9:16)	0.70	0.70
$CHCl_3$–CH_3OH–HOAc (100:5:10)	0.30	0.30
$CHCl_3$–CH_3OH–NH_3 (100:15:2)	0.52	0.52

[a] Enzyme-bound peptide intermediate was prepared as described in Figure 3. It was washed in ether, dried, and incubated with 50 mg of aminoethanol (60°, 1 hr) to effect the aminoethanolysis of the carboxyl of the peptidyl tryptophan bound to the enzyme by an activated thioester linkage. After addition of 1 ml of 90% methanol and centrifugation at 1000*g* for 10 min, the supernatant was aspirated and dried under a stream of air. For the formylation reaction (after Sarges and Witkop, 1965), about 4 ml of 98% formic acid was added to the residue. The supernatant was cooled to 0°, and 1.2 ml of acetic anhydride was added. After 30 min on ice, the mixture was incubated at ambient temperature for 4 hr. Then 1.2 ml of ice-cold water was added, and the mixture was evaporated *in vacuo*. The *O*-formyl group was saponified by addition of 5 ml of methanol and 0.55 ml of 1 N KOH for 1 hr (ambient temperature). The solution was neutralized with $HClO_4$. The supernatants were chromatographed on silica gel thin-layer plates with authentic linear gramicidin (Sigma Chemical Co.). [b] Biosynthetic product. [c] Standard linear gramicidin.

TABLE IV: Aminopterin Inhibition of LG Synthesis *in Vivo*.[a]

Aminopterin (μg/ml)	dpm/2.5 ml of culture medium $\times 10^{-3}$		
	LG	Ty	Protein
0	2.1	12.0	92
20	1.9	13.0	90
100	1.3	15.5	85
500	0.6	20.0	65
1000	0.0	24.0	52.5

[a] A minimal medium of 200 ml of asparagine–glycerol (Mach and Tatum, 1964) was inoculated with 0.1 ml of a spore suspension in distilled water. It was incubated for 30 hr at 37° in a New Brunswick rotatory shaker at 300 rpm. Then 25 ml of the grown culture was transferred to 250-ml flasks to which the specified amounts of aminopterin (Lederle) were added. After 20 min, 2.5 μCi of [^{14}C]valine (10 Ci/mole) in 0.2 ml of water was added to each flask. The cells were incubated for an additional hour. Ty and GS were isolated from 2.5 ml of medium as described by Fujikawa *et al.* (1968). Because a precipitate formed during acid precipitation (pH 4) with high concentrations of aminopterin, the precipitate was extracted with butanol–chloroform (4:1, v/v) before thin-layer chromatographic resolution of the respective antibiotics (Roskoski *et al.*, 1970a). Incorporation into protein was measured as described by Mach and Tatum (1964).

produce LG. Supplementation of the extracts with ethanolamine, ethanolamine phosphate, CDP-ethanolamine, in addition to ATP, Mg^{2+}, and amino acids, failed to activate extracts for LG bioformation. Furthermore, it was considered that serine might be incorporated at the carboxyl terminus and undergo decarboxylation to produce peptidically linked ethanolamine. However, labeled serine was not incorporated into LG *in vitro* even when the incubation medium was supplemented with pyridoxal phosphate which might have been required for decarboxylation. During LG biosynthesis *in vivo*, labeled ethanolamine was not incorporated, nor was it possible to find it inside the cells. When the cells were incubated with labeled serine, which is transported into them, label spread into both LG and Ty with no increased specific activity in ethanolamine.

As previously reported (Roskoski *et al.*, 1970a), the early eluting Sephadex G-200 filtrate contained enzymes that catalyze ATP–[^{32}P]PP_i exchanges and that are dependent upon the LG and Ty constituent amino acids. These exchanges were not related to the amino acid–tRNA ligase activities, which elute later. As previously demonstrated (Roskoski *et al.*, 1970a), the amino acids in Ty and LG bind to the Sephadex enzyme covalently as thioester. Thus the mode of amino acid activation appears to be the same in the two systems and parallels that of the GS system (Gevers *et al.*, 1968). The number of enzyme fractions required for LG biosynthesis is unknown. In GS two fractions and in Ty three fractions are required. The GS and Ty heavy fractions contain 4′-phosphopantetheine (Kleinkauf *et al.*, 1970). We suspect that this cofactor also participates in LG biosynthesis but have not yet examined this point.

The crude cell-free extracts catalyzed the formation of the formylated pentadecapeptide, and the early eluting Sephadex G-200 fractions were active in the biosynthesis of the unformylated intermediate. The formylation reaction has not yet been identified in detail; however, aminopterin *in vivo* inhibited LG synthesis more than 95% and stimulated Ty synthesis twofold.

The evidence that the R_F 0.45 peptide, an enzyme-bound pentadecapeptide, is an intermediate in LG biosynthesis includes: (1) incorporation of all the LG amino acids with the stoichiometry found in LG; (2) conversion of the peptide into the precursor amino acids by acid hydrolysis; (3) the optical configuration of the amino acids in the pentadecapeptide is the same as that in LG; (4) organic ethanolaminolysis of enzyme-bound product followed by organic formylation and deformylation of the *O*-formylethanolamine derivative gave a product not distinguishable from LG after thin-layer chromatography on four different solvent systems.

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Effect of Polypeptide Chain Length on Dissociation of Ribosomal Complexes†

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ABSTRACT: The selectivity of Na^+ in distinguishing free ribosomes from those complexed with peptidyl-tRNA and mRNA (Beller, R. J., and Davis, B. D. (1971), *J. Mol. Biol. 55*, 477) suggested its usefulness in assessing the possible effect of the length of the nascent peptide on ribosome stability. Accordingly, ribosomes from *Escherichia coli* bearing peptides of defined length and composition were prepared *in vitro* with phage R17 RNA as messenger. Ribosomes bearing hot trichloroacetic acid precipitable nascent peptides were stable in Na^+ gradients, while the 70S initiation complex containing fMet-tRNA was completely dissociated under these conditions. However, gradients containing a high K^+ concentration showed that the completed initiation complex was more stable than free 70S ribosomes. Ribosomes complexed with tripeptidyl-tRNA (fMet-Ala-Ser-tRNA), prepared in the presence of fusidic acid, showed intermediate stability; when such ribosomes were analyzed in a Na^+ gradient the majority of the ribosomes was dissociated, but the tripeptide remained associated with the 50S subunit peak. Thus the degree of resistance to dissociation by salt, conferred on ribosomes by complexed peptidyl-tRNA, increases with increasing peptide length, and tripeptidyl-tRNA seems to have a greater affinity than fMet-tRNA for the peptidyl binding site on the 50S subunit. In contrast to the normal initiation complexes, those prepared with a nonhydrolyzable analog of GTP could not be distinguished in their stability from free ribosomes.

It is known that ribosomes complexed with mRNA and peptidyl-tRNA are not dissociated under various conditions that do cause dissociation of free ribosomes. These conditions include the addition of the ribosome dissociation factor (Subramanian *et al.*, 1969; Albrecht *et al.*, 1970), replacement of K^+ by Na^+ in sucrose gradient buffers (Beller and Davis, 1971), lowering of the Mg^{2+} concentration (Ron *et al.*, 1968; Oppenheim *et al.*, 1968; Kelly and Schaechter, 1969), elevation of the K^+ concentration (Edelman *et al.*, 1960; Martin *et al.*, 1969), and exposure of ribosomes to air in the absence of sulfhydryl compounds (Miyazawa and Tamaoki, 1967; Beller and Davis, 1970). This stabilization against dissociation might depend only on the presence of a bound tRNA, or it might also depend on the nature of the nascent peptide chain. Accordingly, we have prepared ribosomal complexes carrying nascent peptides of various defined lengths in extracts of *Escherichia coil*, with R17 phage RNA as messenger, and have compared them for their ability to survive gradient centrifugation under various ionic conditions. Furthermore, we have compared the stability of initiation complexes prepared with GTP or with the nonhydrolyzable analog, GMP·PCP.[1,2]

Materials and Methods

Preparation of Ribosomes and Factors. All subcellular components were prepared from *E. coli* strain MRE600 (Cammack and Wade, 1965), grown at 37° in minimal medium A (Davis and Mingioli, 1950), supplemented with 0.2% glucose and 0.2% Casamino Acids.

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[2] Abbreviations used are: GMP·PCP, 5′-guanylylmethylenediphosphonate; IF, initiation factors; TKM, 10 mM Tris·HCl (pH 7.6)–50 mM KCl–5 mM magnesium acetate; TNaM, TKM with NaCl instead of KCl.