Tyrocidine Biosynthesis by Three Complementary Fractions from *Bacillus brevis* (ATCC 8185)*

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ABSTRACT: Tyrocidines are cyclic peptide antibiotics with the sequence

D-Phe-L-Pro-L-Phe-D-Phe-L-Asn
↑ ↓
L-Leu-L-Orn-L-Val-L-Tyr-L-Gln

where the second and third phenylalanines may be replaced by a corresponding tryptophan, and tyrosine by phenylalanine or tryptophan. An enzyme system prepared from *Bacillus brevis* (ATCC 8185), active in tyrocidine biosynthesis, was resolved into three complementary fractions by Sephadex G-200 gel filtration. A light (mol wt 100,000) and an intermediate component (mol wt 230,000) activate phenylalanine and proline, respectively. A heavy fraction (mol wt 460,000) activates the remaining tyrocidine constituent amino acids, including phenylalanine. As found in gramicidin S synthesis, each fraction catalyzes ATP-[^{32}P]P_i and ATP-[^{14}C]AMP exchanges dependent on its amino acid substrates. The activated amino acids are protein bound, and the complexes can be isolated by Sephadex G-50 gel filtration. We find that the enzyme–amino acid complexes each contain aminoacyl adenylate and an equivalent amount of amino acid covalently linked as thio ester.

The tyrocidines are antibiotics produced by specific strains of *Bacillus brevis* (ATCC 8185 or Dubos strain; ATCC 10068). Figure 1 shows the structure of these cyclic peptides, which differ only in analog-type substitutions of aromatic residues. In two places D-amino acids are present, and, at one, a nonprotein amino acid, L-ornithine. The tyrocidine analogs are not produced by different sets of enzymes, but rather by an enzyme system which is capable of incorporating structurally similar amino acids at certain sites (Mach and Tatum, 1964; Fujikawa *et al.*, 1968b). Gramicidin S, which is a related antibiotic (Figure 2), is produced by different strains of *B. brevis* (ATCC 9999; Nagano strain). It is likewise a cyclic decapeptide, but it contains only five different amino acids, including D-phenylalanine and L-ornithine. The same five amino acids are also present in tyrocidines.

The development of cell-free systems active in the biosynthesis of gramicidin S and the tyrocidines has played an important role in attempts to decipher the mechanism of peptide antibiotic synthesis. Many laboratories agreed (Yukioka *et al.*, 1965; Berg *et al.*, 1966; Bhagavan *et al.*, 1966; Tomino *et al.*, 1967; Fujikawa *et al.*, 1968a), and we confirmed (Gevers *et al.*, 1968) that this type of synthesis occurs in the complete absence of polynucleotides. This indicates that the amino acid sequence in these antibiotics is determined by enzyme specificity and organization and not by RNA templates. The overall stoichiometry for tyrocidine synthesis is as follows (Fujikawa *et al.*, 1968a)

$$10\text{ATP} + 10\text{amino acids} \longrightarrow \text{tyrocidine} + 10\text{AMP} + 10\text{PP}_i$$

The enzyme system producing tyrocidine, like the one for gramicidin S, catalyzes ATP-[^{32}P]P_i exchanges dependent on the constituent amino acids, including D-phenylalanine and L-ornithine (Gevers *et al.*, 1968; Fujikawa *et al.*, 1968a). The first indication of a transfer of the activated amino acids from aminoacyl adenylates to the enzyme proteins, with retention of activation, was the detection of amino acid dependent ATP-[^{14}C]AMP exchanges catalyzed by the gramicidin S forming enzymes in the absence of tRNA (Gevers *et al.*, 1968, 1969). The studies in the present paper show that each of the three enzyme fractions required for tyrocidine biosynthesis also catalyzes ATP-[^{14}C]AMP exchanges dependent on its corresponding substrate amino acids. As in the case of gramicidin S synthesis (Gevers *et al.*, 1969; Kleinkauf and Gevers, 1969; Froshøv *et al.*, 1970), the second forms of bound amino acid in tyrocidine synthesis, isolated by trichloroacetic acid precipitation of Sephadex G-50 eluents, show the characteristics of thio esters (Gevers *et al.*, 1969; Kleinkauf and Gevers, 1969).

Experimental Section

Materials. The unlabeled amino acids were obtained from Calbiochem. Pyruvate kinase, β-galactokinase, catalase, yeast alcohol dehydrogenase, and triethanolamine-HCl were purchased from Boehringer-Mannheim Corp. Tyrocidine, β-glucuronidase, and amino acid hydroxamates were obtained from Sigma Chemical Co. DNase, pancreatic RNase, and egg-white lysozyme were purchased from Worthington Biochemical Corp. Puromycin-HCl came from Nutritional Biochemicals Corp. Serva DEAE-cellulose (0.7 mequiv/g) was from Gallard-Schlesinger Chemical Manufacturing Corp., and the Sephadex gels from Pharmacia Fine Chemicals Inc. The chromatogram-developing apparatus and silica gel thin-layer plates were obtained from Eastman Kodak. Liquifluor was a product of T. M. Pilot Chemicals Inc.

* From The Rockefeller University, New York, New York 10021. *Received August 20, 1970.* Supported by Grant GM-13972 from the U. S. Public Health Service. A preliminary report of these results has appeared (Roskoski *et al.*, 1970).

† Recipient of a special fellowship (F03 CA22798) from the U. S. Public Health Service.

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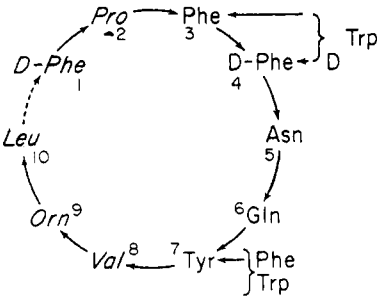

FIGURE 1: Primary structure of the tyrocidines. Residues are numbered arbitrarily.

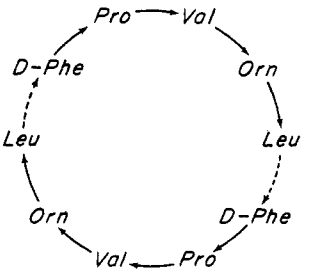

FIGURE 2: Gramicidin S.

Labeled amino acids were purchased from New England Nuclear Corp. or from Schwarz BioResearch, Inc. The specific activities were adjusted to 80 μCi/μmole (^{14}C) and 400 μCi/μmole (^{3}H). All other isotopic compounds were purchased from New England Nuclear Corp.

Radioactivity Determinations. Radioactivity on chromatograms was measured with a Varian Aerograph radio scanner. Radioactivity on Whatman No. 3MM glass fiber and Millipore filters was determined in a Packard Tri-Carb liquid scintillation counter in vials containing 5 ml of toluene with 0.42% liquifluor. Radioactivity in aqueous solutions was measured similarly using 10 ml of Bray's solution (1960).

Standard Mixtures. Tyrocidine and gramicidin S synthesis, and the formation of protein-bound amino acids and peptides, were assayed with the designated enzyme fractions and amino acids, supplemented with the following medium (M). In medium M the components were present to give the following concentrations in the assay mixtures: 50 mM triethanolamine-HCl (pH 7.8), 20 mM magnesium acetate, 5 mM KCl, 1 mM dithiothreitol, 4 mM ATP, 4 mM phosphoenolpyruvate, and 2 μg/ml of pyruvate kinase (from 10 mg/ml of suspension in 2.8 M ammonium sulfate). The buffer (A) used during enzyme purification, contained 20 mM triethanolamine-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.25 mM EDTA.

Assay of Tyrocidine Biosynthesis. Tyrocidine formation was measured in incubation mixtures (100 μl) containing the designated enzyme fractions, medium M, and each of the following L-amino acids: phenylalanine, proline, asparagine, glutamine, tryptophan, tyrosine, valine, ornithine, and leucine. The concentration of one amino acid, which was labeled, was 50 μM, and that of the remaining, 100 μM. Incubations were at 37° for 15 min unless specified otherwise. Reactions were stopped by placing the assay tubes in boiling water for 2 min which removes enzyme-bound intermediates. After cooling, 2 ml of 7% trichloroacetic acid was added; the mixtures were filtered through Millipore filters, pore size 0.45 μ, and washed five times with 7-ml portions of 7% trichloroacetic acid. The filters were dried at 110° for 10 min before liquid scintillation counting. The Millipore filter assay was corroborated by the following procedure in selected cases. After addition of 2 ml of 1-butanol–chloroform (4:1, v/v) and 0.4 ml of water to the heated reaction mixture, the suspension was agitated before centrifugation at 1500*g* for 5 min. A portion of the upper layer was removed and dried under a stream of air at 37°. The residue was taken up in 100 μl of ethanol–0.2 N HCl (9:1, v/v), and 60–80 μl was chromatographed on thin layers of silica gel, using solvent 1 for development (see below). The incorporations measured by the filter assay agreed to within 5% with those determined by chromatography, using standard tyrocidine for product identification.

Isolation of Bound Substrates. Reaction mixtures (100 μl) contained the designated enzyme fractions, medium M, and the specified amino acids at 100 μM. Incubations were for 30 min at 37°, at which time the incorporations had reached a constant value. The reaction mixtures were chilled in ice, passed through a Sephadex G-50 column (35 × 0.8 cm), and eluted with buffer A at 2°. The eluate (about 2.2 ml) containing the enzyme protein was collected, 0.1 ml was removed for determination of radioactivity by liquid scintillation counting, and the remainder treated with trichloroacetic acid (final concentration 7%). Carrier bovine serum albumin (0.2 ml of a 0.5% solution) was added prior to precipitation. After 20 min, the suspension was centrifuged at 1500*g* for 10 min. The precipitate was resuspended in 2 ml of 7% trichloroacetic acid and recentrifuged. It was similarly washed in 2 ml of ethanol–ether (25:75, v/v) and 2 ml of ether, before drying at 37° and storage at room temperature.

Treatment of the Washed Precipitates. Amino acid hydroxamates were formed by incubating the washed precipitates with 0.1 ml of neutral salt-free hydroxylamine (3 M, pH 6.1) at 60° for 20 min, with occasional agitation. A portion of the supernatant was chromatographed on paper (see below). Some precipitates were treated with 1% mercuric acetate in 0.1 M sodium maleate (pH 6.6) at 37° for 30 min, with controls from which mercuric acetate was omitted. The supernatants were then also analyzed by paper chromatography.

Chromatography. Amino acids and amino acid hydroxamates were separated by ascending paper chromatography on Whatman No. 1 paper using 1-butanol–formic acid–water (75:15:10, v/v) as solvent. The front migrated about 18 cm in 4 hr. After drying at 80° for 20 min, radioactivity was determined with a strip scanner. Standard amino acids and hydroxamates were used for product identification and located with ninhydrin reagent.

Thin-layer chromatography was carried out on silica gel plates (20 × 20 cm). The solvent (1) was ethyl acetate–pyridine–acetic acid–water (60:20:6:11, v/v).

Preparation of Extracts. B. brevis (ATCC 8185), obtained from the American Type Culture Collection, was grown according to the method of Fujikawa *et al.* (1968a). The cells were harvested in the late logarithmic phase, and could be stored for at least 2 months in liquid nitrogen without apparent loss of activity. The following extraction procedures and ammonium sulfate fractionations were similar to those described by these authors. The operations were conducted

at 2–4°. The cells were thawed (100 g) and suspended in 400 ml of 0.02 M potassium phosphate buffer (pH 7) containing 2.5 mM EDTA, 1 mM dithiothreitol, 400 μg of DNase, and 160 mg of lysozyme. After incubation at 30° for 20 min, the suspension was centrifuged at 10,000*g* for 60 min. The sediment was resuspended in another 400 ml of the same solution. The incubation and centrifugation were repeated. A saturated solution of ammonium sulfate (pH 7) was added slowly, with stirring, to the combined supernatants to give 33% saturation. After 15 min, the suspension was centrifuged at 10,000*g* for 15 min, and the sediment discarded. The supernatant was brought to 41% saturation with ammonium sulfate and centrifuged after 15 min. The precipitate was dissolved in buffer A to give a concentration of 15 mg/ml of protein, as determined spectrophotometrically (Warburg and Christian, 1941). Molar acetic acid was added to bring the pH to 5.2 (0°). The suspension was then centrifuged at 27,000*g* for 10 min, and yielded a sizeable precipitate with little activity. The supernatant was adjusted to pH 7.4 with solid $KHCO_3$, and ammonium sulfate solution was added to 25% saturation. After 15 min, the suspension was centrifuged, the precipitate taken up in a small volume of buffer A containing 10% sucrose, and passed through a Sephadex G-50 column (35 × 0.8 cm) equilibrated with the same solution. The partially purified unresolved enzymes (about 10 mg/ml of protein) were stored in liquid nitrogen, and were stable for at least 8 months.

Sephadex G-200 Filtration. A total of 60–90 mg of the above material was applied to a Sephadex G-200 column (100 × 5 cm) equilibrated with buffer A, which was used for elution. Fractions (6 ml) were collected and absorbancies at 280 mμ determined; L-proline-, L-ornithine-, and D-phenylalanine-dependent ATP–[^{32}P]P_i exchanges were measured (Figure 3). Solid ammonium sulfate was added to the specified fractions to bring them to 80% saturation. After 15 min the suspensions were centrifuged at 10,000*g* for 10 min, the precipitates dissolved in a small volume of buffer A containing 10% sucrose, and dialyzed against 200 volumes of the solvent for 2 hr. In the cases where protein concentrations were low, carrier serum albumin was added to a final concentration of 10 mg/ml before precipitation. The fractions were stored in liquid nitrogen and were stable for at least 8 months.

Results

General Characteristics of Tyrocidine Biosynthesis. The time course of amino acid incorporation into tyrocidine was linear for at least 30 min. Synthesis of the product required the addition of ATP, Mg^{2+}, and the tyrocidine-constituent amino acids. Optimum concentrations of ATP and Mg^{2+} were 4 and 20 mM, respectively. In agreement with the findings of other laboratories, as mentioned above, we found that tyrocidine biosynthesis was insensitive to addition of RNase (10 μg/ml) and puromycin (0.3 mM) (Table I). Thus, antibiotic biosynthesis occurs independently of ribosomal polypeptide synthesis. As in the case of gramicidin S (Gevers *et al.*, 1968), tyrocidine formation was inhibited by AMP and inorganic pyrophosphate (Table I).

Resolution of the Enzyme System into Three Complementary Fractions. The partially purified enzyme system was resolved into three components involved in tyrocidine biosynthesis by filtration through Sephadex G-200 (Figure 3). The first

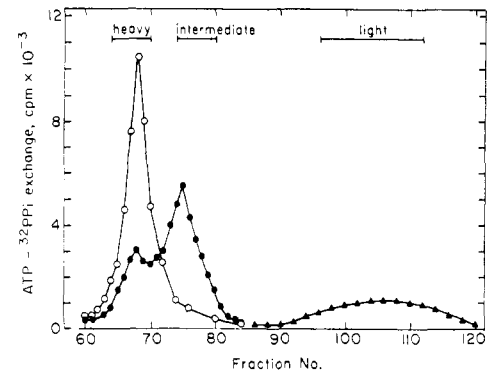

FIGURE 3: Resolution of the tyrocidine-synthesizing system into three complementary fractions by Sephadex G-200 gel filtration. ○—○, ●—●, and ▲—▲ represent ATP–[^{32}P]P_i exchanges dependent on L-ornithine, L-proline, and D-phenylalanine, respectively, determined as described previously (Gevers *et al.*, 1968). About 80% of the protein, measured by the absorbancy at 280 mμ, emerged in the void volume with the L-ornithine-dependent exchange activity. The method for gel filtration and the isolation of the separated fractions is given in the Experimental Section.

of these fractions to emerge from the column, which was nearly coincident with the main protein peak, was detected by its L-ornithine-dependent ATP–[^{32}P]P_i exchange activity. Exchange activities dependent on all the remaining tyrocidine amino acids gave profiles similar to that of the L-ornithine-dependent activity. Only in the case of L-proline was there a second large peak separated into an intermediate fraction, while a third component, detected by its D-phenylalanine-dependent ATP–[^{32}P]P_i exchange activity, eluted last. All three fractions were required for antibiotic synthesis, and a combination of any two fractions was less than 16% as active as the three combined fractions (Table II).

Molecular Weight Estimations. The sedimentation coefficients of the complementary components were measured by sucrose density gradient centrifugation (Figure 4). The fractions with D-phenylalanine-, L-proline-, and L-ornithine-dependent ATP–[^{32}P]P_i exchange activities had sedimentation coefficients of 5.7, 11, and 15 S, respectively. The following molecular weights were calculated, using the formula given by Martin and Ames (1961), and adopting the standards specified: light, 100,000; intermediate, 230,000; heavy,

TABLE I: General Characteristics of Tyrocidine Biosynthesis.[a]

Addition	Tyrocidine Synthesis (μμmoles)
Control	31.4
RNase (10 μg/ml)	32.1
Puromycin (0.3 mM)	30.8
AMP (1 mM)	17.8
PP_i (1 mM)	13.5

[a] Tyrocidine biosynthesis was assayed by the Millipore procedure described in the Experimental Section using proline as the labeled precursor. The second $(NH_4)_2SO_4$ fraction (100 μg) was used as the enzyme source.

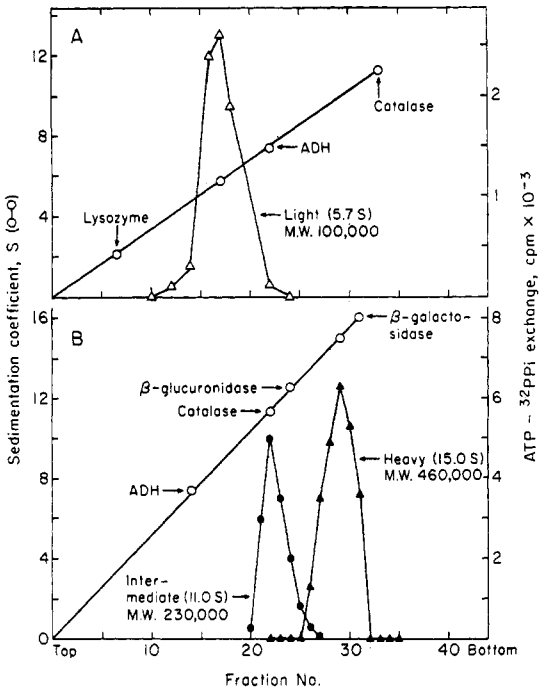

FIGURE 4: Estimation of the molecular weights of the three complementary fractions by sucrose density gradient centrifugation. The procedures were those previously described (Kleinkauf *et al.*, 1969), with the following changes. Linear 5–20% sucrose gradients were used. (A) The light fraction (40 μg in 0.1 ml), which was measured by its D-phenylalanine-dependent ATP-[^{32}P]P_i exchange activity (△—△), was centrifuged for 10 hr. (B) The intermediate and heavy fractions (60 μg of each together in 0.1 ml) were centrifuged for 6 hr and detected by their respective exchange activities dependent on L-proline (●—●) and L-ornithine (▲—▲). Lysozyme (50 μg/gradient) and β-galactosidase (25 μg/gradient) were assayed by the procedures of Schweiger and Gold (1969) and Pardee *et al.* (1959), respectively.

460,000. The respective standards were yeast alcohol dehydrogenase (7.4 S and 150,000) and catalase (11.3 S and 244,000) (Martin and Ames, 1961), and also β-galactosidase (16 S and 518,000; Sund and Weber, 1963).

ATP-[^{32}P]P_i Exchanges and Amino Acid tRNA Ligase Activities of the Three Fractions. The light component displayed D- and L-phenylalanine-dependent ATP-[^{32}P]P_i exchange activity (Table III). This appeared to be related to antibiotic synthesis since there was no demonstrable phenylalanine-tRNA ligase activity (Table IV). However, the fraction did catalyze several other amino acid dependent exchanges which corresponded to demonstrable amino acid tRNA ligase activities (Tables III and IV). The intermediate fraction activated L-proline, but was free of proline-tRNA ligase activity. The heavy fraction contained exchange activities dependent on the amino acids which occur in positions 3–10 in tyrocidine molecules (Figure 1), and was free of the corresponding tRNA-acylating enzymes. The rates varied greatly with different amino acids (compare L-tryptophan and L-glutamine). This is interesting when one considers the general absence of such differences in the binding of amino acids (see below).

Amino Acid Dependent ATP-[^{14}C]AMP Exchanges Catalyzed by the Three Fractions. All three components catalyzed ATP-[^{14}C]AMP exchanges dependent on their corresponding amino acid substrates in the absence of tRNA (Table V). The light fraction was about 20 times as active as the heavy component, and about five times as active as the intermediate fraction. The reason for the different specific activities is not known, but the results are similar to those obtained in the

TABLE II: Requirement for Three Enzyme Fractions in Tyrocidine Biosynthesis.[a]

Added Fraction	Tyrocidine Synthesis (μμmoles)
Light	0.90
Intermediate	0.99
Heavy	0.95
Light + intermediate	4.40
Light + heavy	3.40
Intermediate + heavy	1.70
Light + intermediate + heavy	29.60

[a] Tyrocidine formation was measured as described in Table I with light (10 μg), intermediate (25 μg), and heavy (37.5 μg) components. These amounts of added enzymes were saturating for antibiotic synthesis; a decrease in any of the three led to a corresponding decrease in net synthesis. This equivalence parallels that provided by substrate binding studies of the fraction (see Table V).

TABLE III: Amino Acid Dependent ATP-[^{32}P]P_i Exchanges Catalyzed by the Light, Intermediate, and Heavy Fractions.[a]

Amino Acid Added	ATP Formed (mμmoles)		
	Light	Intermediate	Heavy
L-Phenylalanine	20.3	3.4	30.7
D-Phenylalanine	16.7	2.1	9.3
L-Proline	1.6	12.5	0.5
L-Tryptophan	2.0	3.6	68.0
D-Tryptophan	0.1	0.5	9.3
L-Asparagine	8.3	2.0	32.0
L-Glutamine	0.1	0.5	9.9
L-Tyrosine	6.0	0.9	28.6
L-Valine	21.7	5.7	70.8
L-Ornithine	0.0	0.2	13.2
L-Leucine	12.0	2.0	62.0
L-Alanine	18.4	2.1	23.3
Glycine	1.0	1.8	14.0
L-Aspartate	12.6	0.1	0.4
L-Glutamate	12.3	0.0	0.8

[a] The exchanges were carried out as described in Figure 3 with 10, 25, and 37.5 μg of the light, intermediate, and heavy fractions, respectively. Control values (<0.3 mμmole) obtained in the absence of added amino acids were subtracted in each case.

TABLE IV: Amino Acid tRNA Ligase Activities in the Light, Intermediate, and Heavy Fractions.[a]

Amino Acid Tested	Aminoacyl-tRNA Formed ($\mu\mu$moles)		
	Light	Intermediate	Heavy
L-Phenylalanine	0	0	0
D-Phenylalanine	0	0	0
L-Proline	3.2	0	0
L-Tryptophan	5.0	0.6	0
D-Tryptophan	0	0	0
L-Asparagine	3.1	0	0
L-Glutamine	0	0	0
L-Tyrosine	9.0	0.2	0
L-Valine	37.0	0.2	0
L-Ornithine	0	0	0
L-Leucine	9.6	0	0
L-Alanine	8.1	0	0
Glycine	0.8	0	0
L-Aspartate	101.0	0	0
L-Glutamate	12.4	0	0

[a] The assays were carried out by the procedure of Muench and Berg (1966), except that dithiothreitol (1 mM) replaced glutathione. Incorporation in the absence of added *E. coli* tRNA (gift of M. Schweiger) was used as blank. The light (10 μg), intermediate (25 μg), and heavy (37.5 μg) fractions were prepared by Sephadex G-200 gel filtration as described in the Experimental Section.

TABLE V: Amino Acid Dependent ATP-[^{14}C]AMP Exchange.[a]

Fraction Tested	Amino Acid Tested	[^{14}C]ATP Formed ($\mu\mu$moles)
Light	L-Phenylalanine	9300
Intermediate	L-Proline	2100
Heavy	L-Ornithine	500
	L-Ornithine + L-asparagine + L-glutamine	1700

[a] The assays were carried out by incubating 20, 50, and 75 μg of light, intermediate, and heavy fractions in a medium with the following composition: 35 mM triethanolamine-HCl (pH 8.0) 5 mM magnesium acetate, 0.5 mM KF, 200 μg/ml of bovine serum albumin, 0.5 mM EDTA, 0.25 mM pyrophosphate (sodium salt), 1.0 mM ATP (sodium salt), and 0.25 mM [^{14}C]AMP (0.5 μCi/μmole). The incubations were for 15 min at 37° in a final volume of 200 μl. Incorporation into ATP was determined as previously described (Gevers *et al.*, 1968). Fraction I, which was relatively free of adenylate kinase activity, was prepared by DEAE-cellulose chromatography according to Fujikawa *et al.* (1968a) and used as the source of the light component. The intermediate and heavy components were prepared by Sephadex G-200 gel filtration as described in the Experimental Section.

gramicidin S system (Gevers *et al.*, 1969). The occurrence of these exchanges is consistent with the notion that each of the aminoacyl moieties is transferred to a second energy-rich bonding in the enzyme fraction.

Formation of Amino Acid Complexes with the Tyrocidine-Synthesizing Fractions. When the light component was incubated with ATP, Mg^{2+}, and L-[^{14}C]phenylalanine, and then applied to a column of Sephadex G-50, enzyme-bound radioactivity was found in the eluate. Trichloroacetic acid precipitation of the protein discharged about half the amino acid into solution (Table VI); the remaining label was associated with the precipitate despite repeated washings with acid. The bound radioactivity, however, was quantitatively discharged by treatment with dilute alkali (pH 9), and recovered as free amino acid on paper chromatographic analysis. Treatment of the washed precipitate with hydroxylamine (pH 6) quantitatively discharged the radioactivity as phenylalanine hydroxamate, also identified by paper chromatography. Labeled proline was bound to the intermediate component in an analogous manner, whereas the remaining tyrocidine amino acids formed complexes with the heavy fraction (Table VII), and in these cases there was also a bimodal form of binding (Table VI). Further, in all instances the same sensitivity to dilute alkali and to hydroxylamine was evident. These results are similar to those found in the gramicidin S system (Kleinkauf and Gevers, 1969), and are consistent with the notion that the amino acids are bound to the

TABLE VI: Isolation of Protein-Bound Amino Acids Free of Aminoacyl Adenylates.[a]

Binding System	Material Assayed	Bound Substrate ($\mu\mu$moles)	
		[^{14}C]Amino Acid	[^{3}H]AMP
Light fraction + L-[^{14}C]phenylalanine + [^{3}H]ATP	Sephadex eluate	9.6	4.6
	Protein precipitate	4.7	0.1
Intermediate fraction + [^{14}C]proline + [^{3}H]ATP	Sephadex eluate	10.0	5.1
	Protein precipitate	5.1	0.0
Heavy fraction + L-[^{14}C]ornithine + [^{3}H]ATP	Sephadex eluate	9.8	5.0
	Protein precipitate	4.9	0.0

[a] The procedures for the formation and isolation of the enzyme–substrate complexes by Sephadex G-50 gel filtration and trichloroacetic acid precipitation were as described in the Experimental Section. The specific activity of [^{3}H]ATP was adjusted to 400 μCi/μmole. The final precipitate was dissolved in 50 μl of 0.1 N KOH by heating at 50–60° for 5 min, and the radioactivity was determined by liquid scintillation counting. The light (20 μg), intermediate (50 μg), and the heavy (75 μg) fractions were prepared as described in the Experimental Section.

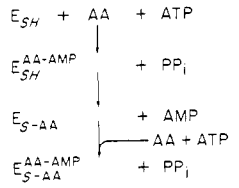

FIGURE 5: The mechanism of activation of each of the amino acids in tyrocidine biosynthesis. The details are described in the text.

enzymes both noncovalently as aminoacyl adenylates and covalently by thio ester linkages.

Glycine and alanine, which are not present in tyrocidines, were also bound to the heavy component (Table VII). These amino acids occur in the linear gramicidins (A–C) which are also produced by *B. brevis* (ATCC 8185), and their binding might be related to contaminant enzymes involved in linear gramicidin synthesis. Threonine, aspartate, and glutamate, which do not occur in any of these antibiotics, did not form complexes with the heavy fraction. Amino acids, such as asparagine, glutamine, valine, ornithine, and leucine, which each occur once in a tyrocidine molecule, were bound to the heavy fraction in approximately equal amounts. Phenylalanine, which may be found at several positions (Figure 1), was bound in larger amounts, but this did not apply to tryptophan which was bound only to the same extent as the others;

TABLE VII: Binding of Amino Acids to Three Fractions in Thio Ester Linkage.[a]

Amino Acid Tested	Amino Acid Bound ($\mu\mu$moles)		
	Light	Intermediate	Heavy
L-Phenylalanine	4.60	0.95	9.80
L-Proline	0.15	3.10	0.11
L-Ornithine	0.30	0.40	3.30
L-Leucine	0.20	0.35	4.20
L-Tryptophan			2.45
L-Asparagine			3.10
L-Glutamine			4.50
L-Tyrosine			3.05
L-Valine			4.70
L-Alanine			2.90
Glycine			3.70
L-Threonine			0.10
L-Aspartate			0.30
L-Glutamate			0.20

[a] Covalent binding of labeled amino acids to light (20 μg), intermediate (50 μg), and heavy (75 μg) enzyme fractions was determined by incubation with medium M and 5.0 mμmoles of the specified amino acid for 30 min at 37° in a total volume of 50 μl. Then 40-μl aliquots were transferred to Whatman No. 3MM filter disks which were immersed in 10% trichloroacetic acid. The radioactivity in the precipitated material was determined by liquid scintillation counting after the disks had been treated by the method of Mans and Novelli (1960).

TABLE VIII: Complementation Studies with the Tyrocidine Light, Intermediate, and Heavy Fractions, and the Gramicidin S Fractions II and I.[a]

Added Fractions	Antibiotic Synthesis ($\mu\mu$moles)
Tyrocidine light, intermediate, and heavy	32.5
Gramicidin S fraction I + tyrocidine intermediate and heavy	2.8
Tyrocidine light + gramicidin S fraction II	2.4
Gramicidin S fractions I + II	35.3
Each fraction alone	<0.5

[a] Tyrocidine and gramicidin S syntheses were determined by the Millipore filter assay procedure outlined in the Experimental Section. The tyrocidine light (10 μg), intermediate (25 μg), and heavy (37.5 μg) fractions were prepared by Sephadex G-200 gel filtration (Figure 3), and the gramicidin S components (5 μg of fraction I and 5 μg of fraction II) were prepared as previously described (Gevers *et al.*, 1969).

this might be related to a low affinity for tryptophan of the binding sites. Again, the data are similar to the 1:1:1:1 stoichiometry in the binding of four gramicidin S constituent amino acids to a multienzyme complex previously described (Kleinkauf *et al.*, 1969).

Lack of Heterologous Complementarity between the Tyrocidine- and Gramicidin S Synthesizing Fractions. When fraction II of the gramicidin S system, which activates phenylalanine, was substituted for the tyrocidine light component in an otherwise complete tyrocidine-synthesizing assay mixture, there was no antibiotic synthesis (Table VIII). Similarly, the tyrocidine light fraction, which also activates and racemizes phenylalanine, did not promote gramicidin S formation in the presence of the multienzyme complex (fraction I) specific for the latter antibiotic. Thus, the enzyme systems, which are derived from related strains of the same organism and which synthesize structurally similar antibiotics, are unable to cross-react.

Discussion

The mechanism for amino acid activation in peptide antibiotic biosynthesis is outlined in Figure 5. Each enzyme fraction reacts with one molecule of the substrate amino acid and one molecule of ATP to form an intermediate aminoacyl adenylate–enzyme complex and inorganic pyrophosphate. The adenylate-bound amino acid is then transferred in an equilibrium to an enzyme-bound sulfhydryl group to form a thio ester, with liberation of AMP. Under the saturation binding conditions of our experiments, the aminoacyl–enzyme complex reacts with a second molecule of amino acid and a second molecule of ATP to form an aminoacyl adenylate–enzyme–amino acid complex and a second molecule of inorganic pyrophosphate. This postulated intermediate is in accord with our results since the ratio of total bound amino acid to AMP is 2:1. The rapid equilibration between the two

forms is in line with the presence in both of high-energy bonds. Precipitation of the protein with trichloroacetic acid would be expected to discharge the adenylate but not the amino acid bound as thio ester, that is, all of the AMP and half the amino acid. This is indeed the case (Table VI).

The evidence that the second form of bound amino acid is a thio ester includes: acid stability, cleavage by dilute alkali and by mercuric salts at neutral pH, and cleavage by neutral salt-free hydroxylamine with formation of the amino acid hydroxamate.

The data on ATP-[^{32}P]P_i exchanges, substrate binding, and enzyme complementation indicate that relative cross-contamination of the three fractions is about 5%. Fujikawa *et al.* (1968a) reported that their enzyme system active in tyrocidine biosynthesis could be resolved into two complementary fractions by DEAE-cellulose chromatography. Confirming these observations (results not presented), we found, however, by amino acid binding studies, that their fraction I contains the light component and about one-third of the intermediate fraction, whereas their fraction II contains the heavy component and two-thirds of the intermediate fraction. The specific activity of the ATP-[^{32}P]P_i exchanges dependent on the various amino acid substrates of our heavy fraction varied over an eightfold range (Table III; compare glutamine and valine). The high specific activities associated with phenylalanine, tryptophan, tyrosine, valine, and leucine might be due, *inter alia*, to the presence of contaminant enzyme fractions concerned with the biosynthesis of the linear gramicidins (A–C). The heavy fraction also contains alanine- and glycine-dependent ATP-[^{32}P]P_i exchanges, which may have a similar origin. Moreover, these amino acids form thio ester links with this fraction. We have been unable to demonstrate synthesis of the linear gramicidins, but it is probable that similar mechanisms of activation and polymerization occur in all these antibiotic biosyntheses.

Despite the similarities in the properties of the two light fractions so far examined, we have been unable to demonstrate the substitution of the one active in gramicidin S synthesis for that active in tyrocidine synthesis. Kurahashi *et al.* (1969) have reported that the purified phenylalanine "racemase" from their gramicidin S system can substitute for the tyrocidine light fraction (present in their fraction I). The reason for this discrepancy is not clear, but may arise from differences between the ATCC 9999 and Nagano strains used in the two studies.

There is, at present, no explanation for the relatively high molecular weight (230,000) of the intermediate fraction, considering its limited contribution to the overall decapeptide synthesis in comparison with that of the heavy fraction (460,000). This might represent subunit aggregation, although there is no evidence for it. The intermediate fraction migrates independently of the light and heavy fractions on sucrose density gradients. However, as the heavy fraction elutes from a Sephadex G-200 column it contains proline-activating enzyme, which may also represent an association between the two fractions occurring at higher concentrations of protein. Disregarding the unexplained high molecular weight of the proline-activating protein, there appears otherwise to be fair proportionality between the molecular weight and the number of amino acids activated. This is true, at least when one compares the molecular weight of the two large fractions for gramicidin S and tyrocidine, which are 280,000 for a sequence of four and 460,000 for a sequence of eight amino acids, respectively. This might be taken as an indication of the existence of subunits specific for each amino acids. So far, however, attempts to obtain subunits from the larger enzyme fractions have been unsuccessful.

Acknowledgment

We thank Mr. B. Fedyniak for help in the growth and harvest of the cells.

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