BIOSYNTHESIS OF GRAMICIDIN AND TYROCIDINE IN THE DUBOS STRAIN OF *BACILLUS BREVIS*

I. Experiments with Growing Cultures

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Abstract

Okuda, Kiyoshi (Department of Biochemistry and Biophysics, University of Hawaii, Honolulu), Gordon C. Edwards, and Theodore Winnick. Biosynthesis of gramicidin and tyrocidine in the Dubos strain of *Bacillus brevis*. I. Experiments with growing cultures. J. Bacteriol. **85**:329–338. 1963.—A simple chromatographic method was developed for the isolation of gramicidin and tyrocidine from tyrothricin of *Bacillus brevis*. A Tryptone-yeast extract-glucose medium containing mineral salts gave the best yields of peptides in a stationary culture of the organism. The incorporation of suitable C^{14}-labeled amino acids into gramicidin and tyrocidine was studied. Several analogues of tyrocidine amino acids (β-hydroxyglutamic acid, pipecolic acid, β-2-thienylalanine, *p*-fluorophenylalanine, and phenyl glycine) selectively reduced tyrocidine synthesis, when added to the nutrient medium. At the same time, the production of gramicidin was augmented. Growth and protein synthesis were not affected. Two analogues in isotopic form, β-2-thienylalanine and isoleucine, were shown to give rise to high degrees of labeling in tyrocidine.

A number of papers have appeared dealing with the metabolism and biosynthesis of gramicidin S (Winnick, Lis, and Winnick, 1961; Winnick and Winnick, 1961) and of the closely related peptide, gramicidin J (Uemura, 1960; Chern, 1960). However, no comparable studies have been reported for the tyrocidine and gramicidin antibiotic polypeptides produced by the Dubos strain of *Bacillus brevis*.

Like gramicidin S, the tyrocidines are basic cyclic decapeptides containing L-valine, L-ornithine, L-leucine, D-phenylalanine, and L-proline. In addition, they are comprised of L-phenylalanine (or tryptophan), L-asparagine, L-glutamine, and L-tyrosine. A recent investigation (Okuda, Lin, and Winnick, Nature, *in press*) supports the view that the gramicidins are larger molecules, containing 30 amino acid residues and two ethanolamine groups. The major component, gramicidin A, contains five different amino acids: glycine, L-alanine, D- and L-valine, D- (and possibly L-) leucine, and L-tryptophan (Synge, 1949). In gramicidins B and C, L-phenylalanine and L-tyrosine, respectively, replace a part of the tryptophan residues (Craig, Gregory, and Barry, 1949).

The present study is concerned with the conditions which control the production of this interesting group of polypeptides in *B. brevis* cells. (For convenience, the singular terms gramicidin and tyrocidine will be used in the text.) The factors studied include variations in the nutrient medium and the introduction of amino acid analogues.

Materials and Methods

Peptides. Gramicidin, tyrocidine, and tyrothricin were kindly donated by the Wallerstein Co., Staten Island, N.Y.

Radioactive amino acids. DL-Pipecolic acid-H^3 was a previously described preparation (Winnick and Winnick, 1961); DL-β (2-thienyl) alanine-3-C^{14} and glycine-1-C^{14} were obtained from the Commissariat à l'Energie Atomique, France. DL-Proline-1-C^{14}, DL-ornithine-2-C^{14}, DL-valine-1-C^{14}, and ethanolamine-1,2-C^{14} were from the California Corporation for Biochemical Research. L-Isoleucine-U-C^{14} was purchased from the Radiochemical Centre, England.

Amino acid analogues. DL-*p*-Fluorophenylalanine, phenylglycine, DL-pipecolic acid, and DL-β-2-thienylalanine were purchased from the California Corporation for Biochemical Research.

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Thioproline and 5-methyl-DL-tryptophan were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; and β-hydroxyl-DL-glutamic acid, from Light and Co., England.

Cultivation procedure. B. brevis (ATCC 8185, Dubos strain BG) was prepared in spore form, and stored as a concentrated suspension at −20 C. Nutrient medium (100-ml quantities in 500-ml Erlenmeyer flasks) was inoculated with 0.2 ml of this spore suspension. The usual medium was 1% Tryptone in 0.5% NaCl, made up in tap water and adjusted to pH 7.0 (Dubos and Hotchkiss, 1941). However, a number of other media were tested in which a mineral mixture (pH 7.0), of the following composition per liter, was employed: KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 10 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 4H_2O$, 10 mg; and $CaCl_2 \cdot 2H_2O$, 22 mg. In these media, the Tryptone was usually replaced with different combinations of yeast extract (Difco), Casamino Acids (Difco), glutamic acid, and glucose. In some experiments, various mixtures of the component amino acids of gramicidin and tyrocidine were included in the nutrient solution. The exact details are given in Results and Discussion.

The cultures were incubated for 4 days at 37 C, without aeration or shaking. Under optimal conditions, the final turbidity corresponded to an optical density reading (Klett colorimeter) of approximately 0.5. When C^{14}-labeled amino acids or amino acid analogues were tested, these were added either at the beginning of the incubation period or after 24 to 30 hr.

Isolation of tyrothricin. The following procedure was adapted from the original method (Hotchkiss and Dubos, 1941). The medium was acidified to pH 4.5 by adding 1 N HCl. It was then heated for 5 min at 50 to 60 C (to promote coagulation of the cells), and centrifuged for 20 min at 5,000 × *g*. The supernatant solution was discarded. The sediment was extracted for 2 hr at room temperature with 25 ml of 95% ethanol-0.2 N HCl (9:1). After centrifugation (20 min at 5,000 × *g*), the residue was retained for protein determination, and the extract was evaporated (with an air stream) to a volume of about 2 ml. To the concentrate were added 10 volumes of 2% NaCl. After 2 hr in the cold, the precipitate of crude tyrothricin was collected by centrifugation. The tyrothricin was again dissolved in 0.5 ml of the ethanol-HCl solvent, and then precipitated by the addition of 10 ml of 2% NaCl. The precipitate was stirred with 2 ml of ethanol, and an insoluble residue was removed by centrifugation and discarded. The extract usually contained 15 to 20 mg of tyrothricin per 100 ml of original medium.

Chromatographic separation of gramicidin and tyrocidine. A portion (1 g) of a mixture of 95 parts (by weight) of Whatman standard grade cellulose powder and 5 parts of Darco G 60 activated charcoal was suspended in 10 ml of chloroform. The suspension was poured into a glass column (inside diam, approximately 8 mm), and was allowed to pack under gravity. From 0.1 to 0.2 ml of tyrothricin solution (1 to 2 mg of peptides) was applied to the moist column, followed by 2 ml of chloroform. The first eluate was discarded. Gramicidin was then eluted by 18 ml of chloroform-ethanol (95:5). Next, 2 ml of ethanol-0.2 N HCl (9:1) were passed through the column and combined with the preceding eluate. Lastly, tyrocidine was eluted by an additional 10 ml of the ethanol-HCl. In certain experiments, small successive quantities of the eluates were collected, and their absorbancies were determined spectrophotometrically at 280 mμ.

Color tests for purity of separated peptides. The tyrocidine and gramicidin, isolated by the above procedure, were tested as follows. Samples of each fraction were subjected to chromatography on Whatman no. 1 paper, using the solvent system pyridine-isoamyl alcohol-water (3:3:2). The R_F of tyrocidine was 0.73; that of gramicidin was 0.96. The former peptide was strongly revealed by dipping the dried chromatogram briefly into 1% aqueous bromophenol blue, and then washing out the excess dye with 1% acetic acid. Gramicidin is stained a bright yellow when the chromatogram is dipped into 7 N HNO_3 (and then washed with water). Such tests showed that each peptide fraction was contaminated by a very small proportion of the other.

Determination of protein. The residue from the extraction of the washed cells with alcohol-HCl was stirred with 20 volumes of hot 5% trichloroacetic acid. After centrifugation, this treatment was repeated with 95% ethanol, absolute ethanol, and ether. The resulting powder was dried in vacuo and weighed. These weights agreed closely with colorimetric determinations by the Lowry method.

Distribution of C^{14} in peptides. Samples of radioactive gramicidin or tyrocidine were hydrolyzed

at 110 to 114 C with glacial acetic acid-HCl (1:1) in sealed ampules, for specified periods of time. After removal of acids, suitable samples of the hydrolysates were subjected to chromatography on Whatman paper, with *n*-butanol-acetic acid-water (75:15:10) as solvent. Unusual amino acids such as thienylalanine were located with the aid of parallel markers, developed with ninhydrin. Generally, the radioactive regions along the chromatogram were cut out and eluted with water.

Radioactivity measurements. Solutions of labeled amino acids or peptides were evaporated as thin uniform layers on metal planchets. The dry residues were measured in a windowless gas-flow Geiger counter. When necessary, corrections were applied for self-absorption of radiation.

Results and Discussion

Chromatography of polypeptides on cellulose-charcoal. When commercial samples of tyrocidine were purified by passage through columns, 65 to 70% of the initial absorbance at 280 mμ was recovered in the eluted tyrocidine peak. The corresponding recovery for commercial gramicidin was 80 to 85%.

Figure 1 illustrates the type of separation obtained. It can be seen that a mixture of equal amounts of the two peptide fractions was reasonably well resolved. It is estimated that the tyrocidine eluate contained approximately 7% of gramicidin, while the gramicidin was contaminated by 5% of tyrocidine.

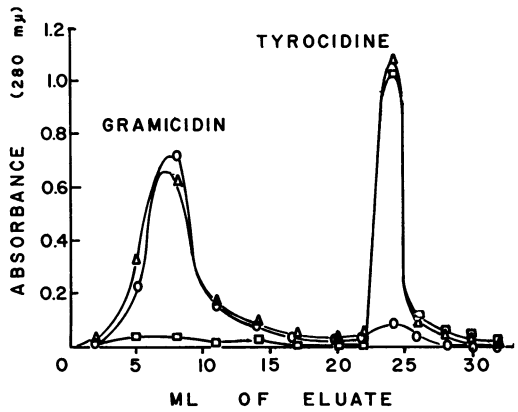

FIG. 1. *Chromatography of Wallerstein gramicidin and tyrocidine on a cellulose-charcoal column.* ○, *1 mg of gramicidin;* □, *1 mg of tyrocidine;* △, *mixture of 1 mg of each peptide.*

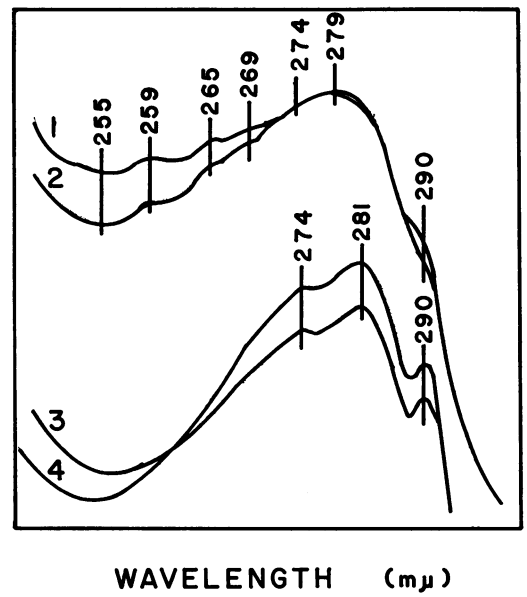

FIG. 2. *Absorption spectra of peptide preparations, separated by cellulose-charcoal chromatography.* 1, *tyrocidine from tyrothricin, isolated from Bacillus brevis cells;* 2, *tyrocidine isolated from an artificial mixture of commercial tyrocidine and gramicidin;* 3, *gramicidin derived from tyrothricin, isolated from B. brevis cells;* 4, *gramicidin isolated from a mixture of commercial gramicidin and tyrocidine. The solvent was 95% ethanol.*

This cross contamination confirms the results with sensitive staining tests on paper chromatograms, mentioned in a previous section. In our experience, the charcoal-cellulose procedure is simpler and more rapid than the original separation method based upon differential solubilities in organic solvents, and is sufficiently quantitative for present purposes.

Comparison of spectra. The ultraviolet-absorption curves of gramicidin and tyrocidine, derived from the present cultures, were virtually indistinguishable from those of the corresponding commercial preparations. Figure 2 shows that the tyrocidine spectrum exhibited several minor peaks and had an absorption maximum at 279 mμ. Gramicidin had three well-defined peaks, with strongest absorption at 281 mμ. The absorbancy indices, defined in terms of g of peptide per liter (in ethanol) per cm, were 10.3 and 4.50 for gramicidin and tyrocidine, respectively, at 280 mμ. The stronger absorbance of the former peptide reflects its higher tryptophan content.

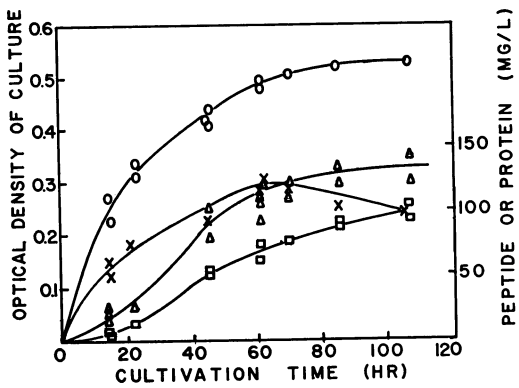

FIG. 3. *Rates of growth, protein synthesis, and polypeptide synthesis in cultures of Bacillus brevis.* ○, *optical density;* ×, *protein* × *0.1;* △, *tyrocidine;* □, *gramicidin.*

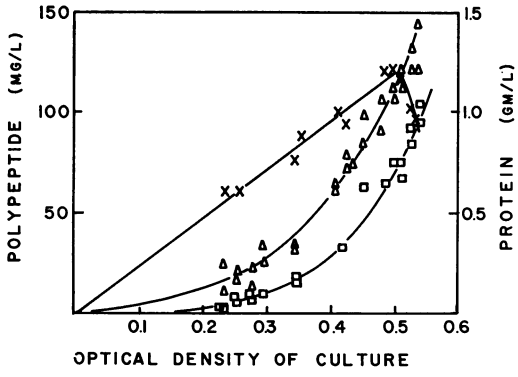

FIG. 4. *Protein and polypeptide production, in relation to growth of culture.* ×, *protein;* △, *tyrocidines;* □, *gramicidins.*

Rates of polypeptide production. Figure 3 gives results obtained with a simple Tryptone-NaCl medium. Gramicidin and tyrocidine synthesis was slow during the initial period of growth. However, the rates accelerated in the log phase, and the final yields were 130 mg of tyrocidine and 95 mg of gramicidin per liter of medium, at 108 hr. The protein content of the cells decreased somewhat after 60 hr, although the turbidity of the cultures continued to rise slowly. In some experiments, cited later, the quantities of protein and of peptides produced varied greatly, depending upon experimental conditions. In most cases the tyrocidine to gramicidin ratio at 96 hr was greater than 3:1.

The contrasting rates of protein and of peptide synthesis are accentuated if the yields are plotted against optical density (Fig. 4). These curves, which are composites of several experiments, resemble those for the *B. brevis* strains that produce gramicidin S and J.

Influence of medium on polypeptide synthesis. In experiment 1 (Table 1), the 4-day yields of gramicidin and tyrocidine for *B. brevis* cells grown in Tryptone-NaCl medium are comparable to those originally reported by Hotchkiss and Dubos (1941). When the saline was replaced by a mixture of mineral salts (Materials and Methods), and the Tryptone was supplemented with glucose and yeast extract, the production of both peptides was increased (experiment 2). The quantity of tyrocidine was almost doubled, and the ratio of total peptide to protein rose to approximately 1:7.

Other media, lacking Tryptone, gave inferior yields of peptides. Moderate growth and high protein synthesis occurred in the presence of either Casamino Acids (experiment 3) or a high level of glutamic acid (experiment 4) in mineral salt media supplemented with glucose and yeast extract. However, peptide synthesis (particularly gramicidin) was markedly depressed. Omission of the glucose from the medium had little effect (results not shown), but a reduction in the level of yeast extract (experiment 5) led to relatively poor growth, and the isolation of peptides was not feasible.

The nature of the substances in the Tryptone, which promote polypeptide synthesis, was not determined, but they are apparently other than amino acids. In Table 2 further attempts were made to stimulate gramicidin and tyrocidine synthesis by the addition of amino acid mixtures to a glutamic acid-glucose-yeast extract medium. The addition of all the component amino acids of gramicidin and tyrocidine (experiment 2) did not greatly alter the yields of peptides, as compared with the control culture (experiment 1). A mixture of the tyrocidine group of amino acids, containing a high proportion of L-phenylalanine (experiment 3), stimulated peptide synthesis slightly, but the substitution of D- for L-phenylalanine (experiment 4) depressed both peptide levels and growth. Similarly, excess D-tryptophan had a slight inhibitory effect in experiment 5, in which the component amino acids of gramicidin were added to the basal medium. Experiment 6, in which isoleucine appeared to substitute successfully for leucine, will

TABLE 1. *Influence of the medium on growth, protein, and peptide synthesis by Bacillus brevis*

Expt no.	Basal medium	Supplemental components	Final optical density (96 hr)	Yield (mg/100 ml of medium)		
				Protein	Gramicidin	Tyrocidine
1	1% Tryptone in 0.5% NaCl	None	0.50	124	3.28	9.65
2	1% Tryptone in mineral salt mixture	0.5% Yeast extract + 1% glucose	0.60	166	4.75	18.10
3	3% Casamino Acids in mineral salt mixture	0.5% Yeast extract + 1% glucose	0.40	151	0.38	1.37
4	1.5% L-Glutamic acid in mineral salt mixture	1% Glucose + 0.5% yeast extract	0.47	180	0.77	4.64
5	1.5% L-Glutamic acid in mineral salt mixture	1% Glucose + 0.1% yeast extract	0.263	—	—	—

TABLE 2. *Effect of amino acid supplementation on growth and peptide synthesis*

Expt no.	Additions to basal medium*	Final optical density (96 hr)	Yield (mg/100 ml)	
			Gramicidin	Tyrocidine
1	L-Glutamic acid (100 mg)	0.485	0.66	5.42
2	L-Orn, L-val, L-leu, L-pro, L-phe, L-tyr, L-try, L-asp, L-glu, gly, L-ala	0.43	0.82	4.40
3	L-Orn, L-val, L-leu, L-pro, 5 L-phe, L-tyr, L-try, L-asp, L-glu	0.45	1.59	7.50
4	Same, but with L-phe replaced by 5 D-phe	0.35	0.44	3.42
5	Gly, L-ala, L-val, L-leu, L-phe, L-tyr, 4 D-try	0.41	0.41	2.98
6	Same as expt 2, but with L-leu replaced by 3 L-isoleu	0.43	0.82	5.42

* This medium consisted of mineral salts, 1.5% L-glutamic acid, 1% glucose, and 0.5% yeast extract. The indicated amino acid mixtures totaled 100 mg and were in equimolar proportions, except where otherwise indicated. The additions were made at 24 hr; optical density, approximately 0.23.

be referred to later in connection with an experiment employing isotopic isoleucine in the growth medium.

In connection with the above experiments, an analogous situation may be recalled in the case of tetanus toxin formation. Mueller and Miller (1956) found that certain acid-labile substances (probably peptides), present in a pancreatic digest of casein, were necessary for production of high titers of toxin in *Clostridium tetani*. An acid hydrolysate of casein was not effective.

Efficiencies of utilization of C^{14}-labeled amino acids for gramicidin and tyrocidine synthesis. The results obtained when various isotopic amino acids were added to the medium are indicated in Table 3. Valine, which is a constituent of both gramicidin and tyrocidine, was well incorporated into both types of peptides. Slightly higher efficiency resulted when the labeled amino acid was added in the most rapid period of growth and polypeptide formation. Ornithine and proline, which are components of tyrocidine but not of gramicidin, were readily incorporated into the former peptide fraction, but gave rise to only low C^{14} concentrations in gramicidin. These traces of radioactivity may reflect either cross contamination of gramicidin by tyrocidine, or may represent the incorporation of small amounts of other amino acids (such as glutamic acid) derived from metabolic transformations of the proline and ornithine (Winnick et al., 1961). Glycine and ethanolamine, both of which occur only in gramicidin, were preferentially utilized for gramicidin synthesis. However, these labeled compounds were extremely active metabolically. Indications have been obtained that their C^{14} was utilized for the formation of serine and the side chain of tryptophan, so that the latter amino acid could account for a substantial part of the observed radioactivity in gramicidin. Further

TABLE 3. *Efficiencies of C^{14}-amino acid utilization for polypeptide synthesis**

Labeled amino acid	Isotope incorporated (% of total)	
	Gramicidin	Tyrocidine
DL-Valine-1-C^{14}	3.70	3.05
DL-Valine-1-C^{14}	2.72	2.76
L-Ornithine-2-C^{14}	0.13	1.88
DL-Proline-1-C^{14}	0.10	2.85
Glycine-1-C^{14}	0.98	0.17
Ethanolamine-1,2-C^{14}	1.15	0.15

* The nutrient medium was 1% Tryptone in 0.5% NaCl. Approximately 1 μc of C^{14} was used in each experiment. The isotopic amino acid was added initially, except for the first experiment in which the valine-C^{14} was added at 24 hr.

TABLE 4. *Specific radioactivities of peptides and of valine**

Expt no.	C^{14} concn (counts per min per mg)			
	Gramicidin	Tyrocidine	Valine of gramicidin	Valine of tyrocidine
1	2,260	1,210	11,000	16,000
2	2,230	1,110	11,000	14,000
3	5,300	3,100	26,000	40,000

* The cells were grown in Tryptone-NaCl medium. Valine-1-C^{14} (500,000 count/min) was added in the log phase in experiments 1 and 2, and at the beginning of the cultivation in experiment 3. The values in the last two columns were calculated from the percentages of valine in the peptide preparations.

experiments are in progress to elucidate the pathway of introduction of ethanolamine into gramicidin.

When the C^{14} concentrations in gramicidin and tyrocidine were compared, after cultivation in the presence of isotopic valine, it was found that gramicidin invariably had a higher specific radioactivity (Table 4). However, when the valine contents of each peptide fraction were taken into account (gramicidin has approximately 21% valine; tyrocidine, 7.7%), the ratio of isotopic to total valine was higher in the tyrocidine, as compared with the gramicidin. Actually, the differences are not large, all complications considered, and the results suggest that the pool of labeled valine was utilized in a similar manner for the synthesis of both peptides.

Effect of amino acid analogues on polypeptide formation. In a previous study concerned with gramicidin S (Winnick and Winnick, 1961), several structural analogues of the component amino acids of this peptide were found to inhibit strongly the biosynthetic process in cultures of *B. brevis* ATCC 9999. Thus, norleucine, norvaline, and *p*-fluorophenylalanine each caused a 60 to 70% decrease in antibiotic activity, while depressing growth and protein synthesis to a lesser extent. It was of interest to employ various analogues in the present study, with the object of selectively blocking tyrocidine or gramicidin formation, and to attempt the uncoupling of protein and polypeptide synthesis (Table 5).

Certain analogues, such as 5-methyltryptophan and thioproline, exerted a generalized inhibitory action on growth and peptide synthesis, and on the incorporation of labeled valine into gramicidin and tyrocidine. The effect on peptide formation was probably indirect. Of a considerable number of other unnatural amino acids tested, none was found to completely abolish polypeptide synthesis without an accompanying inhibition of growth (data not included in Table 5).

However, an unexpected observation was that several analogues, including β-hydroxyglutamic acid, pipecolic acid (proline analogue), phenylglycine, thienylalanine, and *p*-fluorophenylalanine, all caused a considerable increase in gramicidin production, without any significant influence on the growth of the cultures (Table 5). The stimulations were paralleled by an elevated incorporation of radioactive valine into gramicidin in the three cases tested.

In the case of tyrocidine, the second group of five analogues in Table 5 had no effect, or caused a slight to moderate depression of synthesis. The incorporation of valine-C^{14} into tyrocidine exhibited a varied pattern, ranging from a slight stimulation with β-hydroxyglutamic acid and β-thienylalanine to an inhibition with pipecolic acid. With proline-C^{14} in the medium, there was an inhibition of incorporation into tyrocidine, with all analogues tested.

The finding that gramicidin synthesis can proceed actively, under conditions in which tyrocidine formation is impaired, has interesting implications. The analogues appear to have somehow shifted the peptide-synthesizing apparatus of the cell more in the direction of gramicidin synthesis. It should be pointed out that

TABLE 5. *Effect of amino acid analogues on growth, polypeptide synthesis, and incorporation of isotopic valine and proline into peptides of Bacillus brevis**

Analogue (50 mg/100 ml)	Turbidity of culture (96 hr)	Polypeptide synthesis		Incorporation of valine-1-C^{14}		Incorporation of proline-1-C^{14} into tyrocidine
		Gramicidin	Tyrocidine	Gramicidin	Tyrocidine	
5-Methyltryptophan	−31†	−42†	−57†	−45†	−78†	
	−38	−57	−66	−42	−72	
Thioproline‡	−45	−81	−63	−61	−40	−23
β-Hydroxyglutamic acid	0	55	−15	16	13	
Pipecolic acid	−5†	44†	−8†	41†	−1†	
	−1	90	−13	24	−16	−32
Phenylglycine	2	113	−19			−25
Thienylalanine	0†	57†	−12†	44†	21†	−15
	0	23	−1	19	4	
p-Fluorophenylalanine	−3	58	2			−25

* The figures are averages of two or three separate cultivation experiments, and are all expressed as per cent increase or decrease, relative to standard values (in the absence of analogues). The medium was 1% Tryptone in 0.5% NaCl. Labeled amino acid (1 μc) was added at the start of each experiment. Except where otherwise indicated, the analogues were added to the medium in the middle of the log phase.

† Analogue introduced at the beginning of the incubation period.

‡ Concentration of 20 mg/100 ml of medium.

TABLE 6. *Comparison of two labeled amino acid analogues in peptide synthesis**

Determination	Concn of analogue in medium (mg/100 ml)							
	DL-Thienylalanine-C^{14}				DL-Pipecolic acid-H^3			
	0.1	25	50	100	0.1	25	50	100
Final optical density of culture	0.50	0.48	0.48	0.48	0.45	0.43	0.40	0.38
Gramicidin (mg)	—†	2.7	3.4	3.9	2.0	2.5	2.5	2.6
Tyrocidine (mg)	11.3	10.6	11.2	12.4	6.4	6.5	4.6	4.6
Total isotope incorporated into gramicidin (%)	0.06	0.05	0.04	0.04	0.06	0.02	0.01	0.005
Total isotope incorporated into tyrocidine (%)	1.05	0.98	0.94	0.88	0.34	0.07	0.06	0.04

* The nutrient medium was 1% Tryptone in 0.5% NaCl. Quantities of 2, 2, 4, and 8 μc of the radioactive form of each analogue were mixed, respectively, with the 0.1, 25, 50, and 100 mg of corresponding nonlabeled compound, and were added to the medium at the beginning of the cultivation period.

† Value not available.

hydroxyglutamic acid, pipecolic acid, phenylglycine, thienylalanine, and fluorophenylalanine are all analogues (and apparently metabolic antagonists) of the tyrocidine, but not of the gramicidin, component amino acids. It may be mentioned that several glycine and alanine analogues (such as sarcosine and α-amino-isobutyric acid) were found to have no effect on gramicidin (or tyrocidine) production.

Incorporation of radioactive amino acid analogues into gramicidin and tyrocidine. It was found that labeled thienylalanine was incorporated into gramicidin S to an extent equivalent to about 5% of the phenylalanine residues, with *B. brevis* ATCC 9999 (Winnick and Winnick, 1961). Other analogues, such as norleucine and *p*-fluorophenylalanine, were less effectively utilized. Also, Katz (1960) found that the proline analogues, pipecolic acid and azetidine-2-carboxylic acid, could replace proline in the actinomycin peptides, though not in the protein of *Streptomyces antibioticus*.

TABLE 7. *Incorporation of radioactive thienylalanine and isoleucine into tyrocidine*

Expt no.	Growth medium	Labeled amino acid added per 100 ml of medium	Final optical density (96 hr)	Tyrocidine (mg/100 ml)	Specific activity (counts per min per μmole)		Apparent μmoles of amino acid incorporated/μmole of tyrocidine
					Original amino acid	Isolated tyrocidine	
1*	Tryptone-NaCl	DL-Thienylalanine (100 mg)	0.48	12.4	6,750	3,780	0.56
2	Glutamic acid-glucose-yeast extract-mineral salt mixture	DL-Thienylalanine (60 mg)	0.33	3.4	7,050	4,800	0.68
3	Glutamic acid-yeast extract-mineral salt mixture-amino acid mixture†	L-Isoleucine (30 mg)	0.45	6.92	3,050	3,420	1.12

* Based on data of Table 6.

† A total of 100 mg of the following amino acids in equimolar proportions: L-orn, L-val, L-pro, L-asp, L-glu, L-phe, L-tyr, L-try, gly, L-ala (leu omitted).

In Table 6 a phenylalanine and a proline analogue, both in isotopic form, have been compared with respect to their utilization for gramicidin and tyrocidine synthesis in the Dubos variety of *B. brevis*. Higher concentrations of the thienylalanine had very little influence on growth, but pipecolic acid exerted a slight inhibition. The stimulation of gramicidin synthesis by high levels of thienylalanine is in agreement with the results in Table 5, but pipecolic acid had a lesser positive effect. Tyrocidine formation was inhibited somewhat by large amounts of pipecolic acid, although not markedly affected by thienylalanine.

At the 0.1 mg level, pipecolic acid was incorporated into tyrocidine to a moderate extent, but the efficiency of utilization decreased markedly at higher concentrations of the proline analogue; thus, the total quantity taken up was never large. By contrast, the efficiency of thienylalanine utilization for tyrocidine synthesis fell relatively little with increasing concentrations, indicating that a comparatively large amount of the phenylalanine analogue could be incorporated. The great disparity between the degrees of utilization of thienylalanine for gramicidin, as compared with tyrocidine synthesis, is noteworthy. It probably reflects the abundance of phenylalanine in the latter group of peptides, whereas this amino acid occurs in only one minor (B) component of the gramicidin peptide mixture. Pipecolic acid resembles proline (Table 3) in being very poorly incorporated into gramicidin, as would be expected.

Assuming that thienylalanine substituted for phenylalanine residues in tyrocidine, it is possible to estimate the extent of such replacement. In experiment 1 of Table 7, it would follow from the known specific radioactivity of thienylalanine and of the isolated tyrocidine that 0.56 μmole of the analogue was incorporated per μmole of peptide. The major (A) component of the tyrocidine peptides contains two D- and one L-phenylalanine residues, while tyrocidine B has two D-phenylalanines (King and Craig, 1955) in its molecule. Accordingly, the thienylalanine could have replaced approximately 20 to 25% of the phenylalanine groups of the tyrocidine, in cells grown in the Tryptone medium. Experiment 2 of Table 7, in which the phenylalanine content of the medium was relatively low (1% Tryptone replaced by 0.5% yeast extract), gave a small yield of tyrocidine but a slightly higher C^{14} concentration in this peptide, corresponding to about 25 to 30% of the normal phenylalanine content.

Quantitative amino acid analysis (Okuda et al., *in press*) has indicated the presence of small proportions of isoleucine in gramicidin. (Tyrocidine was not analyzed.) It appeared of interest to determine whether isotopic isoleucine, added in high concentration to a medium of low leucine content, could readily be incorporated into the polypeptides of *B. brevis*. Such experiments showed that isolated gramicidin and tyrocidine were both highly radioactive. The yield of gramicidin was low so that accurate measurements were not possible, but an adequate quantity of labeled

TABLE 8. *Analysis of tyrocidine hydrolysates*

Recovery of C^{14} from paper chromatogram (% of total)		
Leucine-isoleucine* region	Thienylalanine region†	
	5-hr hydrolysis	20-hr hydrolysis
69	36	28

* Experiment 3, Table 7.

† Experiment 2, Table 7. Values uncorrected for losses due to destruction of thienylalanine.

tyrocidine was obtained (experiment 3, Table 7). This preparation had a surprisingly high isotopic content, corresponding to about one isoleucine per molecule of tyrocidine. Such a value implies a complete replacement of leucine by isoleucine. It will be recalled that the latter amino acid was able to replace leucine in promoting peptide synthesis in a medium supplemented with an amino acid mixture (experiments 2 and 6, Table 2). Also, we found that washed cells of *B. brevis* readily incorporate isoleucine-C^{14} into peptides.

In chromatography of an acid hydrolysate of isoleucine-C^{14}-labeled tyrocidine (Table 8), 69% of the isotope appeared in the leucine-isoleucine region. Although these two amino acids were not resolved, it seems fairly safe to assume that no extensive metabolic interconversion occurred. However, since the original isoleucine contained C^{14} in all of its carbon atoms, it is not surprising that the remaining 31% of the radioactivity on the chromatogram was distributed among several amino acids, such as proline, alanine, glutamic acid, and aspartic acid, apparently derived from the catabolism of the isoleucine chain. The value of 1.12 in Table 7 for the μmoles of isoleucine incorporated per μmole of tyrocidine may be corrected by the factor 0.69 (from Table 8) to give 0.77.

In analyzing hydrolysates of thienylalanine-C^{14}-tyrocidine, the measurements were complicated by the instability of the sulfur-containing amino acid to the conditions of hydrolysis. However, by heating a sample of radioactive thienylalanine in acetic acid-HCl for varying time intervals, and subjecting samples to chromatography and counting, a series of correction factors for loss of C^{14} was obtained. When such corrections are applied to the results in Table 8, the 5- and 20-hr values become 95 and 105, respectively. Accordingly, it may be concluded that virtually all of the C^{14} in the tyrocidine hydrolysate represented thienylalanine, possibly both the D and L forms of the compound. Furthermore, it is significant that thienylalanine could be detected colorimetrically on the chromatogram, after treatment with ninhydrin reagent. The analogue (R_F, 0.53) gave a distinctive blue-gray color, and the phenylalanine (R_F, 0.60) spot was blue-purple. Similar results were obtained when chromatograms were developed with *n*-butanol-pyridine-water (1:1:1).

It seems reasonable to conclude that isoleucine and thienylalanine could substitute extensively for leucine and phenylalanine, respectively, in tyrocidine. It will be of interest to determine the effect of such modifications in structure on the antibiotic activity of the polypeptide molecule. The extensive incorporation of such analogues as *p*-fluorophenylalanine and thienylalanine into cellular proteins of microorganisms has been observed (Munier and Cohen, 1959), but similar findings have not been reported in the case of polypeptides.

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