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Biosynthesis of Gramicidins and Tyrocidines in Cell-Free Preparations from *Bacillus brevis**

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A cell-free system, prepared from *Bacillus brevis* and consisting of ribosomes plus a 140,000 × *g* supernatant solution, was active in incorporating suitable C^{14}-amino acids into gramicidins and tyrocidines in the presence of magnesium ions, ATP plus an ATP generator, glutathione, and an amino acid mixture. The process proceeded optimally at approximately *p*H 7.5–7.9, and continued at undiminished rate for at least 4 hours. L-Ornithine, D-valine, and D-phenylalanine were among the isotopic amino acids found to be incorporated into the polypeptides. Glutamic acid was better utilized than was glutamine for tyrocidine synthesis. By reisolating the ribosomes at the end of the incubation period, it was shown that these particles retained all the labeled peptide molecules, apparently in bound form. In prolonged experiments the ribosomes synthesized greater quantities of polypeptides than of protein. The synthesizing activity of the whole system was abolished by pretreatment of either ribosomes or supernatant phase with pancreatic ribonuclease. The results suggested that the process of gramicidin and tyrocidine formation resembles that of protein biosynthesis.

In view of the great number and variety of polypeptides found in living cells, the elucidation of the biosynthetic pathways of these substances presents a challenging problem. It is of particular interest to determine whether or not the mechanisms of polypeptide biogenesis resemble the Zamecnik cycle of reactions, which is commonly believed to describe protein synthesis.

The gramicidin and tyrocidine groups of antibiotic peptides found in the Dubos-Hotchkiss strain of *B. brevis* offer a number of attractive features for the above purpose (Okuda *et al.*, 1963). The composition and structures of the cyclic tyrocidines A, B, and C are known (Craig *et al.*, 1949), while the amino acid composition (although not the sequences) of the gramicidin peptides is fairly well worked out (Ramachandran, 1963; Ishii and Witkop, 1963). The latter substances are commonly thought to have closed rings, but this has not been proved.

A previous study (Okuda *et al.*, 1963) was concerned with the biosynthesis of gramicidins and tyrocidines in growing cultures. The present paper describes a cell-free system, prepared from sonicates of *B. brevis*, which is active in incorporating component amino acids into the two groups of polypeptides. The roles of major components of this system, the energy requirements, cofactors, and optimal conditions have all been investigated. In view of the presence of ornithine, certain D-amino acids, and amino acid amides in the peptide molecules, it was of added interest to investigate the modes of utilization of these components in the biosynthetic process.

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EXPERIMENTAL

Isotopic Amino Acids.—The sources and specific activities ($\mu c/\mu m$) were as follows: New England Nuclear Corporation: DL-leucine-1-C^{14}, 17; ethanolamine-2,3-C^{14}, 23; D-valine-U-H^3, 2.0; D-phenylalanine-U-H^3, 4.0. California Corporation for Biochemical Research: DL-valine-1-C^{14}, 9.9; DL-ornithine-2-C^{14}, 1.14; DL-phenylalanine-1-C^{14}, 12. Schwarz Bio-Research, Inc.: L-alanine-1-C^{14}, 78; L-proline-1-C^{14}, 97; L-aspartic acid, 113. Volk Radiochemical Company: L-glutamic acid-U-C^{14}, 2.0. Radiochemical Centre (England): L-isoleucine-U-C^{14}, 6.0; glycine-1-C^{14}, 8.0; DL-tryptophan (indoyl [alanine-3-C^{14}]), 1.0.

The two D-amino acids were originally purchased from the California Corporation for Biochemical Research. They were tested for optical purity with L-amino acid oxidase of snake venom (Sigma Chemical Company). Warburg determinations indicated insignificant quantities of O_2 consumption under conditions which resulted in 100% oxidation of L-phenylalanine and L-valine. Following tritiation by the method of Wilzbach (1959), the preparations were exhaustively freed of labile H^3 by the usual exchange procedures and then rigorously purified by paper chromatography. The final products contained about 0.01% impurity, as judged by zero-time incorporation experiments. Also, automatic scanning of chromatograms indicated the absence of impurities in the final preparations.

Biochemicals.—Crystalline pyruvate kinase enzyme, phospho(enol) pyruvic acid, adenosine triphosphate (ATP), and reduced glutathione were purchased from Sigma Chemical Company. Crystalline pancreatic deoxyribonuclease and ribonuclease were obtained from Worthington Chemical Company. Samples of Dubos-Hotchkiss gramicidin, tyrocidine, and tyrothricin were kindly provided by the Wallerstein Company.

TABLE I
STANDARD SYSTEM FOR INCORPORATION OF ISOTOPIC AMINO ACIDS INTO POLYPEPTIDES

Components (each adjusted to *p*H 7.5)	Quantity (per 2 ml of final mixture)
Sonic extract[a]	1.0 ml
C^{14}-labeled amino acid	0.5 to 1.0 μc
Adenosine triphosphate (ATP)	5 μmoles
Phosphoenolpyruvic acid (PEP)	5 μmoles
Pyruvic kinase	20 μg
$MgCl_2$	10 μmoles
Glutathione	20 μmoles
Amino acid mixture	[b]
Potassium phosphate	10 μmoles

[a] Contained 6.2 mg of protein per ml. [b] This mixture provided the following μmoles of amino acids: 1 L-ala, 1 L-asp, 1 L-glu, 1 gly, 3 L-leu, 2 D-leu, 1 L-orn, 1.3 L-phe, 2 D-phe, 1 L-pro, 4 L-try, 1 D-try, 1.2 L-tyr, 3 L-val, 2 D-val.

Cultivation of Cells.—*B. brevis* (ATCC 8185, Dubos strain BG) was grown in stationary culture in a tryptone–mineral salt medium enriched with yeast extract (Okuda *et al.*, 1963). The cells were harvested near the end of the log phase of growth, corresponding to an optical density reading of 0.48–0.52 with the Klett colorimeter.

Cell-Free Preparation.—All operations were performed at 0° to 2°. The cells from 400 ml of medium were collected by centrifugation (15 minutes at 7,000 × *g*), resuspended in 100 ml of 0.005 M $MgCl_2$, and resedimented. This washing was repeated twice. The cells were then suspended in 20 ml of 0.005 M $MgCl_2$ solution[1] containing 10 μmoles of glutathione per ml and subjected to sonic disruption for 15 minutes at 60 w and 20 kc. Intact cells and debris were removed by centrifuging for 15 minutes at 11,000 × *g*. The supernatant phase (hereafter called "sonic extract") was centrifuged for one hour at 140,000 × *g*. The particle-free supernatant solution was siphoned from the pellet (hereafter referred to as ribosomes). The latter was washed by resuspension in 20 ml of the $MgCl_2$-glutathione solution and sedimented as before. The subcellular fractions were used for incorporation experiments within hours after they were prepared.

Incubation Procedure.—The complete standard system had the composition shown in Table I. However, a number of variations were tested. In most experiments the sonic extract was replaced by ribosomes (2.5 mg protein) suspended in 1 ml of 140,000 × *g* supernatant (4.5 mg protein). In all cases, the volume of the final mixture was adjusted to 2 ml with $MgCl_2$-glutathione solution. Incubations were performed in small centrifuge tubes at 37°, for 4 hours (unless otherwise stated). The amino acid mixture in Table I simulated (roughly) the proportions of various D- and L-amino acids present in gramicidins and tyrocidines. DL-Leucine-C^{14} was the labeled amino acid usually employed. Following incubation, the reaction was terminated by addition of HCl, as described in the next section.

In certain of the experiments with ribosomes, at the end of the incubation period the reaction mixture was centrifuged at 0° and 140,000 × *g* for one hour. After decantation of the supernatant phase, the ribosomal pellet was washed twice with $MgCl_2$ buffer, and recentrifuged each time at 140,000 × *g*. Ribosomes and supernatant phase were then separately subjected to the following procedure.

[1] Slightly lower or higher Mg concentrations are deleterious.

Isolation of Labeled Peptides by Paper Chromatography.—Sufficient 1 N HCl was added to the incubation mixture to bring the *p*H to 3.6. The tubes were heated for 5 minutes at 100° and then centrifuged for 15 minutes at 5,000 × *g*. The precipitates were washed twice by suspension in 4 ml of 1% NaCl and recentrifuged. They were then extracted for about 2 hours with 3 ml of 9:1 ethanol–0.2 N HCl, with the addition of 1-mg quantities of commercial gramicidin and tyrocidine as carriers. After centrifugation, the supernatant solution was decanted and the sediment re-extracted with 2 ml of ethanol. The two extracts were combined and concentrated *in vacuo* to a volume of about 0.1 ml. "Tyrothricin" (gramicidin-tyrocidine mixture) was precipitated by adding 6 ml of 2% NaCl. After centrifugation, the recovered tyrothrycin was redissolved in 0.2 ml of ethanol and reprecipitated by addition of 4 ml of 2% NaCl solution. The final precipitate was dissolved in a minimal quantity of ethanol, and the solution was applied to the origin of a Whatman number 1 paper chromatogram. The latter was developed using 2:2:1 cyclohexane-isopropanol-H_2O (upper layer). Because of the great difference in R_F values (gramicidins, 0.77; tyrocidines, 0.08), short runs were adequate for complete separation of the two peptide fractions. The exact positions of the latter on the paper chromatograms were determined as previously described (Okuda *et al.*, 1963). This separation procedure was employed in all experiments, unless otherwise indicated.

Separation of Peptides by High-Voltage Electrophoresis.—The tyrothrycin, prepared from cellular extracts, was applied to Whatman number 1 paper. A conventional apparatus, cooled by circulating (20°) water, was employed. The "buffer" system was 50% acetic acid. A current of 0.25 ma/cm at 1500 v was then applied. In 1.5 hours gramicidins migrated less than 0.5 cm toward the anode, tyrocidines migrated approximately 3.5 cm toward the cathode, while free phenylalanine and leucine were observed to migrate 7–8 cm toward the cathode.

Isolation of Peptides by Column Chromatography.—A charcoal-cellulose column was used to separate gramicidins and tyrocidines from tyrothricin in certain experiments (Okuda *et al.*, 1963).

Isolation of Proteins.—The residue from the alcohol-HCl extraction of coagulated cellular material was treated as previously described (Okuda *et al.*, 1963).

Radioactivity Measurements.—The peptide regions were carefully eluted from the paper chromatograms with ethanol-HCl solvent. The eluates were dried on metal planchets and the residues counted in a gas-flow Geiger counter with an ultrathin window. When paper electrophoresis was employed, C^{14} analysis was first performed in an automatic scanning apparatus, with double counting chambers, equipped with gas-flow and ultrathin windows. The slit width was 3 mm, for a scanning rate of 6 in./minute and a sensitivity of 10,000 cpm. Subsequently, the radioactive peaks were eluted and counted on planchets, as with paper chromatograms. In comparing amino acids of different specific radioactivities, counts per minute were converted to mμmoles. Protein samples were uniformly layered on planchets for counting and were corrected for self-absorption. All experimental results were corrected for small zero-time radioactivities. These usually were of the order of 0.01 the magnitude of the active incorporation values.

Tritium-labeled compounds were suspended in the phosphor mixture of Bray (1960) and measured in a liquid scintillation counter

TABLE II

ESSENTIALITY OF VARIOUS SUBSTANCES FOR LABELED LEUCINE INCORPORATION INTO PEPTIDES

Constituent Omitted from Standard System	Leucine-C^{14} Incorporated (cpm)	
	Gramicidins	Tyrocidines
None	3200	4400
ATP	250	700
PEP plus pyruvic kinase	230	700
Glutathione	1260	3000
Mg ions	125	740
Mg plus glutathione	20	490
Amino acid mixture	340	2700

TABLE III

IMPORTANCE OF PARTICULATE AND SOLUBLE FRACTIONS IN THE INCORPORATION PROCESS

Substituent for Sonic Extract	Leucine-C^{14} Incorporated (cpm)	
	Gramicidins	Tyrocidines
None (standard conditions)	3050	3100
1 ml of 140,000 × *g* supernatant	195	555
2.5 mg ribosomes in 1 ml phosphate buffer	200	800
2.5 mg ribosomes in 1 ml of 140,000 × *g* supernatant (4.5 mg protein)	1510	2290

RESULTS

Importance of the Individual Components. – In experiments with unfractionated sonicates (freed of debris), isotopic DL-leucine was found to incorporate readily into peptides (Table II). It may be seen that the omission of either ATP or the pyruvate kinase system resulted in a marked decrease in leucine-C^{14} uptake. Apparently both ATP and an ATP generator were needed for optimal results. When glutathione was withheld, a moderate decrease in radioactivity in the two peptide fractions resulted. In the absence of magnesium, labeled amino acid incorporation into gramicidins was particularly depressed. The omission of both glutathione and Mg led to a further lowering of radioactivity in tyrocidines and virtual absence of isotope in gramicidins. The amino acid mixture appeared to be less essential in the case of tyrocidines and more important for the biosynthesis of gramicidins.

The results in Table III are comparable to those of Otani *et al.*, (1963) for gramicidin J biosynthesis in cell-free extracts of the Nagano strain of *B. brevis*. The soluble phase and the ribosomes had relatively low activities when tested separately, but leucine incorporation into gramicidins and tyrocidines was restored to a considerable extent when the two cellular fractions were recombined. All subsequent experiments were performed with this reconstituted system.

In seeking optimal conditions, experiments were performed in which the proportions of ribosomes and of 140,000 × *g* supernatant phase were varied. When the latter fraction was maintained at a relatively high concentration (Fig. 1A), gramicidin and tyrocidine synthesis rose at comparable rates in response to increasing amounts of ribosomes. Figure 1B, in which the level of ribosomes was held constant, shows an experiment based on a separate batch of cells and illustrates the type of variation occasionally encountered in the relative degrees of labeling of tyrocidines and gramicidins. It may be seen that incorporation of leucine-C^{14} into tyrocidines was much more responsive to changes in the quantity of soluble phase than in the case of gramicidins.

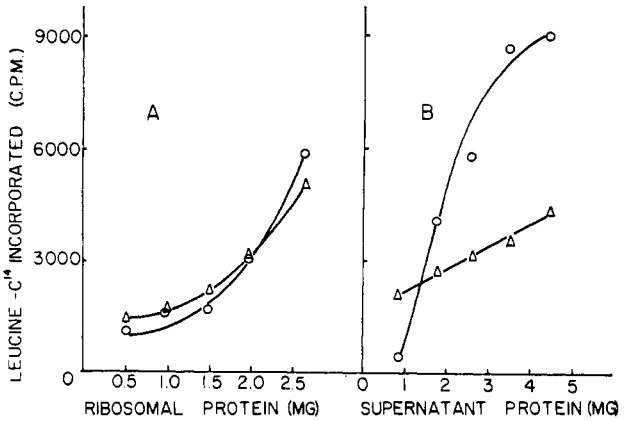

FIG. 1.—Effect of variations in the quantity of ribosomes and of 140,000 × *g* supernatant on the degree of incorporation of leucine-C^{14} into peptides. The sonic extract was omitted from the standard incubation mixture and sufficient $MgCl_2$-glutathione solution added to make the final volume 2 ml. A, with constant amount of supernatant phase (equivalent to 4.6 mg of protein). B, with constant amount of ribosomes (2.5 mg protein). △, gramicidins; ○, tyrocidines.

In order to further demonstrate the participation of the other amino acids in the leucine-C^{14} incorporation process, certain components were omitted from the standard mixture (Table IV). These experiments were complicated by the presence of endogenous amino acids in unknown concentrations. However, the desired effects could be demonstrated. The omission of the whole tyrocidine group of amino acids depressed the utilization of isotopic leucine for tyrocidine, but not appreciably for gramicidin synthesis. Conversely, the omission of the gramicidin amino acids reduced leucine incorporation into tyrocidine relatively little, while the inhibition amounted to 99% for gramicidin labeling. The omission of L-ornithine (found only in tyrocidines) did not alter gramicidin greatly, but reduced tyrocidine synthesis significantly. Withdrawal of L-glutamic acid (actually present in the form of glutamine in tyrocidine) gave similar results. When either glycine or L-alanine (constituents of gramicidins) was omitted, gramicidin was depressed to a relatively greater degree than tyrocidine synthesis. The most striking result was that with L-tryptophan. Deletion

TABLE IV

IMPORTANCE OF VARIOUS AMINO ACIDS FOR THE INCORPORATION OF LEUCINE-C^{14} INTO PEPTIDES

The standard incubation mixture was used, but with sonic extract replaced by ribosomes plus 140,000 × *g* supernatant.

Amino Acids Omitted from Mixture	Radioactivity (cpm)	
	In Gramicidins	In Tyrocidines
None (control)	5,300	13,000
Tyrocidine group[a]	5,000	5,500
Gramicidin group[b]	50	10,300
L-Ornithine	5,100	6,900
L-Glutamic acid	4,800	7,300
Glycine	750	10,400
L-Alanine	1,550	10,700
L-Tryptophan	50	6,000

[a] Asp, glu, leu, orn, phe, pro, tyr, and val. [b] Ala, gly, leu, phe, try, tyr, and val.

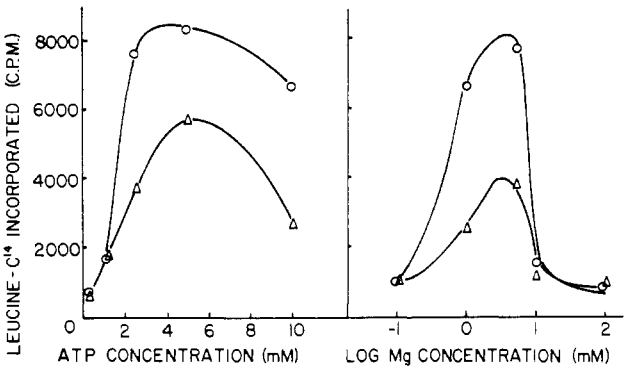

FIG. 2.—Dependence of leucine-C^{14} incorporation process upon ATP and magnesium ion concentration. In this and in all subsequent figures, the sonic extract was replaced by standard amounts of ribosomes plus 140,000 × *g* supernatant. △, gramicidins; ○, tyrocidines.

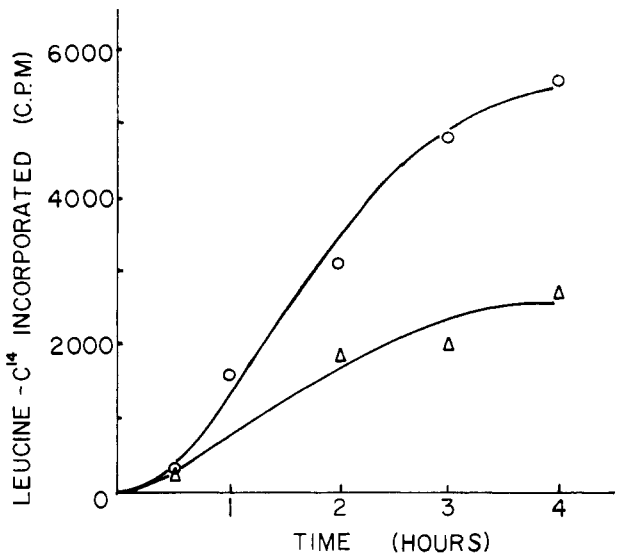

FIG. 3.—Incorporation of labeled leucine into peptides with increasing time of incubation. △, gramicidins, ○, tyrocidines.

of this one amino acid from the mixture was equivalent to omitting the whole gramicidin group insofar as gramicidin formation was concerned. Incorporation into tyrocidine was decreased relatively far less. L-Tryptophan is present to the extent of 4–6 residues in each of the gramicidins (Ramachandran, 1963) but occurs only as a single residue in tyrocidine B.

A detailed examination of the ATP and Mg^{++} requirements for the ribosomal–140,000 × *g* supernatant system is shown in Figure 2. An ATP concentration of 5×10^{-3} M was optimal for both gramicidin and tyrocidine synthesis. Higher levels of ATP were inhibitory, as is generally the case in peptide bond biosynthesis. The concentration of magnesium was rather critical. It was optimal at about 5×10^{-3} M for both groups of peptides, corresponding to a 1:1 ATP-Mg ratio.

Influence of Reaction Time and pH.—Whereas ribosomal systems are generally active for about 1 hour (or less) in protein biosynthesis, it may be seen in Figure 3 that labeled leucine lncorporation into the polypeptide fractions continued for at least 4 hours. After a slight lag, the tyrocidine curve rose much more rapidly than that of gramicidin.

With Tris buffer, the *p*H optima (Fig. 4) were about 7.6 for gramicidin and 7.9 for tyrocidine formation.

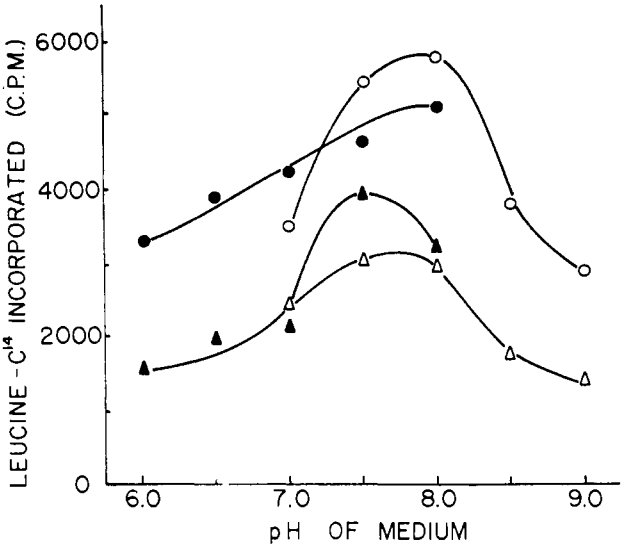

FIG. 4.—Effect of *p*H on the extent of leucine-C^{14} incorporation into polypeptides. Open symbols, 0.01 M tris buffer; solid symbols, usual 0.005 M phosphate: triangles, gramicidins; circles, tyrocidines.

With phosphate buffer, gramicidin synthesis was optimal at *p*H 7.5. The corresponding *p*H for tyrocidine appeared to be slightly more alkaline but could not be definitely ascertained from the available data.

Comparison of Various Radioactive Amino Acids.—Table V is a compilation of data obtained with three groups of isotopic amino acids. The comparisons are rendered difficult in that the labeled compounds frequently differed in optical form, and also, they were isotopically diluted to different extents by the nonlabeled amino acids employed in the standard incubation mixture (Table I). In addition, endogenous amino acids were undoubtedly present in the 140,000 × *g*

TABLE V

COMPARISON OF DIFFERENT LABELED AMINO ACIDS IN THE INCORPORATION PROCESS

The sonic extract in the standard medium was replaced by ribosomes plus supernatant phase. Only one amino acid was used in each experiment.

C^{14}-Labeled Amino Acid	% of Total C^{14} Incorporated		mμmoles Incorporated	
	Gramicidins	Tyrocidines	Gramicidins	Tyrocidines
DL-Leucine	0.82	0.96	41	48
DL-Valine	0.86	1.32	43	66
Glycine	1.45	0.08	14	0.8
L-Alanine	1.68	0.30	17	3.0
L-Isoleucine	0.39	0.71	0.4	0.7
DL-Tryptophan	0.92[a]	0.84[a]	46[a]	42[a]
D-Valine	0.36	0	14	0
Ethanolamine	1.35	0.16	0.4	0.04
D-Phenylalanine	0.24	1.07	4.8	21
L-Proline	0.06	2.00	0.6	20
DL-Ornithine	0.03	0.99	0.3	9.9
L-Aspartic acid	0.31	3.20	3.1	32
L-Glutamic acid	0.60	2.55	6.0	25.5
L-Glutamine[b]	0.11[a]	1.17[a]	1.1[a]	11.7[a]
L-Glutamine[c]	0.01	0.07[a]	0.1	0.7[a]

[a] Isolation by the paper electrophoresis method gave values higher by approximately 5%. [b] The usual nonisotopic glutamic acid in the amino acid mixture was replaced by 1 μmole of L-glutamine. [c] In this experiment, 1 μmole of L-glutamic acid was also present.

TABLE VI

COMPARISON OF DIFFERENT PEPTIDE SEPARATION PROCEDURES FOLLOWING AMINO ACID INCORPORATION

Ribosomes plus 140,000 × *g* supernatant were used in place of sonic extract, under standard conditions.

C^{14}-Labeled Amino Acid	Radioactivity Incorporated, as Per Cent of Total C^{14}						
	Paper Chromatography		Paper Electrophoresis		Charcoal-Cellulose Column		
	Gram.	Tyroc.	Gram.	Tyroc.	Gram.	Tyroc.	Protein
DL-Leucine	0.95	1.29	1.03	1.55	0.75	1.09	0.67
DL-Phenylalanine	0.64	2.82	0.83	3.35	0.69	2.13	0.70
DL-Histidine	0.01	0	0	0	0.03	0.02	0.64

supernatant fraction. Despite these complications, the values are generally consistent when expressed in the two ways shown in Table V.

Considering first DL-leucine and DL-valine (components of both groups of peptides), it can be seen that the C^{14} of each of these amino acids was utilized to the extent of about 0.8% for gramicidin and 1% or slightly more for tyrocidine formation. Since 5 μmoles of each amino acid was present in the incubation mixture, the values in the last two columns are readily derived. It should be mentioned that gramicidin A contains 2 L- and 2 D-valine residues and 1 L- and 3 D-leucines (Ishii and Witkop, 1963), while tyrocidine A contains 1 L-leucine and 1 L-valine (Craig *et al.*, 1949).

With reference to the second group in Table V (representing the gramicidin amino acids), isotopic glycine and L-alanine were somewhat more efficiently utilized for gramicidin synthesis than were the DL-leucine and DL-valine. However, the former were used at considerably lower concentrations in the medium (1-μmole quantities), and smaller amounts of these two amino acids were incorporated into gramicidins. As expected, the concentrations of glycine-C^{14} and alanine-C^{14} found in tyrocidines were low.

Isoleucine was shown by Okuda *et al.*, (1962) to occur to a small extent in mixed gramicidins. Also, the amino acid in isotopic form was observed to incorporate into peptides in growing cultures of *B. brevis* (Okuda *et al.*, 1963). When tested in the cell-free system, L-isoleucine-C^{14} (isotopically undiluted) was readily incorporated into both peptide fractions, but the quantities were small when expressed in mμmoles.

DL-Tryptophan-C^{14} was found to be utilized almost as well for tyrocidine as for gramicidin synthesis. This result seems at variance with the marked tryptophan requirement for leucine-C^{14} incorporation into gramicidins, as compared to tyrocidines (Table IV). Furthermore, as already pointed out, this aromatic amino acid is much more abundant in the gramicidin peptides.

It has been mentioned that D-valine is found only in gramicidins. It may be seen in Table V that the isotopically labeled D-amino acid gave rise to moderate radioactivity in the gramicidin fraction but none in tyrocidines.

The mode of occurrence of ethanolamine in gramicidin is presently not clear. The amino alcohol, when employed in undiluted isotopic form, was taken up to the extent of 1.35% into gramicidin. However, the corresponding quantity in mμmoles is quite small. The incorporation into tyrocidine was not of significant proportions. Further experiments will be needed to determine how ethanolamine is introduced, or originates in gramicidin.

The third group in Table V represents amino acids found only in tyrocidines. D-Phenylalanine was well utilized for tyrocidine synthesis, but was also incorporated to a limited extent into gramicidins. This latter effect probably reflects a certain degree of racemization of the amino acid, since it was observed that 0.20% of the total isotope was incorporated into protein in the same experiment. On the other hand, D-valine gave zero radioactivity in protein.

Tyrocidines contain one residue each of L-proline and L-ornithine. Accordingly, it seems consistent that DL-ornithine-C^{14} was utilized only half as well as an equivalent concentration of L-proline-C^{14}. These amino acids gave rise to only minor labeling in gramicidins.

L-Aspartic acid-C^{14}, L-glutamic acid-C^{14}, and L-glutamine-C^{14} yielded relatively low radioactivities in gramicidins and were all used much more efficiently for tyrocidine synthesis. The glutamine-C^{14} was less readily utilized for tyrocidine formation than was the corresponding acid. Also, the incorporation of the labeled amide was severely inhibited by the presence of nonisotopic glutamic acid. In addition, it will be recalled (Table IV) that glutamic acid, as a component of the amino acid mixture, stimulated tyrocidine synthesis significantly (as measured by leucine-C^{14} uptake).

Application of Three Separation Methods to Peptides.—In order to demonstrate with greater certainty that the radioactivity of the alcohol-HCl extracts represented incorporation of amino acids specifically into the polypeptides and not into protein or other substances, it was important to employ other isolation methods in addition to paper chromatography. Such experiments are described in Table VI. With either isotopic DL-leucine or DL-phenylalanine, both paper electrophoresis and charcoal-cellulose–column chromatography gave results comparable to those with paper chromatography. Of the three methods, electrophoresis gave the highest values. The lowest incorporation figures were obtained by the column method. This accords with the observation (Okuda *et al.*, 1963) that gramicidin and tyrocidine recoveries may be as low as 80% with the charcoal-cellulose procedure. The experiment with histidine-C^{14} is intended to demonstrate that an amino acid which is not a component of the antibiotic polypeptides does not appear in the gramicidin or tyrocidine fractions, even though it may be well utilized for protein synthesis.

The excellence of the separation by high-voltage paper electrophoresis was confirmed by the automatic C^{14} scanning technique (Fig. 5). The exaggerated disparity in the sizes of the tyrocidine and gramicidin peaks in the isotopic phenylalanine experiment is probably due to the fact that tyrocidine A contains 2 residues of D- and one of L-phenylalanine, while L-phenylalanine occurs to only a small extent in the mixed gramicidins. Free radioactive leucine and phenylalanine were absent in the paper electropherograms. As in Table VI, no appreciable C^{14} was detected in the peptide zones in the experiment with labeled histidine.

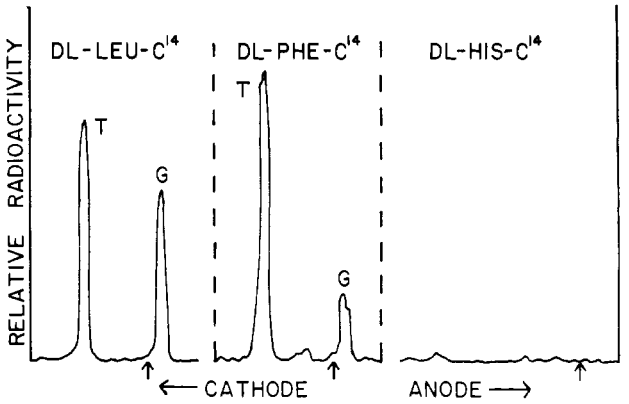

FIG. 5.—Paper electrophoretic analysis of incorporation experiments, as revealed by C^{14} scanning. The preparations were those in Table VI. The vertical arrows indicate the point of application of the substances to the paper.

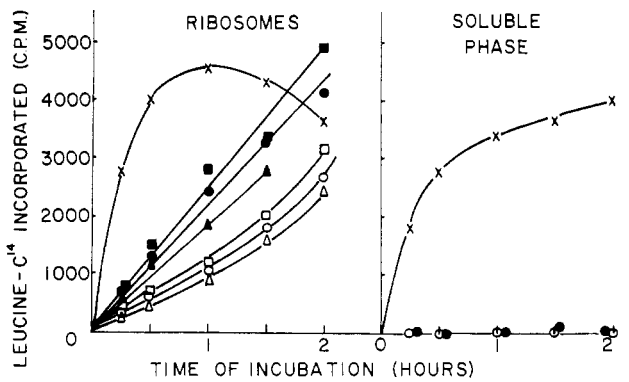

FIG. 6.—Distribution of gramicidins and tyrocidines between ribosomal and soluble phases, as a function of time. Following incubation, ribosomes were sedimented and washed, and protein and peptide fractions were prepared as described under Experimental. Open symbols, gramicidins; solid symbols, tyrocidines. Methods of peptide separation: O, paper chromatography; △, charcoal-cellulose–column chromatography; □, paper electrophoresis; ×, protein.

Recovery of Ribosomes Following Incubation.—Additional evidence that ribosomes were the site of polypeptide synthesis was obtained by resedimenting these particles after varying times of incubation under usual conditions (Fig. 6). As before, three methods were used to isolate the gramicidins and tyrocidines. There were several interesting findings. The recovered washed ribosomes were highly radioactive, due to a retention of both labeled protein and peptides. The C^{14} content of the protein reached a peak in about one hour and thereafter declined (due perhaps to a selective release, or else to a disintegration of ribosomal structure at 37°). Correspondingly, the isotopic content of the soluble protein rose rapidly from the very beginning, and continued to increase slowly between the first and second hours. The radioactivity due to ribosome-bound gramicidins and tyrocidines continued to increase throughout the incubation period. No labeled peptides were detected in the soluble phase.

Effect of Ribonuclease and Deoxyribonuclease.—When either ribosomes or supernatant fraction were preincubated with crystalline ribonuclease, the ability to promote gramicidin or tyrocidine synthesis was destroyed (Table VII). Similar treatments with deoxyribonuclease had a much lesser effect on the activities of the two cellular components.

TABLE VII

EFFECT OF RIBONUCLEASE AND DEOXYRIBONUCLEASE ON POLYPEPTIDE SYNTHESIS

Standard amounts of ribosomes, or of 140,000 × *g* supernatant phase, were pretreated for 30 minutes at 37° with 0.1 mg of either ribonuclease or deoxyribonuclease. The complete system was then employed for assay. In the control experiments, the indicated fraction was preincubated in the absence of enzyme. The peptides were isolated by paper electrophoresis.

Preliminary Enzymic Treatment	Leucine-C^{14} Incorporated (cpm) Gramicidins	Tyrocidines
None (no preincubations)	5200	7200
Ribosome control	5110	6880
Ribosomes with Deoxyribonuclease	4280	5300
Ribosomes with ribonuclease	130	10
Supernatant control	3030	5050
Supernatant with deoxyribonuclease	2300	3400
Supernatant with ribonuclease	0	10

DISCUSSION

Ito and Strominger (1960) have shown that three amino acids and a terminal dipeptide are added in a stepwise fashion to a uridine nucleotide, under the influence of soluble enzymes. The synthesis of the high-molecular, open-chain γ-glutamyl polypeptide of *B. lichenformis* also requires a soluble type of enzyme, and a primer as well (Leonard and Housewright, 1963). Previous attempts to detect intermediates in the biosynthesis of the cyclic polypeptide, gramicidin S, by inhibiting cultures of *B. brevis* (ATCC 9999) with amino acid analogs were unsuccessful (Winnick and Winnick, 1961). The present study, together with that of Otani *et al* (1963), offers the first indication that the biosynthesis of proteins and of polypeptides can follow similar pathways.

While our experiments are based solely upon the uptake of labeled amino acids into cyclic peptides, a net synthesis of gramicidin J (measured by antibiotic assay) has been achieved by Otani *et al.* (1963) with their cell-free system. The process of C^{14}-amino acid incorporation into polypeptides bears striking similarity to the corresponding general requirements for protein biosynthesis: Both particulate and soluble cellular components are necessary, and each fraction can be inactivated by ribonuclease enzyme. The reaction is promoted by magnesium ions, ATP plus an ATP generator, an amino acid mixture, and by glutathione. The resemblance extends even to the binding of the labeled products to the ribosomes. All of these correlations strongly indicate that the present measurements do, in fact, reflect polypeptide synthesis.

The magnitude and the selective character of the incorporation process with the various isotopic amino acids appear highly significant. Particular mention may be made of the utilization of labeled L-ornithine and the D-forms of phenylalanine and valine. The fact that neither the *tyrocidine* nor the *protein* became radioactive in the experiment with D-valine in Table V indicates that extensive racemization of this amino acid had not occurred. However, as already mentioned, D-phenylalanine gave rise to a small degree of labeling in protein. The observations with D-amino acids suggest that they may be derived from the corresponding L- forms in the *B. brevis* cell, prior to

incorporation into peptide structures. On the other hand, Katz and Weissbach (1963) believe that during biogenesis of an actinomycin molecule, the D-valine arises from the L-isomer only after the latter is incorporated into a peptide chain. Additional experiments with L- and D-amino acids at different stages in the pathway are being conducted to clarify these relationships for gramicidin and tyrocidine synthesis.

Zubay (1962) observed that glutamine-C^{14} was more extensively incorporated into acceptor-RNA of *E. coli* than was glutamic acid-C^{14}. An equivalent quantity of nonisotopic glutamine strongly inhibited the uptake of labeled glutamic acid, while the reverse situation was not true. He concluded that glutamine, rather than glutamic acid, was coded for in protein synthesis. On the other hand, Coles and Meister (1962) concluded that dicarboxylic amino acids could be transferred to RNA without obligatory prior amidation. The results in Table V suggest that glutamic acid is utilized preferentially for tyrocidine synthesis and then amidated at a later stage in the process. It should be mentioned also that experiments very similar to those of Zubay and of Coles and Meister have been performed with acceptor-RNA of *B. brevis* which support the view that glutamic acid is utilized as such.[2]

The values for leucine incorporation, expressed in mμmoles in Table V, can be converted into mg of peptides with the aid of the known leucine contents of the two groups of peptides (Okuda *et al.*, 1962). This calculation indicates a synthesis of approximately 20 μgm of gramicidins and 65 μgm of tyrocidines by about 4 mg of ribosomal material in 4 hours. Accordingly, the combined yield of labeled polypeptides comprised some 2% of the ribosomal mass. This minimal value does not take into account any quantities of preexisting nonisotopic peptides on the ribosomes, nor the isotopic dilution of radioactive amino acids by the corresponding endogenous compounds in the supernatant fraction.

It is also instructive to compare the incorporation of labeled amino acids into protein and into peptides. In the experiments of Figure 6, at 1 hour the sum of the gramicidin plus tyrocidine-C^{14} (based on paper electrophoresis) was 3800 cpm, while the combined C^{14} of ribosomal plus soluble protein was 7900 cpm. At 2 hours, the radioactivity of the polypeptides amounted to 7850 cpm, as compared to a total of 7650 cpm for ribosomes and soluble phase. Accordingly, it appears that the incorporation of leucine-C^{14} into protein stopped after about 1 hour, and that by 2 hours the quantity of isotopic amino acid utilized for polypeptide synthesis equaled that recovered in the total protein of the system. In the 4-hour experiments of Table VI, leucine-C^{14} and phenylalanine-C^{14} were each incorporated about three to four times more extensively into polypeptides than into proteins of the incubation mixture. These observations are not too surprising in view of the elevated levels of polypeptides found in *B. brevis* cells at the end of the log phase of growth (Okuda *et al.*, 1963). It may be mentioned that high concentrations of radioactive gramicidin J can be detected in ribosomes isolated from *B. brevis* cells grown in the presence of labeled amino acids (Chern, 1960).

There are several considerations which make it unlikely that the antibiotic peptides are simply absorbed to the ribosomes after synthesis in the soluble phase: (A) the combination of ribosomes and soluble phase is needed for optimum synthesis; (B) the ribosomes are quite sensitive to magnesium ion concentration during the sonication process and the subsequent washing procedure; (C) the synthesizing activity of the complete system shows a sharp optimum Mg concentration, again suggesting that this ion maintains the structural integrity of the ribosomes; and (D) ribosomes lose their ability to supplement the supernatant phase when the former are stored for some days at low temperature.[2]

The present work suggests that the participating macromolecular components in the 140,000 × *g* supernatant solution include *s*-RNA and amino acid activating enzymes. In fact, experiments are in progress to determine whether D-amino acids and L-ornithine are transferred to *s*-RNA.[2] These findings, which bear on the central question of coding mechanisms, will be reported in subsequent papers.

[2] Unpublished work by K. Okuda, I. Uemura, and T. Winnick.

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