Inserting $(1 + p)$ for the denominator of (A 62) and using

$$\Sigma\Delta H = \Delta H_{E,H} - \Delta H_{ER,H} - \Delta H_{E,R} \quad \text{(A 68)}$$

and

$$\frac{\Delta K_{Y,Z}}{K_{Y,Z}} = \frac{\Delta H_{Y,Z}}{RT^2} \quad \text{(A 69)}$$

leads to

$$\frac{(\Delta \bar{c}_{ERH})_2}{\bar{c}_{ERH}} \approx \frac{\Sigma\Delta H}{1 + p} \frac{\Delta T}{RT^2} \quad \text{(A 70)}$$

This last equation may be used for a crude relative evaluation of enthalpies as demonstrated in the main part under Thermodynamics.

Acknowledgments

The authors would like to express their high appreciation to Dr. Britton Chance for his continuous stimulating interest in the progress of this investigation. They would also like to thank Miss Johanna Schwartz and Mr. Albrecht Foelsing for their assistance in checking the derivations of the equations and in the numerical evaluations.

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Experiments on the Mechanism of Gramicidin and Tyrocidine Synthesis in Cell-free Preparations of *Bacillus brevis**

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Received August 1, 1963

Studies have been made of the mechanism of biosynthesis of the gramicidin and tyrocidine polypeptides, with ribosomes and various soluble preparations, derived from sonic extracts of *Bacillus brevis*. The pathway of L-amino acid utilization appeared to proceed through the following stages: activation via the adenylate, incorporation into soluble RNA, and transfer from aminoacyl-s-RNA to ribosomes. While D-amino acids were readily activated, they were poorly incorporated into s-RNA, and the possibility of an alternate type of carrier for these D-isomers was explored. Experiments with mixed fractions from two different strains of *B. brevis* revealed that a soluble factor, distinct from amino acid–activating enzymes and from phenol-extractable RNA, controlled the specificity of polypeptide synthesis.

There is now a strong body of evidence in support of the pathway of protein biosynthesis formulated by Zamecnik (1962). By contrast, no requirement has been established for soluble[1] RNA or ribosomes in the formation of glutathione (Lane and Lipmann, 1961), uridine nucleotide peptide (Strominger, 1962), or γ-glutamyl polypeptide (Leonard and Housewright, 1963).

* Supported by a research grant (GM-09335) from the National Institutes of Health, U. S. Public Health Service. Contribution No. 005 from the Pacific Biomedical Research Center, University of Hawaii.

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‡ Research performed in partial fulfillment of requirements for the degree of Doctor of Philosophy in Biochemistry, University of Hawaii.

[1] This term is used for convenience, to refer to the RNA possessing both amino acid "acceptor" and "transfer" functions.

The cyclic polypeptides with antibiotic properties, found in a variety of microorganisms, provide additional attractive model systems for the study of mechanisms of peptide-bond synthesis. Two papers (Uemura *et al.*, 1963; Okuda *et al.*, 1963b) dealing with cell-free preparations from *Bacillus brevis* (Dubos-Hotchkiss strain) have revealed that the process of gramicidin and tyrocidine biosynthesis resembles protein synthesis in its requirement for both particulate and soluble cellular components, and in its dependence on ATP, magnesium ions, glutathione, and an amino acid mixture. Polypeptide, as well as protein formation was completely inhibited by chloramphenicol and by puromycin. The synthesis of gramicidins and tyrocidines could be measured by incorporation experiments with isotopic L- and D-amino acids, or by a net increase in antibiotic activity. In either case the newly formed peptides were shown to be attached to ribosomes. By employing combinations of ribosomal and supernatant

fractions derived from two different strains of *B. brevis* it was found that the specificity of peptide synthesis resided in the soluble (140,000 × *g*) phase. Pretreatment with pancreatic ribonuclease caused inactivation. However, in these previous publications no evidence was provided for the participation of s-RNA in the biosynthetic mechanism, and it was possible to interpret ribonuclease action as disintegrating ribosomal structures.

The present investigation is concerned with the elucidation of certain of the stages in the path of gramicidin and tyrocidine formation: the activation of L- and D-amino acids, the incorporation of L-amino acids into s-RNA, and the transfer of L-amino acids from s-RNA to ribosomes. The D-enantiomorphs of the amino acids studied do not appear to utilize the s-RNA pathway, and attempts have been made to detect a special carrier for these isomers. Experiments with reconstituted systems have made it possible to examine the requirements for (phenol-extracted) RNA, *p*H 4.8–precipitable enzymes, and *p*H 4.8–soluble substances, and to localize the factor which controls the specificity of polypeptide synthesis. In addition, the incorporation of glutamine and glutamic acid into s-RNA is compared.

Experimental Procedures

Materials.—The biochemicals and radioactive amino acids used were those previously described (Okuda *et al.*, 1963b; Uemura *et al.*, 1963). In addition the following substances were employed: P^{32}-pyrophosphate (inorganic) (Nuclear Chicago Corporation), and L-valine-U-C^{14} (205 mc/mM) and L-leucine-U-C^{14} (246 mc/mM), both from New England Nuclear Corporation.

Amino Acid–activating Enzymes.—All operations were performed at 0°. A 30-g quantity of washed cells of *B. brevis* ATCC 8185 (Dubos-Hotchkiss strain)[2] (Okuda *et al.*, 1963a) was suspended in 30 ml of 0.1 M Tris buffer (*p*H 7.5) containing 0.005 M mercaptoethanol and subjected to sonic disruption (15 minutes at 60 w and 20 kc). After removal of debris and surviving cells by centrifugation for 15 minutes at 11,000 × *g*, the supernatant phase was diluted with two volumes of the Tris buffer. The protein precipitating between 25 and 70% saturation with $(NH_4)_2SO_4$ was collected, and was redissolved in 30 ml of buffer. Using 1 M acetic acid, a protein fraction which precipitated between *p*H 4.8 and 4.6 was sedimented, and was dissolved in 5–10 ml of the Tris buffer. The solution was then passed through a 12 × 0.8-cm DEAE-cellulose[3] column (Bio-Rad Cellex D, 0.8 meq/g), pre-equilibrated with buffer. The hold-up volume was discarded. An additional 10 ml of buffer served to elute the desired enzyme fraction. The effluent was dialyzed for 3 hours against buffer. The final solution was stable for at least 1 week when stored at −20°.

Assay of Amino Acid–dependent Inorganic Pyrophosphate–ATP Exchange.—The method employed was that described by Stulberg and Novelli (1962), with a 10-minute incubation time. The results were expressed in moles of P^{32}-labeled inorganic pyrophosphate exchanged, and all values were corrected for endogenous effects. In most experiments, the added amino acids caused a 3- to 4-fold increase above the endogenous level. With L-tryptophan, the effect was some 10-fold greater than the basal exchange. Approximately 1 mg of enzyme protein was routinely used per incubation tube.

[2] Used in all experiments unless otherwise stated.

[3] Abbreviation used in this work: DEAE-cellulose, diethylaminoethyl-cellulose.

Preparation of Soluble RNA.—The procedure was based on the methods described by Brunngraber (1962), and Von der Decken and Campbell (1962), with a number of modifications. All operations were carried out at 0°, unless otherwise stated. Washed *B. brevis* cells (100 g wet wt) were suspended in 100 ml of 0.1 M *p*H 7.5 Tris buffer, containing 1 M NaCl and 0.005 M EDTA, and then subjected to sonication. An additional 50 ml of the above buffer and 150 ml of water-saturated phenol (redistilled) were added, and the mixture was blended in a Waring Blendor for 2 minutes (at intermediate speed). This treatment was followed by 2 hours of vigorous shaking. After centrifuging the suspension for 20 minutes at 15,000 × *g*, the upper layer was separated by decantation. This extract was treated with three volumes of ethanol and chilled to −20°. The resultant precipitate was sedimented (5 minutes at 5,000 × *g*) and washed with 80% ethanol, 95% ethanol, absolute ethanol, and ether. The dry product weighed 1.5–2.0 g, and will be referred to as crude s-RNA.

For further purification, the above quantity of crude s-RNA was dissolved in 100 ml of 0.05 M Tris buffer (*p*H 9.0), and incubated for 40 minutes at 37°, in order to remove esterified amino acids (Von der Decken and Campbell, 1962). After incubation the solution was adjusted to *p*H 5.0 with 1 M acetic acid, and 10 ml of 20% sodium acetate (*p*H 5.0) and 250 ml of ethanol were added. The precipitate was collected by centrifugation, dissolved in 200 ml of 0.1 M Tris buffer, and further purified on DEAE-cellulose as described by Brunngraber (1962). The yield of purified s-RNA was approximately 100 mg. This (dry) preparation could be stored for at least 2 months at −20° without apparent loss in activity. Ribosomal RNA was also prepared by the above procedure, starting with washed ribosomes.

Assay of Amino Acid Incorporation into Soluble RNA.—The standard reaction mixture contained 0.5 mg activating enzyme, 0.5 μc of labeled amino acid, 2.5 μmoles $MgCl_2$, 1.25 μmoles ATP, 25 μmoles Tris buffer *p*H 7.5, and 0.5 mg s-RNA, all in a total volume of 0.5 ml. All components were adjusted to *p*H 7.5 before mixing. The system was incubated for 30 minutes at 37°, and the radioactive aminoacyl-s-RNA was isolated with the aid of yeast RNA carrier, by the method of Berg *et al.* (1961). All incorporation values were corrected for zero-time radioactivity.

Preparation of C^{14}-Aminoacyl-s-RNA.—The above assay system for measuring C^{14}-amino acid incorporation into s-RNA was employed. However, the scale was increased 8-fold, corresponding to 4 ml of reaction mixture. The level of purified s-RNA was disproportionately increased to 20 mg, while 20–30 μc of L-valine or L-leucine of high specific radioactivity was employed. Also, 40 μg of pyruvic kinase and 5 mM phosphoenolpyruvate were present. At the end of the incubation period the labeled aminoacyl-s-RNA was precipitated (without addition of carrier) by adding 0.4 ml of 20% sodium acetate plus 10 ml of ethanol, and was washed and dried, as described by Berg *et al.* (1961). The product (approximately 18 mg) was stored at −20°, and was stable for at least a week. Its specific radioactivity was of the order of 10,000–30,000 cpm/mg s-RNA.

Determination of Optical Configuration of C^{14}-Amino Acids in Aminoacyl-s-RNA.—A 10-mg quantity of the amino acid–s-RNA complex, prepared by the method just described, was dissociated by a 30-minute incubation in the presence of the same amino acid–activating enzyme and cofactors employed in the incorporation process, but with the addition of 5-μmole quantities of inorganic py-

rophosphate and AMP, instead of ATP. The excess pyrophosphate caused virtually complete dissociation of the aminoacyl-s-RNA, so that more than 90% of the C^{14} was released. Subsequently the s-RNA was precipitated in the usual manner, and the C^{14}-amino acid remaining in solution was treated with specific D- or L-amino acid oxidase, followed by Dowex-column chromatography (Okuda *et al.*, 1963b). The radioactivity of the recovered amino acid was then measured.

Assay of C^{14}-Aminoacyl-s-RNA Utilization for Polypeptide Synthesis.—Labeled L-leucine s-RNA complex (0.5–2 mg) was added to the whole sonicate system, containing all the standard components. However, L-leucine-C^{12} was omitted from the amino acid mixture (Uemura *et al.*, 1963). After specified times of incubation, tyrothricin and protein were isolated and counted for radioactivity (Uemura *et al.*, 1963).

Subfractionation of Soluble Supernatant Phase.—Operations were conducted at 0°. The 140,000 × *g* supernatant, derived from a standard sonicate of 12 g of *B. brevis* cells (Uemura *et al.*, 1963), was adjusted to *p*H 4.8 with 1 M acetic acid, and the resulting precipitate was sedimented. The supernatant solution, hereafter referred to as the "*p*H 4.8 supernatant," was brought to *p*H 7.5 with K_2CO_3. The *p*H 4.8 precipitate was dissolved in 4.5 ml of 0.1 M (*p*H 7.5) Tris buffer, and the solution was passed through a 5 × 0.8-cm DEAE-cellulose column. The hold-up volume was discarded, and the amino acid–activating enzyme fraction was eluted by an additional 10 ml of buffer. The s-RNA remained on the column. The effluent is hereafter referred to as the RNA-free *p*H 4.8 precipitate.

Combinations of the above fractions, together with s-RNA and ribosomes, were assayed in the presence of a specified quantity of labeled amino acid and the various cofactors of the standard reaction mixture (Uemura *et al.*, 1963). In some experiments mixed cellular components from two different strains of *B. brevis* were employed, and in these cases radioactivity was measured in the gramicidin S, as well as in Dubos gramicidin and tyrocidine regions after paper electrophoresis (Uemura *et al.*, 1963).

Preincubation Experiments.—Attempts to detect an accumulation of intermediates in polypeptide formation involved a preliminary incubation of the whole soluble (140,000 × *g*) supernatant phase with a specific radioactive D-amino acid and standard cofactors. Subsequently ribosomes plus an excess of the corresponding nonisotopic amino acid were added, and the rate of labeling of polypeptides was determined. Conversely, the C^{12}-D-amino acid was employed in the preincubation stage, and the corresponding C^{14}-amino acid was subsequently added with the ribosomes.

Experiments of this type were also performed with the *p*H 4.8 supernatant fraction, in place of the whole 140,000 × *g* supernatant. The *p*H 4.8 precipitate and ribosomes were added at the second stage. In alternate cases, the *p*H 4.8 precipitate was used in the preliminary step, followed by addition of *p*H 4.8 supernatant and ribosomes. Radioactive L-amino acid was tested (as a control), as well as the D-isomer, in the above experiments.

Results

Activation of L- and D-Amino Acids.—The ability of enzymes of *B. brevis* to catalyze the incorporation of P^{32}-pyrophosphate into ATP in the presence of amino acids is shown in Table I. The degree of activation of the D-amino acids which occur in gramicidins and tyrocidines was comparable to that of the L-isomers of these compounds. The most marked difference between the *p*H 5.0 and 4.6 preparations is in the greater response of the former toward L-phenylalanine; whereas the *p*H 4.6 fraction was more active with the D-form of this amino acid. It may be mentioned that a D-phenylalanine-activating enzyme has been partially purified from extracts of the Nagano strain of *B. brevis* (Okuda *et al.*, 1960).

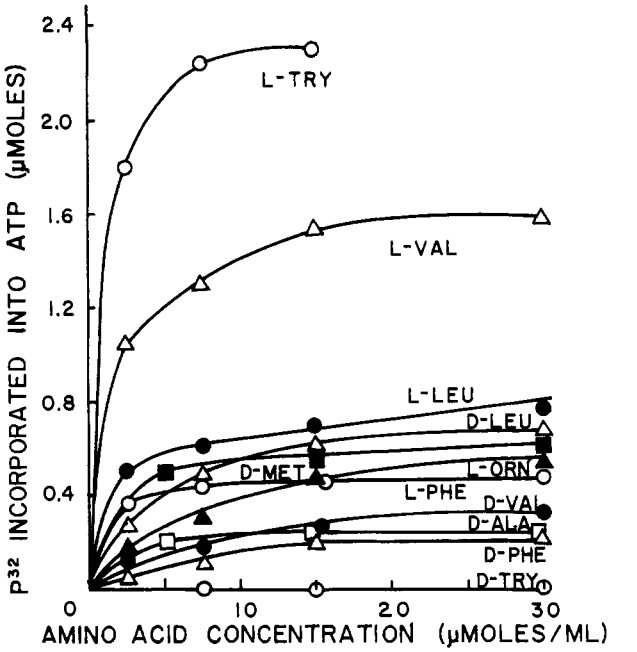

FIG. 1.—Stimulation of P^{32}-pyrophosphate incorporation into ATP by various L- and D-amino acids, in the presence of activating enzyme.

The dependence of the P^{32}-pyrophosphate (inorganic) incorporation into ATP upon amino acid concentration is shown in Figure 1. In addition to D-leucine, D-valine, and D-phenylalanine two other D-amino acids, *not* constituents of gramicidins and tyrocidines, were found to stimulate the exchange process. These compounds were D-alanine and D-methionine. It may also be seen that L-tryptophan, which occurs abundantly in gramicidins, was the most active of the amino acids tested. D-Tryptophan, which is not present in polypeptides, was not activated. L-Ornithine, found in tyrocidines, was moderately effective in stimulating pyrophosphate^{32} exchange. As in earlier studies on gramicidin S (Winnick and Winnick, 1961) the sub-

TABLE I
ACTIVATION OF VARIOUS AMINO ACIDS BY ENZYME FRACTIONS OF *B. Brevis*[a]

Amino Acid (0.01 M)	Labeled Inorganic Pyrophosphate Incorporated into ATP (mμmoles/mg protein)	
	Enzymes Precipitated at *p*H 5.0	Enzymes Precipitated at *p*H 4.6
L-Leucine	369	381
D-Leucine	336	504
L-Valine	460	510
D-Valine	197	112
L-Phenylalanine	501	60
D-Phenylalanine	192	167

[a] The method of preparation of the two enzyme fractions was that described under Experimental Procedures, except that $(NH_4)_2SO_4$ fractionation was omitted, and the procedure was not carried beyond the acetic acid–precipitation step.

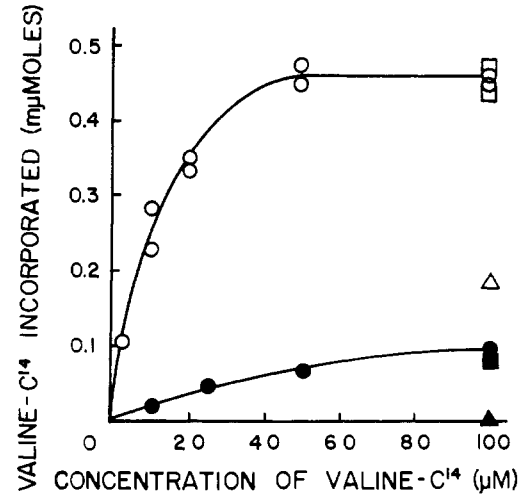

FIG. 2.—Influence of nonisotopic D- and L-valine on the incorporation of D-valine-C^{14} and L-valine-C^{14} into s-RNA. ●, D-valine-C^{14} alone; ■, D-valine-C^{14} plus 0.2 mM D-valine-C^{12}; ▲, D-valine-C^{14} plus 0.1 mM L-valine-C^{12}; ○, L-valine-C^{14} alone; □, L-valine-C^{14} plus 0.1 mM D-valine-C^{12}; △, L-valine-C^{14} plus 0.1 mM L-valine-C^{14}.

stitution of P^{32} ortho- for pyrophosphate resulted in much lower degrees of incorporation into ATP, in the presence of various L- and D-amino acids. Pyrophosphatase activity was not measured. However, the above results with orthophosphate make it appear unlikely that the observed effects with pyrophosphate^{32} reflected inorganic P^{32} incorporation.

Amino Acid Incorporation into s-RNA.—It was important to study the second function of the amino acid–activating enzymes of *B. brevis*, namely, the stimulation of amino acid incorporation into s-RNA. The occurrence of D-amino acids and L-ornithine in the polypeptide molecules, but not in protein, focused attention on these unusual compounds. It proved difficult to prepare radioactive D-amino acids completely free of the L-isomers, although both H^3- and C^{14}-labeled compounds were employed. The former were obtained by titration (followed by purification) of commercial D-amino acids (Uemura *et al.*, 1963); while C^{14}-D-amino acids were prepared by destruction of the L forms of commercial C^{14}-DL-amino acids by L-amino acid oxidase, followed by purification of the surviving D-enantiomorph (Okuda *et al.*, 1963b). However, the final preparations were still contaminated by traces of the L-isomers. An added complication was the relatively high zero-time values in incorporation experiments with labeled D-amino acids, which probably reflected incomplete removal of degradation products resulting from tritiation, or from the L-amino acid oxidase treatment.

It was invariably observed that isotopic L-amino acids were much more efficiently incorporated into s-RNA than were their D-isomers. Figure 2 illustrates this fact in the case of C^{14}-labeled L- and D-valine. With a constant level of s-RNA and activating enzyme, and varying amino acid concentration, the system attained maximum activity (0.45 mμmole of amino acid incorporated) at approximately 50 mμmoles of L-valine-C^{14}/ml. The labeling of s-RNA appeared to increase between 50 and 100 mμmoles/ml in the case of D-valine-C^{14}, reaching an incorporation value of 0.10 mμmole. The uptake of L-valine-C^{14} was reduced by approximately the expected amount in the presence 100 mμmoles of L-valine-C^{12}, while the addition of an equivalent quantity of D-valine-C^{12} had no effect. On the other hand, it is significant that the incorporation of D-valine-C^{14} into s-RNA was *not* greatly depressed by adding two equivalents of unlabeled D-valine, while one equivalent of L-valine-C^{12} *completely inhibited* the reaction. Very similar results were obtained in experiments with isotopic L- and D-leucine.

Additional experiments in Table II indicate that the addition of one part of D-leucine-C^{12} to a system containing L-leucine-C^{14} caused only a slight reduction in the incorporation of the latter into s-RNA; while adding one part of L-leucine-C^{12} lowered the incorporation by the expected 50%. In the case of DL-leucine-C^{14}, the presence of one part of D-leucine-C^{12} had no effect, while an equivalent amount of L-leucine-C^{12} caused a two-thirds inhibition in s-RNA labeling. This would be the theoretical value if it were assumed that no D-leucine-C^{14} were utilized. Calculations showed that the mμmoles of leucine-C^{14} incorporated into s-RNA were approximately the same, when either L- or DL-leucine-C^{14} was employed at saturating concentrations. Again the inference is that no significant transfer of D-leucine to s-RNA occurred in the presence of the L-isomer. With H^3-labeled D-phenylalanine, addition of nonisotopic D-amino acid caused some reduction of radioactivity in s-RNA; but a *complete* suppression was observed upon adding L-phenylalanine-C^{12}.

It was pertinent to determine the optical configuration of labeled amino acids recovered from the aminoacyl-s-RNA complex following incorporation experiments with a D-amino acid. For this purpose, samples of valyl-s-RNA, derived from such an experiment with D-valine-C^{14}, were dissociated, and then subjected to the action of specific amino acid oxidases. Thirty-three per cent of the labeled amino acid was destroyed by L-oxidase, and 76% by D-oxidase treatment. This observation confirmed our suspicions regarding the insufficient purity of the original C^{14}-D-amino acid, and pointed to a very strong affinity of the L form for the s-RNA. The foregoing observations, particularly the marked inhibition of D-aminoacyl-s-RNA formation

TABLE II
EFFECT OF NONLABELED D- AND L-AMINO ACIDS ON THE INCORPORATION OF RADIOACTIVE FORMS INTO RNA[a]

Isotopic Amino Acid	Concentration (mM)	C^{12}-Amino Acid	Concentration (mM)	Incorporation into RNA
L-Leucine-C^{14}	0.10	D-Leucine	0.10	89
L-Leucine-C^{14}	0.10	L-Leucine	0.10	50
DL-Leucine-C^{14}	0.10	D-Leucine	0.10	100
DL-Leucine-C^{14}	0.10	L-Leucine	0.10	31
D-Phenylalanine-H^3	0.30	D-Phenylalanine	0.25	72
D-Phenylalanine-H^3	0.30	L-Phenylalanine	0.25	0

[a] The incorporation values are expressed in terms of control experiments, taken as 100, in which no C^{12}-amino acid was present. In these cases the degree of labeling was of the order of 0.3, 0.3, and 0.1 mμmoles/mg RNA with L-, DL-, and D-amino acids, respectively.

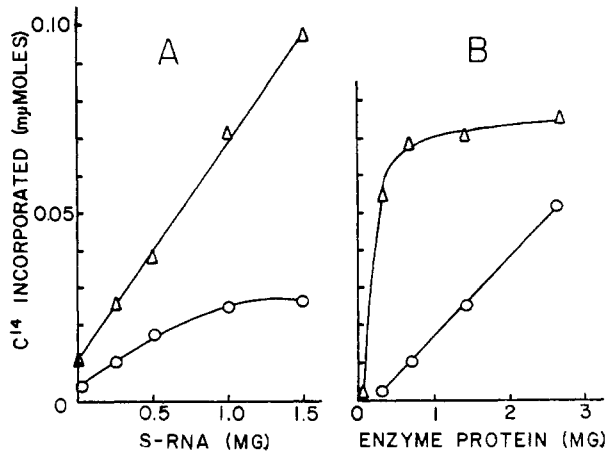

FIG. 3.—Relative incorporation of glutamic acid-C^{14} and glutamine-C^{14} into s-RNA. Incubation time, 10 minutes. △, L-glutamic acid; O, L-glutamine. A, with 1.36 mg enzyme protein. B, with 1 mg s-RNA.

by L-amino acid, made it seem unlikely that s-RNA participated as an intermediate in D-amino acid utilization.

Incorporation of Glutamic Acid and Glutamine into s-RNA.—Coles and Meister (1962) concluded from their studies with yeast preparations that dicarboxylic amino acids could be transferred to s-RNA without obligatory prior amidation. A similar tentative conclusion was reached in our previous experiments, which showed that radioactive glutamic acid was used more readily than was glutamine for tyrocidine synthesis in unfractionated extracts of *B. brevis* (Uemura *et al.*, 1963). It was of interest to extend these experiments to the measurement of the incorporation of glutamic acid and glutamine into s-RNA. The relative degrees of utilization of the two compounds were found to vary with the age of the activating enzyme preparation. When the latter was freshly prepared, L-glutamine and L-glutamic acid were incorporated to comparable extents; however after aging for 2–3 days (at −20°), the enzyme preparations were less active with the amide.

Table III, with fresh activating enzyme, shows that increasing proportions of nonisotopic L-glutamic acid severely inhibited the incorporation of L-glutamine-C^{14} into s-RNA. Conversely, excess quantities of nonlabeled L-glutamine had much smaller effects on L-glutamic acid-C^{14} transfer to s-RNA. As Coles and Meister point out, the interpretation of such experiments may be complicated by some degree of hydrolysis or synthesis of glutamine during the incubations. In any event, the present data do not rule out the possibility that both glutamyl- and glutaminyl-s-RNA may be utilized, either for tyrocidine, or for protein formation.

TABLE III
COMPARISON OF LABELED GLUTAMINE AND GLUTAMIC ACID INCORPORATION INTO RNA[a]

C^{14} Compound (0.05 μmole)	C^{12} Compound (μmoles)	C^{14} Incorporation into RNA
L-Glutamine	L-Glutamic acid, 0.1	55
L-Glutamine	L-Glutamic acid, 2.5	6
L-Glutamine	L-Glutamic acid, 5.0	3
L-Glutamic acid	L-Glutamine, 0.1	100
L-Glutamic acid	L-Glutamine, 2.5	45
L-Glutamic acid	L-Glutamine, 5.0	39

[a] The results are expressed in terms of controls (without C^{12} compounds), taken as 100.

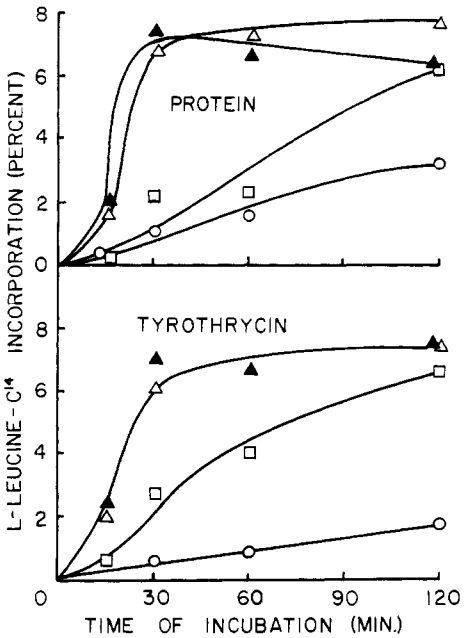

FIG. 4.—Transfer of isotopic leucine from s-RNA to peptide and to protein. 13,000 cpm of either free L-leucine-C^{14}, or s-RNA-L-leucine-C^{14} were used per tube. □, L-leucine-C^{14} alone; O, L-leucine-C^{14} plus 1 mM L-leucine-C^{12}; △, s-RNA-L-leucine-C^{14} alone; ▲, RNA-L-leucine-C^{14} plus 1 mM L-leucine-C^{12}.

In Figure 3, the enzyme had been stored for 3 days and was much less active for glutamine. With constant enzyme (A), the quantity of glutamic acid-C^{14} incorporated was proportional to the amount of s-RNA. With a constant quantity of s-RNA (B), L-glutamic acid-C^{14} uptake approached a maximum with about 1 mg of enzyme, while the utilization of L-glutamine-C^{14} was proportional to enzyme concentration over the range tested. The results suggest that glutamic acid was the form primarily utilized, and that glutamine to glutamic acid conversion was rate limiting.

Utilization of L-Leucyl-s-RNA for Polypeptide Synthesis.—Experiments designed to determine whether L-aminoacyl-s-RNA could serve as an intermediate in polypeptide synthesis are described in Figure 4. It may be seen that, after a slight initial lag, the labeling of both polypeptide and protein proceeded rapidly, reaching a maximum in approximately 30–60 minutes. It is significant that the addition of free L-leucine-C^{12} to the system had no marked effect. When the L-leucyl-C^{14}-s-RNA was replaced by an equivalent quantity of free L-leucine-C^{14}, the latter proved much less effective in tyrothricin and in protein synthesis. The combination of free L-leucine-C^{14} and L-leucine-C^{12} resulted in a further reduction of C^{14} incorporation into peptide and protein, as would be expected. It appears that the labeled leucyl-s-RNA complex was utilized to approximately the same extent for polypeptide and for protein synthesis. The combined processes involved an incorporation of about 15% of the total C^{14} employed in the experiment. Quite similar results to those in Figure 4 were also obtained with L-valyl-C^{14}-s-RNA, leaving little doubt that s-RNA functioned as an intermediate in the utilization of at least two of the component L-amino acids of gramicidins and tyrocidines.

Demonstration of an Intermediate Stage in D-Amino Acid Utilization.—In view of the poor incorporation of labeled D-amino acids into s-RNA, it appeared possible that substances other than s-RNA might function in the transfer of activated D-amino acids to sites of peptide-bond formation. Previously it was found that radioactive D-amino acids were well incorporated into

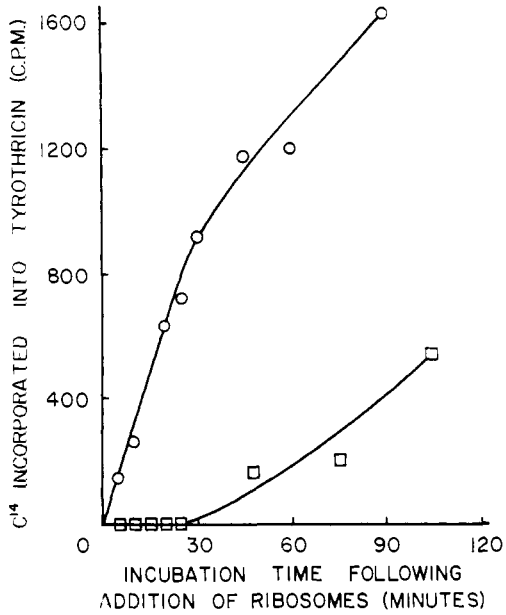

FIG. 5.—Effect of preincubation of soluble fraction on the subsequent rate of D-leucine incorporation into polypeptides. O, 30-minute preliminary incubation of D-leucine-C^{14} with soluble supernatant system; ribosomes plus 2 μmoles of D-leucine-C^{12} added at zero time. □, 60-minute prior incubation of soluble system with 2 mM D-leucine-C^{12}; D-leucine-C^{14} plus ribosomes added at zero time. 0.25 μc of isotopic amino acid was used per tube.

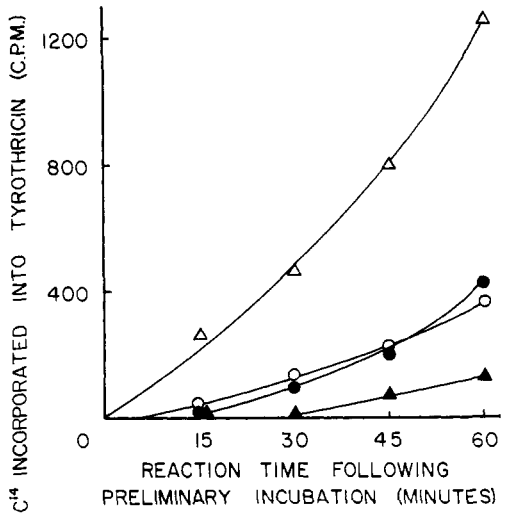

FIG. 6.—Preincubation experiments with isotopic D-leucine and subfractions of the soluble supernatant phase. The quantities of components used were the same as those in Table V. 0.25 μc of C^{14} was employed per tube. The time of prior incubation, before adding ribosomes, was 30 minutes in each case. Triangles, with *p*H 4.8 supernatant; circles, with *p*H 4.8 precipitate; open symbols, preincubation with D-leucine-C^{14} and adding 2 mM D-leucine-C^{12} at zero time. Solid symbols, preincubation with 2 mM D-leucine-C^{12} and addition of D-leucine-C^{14} at zero time.

polypeptides in the whole sonicate system of *B. brevis*, although there was a slight initial lag of several minutes duration (Uemura *et al.*, 1963). This effect was also observed with L-amino acids. It was suggested that the lag reflected the formation of intermediary substances in the biosynthetic pathway.

When the soluble supernatant fraction was preincubated (Fig. 5) in the presence of D-leucine-C^{14} (in order to promote an accumulation of an intermediate), and then ribosomes were added, together with an excess of D-leucine-C^{12}, there was a rapid incorporation of the labeled amino acid into polypeptide, with no suggestion of an initial lag. In the converse situation, preliminary incubation of the soluble phase in the presence of D-leucine-C^{12}, with subsequent addition of ribosomes plus D-leucine-C^{14}, resulted in a marked retardation of C^{14} incorporation into polypeptide; in fact, no incorporation was observed until after 30 minutes, and then the rate remained slow. One interpretation is that a conjugated D-amino acid intermediate was produced, which was not able to exchange rapidly with free D-amino acid.

Additional preincubation experiments to better localize the above postulated D-amino acid carrier are described in Figure 6. It may be seen that the *p*H-4.8 supernatant fraction exhibited the same type of behavior as the whole 140,000 $\times$ *g* supernatant of Figure 5. However, in the case of the *p*H-4.8 precipitate, the order of addition of the C^{12}- and C^{14}-amino acids had no significant effect on the subsequent rate of labeling of polypeptides.

Evidence that the foregoing effects were specific for D-amino acids is provided in Figure 7, in which the *p*H 4.8 supernatant preparation was preincubated with L-leucine. In contrast to the results in Figures 5 and 6, there was no indication of an accumulation of a labeled intermediate. On the other hand, when experiments were performed with L-leucine and the *p*H 4.8 precipitate, it was found that preincubation with non-isotopic L-amino acid did retard subsequent utilization of L-leucine-C^{14}, as compared to the reverse order of addition, although the effect was much less than that exhibited by the D-isomer (data not included). This result probably reflected L-leucyl-RNA formation.

Dependence of Peptide Synthesis on s-RNA.—Although it was shown (Uemura *et al.*, 1963) that polypeptide formation in cell-free systems was completely blocked by the prior treatment of either ribosomal or soluble fractions with pancreatic ribonuclease, there was no *direct* demonstration that s-RNA was essential in the biosynthetic pathway. The experiments in Table IV provide this evidence. Results for protein synthesis are included for purposes of comparison. Experiment 1 gives reference incorporation values for the usual ribosomal–140,000 $\times$ *g* supernatant system. Experiment 2 shows that the recombination of the *p*H 4.8 precipitate and *p*H 4.8 supernatant fractions, derived from the original soluble supernatant phase, retained almost full polypeptide-synthesizing activity when mixed with ribosomes. This activity was not increased by the further addition of purified s-RNA (experiment 3). However, the *p*H 4.8 precipitate fraction, after passage though a DEAE-cellulose column to remove s-RNA, lost all ability to promote leucine-C^{14} incorporation into tyrothricin or protein (experiment 4). The restoration of s-RNA in experiment 5 resulted in a partial recovery of activity for tyrothricin synthesis. This degree of recovery was not significantly altered by increasing the level of s-RNA (experiment 6) or s-RNA-free *p*H 4.8 fraction (experiment 7). Subsequent experiments (see Table VI) showed that full recovery of activity was possible in reconstituted systems, provided that the various component fractions were processed more rapidly during fractionation. Experiments 8 and 9 of Table IV indicate the essentiality of the RNA-free *p*H 4.8 precipitate and *p*H 4.8 supernatant, respectively, while experiment 10 shows that ribosomal RNA could not replace the usual s-RNA in tyrothricin synthesis.

Localization of the Specificity Control for Peptide Synthesis.—It was previously reported (Okuda *et al.*, 1963b) that the soluble supernatant, rather than the particulate fraction of *B. brevis* extracts, exerted the

TABLE IV
DEPENDENCE OF PEPTIDE SYNTHESIS ON RNA IN RECONSTITUTED SYSTEMS[a]

Experiment No.	Quantity of Component (ml)				C^{14}-Incorporated	
	*p*H 4.8 Precipitate	*p*H 4.8 Super.	RNA-free *p*H 4.8 Precipitate	RNA	Tyrothricin	Protein
1[b]					4150	7870
2	0.1	0.3			3400	7840
3	0.1	0.3		0.1	3590	6700
4		0.3	0.17		0	0
5		0.3	0.17	0.1	1310	5520
6		0.3	0.17	1.0	1300	4650
7		0.3	0.51	0.1	1410	6370
8		0.3		0.1	10	0
9			0.17	0.1	0	0
10		0.3	0.17	0.1[c]	0	0

[a] The preparation of the component fractions is described in the experimental section. In addition, a quantity of ribosomes equivalent to that present in 1 ml of original sonicate was employed; 1.0 μc of DL-leucine-1-C^{14} was used in each experiment. The RNA solution contained 4 mg of purified s-RNA/ml. [b] Control, with 0.5 ml of unfractionated 140,000 × *g* supernatant. [c] Ribosomal RNA substituted for usual s-RNA.

TABLE V
EFFECT OF RNA FROM DIFFERENT SOURCES ON THE TYPE OF POLYPEPTIDE SYNTHESIZED[a]

Experiment No.	RNA		Radioactive Leucine Incorporated (cpm)				
	Source	Quantity (mg)	Total Peptide	Gramicidins	Tyrocidines	Gramicidin S	Protein
1[b]			2075	715	1245		4410
2[c]			2020	735	1570		4070
3[d]			1925	930	1360		3830
4	DU-Tryp	0.4	1965	560	1105		4410
5	DU-Cas	4.0	1670	185	170		3630
6	DU-Cas	8.0	2390	290	400		5805
7	9999	4.0	970	230	440	0	2590
8	9999	8.0	1700	535	910	0	4200

[a] Except where otherwise indicated, the reaction mixture contained the following components, all derived from the Dubos strain of *B. brevis*, and in quantities equivalent to those present in 1 ml of original sonicate: ribosomes, *p*H 4.8 supernatant, and *p*H 4.8 precipitate freed of RNA. DL-Leucine-C^{14} (0.5 μc) was used in each experiment, and the incubation time was 2 hours. DU-Tryp, purified RNA from Dubos cells, grown in usual tryptone medium; DU-Cas, crude RNA from Dubos cells grown in Casamino acid medium; 9999, crude RNA from *B. Brevis* ATCC 9999. [b] Control with whole sonicate; no added ribosomes. [c] Control with 0.5 ml of whole supernatant (plus ribosomes). [d] Untreated *p*H 4.8 precipitate substituted for RNA-free *p*H 4.8 precipitate.

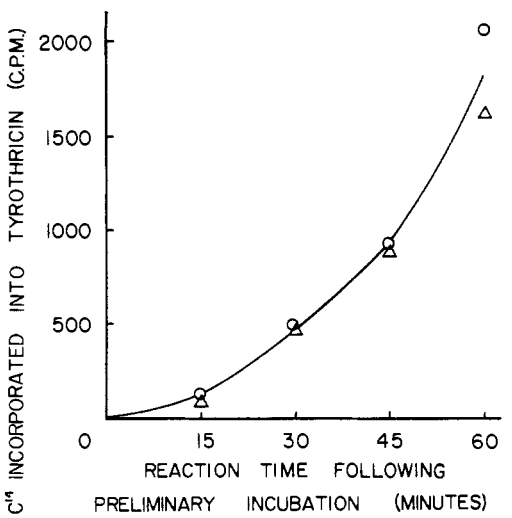

FIG. 7.—Preincubation experiments with L-leucine-C^{14} and *p*H 4.8 supernatant fraction. The quantities of components used were the same as those in Table V. 0.25 μc of L-leucine-1-C^{14} (20 mc/mM) was used per incubation tube. The *p*H 4.8 precipitate fraction plus ribosomes were added after a 30-minute preliminary incubation period. △, preincubation with L-leucine-C^{14}, with 2 mM L-leucine-C^{12} added at zero time; O, preincubation with 2 mM L-leucine-C^{12}, followed by L-leucine-C^{14}.

primary control over the types of peptides synthesized. This conclusion was derived from experiments with combinations of cellular fractions from two different strains of *B. brevis:* ATCC 9999, which produces gramicidin S, and ATCC 8185, which forms Dubos-type gramicidins and tyrocidines. It was of interest to examine this phenomenon more intensively with subfractions derived from the soluble phase of cell extracts.

Table V describes experiments in which different s-RNA preparations were assayed with standard ribosomes, *p*H 4.8 supernatant, and RNA-free *p*H 4.8 precipitate, all derived from the usual Dubos strain cells. Incorporation of DL-leucine-C^{14} into peptide fractions and into protein was measured. Experiments 1, 2, and 3 are controls at different stages of fractionation, which standardize the activity of the regular Dubos system, and reflect the customary distribution of C^{14} among gramicidins, tyrocidines, and protein. Experiment 4 represents a fully reconstituted system, with all components from Dubos cells grown in the usual tryptone-type medium.[4] It may be seen that peptide- and protein-synthesizing activity was well conserved, except that the C^{14} content in gramicidins and tyrocidines was slightly lower than in experiments 1–3. In

[4] The lower radioactivities are simply a consequence of the 2-hour incubation time, as compared to 4 hours in Table IV.

TABLE VI
INFLUENCE OF CELL FRACTIONS FROM DIFFERENT SOURCES ON THE TYPE OF PEPTIDE SYNTHESIZED[a]

Experiment No.	Strain of *B. brevis* Used			Distribution of C^{14} Among Peptide Fractions (%)		
	*p*H 4.8 Supernatant	*p*H 4.8 Precipitate	Ribosomes	Gramicidins	Tyrocidines	Gramicidin S
1	Dubos	Dubos	Dubos	36	61	3
2	Dubos	9999	Dubos	27	70	3
3	Dubos	Dubos	9999	19	80	1
4	Dubos	9999	9999	31	66	3
5	9999	9999	9999	9	15	76
6	9999	Dubos	Dubos	7	16	77
7	9999	9999	Dubos	9	16	75
8	9999	Dubos	9999	2	22	76

[a] All components were employed in quantities equivalent to those present in 1 ml of original sonicate. DL-Leucine-1-C^{14} (0.5 μc) was used in each experiment.

experiments 5 and 6 the s-RNA was derived from Dubos cells grown in a Casamino acid medium, which results in very low production of peptides by the culture (Okuda *et al.*, 1963a). While the degree of labeling in protein and tyrothricin was not too greatly altered, a curious finding was the markedly reduced recoveries of radioactivity in gramicidins and tyrocidines in these two cases. The explanation for these low yields is not presently known. The absence of labeling in the gramicidin S fraction in experiments 7 and 8 demonstrates that the factor controlling specificity of peptide synthesis was *not* localized in the phenol-extracted RNA. Labeling was found instead in Dubos gramicidins and tyrocidines (as well as in protein).

In the further examination of the roles of the cellular component, various combinations of ribosomes, *p*H 4.8 precipitate, and *p*H 4.8 supernatant from the two different *B. brevis* strains were assayed with isotopic leucine (Table VI). The C^{14} recovered in the three peptide fractions after paper electrophoresis is expressed on a percentage basis. In experiment 1, with all components originating from the Dubos strain of *B. brevis*, the radioactivity in the gramicidin S may be considered negligible. The same results were obtained in experiments 2–4, in which the *p*H 4.8 supernatant was derived from Dubos cells, and the other two fractions from either Dubos or ATCC 9999 strains.

When assays were performed with all three cellular fractions originating from ATCC 9999 cells, most of the recovered radioactivity was in gramicidin S, as expected (experiment 5).[5] In experiments 6-8, with *p*H 4.8 supernatant in each case from ATCC 9999 cells, the C^{14} was again found chiefly in gramicidin S, regardless of the source of the *p*H 4.8 precipitate or the ribosomes. Accordingly, it appears that the *p*H 4.8 supernatant contains one or more factors, other than the RNA extracted by the phenol method, which govern the specificity of peptide synthesis.

DISCUSSION

The exchange reaction between inorganic pyrophosphate and ATP in *B. brevis* extracts was found to be stimulated by a variety of L- and D-amino acids, including some which do not occur in gramicidin or tyrocidine molecules. However, it seems reasonable to assume that amino acid adenylate formation, which is reflected in the exchange process, represents a primary step in gramicidin and tyrocidine synthesis (as is generally assumed for protein biosynthesis). It may be mentioned also, at this point, that an additional indication of adenyl amino acid formation was the observation that significant proportions of C^{14}-labeled D-amino acids were converted into a charcoal-absorbable form, following incubation of the superantant fraction in the absence of ribosomal particles. When ribosomes were introduced into the system, the radioactive charcoal-absorbable fraction disappeared.

The ease of incorporation of isotopic L-amino acids into s-RNA (Fig. 2) and the extensive utilization of labeled L-aminoacyl-s-RNA for polypeptide synthesis (Fig. 4) are significant observations in interpreting the pathway of gramicidin and tyrocidine biosynthesis. The inability of an excess of free C^{12}-L-amino acid to inhibit the incorporation of the corresponding s-RNA-bound C^{14}-L-amino acid into polypeptides supports the view that the aminoacyl-s-RNA functioned as the intact complex (Fig. 4). Added confirmation of the essentiality of s-RNA was the demonstration (Table IV) that reconstituted systems exhibited an absolute requirement for this substance.

The present evidence (Fig. 3 and Table III), together with that previously reported (Uemura *et al.*, 1963), suggests that glutamic acid was used preferentially over glutamine, in tyrocidine formation. However, the demonstration of a direct utilization of either L-glutamyl- or L-glutaminyl-s-RNA for polypeptide synthesis has not yet been attempted. Also, the path of L-ornithine incorporation into tyrocidine (or gramicidin S) remains to be clarified.

It was previously found (Okuda *et al.*, 1963b) that labeled D-amino acids were well incorporated into gramicidins and tyrocidines in unfractionated *B. brevis* systems, and an analysis of the isolated polypeptides showed that virtually all the radioactivity could be ascribed to D-amino acid residues. However, the elucidation of the mechanism by which D-amino acids were introduced into peptide chains has proved difficult. While these D-isomers did incorporate to some extent into s-RNA, it is evident that L-amino acids had a much greater affinity for s-RNA (Table II and Fig. 2). It may also be suggested that L-amino acid competitively inhibited the D-amino acid–activating enzyme. In any event, the observation that a small proportion of a nonlabeled L-amino acid *completely blocked* the incorporation of the corresponding radioactive D-form into s-RNA throws much doubt on the possibility of an acceptor-transfer role for s-RNA in D-amino acid utilization. In fact, the data of Figure 6 confirm this suspicion. The *p*H 4.8 supernatant fraction, which was essentially devoid of s-RNA (as was shown in Table IV), but which still contained a considerable quantity of D-leucine-activating enzyme (Table I), was equally effective as the whole 140,000 $\times$ *g* soluble phase (Fig.

[5] The minor quantities of C^{14} found in gramicidin and tyrocidine fractions in experiments 5–8 reflect incomplete separations owing to an inadequate time interval in electrophoresis.

5) in promoting the formation of a D-amino acid intermediate. The *p*H 4.8 precipitate, which contained the s-RNA, did not respond to the assay for this postulated intermediate. The control experiment with L-leucine (Fig. 7) supports our view that the factor in the *p*H 4.8 supernatant fraction is concerned with D-, but not with L-amino acid utilization. Additional research is proceeding, with the aim of isolating and characterizing the carrier of activated D-amino acids.

The experiments in Table IV show that soluble components in addition to s-RNA were necessary for polypeptide synthesis. These components may have included the postulated factor in D-amino acid transfer, and quite possibly messenger RNA. It is conceivable that a special form of m-RNA was the agent which controlled the specificity of peptide synthesis (Tables V and VI). Much of our future effort will be centered about the study of this problem.

As in protein biosynthesis, the exact role of the ribosomes in polypeptide formation requires additional investigation. One particularly intriguing question is whether there exist in *B. brevis* cells unique ribosomes concerned with only gramicidin or tyrocidine (and not protein) synthesis. In view of the great diversity of polypeptides in nature, it will be of much interest to investigate intensively the mode of origin of this class of substances in various organisms, and to compare the biosynthetic mechanisms with those of proteins.

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Further Aspects of Gramicidin and Tyrocidine Biosynthesis in the Cell-free System of *Bacillus brevis**

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Received July 12, 1963

In studying the incorporation of labeled leucine into gramicidins and tyrocidines in cell-free preparations derived from sonicates of *Bacillus brevis* it was concluded that the specificity of peptide biosynthesis resided chiefly in the soluble components of the system, rather than in the ribosomal particles. D-Valine-C^{14} appeared to be incorporated directly into gramicidins, rather than derived from L-valine at a later stage in polypeptide formation. No uncoupling of protein from peptide biosynthesis could be obtained with puromycin, chloramphenicol, or penicillin. However, on prolonged storage at $-20°$ the soluble fraction of the sonicate lost its peptide-synthesizing activity more rapidly than its capacity for protein biosynthesis. A net increase in tyrocidine content could be demonstrated by antibiotic assays, following incubation of the sonic extracts with amino acid mixture, ATP, and cofactors.

A preceding paper (Uemura *et al.*, 1963) has described the incorporation of isotopic amino acids into gramicidins and tyrocidines in sonic extracts, derived from washed cells of *Bacillus brevis* (Dubos-Hotchkiss strain). It was demonstrated that both the soluble phase and the ribosomal particles are involved in this process. In addition to ATP and magnesium ions, glutathione and an appropriate amino acid mixture were required for optimum activity.

Before undertaking a detailed examination of the pathway and mechanism of biosynthesis, it was desired to elucidate several additional characteristics of the cell-free system concerned with polypeptide formation, and to compare this process with protein biosynthesis. The aspects considered in the present paper include the stability of the subcellular components, their specificity of biosynthesis as studied with preparations from different sources, the utilization of D-amino acids, the action of certain antibiotics, and the measurement of net polypeptide synthesis.

* Supported by a research grant (GM09335) from the National Institutes of Health. Contribution No. 004 from the Pacific Biomedical Research Center, University of Hawaii.

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‡ Research performed in partial fulfillment of requirements for the degree of Doctor of Philosophy in Biochemistry, University of Hawaii.

Experimental Procedures

Materials.—The biochemicals and radioactive amino acids were those previously described (Uemura *et al.*, 1963). In addition, the following substances were employed: hog kidney D-amino acid oxidase and snake venom L-amino acid oxidase, both from Worthington Biochemical Corporation; puromycin from Nutritional Biochemicals Corporation; and chloramphenicol from Parke Davis and Company.