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.

References

Berg, P., Bergmann, F. H., Ofengand, E. J., and Dieckmann, M. (1961), *J. Biol. Chem. 236*, 1726.

Brunngraber, E. F. (1962), *Biochem. Biophys. Res. Commun. 8*, 1.

Coles, N., and Meister, A. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1602.

Lane, B. G., and Lipmann, F. (1961), *J. Biol. Chem. 236*, PC80.

Leonard, C. G., and Housewright, R. D. (1963), *Biochim. Biophys. Acta 73*, 531.

Mach, B., Reich, E., and Tatum, E. L. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 175.

Okuda, K., Edwards, G. C., and Winnick, T. (1963a), *J. Bacteriol. 85*, 329.

Okuda, K., Saito, Y., and Otani, S. (1960), *J. Japan. Biochem. Soc. [Seikagaku] 32*, 650.

Okuda, K., Uemura, I., Bodley, J. W., and Winnick, T. (1963b), *Biochemistry 2*, 719.

Strominger, J. L. (1962), *Fed. Proc. 21*, 134.

Stulberg, M. P., and Novelli, G. D. (1962), *in* Methods in Enzymology, Vol. V, Colowick, S. P., and Kaplan, N. O., eds., New York, Academic, p. 703.

Uemura, I., Okuda, K., and Winnick, T. (1963), *Biochemistry, 2*, 719.

Von der Decken, A., and Campbell, P. N. (1962), *Biochem. J. 82*, 448.

Winnick, R. E., and Winnick, T. (1961), *Biochim. Biophys. Acta 53*, 461.

Zamecnik, P. C. (1962), *Biochem. J. 85*, 257.

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

K. OKUDA,† I. UEMURA,† J. W. BODLEY,‡ and T. WINNICK

From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu

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.

† On leave of absence from the Osaka City University Medical School, Osaka, Japan.

‡ 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.

Preparation of D-*Valine.*—The method was that of Katz and Weissbach (1963), starting with DL-valine-1-C^{14} (6 $\mu c/\mu M$), and involved the destruction of the L-component with snake venom oxidase. The product, after the purification by Dowex-column chromatography, was subjected to an extra purification step, consisting of paper chromatography with *n*-butanol–acetic acid–water, 75:15:10.

Bacterial Cultures.—The Dubos-Hotchkiss strain (ATCC 8185) of *B. brevis*, which produces mixtures of gramicidins and tyrocidines, was the usual source of cellular materials. The organism was generally cultivated in a tryptone–yeast extract–mineral salt medium, which promotes high yields of polypeptides (Okuda *et al.*, 1963). However, in one experiment the cells were derived from a medium referred to as "deficient," in which Casamino acids (Difco Laboratories) replaced the tryptone (Okuda *et al.*, 1963). Such cultures grow at the usual rate, but produce very low concentrations of gramicidins and tyrocidines. Use was also made of the variety of *B. brevis* (ATCC 9999) which forms gramicidin S (Winnick *et al.*, 1961).

Cellular Components.—A sonicate of washed *B. brevis* cells was prepared and freed of debris, as previously described (Uemura *et al.*, 1963). This extract, referred to as the complete sonicate system, was used in some incubation experiments. In other instances, ribosomes plus the 140,000 $\times$ *g* supernatant phase were employed instead (Uemura *et al.*, 1963).

Rat Liver Cellular Fractions.—The procedure was carried out at 0°. The liver was homogenized (glass-Teflon Potter homogenizer) with 10 parts of 0.25 M sucrose containing 0.005 M $MgCl_2$. The homogenate was centrifuged at 15,000 $\times$ *g* for 15 minutes. The sediment was discarded. The supernatant phase was then recentrifuged at 140,000 $\times$ *g* for 1 hour. The microsomal pellet and the soluble supernatant solution were used separately in certain experiments.

Amino Acid Incorporation into Peptides and Protein.—The assay method utilized either the complete sonicate, or the ribosomal–140,000 $\times$ *g* supernatant system, with isotopic leucine or valine in the standard reaction mixture (Uemura *et al.*, 1963). The latter contained $MgCl_2$, ATP, glutathione, phosphoenolpyruvate, pyruvic kinase, phosphate buffer, and amino acid mixture. Unless otherwise stated, the incubation time was 4 hours, and 0.5 μc of C^{14} was used per tube. In some experiments the radioactivity of the isolated tyrothrycin was measured. In other cases this peptide mixture was further resolved into gramicidins and tyrocidines by high voltage paper electrophoresis, and the C^{14} content of each of these two polypeptide fractions was determined. The labeling of the protein was also measured.

Isolation of Gramicidin S.—The radioactive peptide was first precipitated with the aid of 1 mg of carrier, as in the tyrothricin isolation procedure. Subsequently the gramicidin S was purified by high voltage paper electrophoresis. This relatively basic peptide migrated more rapidly toward the cathode (4–5 cm in 1.5 hours) than did the tyrocidine peptides, and could be easily distinguished from the latter (and also from neutral Dubos gramicidins, which did not migrate significantly). Lastly, the gramicidine S region, after paper electrophoresis, was eluted and radioactivity was counted in the usual manner (Uemura *et al.*, 1963).

Determination of Optical Configuration of Labeled D-*Valine in Gramicidins.*—A sample of the polypeptide fraction (derived from incubation experiments with D-Valine-C^{14}) was hydrolyzed for 48 hours at 110° with glacial acetic acid–concentrated HCl, 1:1. After removal of solvent, the hydrolysate was treated with 5 mg of L-amino acid oxidase for 18 hours at 37°. The solution was deproteinized with 5% trichloroacetic acid, and extracted with ether (to remove trichloroacetic acid and ketoacids). The solution was then transferred to a Dowex 50-X8 column. The latter was washed with 0.1 M HCl. Finally, the radioactive valine was eluted with 1 M NH_4OH, and the effluent was dried and radioactivity was counted for C^{14}.

Bioassay of Polypeptide Synthesis.—The complete sonicate system was employed at five times the usual scale, i.e., with 10 ml of incubation mixture. The gramicidin and tyrocidine fractions (isolated by paper electrophoresis) were assayed antibiotically against *S. faecalis* ATCC 10541. These determinations were performed by the Wallerstein Company, through the kind cooperation of Drs. E. J. Beckhorn and D. M. Roger.

Intracellular Amino Acid Concentration.—Washed cells were suspended in 20 volumes of distilled water and heated for 30 minutes at 100°. The coagulated solids were removed by centrifugation, and the filtrate was concentrated prior to analysis. Total amino acids were determined by the method of Troll and Cannan (1953), while D-amino acids were measured by oxygen consumption, following 10-hour treatment of samples with D-amino acid oxidase in the Warburg manometric apparatus.

Results

Stability of the B. brevis Preparations.—When washed intact cells were stored for 4 days at −20° and then used for the preparation of sonicates, it was found that approximately 50% of the activity for DL-leucine-C^{14} incorporation into either gramicidins or tyrocidines was lost. After a 14-day storage period, the frozen cells retained only about 10% of their initial activity.

Low-temperature storage tests were also performed on isolated ribosomes and 140,000 $\times$ *g* supernatant solution. The ability of these fractions to promote peptide and protein biosynthesis after varying periods at −20° is shown in Figure 1. It may be seen that

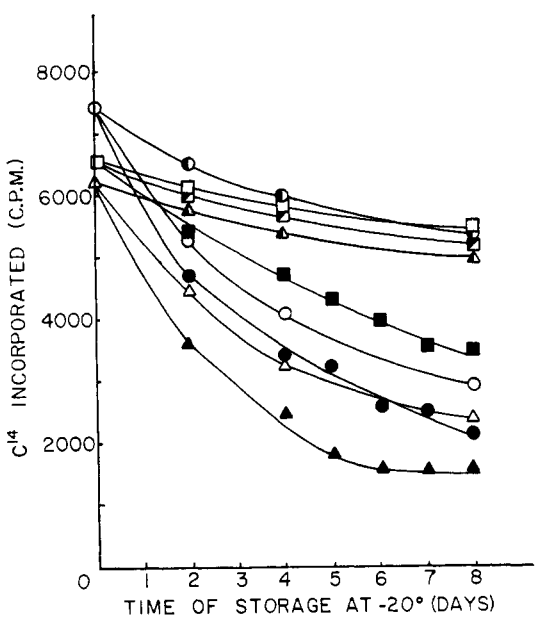

FIG. 1.—Loss of activities on storage of cellular components at −20°. Circles, tyrocidin synthesis; triangles, gramicidin synthesis; squares, protein synthesis. Open symbols, freshly prepared ribosomes plus stored 140,000 $\times$ *g* supernatant; half solid symbols, stored ribosomes plus fresh 140,000 $\times$ *g* supernatant; solid symbols, both ribosomes and supernatant stored. DL-Leucine-1-C^{14} was employed in the assays.

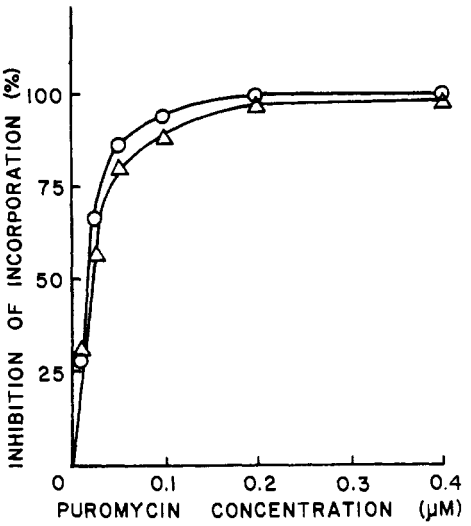

FIG. 2.—Effect of varying puromycin concentration on the incorporation of DL-leucine-1-C^{14} into tyrothricin (△) and protein (○). The whole sonicate system was employed.

aged supernatant, assayed with fresh ribosomes, retained less than 40% of its activity for gramicidin or tyrocidine biosynthesis after 8 days, although still 85% as active as initially, for protein synthesis. In the reverse situation (with stored ribosomes and fresh supernatant), the activities for tyrocidine, gramicidin, and protein synthesis were conserved to comparable extents, namely, 72%, 80%, and 81%, respectively, after 8 days. With both the ribosomal and the supernatant fractions stored (separately) at −20°, the assays after 8 days showed that the incorporation of isotopic leucine into tyrocidine, gramicidin, and protein was 29%, 24%, and 54%, respectively, as high as with fresh preparations. Apparently, certain of the soluble components were more labile with respect to polypeptide, as compared to protein biosynthesis.

It may be mentioned that the soluble supernatant fraction lost peptide-synthesizing activity upon dialysis against water. Reactivation could be effected by addition of a protein-free extract of whole cells. The nature of the dialyzable components is being investigated.

Effect of Antibiotics on Peptide and Protein Biosynthesis.—In further attempts to uncouple the processes of polypeptide and protein synthesis, three antibiotic substances were tested (Table I). Penicillin, which acts primarily to block cell-wall synthesis (Park, 1958), depressed the incorporation of labeled valine into both tyrothricin and protein by approximately one-third (at 15 μg/ml). However, both chloramphenicol and puromycin virtually abolished polypeptide, as well as protein synthesis. Chloramphenicol is believed to inhibit the transfer of amino acids from s-RNA to ribosomal protein, while puromycin appears to interrupt the growth of the polypeptide chain (Nathans *et al.*, 1962). The effect of varying concentration of puromycin on C^{14}-amino acid incorporation into polypeptide and protein is given in Figure 2. It can be seen that both incorporation processes were affected in a very similar manner, and that complete inhibition was achieved at about 200 mμmoles/ml. It may be mentioned that small amounts of radioactivity were detected in several regions after paper electrophoresis runs on protein- and polypeptide-free extracts of the reaction mixtures after incubation. However, more work would be needed to demonstrate "incomplete" peptides (Zamecnik, 1962), if such indeed resulted from the action of puromycin in the *B. brevis* cell-free system.

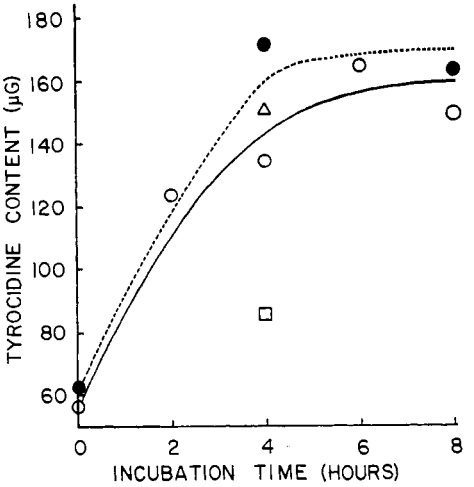

FIG. 3.—Net synthesis of tyrocidines in the whole sonicate system, as measured by bioassay of antibiotic activity. The peptide content is expressed in terms of standard conditions, i.e., a volume of 2 ml. ○, tyrocidines (solid line); △, tyrocidines, with omission of component gramicidin amino acids; □, tyrocidines, with tyrocidine component acids omitted; ●, tyrocidines extracted from ribosomes, which were sedimented and washed after the incubation period (broken line).

TABLE I

EFFECTS OF ANTIBIOTICS ON AMINO ACID INCORPORATION INTO PEPTIDE AND PROTEIN[a]

Antibiotic Added	Concentration (μg/ml)	Radioactivity (cpm) Tyrothricin	Protein
None	—	3210	9200
Penicillin G	7.5	2660	6400
Penicillin G	15	2340	6100
Chloramphenicol	7.5	65	115
Chloramphenicol	15.0	90	50
Puromycin	200	80	60

[a] The whole sonicate system was employed. The labeled amino acid was DL-valine-1-C^{14}.

Net Synthesis of Polypeptides.—The bioassay method against *S. faecalis* was used to demonstrate an increase in tyrocidine content of the sonicate system from approximately 60 to 160 μg in about 6 hours (Fig. 3). As in radioactivity measurements (Uemura *et al.*, 1963), the antibiotic activity remained associated with the ribosomal fraction. It may be seen that the omission of the gramicidin group of amino acids from the incubation mixture had no effect on tyrocidine synthesis. On the other hand, when the component tyrocidine amino acids were withheld net tyrocidine production was reduced by more than two-thirds. A definite (3- to 4-fold) net synthesis of gramicidins (the less abundant peptide fraction) was also achieved, but the data were less accurate, and have not been included in Figure 3.

Utilization of D-*Amino Acids.*—It was of interest to demonstrate the existence of a pool of D-amino acids in *B. brevis* cells. In Figure 4 it may be seen that the total concentration of intracellular amino acids (plus other ninhydrin-reactive substances) rose parallel to the growth rate of the cultures. With the cessation of growth the amino acid level fell rapidly. The production of D-amino acids lagged somewhat initially, but reached a peak at about the same time as that for total amino acids. This point also corresponds to a high level of polypeptide synthesis in the cells (Okuda *et al.*, 1963). Thereafter the D-amino acid concentration slowly declined.

TABLE II

UTILIZATION OF L- AND D-VALINE C^{14} FOR PEPTIDE AND PROTEIN SYNTHESIS[a]

Experiment Number	Isotopic Amino Acid	Non-labeled Isomer Added	Radioactivity (c.p.m.)							
				Tyrocidines			Gramicidins			
				Unhydrolyzed	Hydrolysate		Unhydrolyzed	Hydrolysate		
			Tyrothricin		L-Oxidase	D-Oxidase		L-Oxidase	D-Oxidase	Protein
1	L-Valine-U-C^{14}	None	4630	2740	40	1910	1870	40	1730	1070
2	L-Valine-U-C^{14}	2 μmoles D-valine	4860	2800	50	1785	1845	40	1970	1220
3	D-Valine-1-C^{14}	None	2090	40	60	70	1890	1930	40	60
4	D-Valine-1-C^{14}	3 μmoles L-valine	1870	30	80	90	1740	2160	50	20

[a] The whole sonicate system was employed in 2-hour incubations. C^{14}-amino acid (0.5 μc), representing 0.05–0.10 μmole, was used in each experiment.

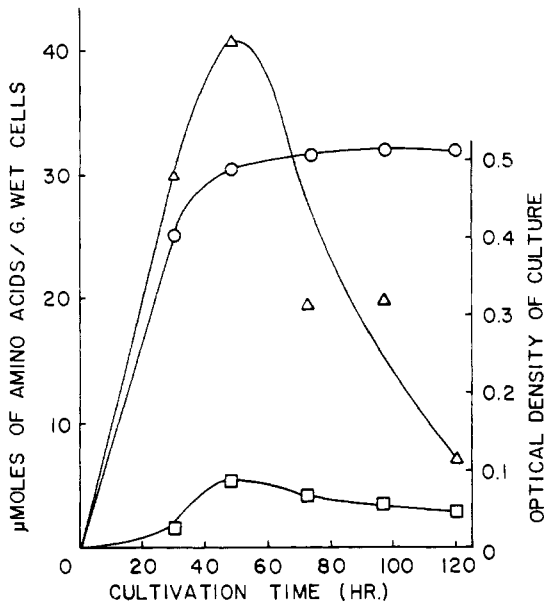

FIG. 4.—Amino acid levels during cultivation of *B. brevis* cells. O, optical density of culture; △, total free amino acids (sum of D- and L-); □, D-amino acids.

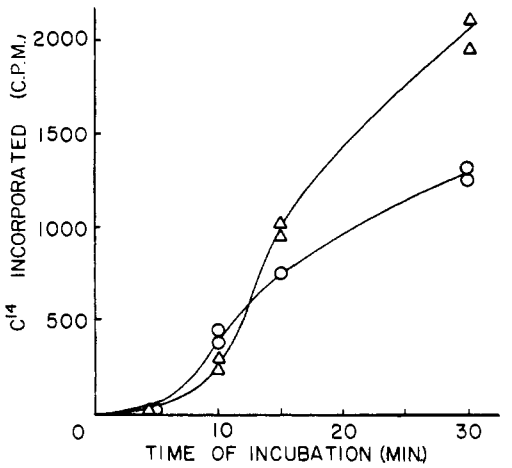

FIG. 5.—Rates of incorporation of D-valine-1-C^{14} (O) and L-valine-1-C^{14} (△) into tyrothricin. The whole sonicate system was employed.

The presence of a sizable quantity of D-amino acids (at least one-seventh of the total amino acid pool in *B. brevis*) suggests that these D-isomers were available for direct utilization in gramicidin and tyrocidine synthesis. Previously (Uemura *et al.*, 1963) it was shown that tritiated D-valine and D-phenylalanine were readily used for polypeptide formation, although not for protein biosynthesis. In Figure 5 the rates of incorporation of C^{14}-labeled D- and L-valine into polypeptides are compared. The L-isomer was utilized to a somewhat greater extent than the D-enantiomorph in 30 minutes. It is possible that the initial lags reflect delays associated with intermediate stages in the pathway of peptide formation.

The above results with isotopic L- and D-amino acids do not actually reveal the nature of the labeling in the final products. The experiments in Table II were designed to answer this question. Experiment 1 shows that L-valine was utilized for both protein and polypeptide synthesis, and that radioactivity of the tyrothricin was quantitatively recovered in tyrocidines plus gramicidins. The C^{14}-valine in hydrolysates of these peptide fractions was found to be relatively resistant to D-amino acid oxidase, but was almost completely decarboxylated by the L-oxidase. This showed that little, if any, inversion had occurred. In experiment 2, the presence of a comparatively large excess of non-isotopic D-valine had no effect on the utilization of the L-valine-C-^{14}. When D-valine-C^{14} was employed in the incubation mixture (experiment 3), very little C^{14} was found in protein, and almost all the tyrothricin-C^{14} was recovered in gramicidins. Treatment of the hydrolyzed gramicidins with oxidases revealed that the radioactive valine was virtually all in the D form. The absence of significant labeling in tyrocidines is in agreement with the fact that these peptides do not contain D-valine in their molecules. (King and Craig, 1955). The last experiment in Table II shows that a relatively large quantity of L-valine did not markedly diminish the utilization of D-valine-C^{14} for gramicidin synthesis. This observation confirms the optical purity of the isotopic D-amino acid.

Combinations of Ribosomal and Supernatant Fractions from Different Sources.—The experiments in Table III were performed in order to determine whether the specificity of peptide biosynthesis resided in the particulate or in the soluble phase of the *B. brevis* system. The reference experiment (number 1), with ribosomes and supernatant phase both derived from the Dubos strain (cultivated under optimum conditions), showed typically good incorporation of labeled leucine into gramicidins, tyrocidines, and protein. The gramicidin S region shown by paper electrophoresis had insignificant radioactivity. When the standard ribosomes were used with supernatant derived from cells grown in medium containing Casamino acids (experiment 2), the C^{14} uptake into polypeptides was reduced by about 90%, while protein synthesis was less affected. It had been previously shown (Okuda *et al.*, 1963) that gramicidin and tyrocidine formation was greatly depressed in cultures containing Casamino acids (in place

TABLE III

INFLUENCE OF THE SOLUBLE CELLULAR COMPONENTS ON THE BIOSYNTHESIS OF POLYPEPTIDES[a]

Experiment Number	Source of Ribosomes	Source of 140,000 × *g* Supernatant	DL-Leucine-1-C^{14} Incorporated (cpm)			
			Gram.	Tyroc.	Gram. S.	Protein
1	DU-Tryp	DU-Tryp	4730	5700	20	5850
2	DU-Tryp	DU-Cas	530	460	0	3700
3	DU-Cas	DU-Tryp	5050	3810	0	4760
4	DU-Tryp	9999	40	500	4360	4800
5	9999	DU-Tryp	4930	5730	50	4710
6	DU-Tryp	Rat liver	0	10		100
7	Rat liver[b]	DU-Tryp	50	30		0

[a] DU-Tryp, *B. brevis* Dubos strain grown on tryptone medium; DU-Cas, *B. brevis* Dubos grown on Casamino acid medium; 9999, *B. brevis* ATCC 9999 strain. [b] Microsomal preparation.

of tryptone). Experiment 3 indicates that polypeptide synthesis was almost normal when ribosomes from the deficient medium were combined with the standard supernatant. Experiment 4 demonstrates that a combination of Dubos-strain ribosomes and supernatant from ATCC 9999 cells resulted in the extensive incorporation of leucine-C^{14} into gramicidin S (as well as into protein); however, no biosynthesis of Dubos gramicidins occurred, while labeling in tyrocidines was rather low. In the reverse situation, with ATCC 9999 ribosomes and Dubos-strain supernatant (experiment 5), gramicidin and tyrocidine synthesis was normal, but no isotope appeared in gramicidin S. Protein synthesis remained high. As might be expected, rat liver preparations were ineffective when combined with *B. brevis* fractions (experiments 6 and 7).

DISCUSSION

A preceding paper (Uemura *et al.*, 1963) stressed a broad resemblance of the process of gramicidin and tyrocidine formation to that of protein biosynthesis: the common requirement for both particulate and soluble cellular components; the stimulation by ATP, magnesium ions, glutathione, and amino acid mixture; and the binding of the reaction products to ribosomes. Generally speaking the present results strengthen this similarity. Both protein and polypeptide synthesis in the *B. brevis* preparations were partially inhibited by penicillin, and completely suppressed by either chloramphenicol or puromycin. On storage at −20°, both protein- and peptide-synthesizing activities were slowly lost by ribosomes and by the supernatant phase, although the rates differed somewhat for the two processes. In demonstrating *de novo* synthesis of tyrocidine by net increase in antibacterial activity (against *S. faecalis*), the newly formed peptide was shown to be associated with ribosomes. This situation is generally observed in protein biosynthesis.

The previous (minimal) estimate of 65 μg of tyrocidines synthesized in 4 hours, based purely upon radioactivity measurements (Uemura *et al.*, 1963), agrees reasonably well with the value of 90 μg calculated from the bioassays of Figure 3. There have been reports of net protein (enzyme) synthesis in cell-free systems, notably for β-galactosidase (Kameyama and Novelli, 1962). To the best of our knowledge, the data in Figure 3 represent the first such instance in the case of natural polypeptides.

With reference to the mode of introduction of D-amino acid residues into bacterial peptide antibiotics, Arnstein and Margreiter (1958) proposed that the conversion of L- to D-valine during penicillin biogenesis in *Penicillium chrysogenum* may occur after synthesis of the L-cystinyl-L-valine peptide. Katz and Weissbach (1963) suggested that, in a similar fashion, L-valine might form a peptide with L-threonine or L-proline during actinomycin synthesis in *Streptomyces antibioticus*, and that in a subsequent reaction inversion of the configuration of L-valine might occur. On the other hand, Ito and Strominger (1960) showed that D-glutamic acid and D-alanyl-D-alanine were introduced as such into the uridine nucleotide peptide of *Staphylococcus aureus*. While the mode of origin of the D-amino acids within the *B. brevis* cell has not yet been determined, the present results with the cell-free system suggest strongly that D-valine is directly incorporated into polypeptide linkage, as is the case with D components of the uridine peptide.

In earlier work with growing cultures of *B. brevis* ATCC 9999 (Winnick and Winnick, 1961), unsuccessful attempts were made to detect accumulated intermediate peptide precursors of gramicidin S in media containing amino acid analogs. In the present study, two labeled pentapeptides were tested in the cell-free system to determine whether these substances could be utilized for polypeptide synthesis without prior hydrolysis. The peptides were tritiated L-val-L-orn-L-leu-D-phe-L-pro,[1] and L-orn-L-leu-D-phe-L-pro-L-val-C^{14} (prepared by the action of nagarse enzyme on valine-C^{14}-labeled gramicidin S (Yukioka, 1962). However, neither pentapeptide was utilized to a significant extent (data not included in this paper).

In studying the specificity of hemoglobin synthesis in cell-free systems of reticulocytes of different species, Lamfrom (1961) concluded that the microsomal component was important in determining the type of protein synthesized. The "*p*H 5" fraction was not species specific. However, a soluble supernatant fraction, possibly messenger RNA, also influenced the type of hemoglobin formed in mixed preparations. The present results with the two different strains of *B. brevis* (Table III) suggest that the specificity resides largely in the supernatant phase and while it would be premature to stress m-RNA, it is possible that this substance is implicated. The next paper of this series will deal with the detailed mechanism of gramicidin and tyrocidine formation, with emphasis on the role of the soluble components of the *B. brevis* system, including RNA.

ADDENDUM

Since this work was submitted for publication, a paper (B. Mach, E. Reich, and E. L. Tatum (1963), *Proc. Nat. Acad. Sci. U.S., 50,* 175) has come to our attention in which the authors report on the lack of inhibition by puromycin and chloramphenicol of tyrocidine synthesis in cultures of *B. brevis* ATCC 10068. In view of this apparent contradiction, the antibiotic

[1] A sample of the synthetic peptide was kindly supplied by Dr. T. S. Work, National Institute for Medical Research, Mill Hill, London.

data reported here was reinvestigated, both with the cell-free system and growing cultures of *B. brevis* ATCC 8185. In both cases more than 98% inhibition of peptide and protein synthesis was observed with 10 μg/ml chloramphenicol and 100 μg/ml puromycin. Moreover, the curves for inhibition of biosynthesis with varying concentrations of puromycin and chloramphenicol were virtually identical for both protein and tyrothricin in both types of experiments. The reason for the discrepancy between the present results and those of Mach and co-workers is not presently understood, but further work is in progress to solve this question.

References

Arnstein, H. R. V., and Margreiter, H. (1958), *Biochem. J. 68*, 339.

Ito, E., and Strominger, J. L. (1960), *J. Biol. Chem. 235*, PC7.

Kameyama, T., and Novelli, G. D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 659.

Katz, E., and Weissbach, H. (1963), *J. Biol. Chem. 238*, 666.

King, T. P., and Craig, L. C. (1955), *J. Am. Chem. Soc. 77*, 6627.

Lamfrom, H. (1961), *J. Mol. Biol. 3*, 241.

Nathans, D., von Ehrenstein, G., Monro, R., and Lipmann, F. (1962), *Fed. Proc. 21*, 127.

Okuda, K., Edwards, G. C., and Winnick, T. (1963), *J. Bacteriol. 85*, 329.

Park, J. T. (1958), *in* The Strategy of Chemotherapy, Society for General Microbiology, 8th Symposium, Cambridge, Cambridge University Press, p. 49.

Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.

Uemura, I., Okuda, K., and Winnick, T. (1963), *Biochemistry 2*, 719.

Winnick, R. E., Lis, H., and Winnick, T. (1961), *Biochim. Biophys. Acta 49*, 451.

Winnick, R. E., and Winnick, T. (1961), *Biochim. Biophys. Acta 53*, 461.

Yukioka, M. (1962), *J. Osaka City Med. Center [Osaka Shiritsu Daigaku Igaku Zasshi] 11*, 239.

Zamecnik, P. C. (1962), *Biochem. J. 85*, 257.

Immunochemical Studies on Blood Groups XXX. Cleavage of A, B, and H Blood-Group Substances by Alkali*

GERALD SCHIFFMAN,† ELVIN A. KABAT, AND WILLIAM THOMPSON

From the Departments of Microbiology and Neurology, College of Physicians and Surgeons, Columbia University and the Neurological Institute, Presbyterian Hospital, New York

Received August 7, 1963

Treatment of A, B, and H substances with 0.2 M NaOH in 1% $NaBH_4$ at room temperature yields dialyzable fragments with high blood-group activity. Partial purification of these materials has been accomplished by paper chromatography; active fractions were found in three regions. The most rapidly migrating active fraction from A and B substances was many times more potent in inhibiting A-anti-A or B-anti-B precipitation, respectively, than the most active oligosaccharides previously studied. While further purification and characterization of these fragments is necessary, evidence is presented indicating that they probably contain the entire antigenic determinant.

Success in isolating the antigenic determinants of blood group substances will depend in large measure on finding a procedure which will maintain their integrity but cleave the bonds holding them to the rest of the molecule. Methods involving enzymes (Buchanan *et al.*, 1957; Howe and Kabat, 1953; Iseki *et al.*, 1959; Iseki and Ikeda, 1956; Iseki and Masaki, 1953; Schiffman *et al.*, 1958; Watkins, 1953, 1956, 1960, 1962; Watkins and Morgan, 1955; Zarnitz and Kabat, 1960; for earlier work see Kabat, 1956), acid (Cheese and Morgan, 1961; Côté and Morgan, 1956; Kabat *et al.*, 1946; Kabat and Leskowitz, 1955; Kuhn and Kirchenlohr, 1954; Schiffman *et al.*, 1960; Schiffman and Kabat, 1961; Tomarelli *et al.*, 1954; Yosizawa, 1949; for other earlier work see Kabat, 1956), alkali (Morgan, 1944, 1946; Knox and Morgan, 1954), hydrazine (Yosizawa, 1961, 1962a,b; Yosizawa and Sato, 1962) and resins (Painter, 1960; Painter and Morgan, 1961a,b; Painter *et al.*, 1962) have been used but no thoroughly satisfactory procedure has yet been reported. However, much useful information about the determinants has been accumulated, largely about terminal nonreducing sequences of sugar residues associated with blood group A, B, and H activity (Watkins, 1962; Morgan, 1960; Kabat, 1956) and with cross reactivity with type XIV antipneumococcal serum (Howe and Kabat, 1953; Watkins, 1953; Howe *et al.*, 1958; see also Kabat, 1956). During the course of these studies a considerable number of active and inactive oligosaccharides has been isolated (Côté and Morgan, 1956; Cheese and Morgan, 1961; Painter and Morgan, 1961a,b; Schiffman *et al.*, 1960; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962a; Painter *et al.*, 1962; Yosizawa, 1949; for other earlier work see Kabat, 1956).

With blood group A substance, *N*-acetylgalactosamine is considered to be the terminal nonreducing end of the antigenic determinant (Watkins and Morgan, 1955; Kabat and Leskowitz, 1955; see also Kabat, 1956) and α-*N*-acetylgalactosaminoyl(1 → 3) galactose, a disaccharide more active than *N*-acetylgalactosamine in inhibiting A-anti-A precipitation and hemagglutination is accepted as a disaccharide sequence of the determinant (Côté and Morgan, 1956; Schiffman *et al.*, 1962a). Two active trisaccharides have been reported in which this disaccharide is linked β1,3 (Cheese and Morgan, 1961; Schiffman and Kabat, 1961; Schiffman *et al.*, 1962a) and β1,4 (Cheese and Morgan, 1961) to *N*-acetylglucosamine and more recently Morgan (1962)

* Aided by the National Science Foundation (G-18727), the Office of Naval Research, and the General Research Support Grant from the National Institutes of Health, USPHS. Reproduction in whole or in part is permitted for any purpose of the United States Government.

† Present address: Department of Research Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.