The Biosynthesis of Gramicidin S. A Restudy*

Nadhipuram V. Bhagavan,† Prema M. Rao, Leonard W. Pollard, Retnambal K. Rao, Theodore Winnick, and John B. Hall

ABSTRACT: A soluble system prepared from late log-phase *Bacillus brevis* cells, consisting of 105,000*g* supernatant, incorporated all five constituent amino acids efficiently into gramicidin S. The synthesis was adenosine triphosphate (ATP) dependent and showed two pH optima with different buffers. Furthermore, it was found that phosphate ion stimulated the biosynthesis. The incorporation of [^{14}C]leucine into gramicidin S was insensitive to the classical inhibitors of protein synthesis: ribonuclease, puromycin, and chloramphenicol. In the cell-free synthesis, a peptide other than gramicidin S, but containing all of its constituent amino acids, was detected. At the present time, no explanation can be given for the previous finding of a ribosome-dependent pathway for the biosynthesis of gramicidin S.

Recent years have seen remarkable advances in our understanding of the mechanisms of protein synthesis. One question that remains to be resolved, however, concerns the level of complexity that a polypeptide structure must attain before the relatively intricate ribosomal mechanisms come to be preferred over the purely enzymatic systems for its synthesis. Glutathione is synthesized by a system requiring only soluble enzymes (Snoke and Bloch, 1952) whereas it is generally assumed that insulin and polypeptides of similar size require ribosomal systems for their formation. In an attempt to define more exactly the minimum size of peptide that would require a ribosomal system for its synthesis, and to obtain a convenient model system for studying the formation of the peptide bond, studies were initiated in this laboratory on the biosynthesis of the antibiotic polypeptides produced by *Bacillus brevis*. Different strains of this organism produce a variety of polypeptides which range from 10 to 17 or 20 amino acids in length. Originally, it appeared that a ribosomal system had been obtained for the synthesis of these peptides (Bodley *et al.*, 1964; Hall *et al.*, 1965), a finding which contradicted reports from other laboratories of a nonribosomal type of pathway (Mach *et al.*, 1963; Eikhom *et al.*, 1964; Yukioka *et al.*, 1965). During the past year, however, it has not been possible to repeat these experiments and the possibility must be considered that the earlier reports should be reassessed. Instead, a highly active extract has been obtained from *B. brevis* 9999 which makes the decapeptide, gramicidin S under conditions similar to those reported from other laboratories (Yukioka *et al.*, 1965; Berg *et al.*, 1965). No ribosomes are required for this synthesis and ribonuclease or puromycin have no effect on the synthetic activity. Furthermore, a soluble enzyme system which synthesizes the cyclic pentapeptide, malformin, has been prepared from *Aspergillus niger* (Yukioka and Winnick, 1966).

The intent of the present paper is to help resolve the controversy that has revolved around the biosynthetic mechanism of the *B. brevis* peptides by confirming the published results of the laboratories referred to above. In addition, certain procedural modifications which give preparations of higher and more reproducible activity are described, and some new observations are presented. No adequate explanation has been found in the course of this work for the previous discrepant results.

Materials and Methods

Chemicals and Enzymes. Puromycin and *p*-mercuribenzoate[1] were obtained from Nutritional Biochemicals Corp. Chloramphenicol was purchased from Parke-Davis Co. Amino acids, phosphoenolypyruvate, and ATP were supplied by Sigma Chemical Co. CTP, GTP, and UTP were products of Calbiochem. The sources and specific activities (millicuries/mmole) of the isotopic amino acids used were as follows; New England Nuclear Corporation: DL-[1-^{14}C]cystine, 4.5; [1-^{14}C]glycine, 25.2; DL-[1-^{14}C]isoleucine, 30; D-[1-^{14}C]leucine, 25, L-[1-^{14}C]leucine, 24; DL-[1-^{14}C]-leucine, 21.9; DL-[1-^{14}C]lysine, 3.4; DL-[5-^{14}C]-ornithine, 9.8; D-[1-^{14}C]phenylalanine, 24.6; L-[^{14}C]-phenylalanine, 367; DL-[1-^{14}C]phenylalanine, 5.5; DL-[5-^{14}C]proline, 5; and DL-[1-^{14}C]valine, 18.5. Schwartz Bioresearch, Inc.: L-[^{14}C]aspartic acid, 111.3; L-[^{14}C]-

* From the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii. *Received August 24, 1966.* This research was supported by grants from the National Institutes of Health (GM 13312 and GM 09335).

† Presently a member of the Department of Anatomy, University of Hawaii, School of Medicine, Honolulu, Hawaii.

[1] Abbreviations used: PMB, *p*-mercuribenzoate; ATP, CTP, GTP, and UTP, adenosine, cytidine, guanosine, and uridine triphosphates; PEP, phosphoenolypyruvate.

serine, 89.3; and L-[^{14}C]tyrosine, 9.1. Calbiochem: L-[1-^{14}C]alanine, 7.5; and DL-[^{14}C]tryptophan, 6.6. Crystalline pancreatic deoxyribonuclease (EC 3.1.4.5), and ribonuclease (EC 2.7.7.16) were products of Worthington Biochemical Corp., while ribonuclease T_1 (EC 3.1.4.8) and crystalline pyruvic kinase (EC 2.7.1.40) were products of Calbiochem.

Bacterial Culture Conditions. *B. brevis* strain ATCC 9999 was grown in 1% tryptone–0.5% beef extract–0.5% NaCl medium, pH 7.2, with shaking in a New Brunswick incubator shaker at 37° (Okuda *et al.*, 1960). The density of the culture was measured at 650 mμ in the Beckman DU spectrophotometer. Optimal results were obtained with cells harvested between absorbancies of 0.86 and 1.0.

Measurement of Radioactivity. Radioactivity was measured in a Nuclear-Chicago gas-flow counter. Paper electrophoresis strips were scanned in a Nuclear-Chicago Actigraph gas-flow counter. Radioactivity on thin layer plates and paper electrophoresis strips was located by autoradiography using Kodak medical X-ray film and leaving the plates in contact with the film for 1 week.

Cell-Free Preparation. All operations were performed at 0–4°. After trying several variations the following optimal conditions were used routinely. The cells were washed twice with 0.02 M magnesium acetate and washed once with 0.02 M magnesium acetate plus 0.02 M mercaptoethanol (solution A) preparatory to cell disruption. The packed cells were suspended in two volumes of solution A and sonicated at 60 w and 20 kcycles for 6 min. Three periods of sonication, each 2 min long, were used, and the cells were chilled to 4° between periods. Unbroken cells and debris were removed by centrifugation at 30,000*g* for 45 min and the supernatant phase was further centrifuged at 105,000*g* for 1 hr. Unless otherwise indicated 1 ml of 105,000*g* supernatant (hereafter referred to as sonic extract) was used/reaction tube in the gramicidin S biosynthesis experiments.

Incubation Procedure. The following optimal conditions were used for the peptide biosynthesis. The final volume of incubation mixture in all cases was adjusted to 2 ml and incubated at 37°, for 3 hr. The standard system consisted of the following per tube: 1 ml of sonic extract (10–14 mg of protein), 10 μmoles of ATP; 5 μmoles of PEP, 20 μg of pyruvic kinase; 20 μmoles of magnesium acetate; 20 μmoles of mercaptoethanol; 0.5 μc of one of the amino acids occurring in gramicidin S, labeled with ^{14}C, plus 1 μmole of each of the four unlabeled amino acids; and 400 μmoles of phosphate buffer, pH 7.0. This system was routinely used unless otherwise indicated.

In experiments where protein synthesis was compared with peptide synthesis, 30,000*g* supernatant was used, and the buffer system was Tris–HCl, pH 7.8. In these cases, the [^{12}C]amino acid mixture contained, in addition to the amino acids present in gramicidin S, 1 μmole each of rhe 15 other commonly occurring amino acids. Protein was determined by the Biuret method.

Isolation of Peptide. The method used was essentially that of Uemura *et al.* (1963). The reaction was terminated by adding 0.4 ml of 50% trichloroacetic acid, and the mixture was centrifuged at 1500*g*. The precipitate was washed twice with 2% NaCl, and extracted overnight with 4 ml of ethanol–0.2 N HCl (9:1). After centrifugation, 2 mg of commercial gramicidin S (a gift from Aktiebolaget Astra, Sodertalje, Sweden) was added as carrier, and the precipitate was reextracted with ethanol–HCl.

The two ethanol–HCl extracts were pooled and concentrated by warming under an air jet to about 0.2 ml. The peptide was precipitated by adding about 5 ml of 10% NaCl. Gramicidin S, recovered by centrifugation, was redissolved in 0.2 ml of ethanol–HCl, and reprecipitated with 10% NaCl. The final precipitate, dissolved in a small quantity of ethanol, was dried on a metal planchet and counted, or else used for electrophoretic and chromatographic analyses. In experiments in which protein synthesis was to be examined, the residue after ethanol–HCl extraction was further treated with hot 5% trichloroacetic acid, washed once with ethanol–ether, dissolved in 1.5 M NH_4OH, dried on a metal planchet, and the radioactivity was measured.

Chromatography. Two-dimensional chromatography on thin layer silica gel plates was carried out using ethyl acetate–pyridine–acetic acid–water (60:20:6:11, v/v) in the first dimension, and the upper phase of 1-butanol–acetic acid–water (100:24:100, v/v) in the second dimension (Holm *et al.*, 1966). Unidimensional chromatography was performed in the upper phase of 1-butanol–acetic acid–water (100:24:100, v/v). The chromatograms were sprayed with 0.2% (w/v) ninhydrin solution in acetone.

Electrophoresis. Electrophoresis was performed in 50% acetic acid, using an apparatus cooled by circulating tap water. The samples were applied to the center of 18 × 58 cm Whatman 3MM paper, and subjected to a current of 18 ma/cm at 34 v/cm, for 45–90 min. The dried chromatograms were sprayed with ninhydrin or bromophenol blue, and scanned in an Actigraph or by autoradiography.

Results

Cultivation of B. brevis. To determine the growth phase at which the maximal rate of peptide synthesis occurred, the cells were harvested at different stages of growth, and the degree of incorporation of the label into the cell-free extracts was measured. The results are reported in Table I. At optical densities of 0.86–1.0, which correspond to the late log phase of cell growth, a high rate of peptide synthesis was observed. It is of interest to note that in the early log phase, in which there is a high rate of protein synthesis, very little synthesis of peptide occurred (see also Berg *et al.*, 1965).

Disruption of Cells. Aliquots of cell suspensions were sonicated for periods of 2–15 min. An extract sonicated for 6 min gave the maximal incorporation

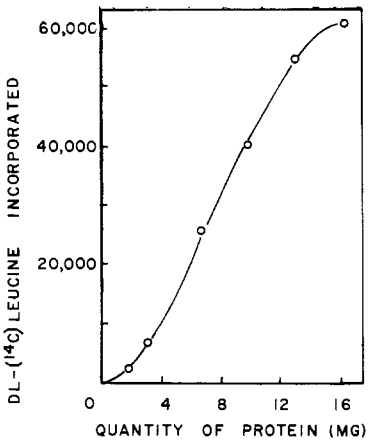

FIGURE 1: Dependence of peptide synthesis on the amount of cell extract added under standard conditions of incubation.

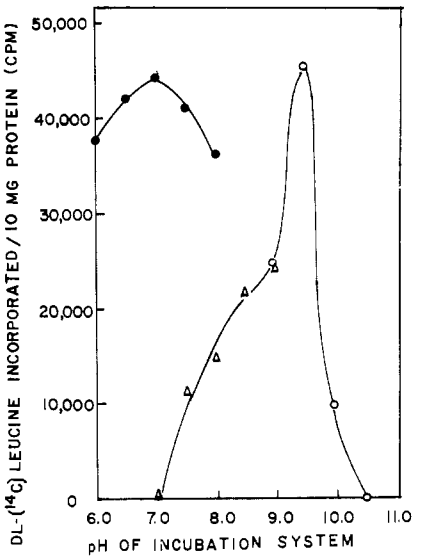

FIGURE 2: Effect of pH on the incorporation of DL-[1-^{14}C]leucine into peptide. ●, 0.2 M phosphate buffer; △, 0.1 M Tris–HCl buffer; ○, 0.1 M glycine–NaOH buffer.

TABLE I: Cell Growth and Gramicidin S Biosynthesis.[a]

Cell Density at Harvest (OD at 650 mμ)	Radioactivity of Labeled Peptide (cpm/10 mg of protein)
0.130	None
0.695	3,200
0.740	28,400
0.860	41,500
0.920	47,800
1.105	34,200
1.130	30,900

[a] The labeled amino acid was DL-[1-^{14}C]leucine.

of [^{14}C]leucine into gramicidin S. Cells subjected to sonication for 15 min retained only 30% of the maximal activity. Cells were also disrupted by passage through a French pressure cell at 10,000–15,000 psi, or by grinding with alumina for 4 min. These extracts were prepared from the same batch of cells, and were then tested for activity. No differences in incorporation were observed with extracts prepared by the various methods of disruption.

Incubation Conditions. The incorporation at the end of 0.5 hr was 30% of the maximal value, and attained a plateau between 2 and 3 hr. Figure 1 shows the relationship between incorporation of [^{14}C]leucine and varying amounts of sonic extract (expressed in terms of milligrams of protein). It is noteworthy that the cell extract under conditions employed contained no apparent inhibitor for the peptide synthesis. The incorporation was not entirely linear, probably due to limiting concentrations of one or more components of the incubation mixture.

To determine the optimum pH for the cell-free biosynthesis of gramicidin S, the assay was carried out using the conditions described for the standard system, except that the buffer was varied (Figure 2). A surprising feature was the observation of two pH optima for the biosynthesis of peptide. In phosphate buffer between pH 6 and 8, optimum activity was obtained at pH 7.0 although there were no large differences in [^{14}C]leucine incorporation throughout this range. Further, it was found that the concentration of the phosphate was important, since the activity dropped significantly if the concentration was lowered to 0.05 M at pH 7.0.

At pH 7.0, using Tris–HCl buffer, the incorporation was nil, but rose steadily thereafter until pH 9.0. It is of interest that the values were identical at pH 9.0 with either Tris–HCl or glycine–NaOH buffer. There was a sharp increase in incorporation at pH 9.5 (glycine–NaOH buffer), and a dramatic decrease at pH 10.0, with virtually no incorporation at 10.5. All of these results were reproducible in repeated assays.

The presence of two distinct pH optima suggested that two different peptides were being observed in these reactions. To test this possibility, the peptide fractions obtained using phosphate buffer (pH 7.0), Tris–HCl buffer (pH 9.0), and glycine–NaOH buffer (pH 9.5) were analyzed by electrophoresis and thin layer chromatography. In all cases labeled peptide migrated with gramicidin S carrier. Throughout the study we have used 0.2 M phosphate buffer (pH 7.0) in the standard system because of the broad pH profile obtained with this buffer.

Importance of Energy Source in the Gramicidin S Synthesis. It was found that optimal activities were

obtained using 10 μmoles of ATP, and that increasing the concentration to as high as 100 μmoles did not significantly alter the degree of incorporation. The level of ATP was particularly important in the Tris or glycine buffer systems, in which 10 μmoles were required to attain the level of activity shown in the pH dependency curves; 5 μmoles gave much less incorporation. Curiously, in phosphate buffer, ATP concentration was not so critical, although 10 μmoles resulted in somewhat better activity than 5 μmoles.

It is evident from Table II that omitting ATP, PEP, and pyruvic kinase completely eliminated the incorporation of the radioactive amino acid into gramicidin S. However, omission of PEP and pyruvic kinase did not significantly decrease the total level of activity. Augmenting the system with CTP, GTP, and UTP did not significantly change the uptake of the [^{14}C]leucine, as compared to the standard value. It is also evident that the substitution of CTP, GTP, or UTP for ATP reduced the incorporation by about 90%. The latter result may be indicative of low levels of kinase activities for the conversion of CTP, GTP, or UTP into ATP; or of incomplete specificity of the system for ATP; but it is clear that ATP was the preferred energy source.

TABLE II: The Effect of Ribonucleoside Triphosphates and an Energy-Generating System for ATP on DL-[1-^{14}C]Leucine Incorporation into Gramicidin S.

Energy Source	Radioactivity (cpm of peptide/10 mg of protein)
Standard system	45,600
ATP, PEP, and pyruvic kinase omitted	27
PEP and pyruvic kinase omitted	43,800
CTP, GTP, and UTP added (2 μmoles each)	49,000
ATP, PEP, and pyruvic kinase omitted; 10 μmoles of CTP added	6,830
ATP, PEP, and pyruvic kinase omitted; 10 μmoles of GTP added	3,860
ATP, PEP, and pyruvic kinase omitted; 10 μmoles of UTP added	3,180

Ionic Requirements. Potassium was neither essential nor inhibitory to [^{14}C]leucine incorporation into peptide in the concentration range tested. The absence of a requirement for K^+ was further shown with fractions of the extract which were precipitated with $(NH_4)_2SO_4$ and dialyzed to removed free endogenous K^+. However, the presence of K^+ bound to nondialysable material cannot be ruled out. The concentration of Mg^{2+} was rather critical, and optimal biosynthesis was obtained with 5 μmoles/tube. Higher concentrations of Mg^{2+}, up to 40 μmoles, neither enhanced nor decreased the incorporation.

Phosphate proved to be required for optimal activity, particularly at neutral pH. Incorporation of label into peptide increased with increasing phosphate concentrations up to 200–300 μmoles/ml.

Effect of Inhibitors. The addition of chloramphenicol (100 μg), puromycin (100 μg), pancreatic ribonuclease (RNAase) (100 μg), or RNAase T_1 (50 units) to the standard system did not alter the incorporation of [^{14}C]leucine into peptide (Table III). In attempts to compare peptide synthesis with protein synthesis, it was essential to employ the inhibitors under conditions in which both processes could occur. This was accomplished by using the 30,000*g* supernatant fraction prepared from mid-log-phase cells with Tris–HCl buffer, pH 7.8 (see methods). Although these conditions were not optimal for gramicidin S synthesis, the antibiotics and pancreatic RNAase did not have any inhibitory effect on the peptide incorporation process, while the protein synthesis was decreased significantly. It was also observed in such systems that removal of ribosomal activity by digestion with pancreatic RNAase, or by prolonged centrifugation at 105,000*g*, slightly increased the incorporation of the labeled amino acid into the peptide fraction. These observations suggest that there are major differences in the biosynthetic pathways for this peptide and for protein.

TABLE III: The Effect of Antibiotics and Nucleases on the Incorporation of DL-[1-^{14}C]Leucine into Gramicidin S.[a]

Addition	Radioactivity (cpm of peptide/10 mg of protein)
None	43,600
Puromycin (100 μg)	43,300
Chloramphenicol (100 μg)	43,900
RNAase, pancreatic (100 μg)	41,200
RNAase T_1 (50 units)	43,300
DNAase I, pancreatic (100 μg)	42,100

[a] The incubation mixture was preincubated for 5 min before adding the energy source and [^{14}C]amino acid.

The sulfhydryl complexing agent, PMB, at a concentration of 3.2×10^{-4} M, completely abolished the peptide biosynthetic activity in sonic extracts prepared in the absence of mercaptoethanol. However, mercaptoethanol, which is a component of the standard system at a concentration of 10^{-2} M, protected completely against the inhibiting effect of PMB.

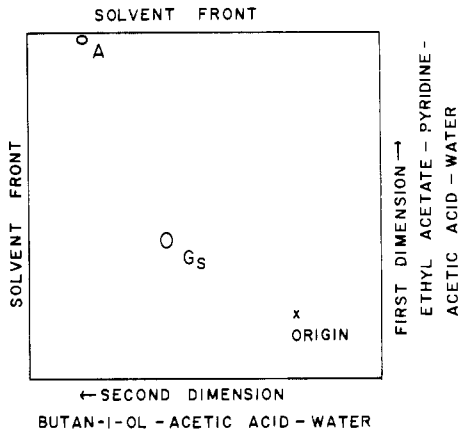

FIGURE 3: A radioautograph of a two-dimensional thin layer chromatogram of the pooled peptide fractions labeled with the [^{14}C]amino acids present in gramicidin S. "G_s" refers to gramicidin S and "A" to the unknown peptide.

TABLE IV: Utilization of Different [^{14}C]Amino Acids in the Synthesis of Peptide.[a]

^{14}C-Labeled Amino Acid	Amino Acid Sp Act. (mc/mmoles)	Peptide (cpm/10 mg of protein)
DL-Leucine	21.9	45,600
DL-Ornithine	9.8	34,000
DL-Phenylalanine	5.5	81,500
DL-Proline	5.0	29,000
DL-Valine	18.5	41,000
L-Aspartic acid	111.3	20
DL-Lysine	3.4	0
DL-Tryptophan	6.6	100
L-Tyrosine	9.1	440
DL-Cystine	4.5	50
Glycine	25.2	0
L-Serine	89.3	14
L-Isoleucine	30.0	680

[a] The amino acid mixture consisted of 0.5 μc of the specified [^{14}C]amino acid and 1 μmole of each of the 19 other [^{12}C]amino acids.

Specificity and Identification of the Peptide Fraction. Table IV is a compilation of data obtained with two groups of amino acids which are present and absent in the gramicidin S molecule. The component gramicidin S amino acids (L-leucine, L-ornithine, D-phenylalanine, L-proline, and L-valine) were efficiently utilized. In some instances the incorporation was not in proportion to specific activities, and this may have been due to isotopic dilution by endogenous amino acid.

The incorporation of amino acids which are not constituents of gramicidin S was not significant. It is also evident from Table IV that there was a rather high incorporation with DL-phenylalanine (despite its low specific activity), suggesting conversion of L isomer to D isomer. To test this possibility, peptide biosynthesis was studied using D-, L-, and DL-phenylalanine. For comparison, it was of interest to find out whether or not D-leucine could be utilized. The results are reported in Table V. It was observed that

TABLE V: Comparison of the Incorporation into Gramicidin S of Labeled Isomers of Phenylalanine and Leucine.[a]

^{14}C-Labeled Amino Acid	Amino Acid Sp Act. (mc/mmoles)	Radioactivity (cpm/10 mg of protein)
DL-Phenylalanine	5.5	77,300
D-Phenylalanine	24.6	68,250
L-Phenylalanine	367.0	40,512
DL-Leucine	21.0	40,150
D-Leucine	25.0	80
L-Leucine	24.0	71,470

[a] Conditions were the same as for Table IV.

L-phenylalanine was incorporated, but less efficiently than the D isomer, which is the form present in the gramicidin S molecule. This result suggests conversion of L to D. While there was no incorporation with D-leucine, the activity with L-leucine was almost twice as high as that with DL-leucine, which indicates that the D isomer was not utilized, and that no racemase for this amino acid was present in the system.

Silica Gel Chromatography. Figure 3 is a replica of an autoradiograph of a two-dimensional chromatogram. Ninhydrin revealed only one spot, corresponding to unlabeled gramicidin S. On autoradiography a faint component (designated as "A") was observed, in addition to the heavily labeled dark spot of gramicidin S, which had an R_F value different from that of any of the free isotopic amino acids used. This unknown substance migrated in the 1-butanol–acetic acid–water system with an R_F value of 0.82 (Figure 4), and was labeled by each of the five constituent amino acids of gramicidin S. In this system gramicidin S had an R_F of 0.45. Since substance A contained all five amino acids of gramicidin S it is probable that it was at least a pentapeptide. The question of its possible role as an intermediate is under study.

Electrophoresis. The electrophoretic pattern of the peptide fractions, along with the distribution of radioactivity as determined by means of an Actigraph tracing, is shown in Figure 5. The rapidly migrating band, which corresponds to carrier gramicidin S,

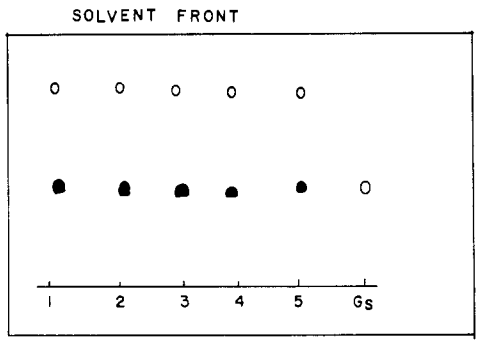

FIGURE 4: A radioautograph of a thin layer chromatogram developed in l-butanol–acetic acid–water. The peptides were labeled with the isotope in Pro (spot 1), Val (2), Phe (3), Orn (4), and Leu (5). The position of unlabeled gramicidin S (G_s), which was located with ninhydrin, is also shown.

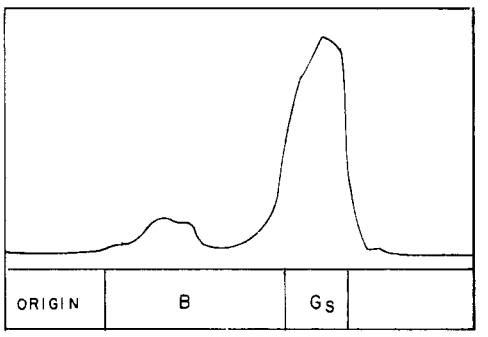

FIGURE 5: A diagram of an electrophoresis strip stained with bromophenol blue, and a radioactivity scan of the same strip. The peptide was labeled with DL-[1-^{14}C]-phenylalanine. "G_s" refers to gramicidin S and "B" to an unidentified peptide.

contained the major portion of radioactivity, suggesting the identity of the radioactive product with gramicidin S. However, it is clear from Figure 5 that there was a slower moving radioactive peak which did not show up on the bromophenol blue stained strips. The slower moving radioactive component (designated B) was found when each of the constituent amino acids of gramicidin S was used as a label, and at the same position. This spot did not correspond to any of the radioactive free amino acids used in the assay system. When substance B was eluted from the electropherograms and submitted to thin layer chromatography, it migrated with substance A, indicating that these two compounds were probably identical.

The Absence of Whole Cells or Spores. The same type of results reported in the present paper might conceivably be obtained if viable cells or spores were contaminating the incubation mixture. This situation could explain the resistance of the system to ribonuclease, and to any other inhibitors which could be excluded by whole cells. When aliquots of the incubation mixture were plated on nutrient agar, bacterial colonies did appear. Their number was, at most a few hundred, even at the end of the incubation period, and far too few to account for the observed synthesis of peptide. However, to exclude any possibility of error from this source, extracts were passed through a Millipore filter (HA, 0.45-μ pore size) before incubation. Aliquots of such extracts contained no colony forming bacteria. Their synthetic capacities were found to be undiminished when compared with an unfiltered control.

Discussion

A highly active and reproducible system for the biosynthesis of gramicidin S is described here. The requirement for high concentrations of phosphate at neutral pH is of interest. A similar requirement was observed by Johnston and Bloch (1951) in their initial studies on glutathione biosynthesis using crude pigeon liver homogenates. It seemed possible that the effect of phosphate was to inhibit ATPases or other degradative enzymes present in the extract. A similar explanation may apply to our system.

The different pH optima seen with two buffer systems may also be explained by the presence of interfering enzymes such as ATPases. This phenomenon seems worthy of further study, but it is felt that further purification of the extracts is desirable in order to obtain clearer results.

Several groups of workers have reported studies on short polypeptides, which appear to be precursors of gramicidin S. Tomino and Kurahashi (1964) observed the synthesis of D-Phe-L-Pro-L-Val in extracts of *B. brevis*, and postulated that this substance was such an intermediate, although the tripeptide itself did not appear to be incorporated into gramicidin S. Holm *et al.* (1966) found that a series of oligopeptides were synthesized in a cell-free system, including D-Phe-L-Pro, the tripeptide mentioned above, L-Pro-L-Val-L-Orn, and the tetrapeptide D-Phe-L-Pro-L-Val-L-Orn. The last substance also occurred in a bound form, in which the carboxyl end of the chain seemed to be masked. We have also observed a peptide in our synthetic system which appears to be an intermediate in gramicidin S biosynthesis, although the possibility cannot yet be excluded that it is a fragment of some larger molecule, containing the five amino acids found in this antibiotic. Since this peptide was labeled by each of the five amino acids found in gramicidin S, it must have been at least a pentapeptide. Since it migrated well behind gramicidin S on electrophoresis, it is probable that some additional substituent was present. Apparently this substituent also enhanced the solubility of the peptide in organic solvents, causing it to migrate near the front in both thin layer chromatography solvents (Figure 3).

The results described in this report are consistent with those that have been published by other laboratories (Yukioka *et al.*, 1965; Berg *et al.*, 1965). The

mass of data accumulated in this laboratory over a period of several years in support of the ribosomal pathway seems too extensive and self-consistent to be explained as the result of simple technical error or misinterpretation of experimental results. However, the fact remains that despite our best efforts, we have not been able to reproduce these experiments, and have found instead a very active enzyme system identical in all essentials with that reported by other laboratories. It is possible that this very active soluble system has masked a less active ribosomal pathway for gramicidin S biosynthesis. Experiments are in progress to investigate this possibility.

Acknowledgments

We wish to express our appreciation to Dr. Lars Nathorst-Westfelt of Aktiebolaget Astra, Soderstalje, Sweden, for his generous gift of gramicidin S.

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Biosynthesis of Adrenocorticotropin and Protein in a Cell-Free System from Bovine Anterior Pituitary Tissue*

P. Radhakantha Adiga, Prema M. Rao, Robert O. Hussa, and Theodore Winnick

ABSTRACT: A cell-free system consisting of ribosomes and pH 5 enzyme fraction, prepared from bovine anterior pituitary tissue, was found to be active in the incorporation of radioactive proline into adrenocorticotropin and mixed proteins. Following isolation by a chemical procedure, the labeled hormone was purified by paper electrophoresis.

The biosynthesis of the polypeptide resembled that of protein synthesis in its requirement for adenosine triphosphate, magnesium ion, guanosine triphosphate, and an amino acid mixture. Also both processes were inhibited by ribonuclease and by puromycin. Deoxyribonuclease and actinomycin D were noninhibitory.

In recent years there has been increasing interest in the biosynthesis of discrete proteins of known chemical constitution. Such investigations offer obvious advantages in the elucidation of basic mechanisms. Among the major types of proteins with well-defined biological activities, the polypeptide and protein hormones of vertebrates offer a number of attractions. Biosynthetic studies of this class of hormones, based on the use of surviving tissue slices, have included insulin (Wagle, 1965a; Taylor *et al.*, 1964; Humbel, 1963), thyroglobulin (Seed and Goldberg, 1963), vasopressin (Haller *et al.*, 1964), and adrenocorticotropin (ACTH)[1] (Adiga *et al.*, 1965). The first paper on the

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[1] Abbreviations used: ACTH, adrenocorticotropin; ATP, adenosine triphosphate; DOC, deoxycholate; GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; PK, pyruvic kinase; TCA, trichloroacetic acid.