The Biosynthesis of Gramicidin S. A New Peptide Intermediate*

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ABSTRACT: The cell-free biosynthesis of the cyclic decapeptide, gramicidin S, in extracts from *Bacillus brevis* strain ATCC 9999 has been described. A second peptide formed in minor amounts in this system has been characterized. It contains the same five amino acids found in gramicidin S, probably in the same repeating sequence, and in addition a residue of ethanolamine and one of formic acid. This peptide is not found in extracts of whole cells but is formed only under conditions in which cell-free synthesis occurs. From its structure and its mode of synthesis, we believe that it is an immediate precursor of gramicidin S. The structure of this intermediate, with formate linked to the N-terminal amino acid and ethanolamine to the C terminus of the chain, is very similar to that of the linear gramicidins produced by other strains of *Bacillus brevis*. This similarity suggests that the two families of antibiotics found in *Bacillus* species, the linear gramicidins and the cyclic tyrocidines, are more closely related to each other than their structures would otherwise suggest.

A number of laboratories have been working on the biosynthesis of the antibiotic polypeptides produced by various strains of *Bacillus brevis*. Several groups which are interested in the synthesis of the cyclic decapeptide, gramicidin S, in cell-free systems have isolated smaller peptides which appear to be intermediates in gramicidin S synthesis. Tomino and Kurahashi (1964) observed the formation of D-Phe-L-Pro-L-Val in cell-free extracts of the Nagano strain of *B. brevis*. The amount of synthesis of this peptide was reduced by the addition of leucine and ornithine to the reaction mixture while the synthesis of gramicidin S was stimulated. The free tripeptide itself, however, did not appear to be incorporated into gramicidin S. Holm *et al.* (1966) isolated a tetrapeptide from extracts synthesizing gramicidin S. It was thought to have the structure D-Phe-L-Pro-L-Val-L-Orn and also occurred in a bound form in which an unidentified substituent was linked to the carboxyl group of the terminal ornithine. They also isolated and characterized the dipeptide D-Phe-L-Pro and two tripeptides which were probably D-Phe-L-Pro-L-Val and L-Pro-L-Val-L-Orn. All of these peptides are possible intermediates in the synthesis of gramicidin S or may represent breakdown products of it. Otani *et al.* (1966) have demonstrated the formation of D-Phe-L-Pro by enzymes from the Nagano strain. They attempted to fractionate the extract and were able to show that synthesis of the dipeptide was stimulated by the combination of two or more fractions.

In this paper, an additional apparent precursor of gramicidin S is described. This peptide, comprising a minor fraction of the reaction products, was labeled by all five of the amino acids incorporated into gramicidin S but could be readily separated from it by thin-layer chromatography or by paper electrophoresis (see components A and B in Bhagavan *et al.*, 1966). It seems likely that this peptide is an intermediate in the biosynthesis of gramicidin S and that the study of its structure and mode of formation will help to elucidate the mechanism of synthesis of this antibiotic.

Materials and Methods

Chemicals and Enzymes. The following radioactive compounds were obtained from New England Nuclear Corp. and had the indicated specific activities (millicuries per millimole): [^{14}C]sodium formate, 4.35; L-[^{14}C]serine, 120.0; [1,2-^{14}C]ethanolamine, 4.34; [^{14}C]-algal protein hydrolysate, 1.8 mCi/mg; L-[^{14}C]leucine, 251.4; [2-^{14}C]sodium acetate, 2.0; DL-[1-^{14}C]phenylalanine, 5.5; DL-[5-^{14}C]proline, 5; DL-[1-^{14}C]valine, 18.5; DL-[5-^{14}C]ornithine, 9.8; DL-[1-^{14}C]isoleucine, 30; L-[^{14}C]tyrosine, 9.1; DL-[^{14}C]tryptophan, 6.6; L-[^{14}C]asparate, 111.3; DL-[^{14}C]lysine, 3.4; DL-[^{14}C]-cysteine, 4.5; and [1-^{14}C]glycine, 25.2. Nagarse was purchased from Amagasaki, Osaka, Japan. ATP, dansyl chloride, and dicyclohexylcarbodiimide were purchased from Sigma Chemical Co. Sephadex G-10, G-15, G-25, G-50, and G-75, and Blue Dextran were obtained from Pharmacia Fine Chemicals; silica gel G from Warner-Chilcott Laboratories; and G. S. polyamide sheets from Gallard-Schlesinger Chemical Co. Chromatography sprays were products of Mann Research Laboratories, as was gramicidin.

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 spectrophotomer. Optimal results were obtained with cells harvested between absorbances of 0.86 and 1.0.

Measurement of Radioactivity. Radioactivity was

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measured in a Nuclear-Chicago gas-flow counter. Labeled compounds on thin-layer plates or paper chromatograms were located by radioautography using Kodak Medical X-ray film and leaving the plates in contact with it for 1 week.

Cell-Free Preparation. The preparation of cell-free extracts followed the procedure reported previously (Bhagavan *et al.*, 1966).

Incubation Procedure. The following conditions were used for biosynthesis of the peptides. In all cases the final volume of the mixture 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;[1] 20 μmoles of magnesium acetate; 20 μmoles of mercaptoethanol; 0.5 μCi of one of the labeled compounds, plus 1 μmole of each of the unlabeled amino acids; and 400 μmoles of sodium phosphate buffer (pH 7.0). As reported earlier (Bhagavan *et al.*, 1966), the addition of phosphoenolpyruvate plus pyruvic kinase to the system did not markedly enhance peptide synthesis over that found in the presence of ATP alone. Therefore these two components were omitted from the reaction mixture. This system was routinely used unless otherwise indicated.

Isolation of Peptide. The isolation procedure was essentially that of Uemura *et al.* (1963) as modified by Bhagavan *et al.* (1966). The unknown peptide was routinely separated from gramicidin S by chromatography on silica gel plates.

Amino acids, peptides, and dansylated derivatives of each were separated on paper, thin-layer silica gel plates, or polyamide sheets. The following solvents were used for the separations: (1) the upper layer of the two-phase system prepared by mixing 1-butanol–acetic acid–water (4:1:5, v/v), (2) chloroform–methanol–17% ammonia solution (2:2:1, v/v), (3) phenol–water (75:25, w/w), (4) the upper layer of a 1-butanol–acetic acid–water mixture (4:1:4, v/v) (Holm *et al.*, 1966), (5) 90% formic acid–water (1.5:100, v/v) (Woods and Wang, 1967), (6) ethyl acetate–2-propanol–concentrated ammonia solution (8:20:6, v/v), (7) ethyl acetate–pyridine–acetic acid–water (60:20:6:11, v/v) (Holm *et al.*, 1966), and (8) benzene–acetic acid (9:1, v/v). Amino acids were detected by spraying the chromatograms with 0.2% ninhydrin in acetone or 0.2% isatin in 1-butanol. Dansyl-containing compounds were localized by their fluorescence under an ultraviolet lamp.

Amino Acid Analyses. An aliquot of the peptide was dried in an hydrochloric acid washed test tube. Redistilled 6 N HCl (1 ml) was added and the tube was evacuated, sealed, and heated to 110° for 16–24 hr. After cooling, the tube was opened and the HCl was removed by evaporation under a stream of nitrogen gas. The residue was then dissolved in 70% ethanol for paper chromatography or analyzed on a Beckman–Spinco Auto Analyzer, Model 120, at 50°.

The instrument was equipped with a high-sensitivity cuvet and an expanded-scale recorder (4–5 mV) for the detection of microgram amounts of amino acids. Standards containing 0.0025 μmole of each amino acid were used to calibrate the analyzer.

Hydrazinolysis. The method of Bradbury (1958) was followed for the determination of the C-terminal amino acid. Approximately 0.02 μmole of peptide containing 8000 cpm of ^{14}C distributed through all five amino acids was dissolved in 0.2 ml of 97% hydrazine and 26 mg of hydrazine sulfate was added. The tube was evacuated and sealed, and heated to 60° for 16 hr. The tube was then opened and the contents were evaporated to dryness in a dessicator over concentrated H_2SO_4 *in vacuo*. HCl (1 ml of 1 N) and benzaldehyde (0.4 ml) were added to the residue and the mixture was shaken for 2 hr at room temperature and then left at 4° overnight. The aqueous phase was removed and the organic layer was extracted twice more with 1 N HCl. The acid solution was then taken to dryness under a stream of nitrogen and the residue was dissolved in 0.02 ml of 70% ethanol and subjected to two-dimensional chromatography on silica gel plates.

Preparation of Dansyl-Amino Acids. The method of Gray and Hartley (1963) was used to prepare the dansyl-amino acids. Amino acids (10 μmoles) in 0.1 ml of water were mixed with 0.1 ml of 0.2 M sodium bicarbonate (pH 7.9), and 0.2 ml of dansyl chloride (1 mg/ml) in acetone was added. After 3 hr, the sample was dried and could then be redissolved in any convenient solvent for chromatography.

Electrophoresis. Dansylated amino acids were separated by high-voltage electrophoresis on Whatman No. 1 paper. The buffer used was 0.8% acetic acid–0.5% pyridine (pH 4.4). The electrophoresis was carried out on a cooled plate for 2.5 hr at 26 V/cm (Gray and Hartley, 1963).

Results

Isolation of the Unknown Peptide. An extra labeled spot or band in addition to that of gramicidin S appeared when products of the incubation mixture were separated on thin-layer plates or by paper electrophoresis (Bhagavan *et al.*, 1966). These extra components observed by several different separation procedures all proved to be identical when chromatographed in the same system. The unknown peptide, called the X peptide hereafter, was readily separated from gramicidin S by thin-layer chromatography on silica gel with solvent 4. The area of the plate occupied by the X peptide as determined by radioautography was scraped off into a test tube and the peptide was eluted into 0.02 N HCl in ethanol.

Incorporation Studies. The incorporation of amino acids and other compounds into gramicidin S and the X peptide is shown in Table I. Incorporation was measured by radioautography and is marked "+" if a spot was clearly visible, "−" if no spot was present, and "±" if a faint spot of doubtful significance was seen. It can be seen that the X peptide is labeled only by those amino acids found in gramicidin S and by no others examined. The slight labeling of gramicidin S by isoleucine can

[1] Abbreviations used: ATP, adenosine triphosphate; dansyl, dimethylaminonaphthalene-5-sulfonyl (DNS); EA, ethanolamine.

TABLE I: The Incorporation of Various Compounds into Gramicidin S and the X Peptide in the Cell-Free System.[a]

Compound Tested	Labeling of: Gramicidin S	Labeling of: X Peptide
Phe	+	+
Pro	+	+
Val	+	+
Leu	+	+
Orn	+	+
Ile	±	−
Ser	−	−
Tyr	−	−
Trp	−	−
Asp[b]	−	−
Lys[b]	−	−
Cys[b]	−	−
Gly[b]	−	−
Formate	−	−
Acetate	−	−
Ethanolamine	−	−

[a] The labeled compound (0.5 μCi) and 1 μmole of each of the other [^{12}C]amino acids found in gramicidin S were included in the incubation mixture. [b] The peptides labeled by these amino acids were not subjected to chromatography since the amount of label in the crude mixture was negligible.

probably be attributed to leucine contamination of the labeled amino acid.

Characterization of the X Peptide. The incorporation studies indicated that the X peptide contained all of the amino acids found in gramicidin S, but its mobility on electrophoresis was not consistent with the hypothesis that is was a simple pentapeptide. The pentapeptide produced from gramicidin S by nagarse digestion had been shown to migrate more rapidly than gramicidin S on electrophoresis in 50% acetic acid (Hall *et al.*, 1965). The X peptide, on the contrary, migrated well behind gramicidin S, indicating that one of its amino groups was masked or that is was larger than a pentapeptide. As a first step in the analysis of the X peptide, an aliquot of it was dried, hydrolyzed with 6 N HCl in a sealed tube, and submitted to paper chromatography on Whatman No. 3MM paper using solvent 1. The dried sheet was sprayed with ninhydrin to reveal the pattern shown in Figure 1. Six spots could be seen, including one corresponding to ethanolamine, and overlapping it, a faint spot which was shown to be proline by spraying it with isatin. The spot believed to be ethanolamine was somewhat unexpected, although the possible presence of this component had been considered because of the occurrence of ethanolamine linked to the C-terminal amino acid in the gramicidins produced by other strains of *B. brevis* (Synge, 1945a).

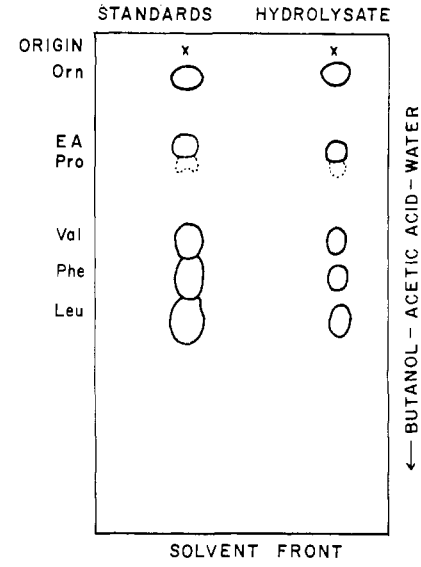

FIGURE 1: Paper chromatography of a hydrolysate of the X peptide.

The presence of ethanolamine in this peptide was confirmed by preparing the dansyl derivatives of the amino compounds in a peptide hydrolysate and chromatographing them in two dimensions on polyamide sheets with solvents 5 and 6 as the first and second developing agents, respectively. Reference compounds were prepared and chromatographed in the same system. A compound was present in the peptide that migrated with dansylated ethanolamine in every instance.

The ninhydrin spots from the paper chromatogram were cut out and eluted into 10% acetic acid. The solution was evaporated and the residue was redissolved in 1 ml of 4 M sodium acetate and its absorbancy was determined at 550 mμ in the Beckman DU spectrophotometer. The results indicated that leucine, phenylalanine, valine, and ornithine were present in the same amounts. To confirm this analysis and obtained values for proline, a sample of hydrolyzed peptide was submitted to the amino acid analyzer. Three runs were made, two on samples hydrolyzed for 24 hr and the third on a sample hydrolyzed for 96 hr. Mechanical difficulties with the instrument were responsible for the loss of the ornithine peak during the first run and the leucine peak in the third. The four remaining amino acids were present in nearly equimolar amounts in each of these runs, however, and gave results consistent with those reported for the second run in Table II. Aspartic acid, serine, glutamic acid, glycine, and alanine were also detected, but each was present in amounts less than one-fourth those reported for any of the five amino acids of gramicidin S, and in view of the molecular weight determined for the X peptide, were not believed to be constituents of it. No other amino acids were found in these analyses.

End Groups. The insolubility of the X peptide in aqueous media made it difficult to use enzymes to study its structure. The C-terminal amino acid was determined by the hydrazinolysis procedure, therefore. The presence of ethanolamine on the carboxyl end of the chain did not present any difficulties for this assay since the carboxyl–

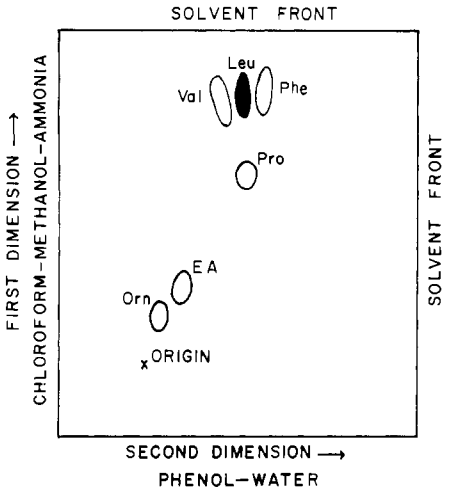

FIGURE 2: A two-dimensional thin-layer chromatogram of the amino acid released from the X peptide by hydrazinolysis. The dark spot on a superimposed radioautogram reveals that only leucine was released as free amino acid from the uniformly labeled peptide.

ethanolamine bond is more acid labile than the peptide bond (see Synge, 1945b), and storage in the ethanol–HCl solution in which the peptide was eluted from the thin-layer plates apparently split much of the ethanolamine from the peptide.

The X peptide was uniformly labeled with [^{14}C]amino acids by incubating *B. brevis* extracts with [^{14}C] algal hydrolysate or with the five appropriate ^{14}C-labeled amino acids. An aliquot of the purified peptide was dried and subjected to hydrazinolysis. The free amino acids obtained by this procedure were mixed with the five unlabeled amino acids and ethanolamine as carriers, and chromatographed in two dimensions on silica gel plates using solvents 2 and 3 in that order. The dried plate was sprayed with ninhydrin and a radioautogram was made. The result is shown in Figure 2. All five amino acids and ethanolamine can be seen as ninhydrin-positive spots on the plate, but radioautography revealed that only the spot corresponding to leucine was labeled by the ^{14}C. The yield of the hydrazinolysis reaction is not quantitative so that the amount of leucine released could not be used to determine the size of the peptide.

An attempt was made to quantitate the amount of ethanolamine in the X peptide by hydrolyzing an aliquot of the peptide, preparing the dansyl derivatives of the amino compounds present, and estimating the amount of each by its fluorescent emission, after separating them by two-dimensional chromatography. The variation in the values obtained by this method was too great to permit any definite conclusions to be drawn, but did show that there was at least 1 mole of ethanolamine present for every 3–5 moles of any one of the amino acids.

To determine the N-terminal amino acid, the peptide was treated with the dansyl chloride reagent using the same procedure that was used to dansylate amino acids, except that the peptide was dissolved in ethanol rather than water. The dansylated X peptide was then hydrolyzed in 6 N HCl and the residue was resolved by electrophoresis. The monodansyl derivative of ornithine was present as expected, but no other dansylated amino acid was observed, confirming the suspicion that the N-terminal amino group was masked. The straight-chain gramicidins produced by other strains of *B. brevis* are characterized by a formyl group on the N-terminal amino acid as well as by ethanolamine at the C-terminal end. By analogy, we suspected that the blocking group on the X peptide might be a formyl group.

TABLE II: Amino Acid Composition of the X Peptide.

Amino Acid	μmoles Obsd
Orn[a]	0.008
Pro	0.011
Val	0.010
Leu	0.011
Phe	0.009

[a] The color factor for Lys was used in calculating the amount of this amino acid.

Sarges and Witkop (1963), who identified the formyl group in the gramicidins, found that it was relatively labile and could be removed from the peptides by treating them with 1.5 N HCl in methanol for 1 hr at room temperature. The X peptide was treated in this way to remove the formate and the analysis was repeated. Ornithine was still the only amino acid labeled by the dansyl reagent. A more vigorous treatment with 1 N HCl in boiling methanol for 4 hr was tried. After this treatment, the peptide was dansylated and separated from other reaction products by chromatography on silica gel plates with solvent 4. The eluted X peptide was then hydrolyzed, dried, and subjected to two-dimensional chromatography on polyamide sheets, using mixtures 5 and 8 as the solvents. Spots corresponding to the dansyl derivatives of ornithine, ethanolamine, and phenylalanine could be seen on the sheets.

The result of these experiments indicated that phenylalanine and ornithine were the only amino acids dansylated by treatment of the peptide. Ornithine migrated as the monodansyl derivative showing that only the δ-amino group was dansylated. Therefore, phenylalanine is believed to be the N-terminal amino acid of the X peptide.

A number of attempts to identify the blocking group on the N-terminal amino acid were made. The color tests for formate were not sensitive enough for the amount of material at our disposal. Radioactive formate was not incorporated into the peptide in the cell-free system, but it seemed possible that an activated form of the compound was required, and that the activating system was destroyed during the extraction procedure. To test this idea, 10 μCi of [^{14}C]sodium formate was added to each of six flasks, containing 300 ml of *B. brevis* culture apiece, 30 min before harvest. Cell-free extracts were made and the components needed for peptide synthesis were added. The resulting peptides were separated

on thin-layer plates and radioautograms were made. It was found that formate labeled the X peptide more heavily than gramicidin S. On elution, the X peptide was found to contain 600 cpm of ^{14}C and gramicidin S to contain 150 cpm. The label in gramicidin S was presumably due to the incorporation of formate into amino acids during the period of incubation with the living bacteria, as this peptide does not contain any constituents other than amino acids. Since the production of X peptide in this system is normally about 1% that of gramicidin S, the labeling of the X peptide by formate-derived amino acids could be estimated to be between 1 and 2 cpm. The bulk of the label must then have been in other components of the peptide, most probably as a formyl group.

This impression was strengthened by hydrolyzing the peptide for 4 hr in boiling methanol–1 N HCl. The mixture was evaporated to dryness under a nitrogen jet and plated for counting. During this treatment, two-thirds of the counts was lost, indicating that they had been present in a volatile material that was liberated from the peptide by mild hydrolysis. Amino acids and ethanolamine are not lost to an appreciable extent under these conditions.

Similar experiments with [^{14}C]acetate failed to label either peptide. Attempts were also made to label the ethanolamine moiety of the X peptide with either [^{14}C]-ethanolamine or [^{14}C]serine. Neither of these compounds were incorporated in either the cell-free extract or in extracts from prelabeled cells, however. The metabolic orgin of the ethanolamine in the X peptide remains obscure.

Size of the Peptide. The amino acid composition of the peptide indicated that it was basically a pentapeptide or a multiple of a pentapeptide unit. Gramicidin S itself is a decapeptide composed of a repeating sequence of five amino acids. To determine the size of the X peptide, ^{14}C-labeled material was passed through a variety of Sephadex columns in 50% acetic acid. Reference compounds were similarly chromatographed. They included gramicidin, a mixture of slightly different polypeptides 15 amino acids in length and with molecular weights around 1850, gramicidin S, the cyclic decapeptide of mol wt 1141, and the pentapeptide obtained from gramicidin S by digesting it with nagarse and half the size of its parent molecule. When the X peptide and the pentapeptide were passed through Sephadex G-10, they emerged with the volid volume as determined with Blue Dextran. Trials were made with G-15 and G-25, but the most satisfactory resolution was achieved with gels of larger pore size. Apparently the acetic acid solvent markedly reduced the effective pore sizes of the Sephadex gels. Gramicidin S and the X peptide were separated from the pentapeptide on Sephadex G-50. When G-75 was used, open-chain gramicidin was eluted well before gramicidin S. As illustrated in Figure 3, a composite diagram of these runs, the X peptide migrated with gramicidin S, or slightly ahead of it, in both of these systems. Clearly, it was very similar to gramicidin S in size and larger than a pentapeptide but smaller than the gramicidins. The X peptide therefore appears to be a decapeptide.

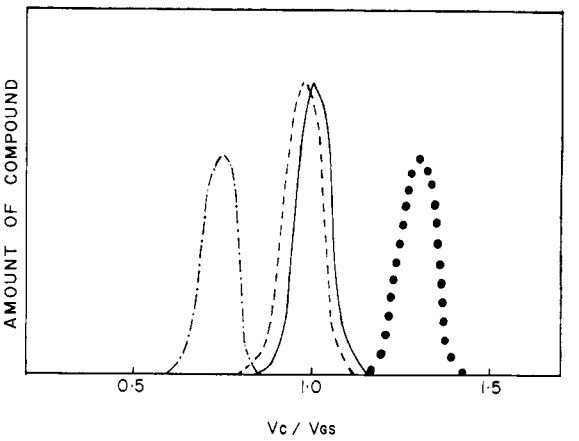

FIGURE 3: A composite, schematic diagram showing the elution volumes (V_C) of the various peptides relative to that of gramicidin S (V_{GS}) when they were passed through columns of Sephadex. (– · –) Gramicidin; (– – –) X peptide; (——) gramicidin S; (· · ·) pentapeptide.

Confirmation of the Structure of the X Peptide. In order to confirm that the X peptide contained the same sequence of amino acids as gramicidin S and was a precursor of it, attempts were made to convert it into gramicidin S. Labeled, isolated, X peptide should transfer label to gramicidin S in high yield when added back to an incubation mixture synthesizing gramicidin S. Unfortunately, the extreme insolubility of X peptide in most aqueous media made this a difficult experiment to carry out. A number of solubilizing agents were examined for their ability to dissolve the X peptide. Among them, ethanol and sodium lauryl sulfate were the most effective. Both of these compounds, however, virtually inactivated the synthetic system at the concentrations necessary to dissolved the peptide.

As an alternative approach to converting the X peptide into gramicidin S, a direct chemical method was tried. The X peptide, stripped of its blocking groups by 4-hr boiling in 1 N HCl–methanol, was dissolved in 4 ml of neutral methanol at 0°, and 2 mg of dicyclohexylcarbodiimide was added in 1 ml of ice-cold methanol. The solution was held at 0° for 4 days, then at room temperature for 3 more days (Hardy *et al.*, 1963). Carrier gramicidin S was added to the solution and it was dried under a nitrogen jet. The peptide mixture was then dissolved in ethanol–0.2 N HCl (9:1) and precipitated with 10% NaCl solution. This procedure was repeated as described for the isolation of the peptides (Bhagavan *et al.*, 1966). The peptides were then separated on a silica gel plate in solvent 4 and a radioautogram was made of the plate. The bulk of the radioactivity migrated as X peptide but a faint band was seen in the gramicidin S position. On elution, it was found that about 6% of the 15,000 cpm of X peptide used had been converted into material migrating with gramicidin S. This material was subjected to two-dimensional chromatography on silica gel plates using solvents 4 and 7 as the first and second developing agents. Radioautography showed that the labeled compounds were separated into two spots with about the same amount of activity in each (Figure 4). One of these did not migrate with any known substance while the

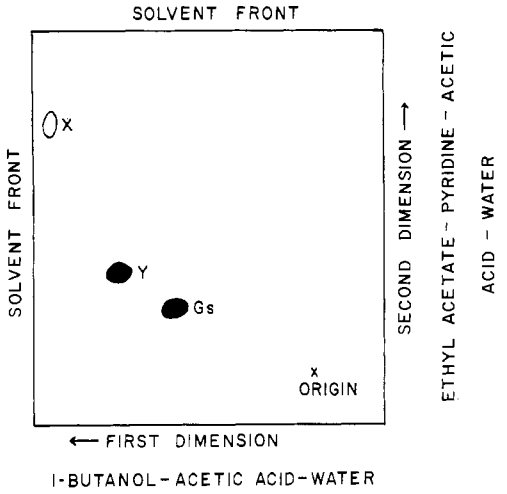

FIGURE 4: Two-dimensional chromatography of the products produced by the reaction between the X peptide and dicyclohexylcarbodiimide. "X" indicates the spot occupied by the X peptide on a control plate. "Y" is the unidentified labeled product and "Gs" the labeled product migrating with gramicidin S.

other was superimposed on carrier gramicidin S. As a control, untreated X peptide ws chromatographed in the same two-dimensional system. No contaminating gramicidin S was observed in this preparation.

These results indicate that treatment of the X peptide which has had the C- and N-terminal blocking groups removed, with dicyclohexylcarbodiimide, converts a significant fraction of it into a peptide closely resembling gramicidin S in chromatographic behavior.

Discussion

The results presented in this paper have led us to believe that we have isolated a new peptide intermediate which serves as an immediate precursor of gramicidin S. The relationship of the X peptide to gramicidin S is supported by the following observations. (1) The X peptide is very similar in solubility properties to gramicidin S, being insoluble in buffer solutions but readily dissolved by acidic ethanol. (2) This peptide contains the same five amino acids as gramicidin S, including the rather unusual amino acid, ornithine, and in the same proportions to each other. Neither peptide appears to contain other amino acids. (The phenylalanine in gramicidin S is in the D configuration, but no attempt was made to determine the configuration of phenylalanine in the X peptide, although the results of the cyclization experiment imply that it is also in the D form.) (3) The studies quoted in the introduction to this paper showed that phenylalanine was the N-terminal amino acid in almost all of the presumed precursors of gramicidin S that have been examined. Our studies of the X peptide indicate that phenylalamine is the N-terminal amino acid of this peptide also. If the X peptide has the same amino acid sequence as gramicidin S, leucine would then be its C-terminal amino acid, and leucine is found at the C terminus of the X peptide. These studies also imply that the direction of synthesis in this peptide is from the N-terminal amino acid to the C-terminal, just as it is in protein synthesis. (4) The X peptide is the same size as gramicidin S, containing ten amino acids. From the data at hand, it is not possible to demonstrate that these ten amino acids are arranged in the same repeating pentapeptide sequences as in gramicidin S, but the occurrence of an N-terminal phenylalanine and a C-terminal leucine suggest that they are. (5) Observation 4 is strengthened by the results of the experiment in which the X peptide was treated with dicyclohexylcarbodiimide. This reagent is an effective condensing agent for the formation of peptide bonds, and when used on peptides in dilute solution, as was done in these experiments, promotes cyclization of the peptide (Hardy *et al.*, 1963). Treatment of the X peptide with this reagent produces a compound which cochromatographs with gramicidin S, suggesting that the X peptide contains the same sequence of amino acids as gramicidin S and only needs to undergo ring closure to be converted into it.

The evidence for the presence of formate is somewhat indirect. The N-terminal amino group is clearly inaccessible to the usual amino-terminal reagents, and the masking group itself contains no amino group that could be detected by ninhydrin or dansylation. The blocking group is small, since the X peptide did not emerge from the molecular sieve columns appreciably ahead of gramicidin S. More significantly, formate activated in whole cells contributed label to the X peptide to an extent far greater than could be accounted for by its conversion into amino acids. It is unlikely that this label was in ethanolamine, which could readily have been labeled *via* serine, since neither ethanolamine nor serine labeled the peptide under the same conditions and since the labeled compound was more volatile than ethanolamine. Therefore it seems likely that the N-terminal blocking group was a formyl residue.

These lines of evidence, together with those for the presence of ethanolamine in the peptide, lead to structure I for the X peptide. A number of interesting ques-

HCO-D-Phe-L-Pro-L-Val-L-Orn-L-Leu-D-Phe-L-Pro-L-Val-L-Orn-L-Leu-$NHCH_2CH_2OH$

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tions are raised by this structure. The presence of formate on the N-terminal amino acid is reminiscent of the recent findings that an *N*-formylated amino acid serves as the initial amino acid in protein sysnthesis. It would be interesting to known whether or not the synthesis of these peptides is also initiated by an *N*-formylamino acid. Holm *et al.* (1966) found no masking group on the D-Phe N terminus of their tetrapeptide, and Otani *et al.* (1966) found that the D-Phe-L-Pro dipeptide readily formed a diketopiperazene indicating that the amino group was not blocked. If these peptides were unaltered intermediates in gramicidin S synthesis, the formyl group must be attached later in the course of synthesis, possibly just prior to cyclization, rather than at the initiation of the chain.

The role of ethanolamine is also somewhat unclear. Holm *et al.* (1966) observed an unidentified substituent on

the ornithine carboxyl end of their tetrapeptide. This group was fairly labile to both acid and alkali. Tomino *et al.* (1967) observed the formation of peptide fractions in their cell-free system which were not bound to Dowex 1 ion-exchange columns and thus appeared to have no free carboxyl groups. They also identified what appeared to be D-phenylalanineamide. It appears from these observations that chain elongation of this peptide may occur *via* the formation of intermediates activated at the carboxyl terminus by conjugation with ammonia, ethanolamine, and perhaps other compounds.

In our X peptide, ethanolamine is probably attached to the C-terminal leucine, presumably the last amino acid to be added to the chain, and must therefore be attached immediately before the cyclization reaction in which it is again eliminated. Possibly both formate and ethanolamine act as activators for the terminal amino acids, which are then joined by a kind of transpeptidation reaction to form the cyclic gramicidin S.

The finding of an open-chain peptide terminated by formate on one end and ethanolamine on the other in *B. brevis* 9999 is of considerable interest with respect to the evolutionary relationships of the antibiotic polypeptides of the various *B. brevis* strains. *B. brevis* 9999 is believed to produce only one antibiotic peptide, the cyclic decapeptide gramicidin S, and indeed, the open-chain X-peptide form is not found in extracts of this bacterium but in only produced in cell-free extracts under peptide-synthesizing conditions. Presumably it is an artifact of the cell-free system in that this peptide would normally never be released from the synthetic complex in the intact cell and is found free in our incubation mixtures only because of damage to the complex during extraction of the system from the bacteria. Other strains of *B. brevis*, on the other hand, produce two families of antibiotic polypeptides. One is made up of cyclic decapeptides closely resembling gramicidin S and called the tyrocidines. The other, unfortunately named the gramicidins, is composed of the open-chain forms mentioned earlier, which are terminated like the X peptide with formate at one end and ethanolamine at the other. The finding of an intermediate in the synthesis of one of the cyclic peptides which bears such an obvious resemblance to the members of the family of linear peptides implies that these two groups of antibiotics are not as distinct as the differences in their structures would suggest, but that they are related, and probably represent but two branched of the same family.

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