The Biosynthesis of Gramicidin S in a Cell-Free System

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1. A cell-free system prepared from *Bacillus brevis* cells, harvested in the late phase of growth and consisting of the 11000g supernatant, has been shown to incorporate into gramicidin S the five constituent amino acids added in labelled form. The results are consistent with complete synthesis and not merely a completion of pre-existing intermediate peptides. 2. The incorporation of ^{14}C -labelled amino acids by the 11000g supernatant into gramicidin S requires an energy source. Omission of phosphoenolpyruvate and pyruvate kinase from the incubation mixture prevents incorporation into gramicidin S. The cell-free system incorporates ^{14}C -leucine, -proline and -phenylalanine over a period of 4hr. With ^{14}C -leucine, incorporation into gramicidin S takes place in the range pH 6–9 with maximum incorporation at pH 7.0. High concentrations of chloramphenicol or puromycin decreased the incorporation into gramicidin S by only about 20%. 3. The 50000g supernatant exhibited no decrease in ability of incorporating ^{14}C -valine into gramicidin S as compared with the 11000g supernatant. About 40% of the incorporating ability remained in the 105000g supernatant after 3hr. centrifugation. When recombining the 105000g sediment with the 105000g supernatant, some increase in incorporation over that obtained with the supernatant alone was obtained. The findings tend to support the view that gramicidin S is synthesized in a different manner from that of proteins.

An increasing amount of interest is being shown in the study of the biosynthesis of bacterial polypeptides such as gramicidin and tyrocidine (Okuda, Edwards & Winnick, 1963; Uemura, Okuda & Winnick, 1963; Okuda, Uemura, Bodley & Winnick, 1964a,b; Mach, Reich & Tatum, 1963), gramicidin S (Winnick & Winnick, 1961; Winnick, Lis & Winnick, 1961; Eikhom, Jonsen, Laland & Refsvik, 1963, 1964; Berg, Frøholm & Laland, 1964a,b; Eikhom & Laland, 1964), bacitracin (Snoke, 1961; Bernlohr & Novelli, 1963), actinomycins (Katz & Weissbach, 1963; Albertini, Cassani & Ciferri, 1964) and the polymyxins (DiGirolamo, Ciferri, DiGirolamo & Albertini, 1964; Paulus & Gray, 1964). Such studies are of interest for several reasons. It is generally believed that the polypeptide chains of proteins are synthesized on ribosomes through the participation of messenger RNA (Brenner, 1961), but the smallest size of polypeptides produced by a ribosomal system is not known, nor is it known whether such a limit exists. In spite of the fact that bacterial polypeptides contain some D-amino acids and some of them are cyclic, a study of the synthesis of polypeptides of different sizes, e.g. in particular the gramicidins, tyrocidins and bacitracins, may shed some light on this problem. In this connexion it is worth remembering that gramicidin A (Sarges

& Witkop, 1964) with a sequence of 15 amino acids is close to the number of amino acids (21) in the A-chain of insulin.

Further, a study of the biosynthesis of some of these bacterial polypeptides may serve as model systems for the synthesis of polypeptide chains in general.

We have been studying the biosynthesis of the cyclic decapeptide gramicidin S produced by *Bacillus brevis*. The synthesis of gramicidin S occurs in whole cells under conditions where protein synthesis is inhibited by chloramphenicol, puromycin or mitomycin C (Eikhom *et al.* 1963, 1964; T. L. Berg, L. O. Frøholm & S. Laland, unpublished work; S. Laland, S. Nossen & T. Smith-Kielland, unpublished work) or actinomycin D (Eikhom & Laland, 1964). These experiments suggest a route of synthesis for gramicidin S that differs from that of bacterial protein. On the other hand, the specific activity of gramicidin S in various cell fractions after a short period of labelling with ^{14}C -labelled amino acid in whole cells was by far the highest in the fraction sedimenting at 105000g, and some relationship between a particulate fraction and gramicidin S synthesis (Eikhom *et al.* 1964) therefore seems to exist. To study the biosynthesis of gramicidin S more closely, a cell-free system has been developed

that will incorporate into gramicidin S the constituent amino acids when added in labelled form. This report presents evidence for the net synthesis of gramicidin S by this system. In addition, some of the characteristics of this system are described.

Some aspects of this work have previously been reported (Berg *et al.* 1964*a,b*).

MATERIALS AND METHODS

Chemicals. Amino acids were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. GTP and CTP were obtained from Pabst Laboratories, Division of Pabst Brewing Co., Milwaukee, Wis., U.S.A. ATP, UTP, phosphoenolpyruvate and GSH were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. The pyruvate kinase was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and was supplied as a suspension (10 mg./ml.) at pH 6 in a solution of 2.1 M-(NH₄)₂SO₄.

Radioactive amino acids. The following amino acids were purchased from The Radiochemical Centre, Amersham, Bucks.: DL-[carboxy-¹⁴C]valine, DL-[carboxy-¹⁴C]phenylalanine and L-[U-¹⁴C]leucine with specific activities 4.76 mc/m-mole, 10.8 mc/m-mole and 41.8 mc/m-mole respectively. DL-[5-¹⁴C]Ornithine hydrochloride, DL-[carboxy-¹⁴C]proline and U-¹⁴C-labelled algal-protein hydrolysate with specific activities 4.0 mc/m-mole, 5.5 mc/m-mole and 1.38 mc/mg. respectively were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.

Growth of the organism. The *B. brevis* strain ATCC 9999 was grown under aeration in a New Brunswick incubator shaker at 37° in a synthetic medium described by Eikhom *et al.* (1963). The density of the culture was measured at 650 mμ in 1 cm. cells in a Coleman Junior spectrophotometer.

Measurement of radioactivity. Radioactivity was measured at infinite thinness with a Tracerlab flow counter. Radioactive paper chromatograms were scanned in a Frieske and Hoepfner strip counter. The distribution of radioactivity on thin-layer chromatograms was measured after suspending 1 cm.² segments of the appropriate thin-layer supporting medium in ethanol-0.2 N-HCl (9:1, v/v) (0.5 ml.) and drying the mixture on the planchets before counting in the flow counter.

Preparation of cell-free extracts. The methods used for preparing cell-free extracts were based on those described by Uemura *et al.* (1963). A culture (340 ml.) of *B. brevis* with extinction about 0.5 was cooled by swirling in ice-water and centrifuged at 2°. The cells were washed with 100 ml. of a 5 mM-MgCl₂ solution and centrifuged again. The washing procedure was repeated once. The cells were then suspended in 17 ml. of 5 mM-MgCl₂ solution containing 10 μmoles of GSH/ml. (pH of solution adjusted to 7.5 with N-KOH) and mixed with 2 vol. of acid-washed glass beads, diam. 0.1 mm. (Edmund Bühler, Tübingen, Germany). The mixture was homogenized for 3 min. with ice cooling in the Vibrogen Zellmühle (Edmund Bühler) and subsequently filtered through a sintered-glass filter. During this treatment the temperature did not rise above 1°. The filtrate was centrifuged in a Servall centrifuge twice at 3000g, for 15 min. each time, and finally at 11000g for 15 min., and the supernatant used as the 'cell-free extract'.

Incubation mixture. Except where otherwise stated, 1 ml. of the 11000g supernatant (containing 5–6 mg. of protein)

was added to 1 ml. of a solution containing ATP (5 μmoles), phosphoenolpyruvate (5 μmoles), pyruvate kinase (20 μg.), MgCl₂ (5 μmoles), GSH (10 μmoles), K₂HPO₄ (10 μmoles), 1 μC of one of the ¹⁴C-labelled amino acids (0.21 μmole of DL-valine, 0.092 μmole of DL-phenylalanine, 0.024 μmole of L-leucine, 0.25 μmole of DL-ornithine and 0.18 μmole of DL-proline) occurring in gramicidin S, and in addition the remaining four as the unlabelled amino acids. Of these, 1 μmole each of L-ornithine and L-proline and 2 μmoles each of DL-leucine, DL-valine and DL-phenylalanine were used. The pH of all the individual solutions except the pyruvate kinase and the solution of the radioactive amino acid was adjusted to 7.5 with N-KOH before mixing. All incubations were carried out at 37°.

Extraction of gramicidin S and protein. The incubation mixture was adjusted to pH 3.5 with N-H₂SO₄ and heated at 100° for 5 min. The mixture was centrifuged, and the residue washed twice with aq. 2% (w/v) Na₂SO₄ solution, 2 ml. each time. The residue was then extracted for 2 hr. at room temperature with 3 ml. of an ethanol-0.2 N-HCl mixture (9:1, v/v). After centrifugation, the residue was washed twice with the above mixture, 2 ml. each time. The combined ethanolic extracts were evaporated to dryness, the residue was redissolved in 0.1 ml. of the ethanol-HCl mixture and the solution was subjected to chromatographic purification.

To extract the protein, the coagulated material after gramicidin S extraction was treated with 3 ml. of 5% (w/v) trichloroacetic acid at 90–100° for 30 min. The remaining residue was extracted with ethanol (3 ml.) for a brief period at 40°, subsequently with boiling methanol (2–3 ml.) for 30 min., washed with ethanol-ether (1:1, v/v) and finally dried with ether. The residue was dissolved in 0.5 ml. of N-NaOH and the solution used for plating and estimation of protein by the biuret method (Zamenhof, 1957).

It was desirable to isolate the protein by an alternative procedure, and therefore in one experiment the incubation mixture was mixed with 2 ml. of ice-cold 10% (w/v) trichloroacetic acid (Siekevitz, 1952; Mans & Novelli, 1961) made 0.1 M with regard to [¹⁴C]leucine and the precipitate centrifuged down after 15 min. at 0°. The precipitate was extracted with 3 ml. of 5% (w/v) trichloroacetic acid for 15 min. at 90–100°. The residue after centrifugation was washed once at 90–100° for a brief period with 3 ml. of the 5% (w/v) trichloroacetic acid. Gramicidin S was extracted from the insoluble material with ethanol-0.2 N-HCl as described above. The residue was then treated as already outlined, with ethanol, methanol, ethanol-ether and ether, and dissolved in N-NaOH.

In some experiments gramicidin S was extracted from the incubation mixture (2 ml.) by shaking three times with a mixture of butan-1-ol-chloroform (4:1, v/v), 2 ml. each time. The combined organic extracts containing gramicidin S were concentrated to dryness before further purification.

Methods used for purifying gramicidin S. Gramicidin S was chromatographed on Whatman no. 1 paper. The solvent system used was butan-1-ol-acetic acid-water (50:11:25, by vol.), where gramicidin S had *R_F* 0.91, and acetone, where it is sparingly soluble and remains at the site of application. Gramicidin S was also purified by thin-layer chromatography on silica (medium H according to Stahl, E. Merck A.-G., Darmstadt, Germany), with the upper phase of butan-1-ol-acetic acid-water (25:6:25, by vol.) as the solvent system, where it had *R_F* 0.46. Grami-

cidin S was eluted from the chromatograms with ethanol-0.2N-HCl (9:1, v/v).

Hydrolysis of gramicidin S. Gramicidin S was hydrolysed in 6N-HCl at 110° for 18 hr., the acid was removed and the hydrolysate was chromatographed on Whatman no. 1 paper in the butan-1-ol-acetic acid-water system.

Development of chromatograms. For spraying with ninhydrin a 0.2% (w/v) solution in acetone was used. For detecting peptides the chlorine-toluidine spray (Brenner & Pataki, 1961) was found suitable.

RESULTS

For the isolation of labelled gramicidin S, extraction with ethanol-HCl has been used (see the Materials and Methods section). When using the ethanol-hydrochloric acid procedure gramicidin S labelled with any of the following ¹⁴C-labelled amino acids, proline, valine, ornithine or leucine, was purified by paper chromatography in butan-1-ol-acetic acid-water (50:11:25, by vol.). Further purification by thin-layer chromatography on silica in butan-1-ol-acetic acid-water (25:6:25, by vol.) did not alter the radioactivity of the gramicidin S. When [¹⁴C]phenylalanine was used, purification by paper chromatography only was not satisfactory, since the isolated gramicidin S contained approx. 25% of radioactive impurities. Further purification by thin-layer chromatography removed the impurities, and this additional purification was always included in experiments with [¹⁴C]phenylalanine.

One experiment with the butan-1-ol-chloroform extraction procedure is described since it seems to offer possibilities when looking for possible intermediates (Fig. 3).

The incorporation of ¹⁴C-labelled amino acids into the hot-trichloroacetic acid-insoluble protein fraction isolated from the system used *in vitro* in the present study appeared to be unusual. For instance, the incorporation is not diminished on the addition of puromycin or chloramphenicol (Table 3). It is therefore questionable whether this incorporation represents 'normal' protein synthesis. With cell-free systems from other micro-organisms, e.g. *Escherichia coli*, it has been emphasized that, for studies on cell-free protein synthesis, the cells should be harvested in the early phase (Lamborg & Zamecnik, 1960; Doerfler, Zillig, Fuchs & Albers, 1962; Tissières, Schlessinger & Gros, 1960). The cells used in the present study were harvested in the late exponential phase at an extinction value of 0.5. With cells harvested in the early exponential phase at an extinction value of 0.1, incorporation of [¹⁴C]valine/mg. of protein in the cell-free system was five times as high as when the cells in the late exponential phase were used (experiments carried out by Miss Unni Spæren). In this case puromycin (10 µg./ml.) and chloramphenicol (100 µg./ml.) decreased incorporation by about 95 and 97% respectively. However, cell-free systems from cells harvested in the early exponential phase gave negligible incorporation into gramicidin S. Thus the preparation of cell-free extracts that would actively carry out incorporation of ¹⁴C-labelled amino acids into gramicidin S as well as 'normal' protein synthesis could not be prepared. To demonstrate the type of incorporation of ¹⁴C-labelled amino acids into the protein fraction that the gramicidin S-synthesizing system would carry out, some results on this have also been included.

Table 1. Incorporation of ¹⁴C-labelled amino acids into gramicidin S synthesized by the 11000g supernatant

Gramicidin S was extracted from the incubation mixture with ethanol-0.2N-HCl, and was chromatographed on paper in the butan-1-ol-acetic acid-water system before counting.

Radioactive amino acid added (1 µc)	Incubation period (hr.)	Radioactivity of gramicidin S (counts/min.)	Radioactivity of gramicidin S (counts/min.)/specific activity of amino acid (µc/m-mole)
DL-Proline	0	0	
	4	2970	540
DL-Valine	0	15	
	4	2880	605
DL-Ornithine	0	15	
	4	2000	500
L-Leucine	0	130	
	4	53400	1280
DL-Phenylalanine	0	125*	
	4	6300*	580

* Gramicidin S purified by paper chromatography was further purified by thin-layer chromatography in the butan-1-ol-acetic acid-water system before counting.

Experiments with the 11000g fraction

The 11000g supernatant used in the incorporation experiments could be stored for several weeks at -20° without loss of activity. In contrast, washed intact cells would lose 50% or more of the incorporating ability when stored overnight at -20° before preparation of the cell-free extract. Storage at -20° for longer periods (3–4 weeks) did not result in any further significant loss of activity. Freezing or thawing of whole cells therefore seems to affect the incorporating ability of the cell-free extract.

Incorporation of the constituent amino acids into gramicidin S. Table 1 demonstrates that the 11000g supernatant has the ability to incorporate all five ^{14}C -labelled amino acids of gramicidin S into the peptide. All the experiments were carried out simultaneously and with samples from the same 11000g batch. Zero-time incubation mixtures were used as controls. Table 1 also shows that the ratio of the radioactivity in gramicidin S to the specific activity of the amino acid is nearly the same in all cases except for leucine, where the higher value could be due to the use of the L-isomer.

Time-course of incorporation of ^{14}C -labelled amino acids into gramicidin S. To examine the incorporation into gramicidin S as a function of time, experiments with [^{14}C]-proline, -phenylalanine and -leucine were carried out (Fig. 1). There is no lag period for the incorporation of any of the three amino acids into gramicidin S and most of the incorporation occurs during the first 2 hr.

Effect of energy sources on the incorporation of [^{14}C]phenylalanine into gramicidin S and the protein fraction. Table 2 clearly shows that the incorpora-

tion of the labelled amino acid into gramicidin S by the cell-free system is energy-dependent. Although ATP and phosphoenolpyruvate + pyruvate kinase to some extent may substitute for each other the omission of both ATP and the energy-regenerating system completely eliminates the incorporation into gramicidin S. As expected, this also affects drastically the incorporation into the protein fraction. The addition of GTP, UTP and CTP does not seem to alter the extent of incorporation. The addition of the 17 amino acids listed in Table 2

Fig. 1. Time-course of incorporation of ^{14}C -labelled amino acids into gramicidin S by the 11000g supernatant. Each mixture was incubated for 3 min. before the addition of the appropriate amino acid: $\circ$, [^{14}C]proline (left-hand scale); Δ , [^{14}C]phenylalanine (left-hand scale); $\square$, [^{14}C]leucine (right-hand scale). Gramicidin S was extracted with ethanol-0.2N-HCl and purified by paper chromatography in the butan-1-ol-acetic acid-water solvent. Gramicidin S labelled with [^{14}C]phenylalanine was subsequently purified by thin-layer chromatography.

Table 2. Effect of energy sources on the incorporation of DL-[^{14}C]phenylalanine into gramicidin S by the 11000g supernatant

The total volume of the incubation mixture was 1 ml. and contained therefore half the amount of those substances described in the Materials and Methods section. A $0.5\mu\text{C}$ sample of DL-[^{14}C]phenylalanine was added 3 min. after the start of the incubation, which lasted 2 hr. Gramicidin S was extracted with the ethanol-0.2N-HCl mixture and purified by chromatography in the butan-1-ol-acetic acid-water solvent, first on paper and subsequently by thin-layer chromatography.

Changes in incubation mixture	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein fraction (counts/min.)
None	9000	1170
None	9650	1150
- ATP	3940	640
- Phosphoenolpyruvate and pyruvate kinase	5750	880
- ATP, phosphoenolpyruvate and pyruvate kinase	80	80
+ $1\mu\text{mole}$ each of GTP, UTP and CTP	8900	1040
+ $1\mu\text{mole}$ each of GTP, UTP, CTP and amino acids*	3200	640

* These amino acids were DL-alanine, L-arginine, DL-asparagine, DL-aspartic acid, L-cysteine, L-cystine, DL-glutamic acid, L-glutamine, glycine, L-histidine, DL-isoleucine, L-lysine, L-methionine, DL-serine, DL-threonine, DL-tryptophan and L-tyrosine.

decreased the incorporation into both gramicidin S and protein.

Effect of pH on the incorporation of [14 C]leucine into gramicidin S. The influence of pH on the incorporation into gramicidin S is shown in Fig. 2. For gramicidin S the pH optimum is at 7.0, but con-

siderable incorporation takes place over the range pH 6.5–8.5.

Effects of puromycin and chloramphenicol on the incorporation of [14 C]leucine into gramicidin S and the protein fraction. In view of the insensitivity of the synthesis of gramicidin S to puromycin and chloramphenicol in whole cells (Eikhom *et al.* 1963), the effect of these substances in the cell-free system has been tested. The concentrations of puromycin and chloramphenicol used decreased the incorporation into gramicidin S only by about 20% (Table 3). The incorporation into the protein fraction, isolated by alternative methods, was only slightly affected.

Effect of amino acid analogues on the incorporation of [14 C]valine into gramicidin S. Table 4 shows that

Fig. 2. Effect of pH on the incorporation of [14 C]leucine into gramicidin S by the 11000g supernatant. In addition to the standard components described in the Materials and Methods section, the incubation mixture contained glycine (10 μ moles). The pH of the mixture was adjusted to the desired value by the addition of N-KOH or N-HCl. A 1 μ C sample of [14 C]leucine was subsequently added and the mixture (total volume 2 ml.) was incubated for 20 min. Gramicidin S was extracted with ethanol-0.2N-HCl and purified by paper chromatography in the butan-1-ol-acetic acid-water system.

Table 4. *Effect of some amino acid analogues on the incorporation of DL-[14 C]valine into gramicidin S by the 11000g supernatant*

In the presence of the amino acid analogue (2 μ moles), the corresponding amino acid was omitted from the medium; otherwise the incubation mixture was as described in the Materials and Methods section. DL- β -2-Thienylserine and DL-*p*-fluorophenylalanine replaced DL-phenylalanine, and DL-norleucine replaced DL-leucine. After 4 hr. incubation, gramicidin S was extracted with the ethanol-0.2N-HCl mixture and purified by paper chromatography in the butan-1-ol-acetic acid-water system.

Addition	Radioactivity of gramicidin S (counts/min.)
None	1360
None	1440
DL- β -2-Thienylserine	185
DL- <i>p</i> -Fluorophenylalanine	1565
DL-Norleucine	1180

Table 3. *Effect of puromycin and chloramphenicol on the incorporation of [14 C]leucine into gramicidin S and protein by the 11 000g fraction*

The standard incubation mixture described in the Materials and Methods section contained in addition GTP (1 μ mole) and 0.5 μ mole each of the 17 amino acids listed in Table 2. The incubation mixtures were preincubated for 3 min. before adding 0.5 μ C of [14 C]leucine. Incubation was for 30 min. Gramicidin S was extracted with the ethanol-0.2N-HCl mixture and chromatographed on paper in the butan-1-ol-acetic acid-water solvent.

Addition	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
None	44200	960
None*	45700	950
Puromycin (200 μ g./ml.)	37600	890
Puromycin (200 μ g./ml.)*	33300	920
Chloramphenicol (100 μ g./ml.)	35400	810
Chloramphenicol (100 μ g./ml.)*	35300	830

* Reaction mixture treated in an alternative manner to that normally used for the isolation of protein (see the Materials and Methods section).

Table 5. *Effect of isotopic dilution on the incorporation of ^{14}C -labelled algal-protein hydrolysate into gramicidin S by the 11000g supernatant*

The volume of all incubation mixtures was 2 ml. and contained: 1 ml. of 11000g supernatant, ATP ($5\mu\text{moles}$), phosphoenolpyruvate ($5\mu\text{moles}$), pyruvate kinase ($20\mu\text{g.}$), MgCl_2 ($5\mu\text{moles}$), GSH ($10\mu\text{moles}$), K_2HPO_4 ($10\mu\text{moles}$) and $10\mu\text{C}$ of the algal-protein hydrolysate. The gramicidin S was extracted after 4 hr. incubation with the butan-1-ol-chloroform mixture and the product chromatographed twice on paper in the butan-1-ol-acetic acid-water solvent and once in acetone.

Addition of unlabelled amino acid	Radioactivity of gramicidin S (counts/min.)
None	44000
$2\mu\text{moles}$ each of DL-phenylalanine, DL-valine, DL-leucine and $1\mu\text{mole}$ each of L-proline and L-ornithine	16000
$20\mu\text{moles}$ each of DL-phenylalanine, DL-valine, DL-leucine and $10\mu\text{moles}$ each of L-proline and L-ornithine	10000

p-fluorophenylalanine and norleucine do not interfere with incorporation of [^{14}C]valine into gramicidin S, whereas β -2-thienylserine does.

Effect of isotopic dilution on the incorporation of ^{14}C -labelled amino acids into gramicidin S. To assess the effect of isotopic dilution on the incorporation into gramicidin S, an experiment in which uniformly labelled algal-protein hydrolysate was used as the source of the ^{14}C -labelled amino acids was carried out. As expected, the addition of the five unlabelled amino acids present in gramicidin S to the incubation mixture resulted in a diminished incorporation (Table 5). The purified gramicidin S fractions isolated from the incubation mixture containing no added unlabelled amino acids and from the one containing the largest amount were hydrolysed to the individual amino acids, chromatographed on paper in butan-1-ol-acetic acid-water and the papers scanned for radioactivity. In the latter case none of the areas on the paper corresponding to the constituent amino acids contained detectable radioactivity. In the other case, however, the areas corresponding to proline, valine, phenylalanine and leucine were radioactive. In neither case could radioactive areas corresponding to other amino acids be detected, whereas in both chromatograms there were radioactive areas near the solvent front originating from contaminants originally present in the algal-protein hydrolysate. These contaminants also explained the residual radioactivity (10000 counts/min.; see Table 2) in gramicidin S isolated from the incubation mixture after dilution with unlabelled amino acids. That this material was present as impurities in the algal-protein hydrolysate and co-chromatographed with gramicidin S was also shown by submitting the algal-protein hydrolysate to the extraction and purification procedure used for gramicidin S.

Fig. 3. Extraction of gramicidin S with butan-1-ol-chloroform from mixtures from zero-time and 4 hr. incorporation of DL- ^{14}C phenylalanine by the 11000g supernatant. In the zero-time experiment, the incubation was stopped immediately after the addition of [^{14}C]phenylalanine. Gramicidin S was extracted with the butan-1-ol-chloroform mixture and purified once on paper in the butan-1-ol-acetic acid-water solvent. Gramicidin S fractions from both zero-time and 4 hr. incubation were finally chromatographed on thin-layer silica and the plates scanned for radioactivity. Broken and continuous lines indicate the distribution of radioactivity after zero-time and 4 hr. incubation respectively. The part of the Figure below the diagram showing the distribution of radioactivity indicates the position of gramicidin S on the chromatogram: A, start of the chromatogram; B, position of the solvent front.

Extraction of gramicidin S with butan-1-ol-chloroform

When gramicidin S was extracted from the incubation mixture with butan-1-ol-chloroform, the gramicidin S region (labelled with [^{14}C]phenylalanine) after chromatography on paper in the butan-1-ol-acetic acid-water solvent contained several other radioactive substances besides gramicidin S. This is shown in Fig. 3, which gives the distribution of radioactivity on the plate after thin-layer chromatography of the gramicidin S fraction previously purified by paper chromatography. In addition, the results from zero-time incubation are recorded. The radioactive material (containing [^{14}C]phenylalanine) moving near the solvent front and detectable in the zero-time incubation experiment seemed to be an artifact and non-polar. When chromatographed in acetone (on paper) it moved with the solvent front, and could also in this way to a large extent be separated from gramicidin S, which then remained at the site of application.

The large radioactive peak in front of gramicidin S and the somewhat smaller one behind it (see Fig. 3) did not give a positive ninhydrin reaction, but gave a positive chlorine-toluidine reaction.

Experiments with the 50 000g and 105 000g fractions

The results for the incorporation of [^{14}C]valine into gramicidin S and protein by 50 000g and 105 000g fractions are presented in Table 6. To ensure a satisfactory resuspension of the 105 000g pellet, short treatment with ultrasonic vibration was used in some experiments. Since the cell-free extract is very sensitive to ultrasonic vibration (U. Spæren, L. O. Frøholm & S. Laland, unpublished work), controls were treated in a similar manner: treatment of the 11 000g supernatant with ultrasonic vibration for 30 sec. decreased the incorporating ability by about 50%.

The 50 000g supernatant incorporates [^{14}C]valine into gramicidin S to the same extent as does the 11 000g supernatant. The incorporating ability of the 105 000g supernatant is, however, considerably diminished as compared with the 50 000g supernatant. On the addition of the 105 000g pellet to the 105 000g supernatant, some stimulation occurred. When centrifuging the 11 000g supernatant for 12 hr. at 105 000g, very little incorporating ability remained in the supernatant.

It is surprising that the 105 000g supernatant (see

Table 6. *Incorporation of [^{14}C]valine into gramicidin S and protein by the 50 000g and 105 000g fractions*

The following fractions were prepared in a Spinco ultracentrifuge (rotor no. 40). A 50 000g supernatant was obtained by centrifugation of the 11 000g fractions for 30 min. In addition, 13 ml. of the 11 000g supernatant was centrifuged at 105 000g for 3 hr. at 0–2°. The pellet was suspended with the aid of a small Potter-Elvehjem homogenizer in 26 ml. of a solution of 5 mM-MgCl₂ containing GSH (10 μ moles/ml.). The suspension was divided into two, and both portions were centrifuged for 1 hr. at 105 000g. The pellets in the two tubes were suspended in 6.5 ml. of the 105 000g supernatant and 6.5 ml. of the MgCl₂-GSH solution respectively. Parts of all fractions were treated separately in an MSE-Mullard Ultrasonic Disintegrator (20 kcy./sec.) for 30 sec. In one experiment the 11 000g supernatant was centrifuged for 12 hr. at 105 000g. As a control the 11 000g supernatant was kept at 2° for 12 hr. Samples (1 ml.) of the fractions were used in the incubation mixtures, which were prepared as described in the Materials and Methods section. The incubation period was 1 hr. Gramicidin S was extracted with ethanol-0.2 N-HCl and purified on paper in the butan-1-ol-acetic acid-water system.

Cell fraction used	Concn. of protein in fraction (mg./ml.)	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
11 000g supernatant	6.7	7240	1000
11 000g supernatant*	6.7	3790	600
50 000g supernatant	5.6	7640	920
105 000g supernatant	4.3	2970	700
105 000g supernatant*	4.3	2100	520
105 000g sediment	1.4	80	120
105 000g sediment*	1.4	20	60
105 000g sediment + supernatant	5.7	3450	760
105 000g sediment + supernatant*	5.7	2900	640
105 000g supernatant†	0.8	20	140
11 000g supernatant‡	6.7	1280	520

* Treated for 30 sec. with ultrasonic vibrations.

† Centrifuged for 12 hr. at 2°.

‡ Kept at 2° for 12 hr.

Table 6) retained the ability to incorporate considerable radioactivity into the protein.

DISCUSSION

The sequence of amino acids in gramicidin S is as follows (Consden, Gordon, Martin & Synge, 1946; Schwyzer, 1958):

and the biosynthesis of gramicidin S therefore consists of joining five different amino acids, each occurring twice, in a cyclic structure.

The present results are strongly indicative of a cell-free synthesis of gramicidin S by the 11000g supernatant as measured by incorporation of radioactive amino acids. The system shows an absolute requirement for an energy source such as ATP or phosphoenolpyruvate + pyruvate kinase. This, together with the nearly identical incorporating ability of the 11000g and 50000g supernatant fractions, should exclude the possibility of whole cells being responsible for the synthesis of the peptide. All five constituent amino acids, when added in labelled form, are incorporated into gramicidin S. It therefore seems very unlikely that one is only dealing with a completion of pre-existing intermediate peptides.

The experiment with the ^{14}C -labelled algal-protein hydrolysate demonstrates that valine, leucine, proline and phenylalanine are the only amino acids incorporated to any measurable extent into the purified gramicidin S. As the algal-protein hydrolysate does not contain ornithine and the synthesis of this amino acid takes place to only a limited extent *in vitro*, little radioactivity in ornithine would be expected. As expected, with dilution of the ^{14}C -labelled algal-protein hydrolysate with a large excess of the unlabelled amino acids of gramicidin S, incorporation is depressed to an extent where no radioactivity corresponding to amino acids can be detected in the hydrolysate of the isolated peptide fraction.

There are several characteristic features of the cell-free system used in the present investigations.

Extensive incorporation into gramicidin S of radioactive leucine, phenylalanine and proline (occurring in this order in the peptide) takes place for 2 hr. continued incorporation, though at diminishing rate, proceeding for the next 2 hr. No lag period for the incorporations could be detected.

The incorporation into gramicidin S takes place over a fairly wide range of pH. Considerable radioactivity was found in the isolated product after incubation between pH 6 and 9. In the experiment described an incubation period of 20 min. was used.

In some other experiments where longer incubation periods were employed, incorporation into gramicidin S was found to take place even at pH 10. It therefore seems unlikely that s-RNA (transfer RNA) plays a role in the biosynthesis of gramicidin S, as the amino acyl-s-RNA intermediates would be expected to be hydrolysed under such alkaline conditions (Decken & Campbell, 1962).

The slight effects of puromycin and chloramphenicol on the incorporation into gramicidin S also suggest a mechanism of gramicidin S biosynthesis different from that of protein biosynthesis. This finding is in agreement with the results of experiments with whole cells (Eikhom *et al.* 1963, 1964).

The experiments with subfractions of the 11000g supernatant give some indications as to the site of gramicidin S formation. The 50000g supernatant exhibited no decrease in incorporating ability as compared with the 11000g supernatant, whereas 40% of the incorporating ability still remained in the 105000g supernatant (3 hr.). When recombining the 105000g sediment and the 105000g supernatant some increase in incorporation over that obtained with the supernatant alone was found. Centrifugation for 3 hr. at 105000g should suffice to sediment most of the ribosomal material. Further, it seems evident that a particulate fraction sedimenting between 50000g and 105000g is important for maximum incorporation into gramicidin S. A possible explanation is that the synthesis of gramicidin S requires some small particles such as the oxidosomes (De Ley, 1963). These particles, which can be identified by their enzymic activity, sediment slowly at 105000g and are therefore partially removed together with the ribosomes. Some support for this view also comes from experiments with whole cells where, after short-time labelling with ^{14}C -labelled amino acids, total radioactivity of gramicidin S as well as specific activity were highest in the 105000g sediment (4 hr.) (Eikhom *et al.* 1964). The RNA/protein ratio of this sediment was between 0.3 and 0.4, which clearly shows that the fraction is contaminated by non-ribosomal material. The high protein content of this fraction could therefore be due to the presence of other particles.

The decreased incorporation obtained with the 105000g supernatant could also be explained if certain amounts of the soluble enzymes involved in the synthesis of gramicidin were adsorbed on the particles of the 105000g sediment.

Studies on the biosynthesis of gramicidins and tyrocidines in cell-free systems prepared from the Dubos BG strain of *B. brevis* (Uemura *et al.* 1963; Okuda *et al.* 1964a,b; Bodley, Uemura, Okuda, Adiga & Winnick, 1964) have recently been published. A considerable net synthesis of tyrocidines in 4 hr. has been demonstrated in this system. Their results so far indicate that the biosynthesis of the

two groups of antibiotics is similar to protein biosynthesis, as they have presented evidence for the participation of s-RNA and ribosomes. The particulate fraction consists, at the optimum Mg^{2+} concentration of two components, of a 56s fraction (90%) and a 36s fraction (10%), both of which have the ability to synthesize gramicidins and tyrocidines. Also, their synthesis is sensitive to puromycin and chloramphenicol *in vitro* as well as *in vivo*. However, Mach *et al.* (1963) have demonstrated in another strain of *B. brevis* the insensitivity *in vivo* of the biosynthesis of tyrocidine to these two inhibitors.

Winnick and his collaborators have also reported a few experiments on the biosynthesis of gramicidin S (Okuda *et al.* 1964a), and one gains the impression that the authors believe that gramicidin S is synthesized in a manner similar to that of gramicidin A and tyrocidine. The fact that we find that gramicidin S synthesis is hardly sensitive to puromycin and chloramphenicol and the finding that the particles that sediment at 105 000g (3 hr.) are not required for synthesis make the discrepancies between their and our results difficult to understand, particularly since our cell-free system and incubation conditions were very similar to theirs, the only difference being in the conditions for growing and disrupting the bacterial cells. We have also attempted preparing the cell-free system from cultures of *B. brevis* grown on a tryptone-yeast medium as used by Winnick and his collaborators. The incorporations obtained with these preparations were very poor. This result was not unexpected as the production of gramicidin S in cultures of *B. brevis* grown on this medium is significantly lower than in cultures grown on the synthetic medium.

The cell-free system used in the present study for incorporating ^{14}C -labelled amino acids into gramicidin S also incorporates these amino acids into the protein fraction. The addition of 17 unlabelled amino acids to the system (Table 2) decreased, somewhat unexpectedly, the incorporation of the ^{14}C -labelled amino acids into the protein. However, a similar effect is found for a 'crude' extract (8000g supernatant) from *E. coli* that incorporates ^{14}C -labelled amino acids into protein (Tissières *et al.* 1960). The incorporation into the protein fraction is not substantially affected by chloramphenicol or puromycin, and a considerable incorporation takes place into the protein fraction by the 105 000g supernatant. It is therefore questionable whether the incorporation into the protein fraction represents 'normal' protein synthesis. However, protein synthesis in a chloramphenicol-inhibited system from *Bacillus megaterium* (Aronsen & Spiegelman, 1961) and incorporation of amino acids by the protein of the post-microsomal fraction of rat liver (Hird, McLean & Munro, 1964) have also been described. These latter results differ from those usually reported

for the incorporation of ^{14}C -labelled amino acids into proteins in bacterial cell-free systems.

The important question in the biosynthesis of gramicidin S is whether the amino acids are put together on some kind of template or whether the amino acids are joined together in the right sequence by enzymes specific for each peptide bond. Further research is required to answer this question. However, the presence in the butan-1-ol-chloroform extract (from the 11000g supernatant incorporating [^{14}C]phenylalanine) of ^{14}C -labelled amino acid-containing peptides (Fig. 3) more radioactive than gramicidin S may be indicative of intermediates and hence favour a stepwise synthesis.

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