Studies on the Biosynthesis of Gramicidin S in a Cell-Free System from *Bacillus brevis*

FURTHER ATTEMPTS TO ELUCIDATE ITS MECHANISM OF SYNTHESIS

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1. The pH optima for the incorporation of ^{14}C -labelled amino acids into gramicidin S by an 11000g cell-free extract from *Bacillus brevis* have been determined. The pH optima for leucine, proline, phenylalanine, ornithine and valine were 7.5–7.7, 7.5–7.7, 7.7–7.9, 7.7–7.9 and 8.0–8.2 respectively. Hence the greatest difference in pH optima existed between leucine and valine, where it was 0.5pH unit. 2. The 11000g cell-free extract incorporated into gramicidin S only the L-isomers of valine, proline and ornithine. However, both isomers of leucine are utilized and the experiments indicate that a leucine racemase exists in the 11000g cell-free extract. With phenylalanine the L-isomer is utilized much more effectively than the D-isomer. This is noteworthy since it is the D-isomer that occurs in gramicidin S. The experiments indicate that conversion of the L-isomer into the D-form takes place at a stage beyond that of the free amino acid.

A cell-free system prepared from *Bacillus brevis* that incorporates the five constituent amino acids in labelled form into gramicidin S in a different way to incorporation into protein was first described by Berg, Frøholm & Laland (1965).

An improved 11000g cell-free system for the synthesis of gramicidin S was developed by Spæren, Frøholm & Laland (1967). Additional support in favour of the view that gramicidin S synthesis is different to that of proteins was also presented. In a further study, it was thought that knowledge of the pH optima for the incorporation of the ^{14}C -labelled constituent amino acids into gramicidin S might throw some light on its mechanism of synthesis. The utilization of both the L- and D-isomers of the constituent amino acids in gramicidin S synthesis was also examined.

The experiments show that for some of the amino acids different pH optima exist. With valine, proline and ornithine only the L-isomers were utilized. Of particular interest is that both isomers of leucine were used and that L-phenylalanine is much more readily incorporated than D-phenylalanine into gramicidin.

Some aspects of this work have been reported by Figenschou, Frøholm & Laland (1965).

MATERIALS AND METHODS

Growth of the organism. The *B. brevis* strain A.T.C.C. 9999 was grown under aeration in the synthetic medium described by Eikhom, Jonsen, Laland & Refsvik (1964) at

38–40°. The extinction of the culture was measured at 650m μ in 1 cm. cells in a Coleman Junior spectrophotometer.

Preparation of cell-free extracts. Cultures were harvested at an extinction value of 0.48–0.50. The 11000g supernatant used as a cell-free extract was prepared as described by Spæren *et al.* (1967) except that the cell suspension was submitted to ultrasonic treatment for 1.5 min. instead of 1 min.

Incubation mixture. The mixture D described by Spæren *et al.* (1967) with small modifications was used. The complete incubation mixture in a total volume of 1 ml. contained: 0.5 ml. of cell-free extract (in most cases the 11000g supernatant), triethanolamine hydrochloride (100 μ moles), KCl (5 μ moles), mercaptoethanol (6 μ moles), magnesium acetate (50 μ moles), $(\text{NH}_4)_2\text{SO}_4$ (100 μ moles), ATP (2.5 μ moles), phosphoenolpyruvate (2.5 μ moles), pyruvate kinase (20 μ g.) and 0.05 μ mole each of L-phenylalanine, L-leucine, L-serine, L-threonine, L-isoleucine, L-tyrosine, L-ornithine, L-proline, L-lysine, L-methionine, L-histidine, L-cysteine, L-cystine, L-aspartic acid, L-asparagine, L-alanine, L-tryptophan, L-arginine, L-glutamine, L-glutamic acid, L-valine and glycine. The labelled amino acid replaced the corresponding unlabelled amino acid in the above list. The incubation mixture was preincubated at 37° for 1 min. before addition of the radioactive amino acid. The incubation period was 15 min.

Isolation of gramicidin S and protein from the incubation mixture. The methods described by Spæren *et al.* (1967) were used. When required, gramicidin S was purified on Whatman no. 1 paper in butan-1-ol-acetic acid-water (50:11:25, by vol.) or by two-dimensional thin-layer chromatography on silica (medium H according to Stahl, E. Merck A.-G., Darmstadt, Germany) with ethyl acetate-pyridine-acetic acid-water (30:10:3:6, by vol.) in one

dimension and butan-1-ol-acetic acid-water (25:6:25, by vol.) in the second dimension.

Measurement of radioactivity. Radioactivity was measured with a Tracerlab SC-16 windowless flow counter.

Estimation of protein. The method used was that described by Zamenhof (1957). Egg albumin was used as a standard.

Chemicals. Amino acids were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A., Sigma Chemical Co., St Louis, Mo., U.S.A., and Hoffman-La Roche and Co. Ltd., Basle, Switzerland. ATP, phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) in a solution of 2.1M-(NH₄)₂SO₄ (10mg./ml.) were obtained from Sigma Chemical Co.

Radioactive amino acids. The following labelled amino acids were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.: L-[U-¹⁴C]leucine (10.0mc/m-mole), DL-[1-¹⁴C]valine (5.0mc/m-mole), L-[U-¹⁴C]proline (4.0mc/m-mole), DL-[5-¹⁴C]ornithine (4.0mc/m-mole), L-[U-¹⁴C]-phenylalanine (10mc/m-mole) and D-[1-¹⁴C]phenylalanine (10.0mc/m-mole). DL-[1-¹⁴C]Phenylalanine (4.0mc/m-mole) was purchased from The Radiochemical Centre, Amersham, Bucks.

Measurement of pH. All pH values were measured at 20° with a pH-meter no. 22 from Radiometer, Copenhagen, Denmark.

RESULTS

The effect of pH on the incorporation of all five amino acids into gramicidin S by using the 11000g extract is shown in Fig. 1. Triethanolamine hydrochloride was used as buffer in all these experiments, since it could be used in the range 6.5–9.0 and did not seem to inhibit the synthesis of gramicidin S. No changes in pH occurred during incubation. The degree of incorporation of the five amino acids into gramicidin S is not quite comparable, since two different batches of cell-free extract were used and the incorporation experiment for each amino acid was carried out on different days. Fig. 1 shows that there are definite pH maxima for each amino acid. The pH optima for leucine, proline, phenylalanine, ornithine and valine were 7.5–7.7, 7.5–7.7, 7.7–7.9, 7.7–7.9 and 8.0–8.2 respectively. The greatest difference in pH optima is between leucine and valine, where it is 0.5pH unit. The pH optimum (8.2–8.4) found previously for valine (Spæren *et al.* 1967) in a glycine buffer is in acceptable agreement with the present value (8.0–8.2). The value of 7 for the pH optimum for leucine in a different incubation mixture reported by Berg *et al.* (1965) is in reasonable agreement with the present result.

The utilization of the D- and L-isomers of phenylalanine, valine, leucine, proline and ornithine in the biosynthesis of gramicidin S by using the 11000g extract is described in Tables 1, 2 and 3. Both isomers of leucine are utilized equally well, but with valine, proline and ornithine only the L-isomers seem to be utilized. Both isomers of phenylalanine

FIG. 1. Effect of pH on the incorporation of all five constituent ¹⁴C-labelled amino acids into gramicidin S by the 11000g extract. The pH was adjusted in the incubation mixture by the addition of NaOH or HCl before the addition of ATP, PEP, pyruvate kinase and the ¹⁴C-labelled amino acid. The results for [¹⁴C]ornithine (□), [¹⁴C]proline (▲) and [¹⁴C]phenylalanine (Δ) were obtained with an extract containing 4.5mg. of protein/ml., and those for [¹⁴C]leucine (○) and [¹⁴C]valine (●) with an extract containing 5.3mg. of protein/ml. In these experiments 0.5μC of DL-[¹⁴C]ornithine, 0.5μC of L-[¹⁴C]proline, 1.0μC of DL-[¹⁴C]phenylalanine, 0.5μC of L-[¹⁴C]leucine or 0.5μC of DL-[¹⁴C]valine was used. The isolated gramicidin S was purified on paper chromatography in butan-1-ol-acetic acid-water.

Table 1. Utilization of the D- and L-isomers of leucine and valine in the biosynthesis of gramicidin S by the 11000g extract

In these experiments 0.5μC of L-[¹⁴C]valine or 1.0μC of DL-[¹⁴C]leucine was used. The incubation mixtures contained 3.6mg. of protein/ml. The isolated gramicidin S was purified on paper by chromatography in butan-1-ol-acetic acid-water. The amount of unlabelled amino acids added was ten times the amount of the corresponding ¹⁴C-labelled isomer. The pH of the incubation mixtures was 7.5.

Amino acids	Radioactivity of gramicidin S (counts/min.)
L-[¹⁴ C]Leucine	7890
L-[¹⁴ C]Leucine + 10 × L-[¹² C]leucine	1187
L-[¹⁴ C]Leucine + 10 × D-[¹² C]leucine	618
DL-[¹⁴ C]Valine	2206
DL-[¹⁴ C]Valine + 10 × L-[¹² C]valine	154
DL-[¹⁴ C]Valine + 10 × D-[¹² C]valine	1784

are utilized, but the L-isomer is incorporated much more effectively.

In the experiments described in Tables 2 and 3

Table 2. Utilization of the D- and L-isomers of phenylalanine, proline and ornithine in the biosynthesis of gramicidin S by the 11000g extract

In these experiments 0.5 μ C of L-[¹⁴C]proline or 1.0 μ C of DL-[¹⁴C]ornithine or DL-[¹⁴C]phenylalanine was used. The incubation mixtures contained 5.6 mg. of protein/ml. The amount of unlabelled amino acids added was ten times the amount of the corresponding ¹⁴C-labelled isomer. The pH of the incubation mixtures was 7.5.

Amino acids	Radioactivity of gramicidin S (counts/min.)
DL-[¹⁴ C]Phenylalanine	14159
DL-[¹⁴ C]Phenylalanine + 10 $\times$ L-[¹² C]-phenylalanine	2574
DL-[¹⁴ C]Phenylalanine + 10 $\times$ D-[¹² C]-phenylalanine	8744
L-[¹⁴ C]Proline	24399
L-[¹⁴ C]Proline + 10 $\times$ D-[¹² C]proline	26448
L-[¹⁴ C]Proline + 10 $\times$ L-[¹² C]proline	1471
DL-[¹⁴ C]Ornithine	15868
DL-[¹⁴ C]Ornithine + 10 $\times$ L-[¹² C]ornithine	1418
DL-[¹⁴ C]Ornithine + 10 $\times$ D-[¹² C]ornithine	13141

Table 3. Utilization of D- and L-isomers of phenylalanine in the biosynthesis of gramicidin S by the 11000g extract

In these experiments 0.5 μ C of L-[¹⁴C]phenylalanine or D-[¹⁴C]phenylalanine was used. The incubation mixtures contained 3.2 mg. of protein/ml. The pH of the incubation mixtures was 7.5. The amount of unlabelled amino acids added was ten times the amount of the corresponding ¹⁴C-labelled isomers.

Amino acids	Radioactivity of gramicidin S (counts/min.)
L-[¹⁴ C]Phenylalanine	76171
L-[¹⁴ C]Phenylalanine + 10 $\times$ L-[¹² C]-phenylalanine	7080
L-[¹⁴ C]Phenylalanine + 10 $\times$ D-[¹² C]-phenylalanine	52677
D-[¹⁴ C]Phenylalanine	5225
D-[¹⁴ C]Phenylalanine + 10 $\times$ L-[¹² C]-phenylalanine	302
D-[¹⁴ C]Phenylalanine + 10 $\times$ D-[¹² C]-phenylalanine	1730

the isolated gramicidin S was not purified by chromatography before being counted. This was not found to be necessary, as control experiments

showed that the error introduced by omitting this step did not exceed $\pm 10\%$.

DISCUSSION

The experiments on the pH optima for the incorporation of the five amino acids into gramicidin S show that differences exist. The differences have repeatedly been reproduced. It is believed that differences in the pH optima of 0.2–0.3 pH unit are significant. With leucine and valine the difference in the optimum is as much as 0.5 pH unit. Further, at the pH optimum for valine (8.0–8.2) the incorporation of leucine is decreased by 25%. Thus for these two amino acids a considerable difference exists. Hence a different mechanism for the incorporation of these two amino acids into the gramicidin S molecule may be operating. Possibly the difference in pH optimum for the incorporation of different amino acids only reflects optimum conditions for the enzymic activation of the amino acids. However, no conclusion is possible at present.

Both the L- and the D-isomers of leucine are about equally well utilized for the biosynthesis of gramicidin S and show that a leucine isomerase exists in the cell-free extract. The experiments with L- and D-phenylalanine show that the L-isomer is much better utilized than the D-isomer in the biosynthesis of gramicidin S, even though the polypeptide contains the D-isomer. This indicates that the conversion of the L-isomer into the D-form takes place at a stage beyond that of the free amino acids. In a similar manner Katz & Weissbach (1965) found for actinomycin D that L-valine is a much better precursor for D-valine in the antibiotic than is the D-isomer.

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