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

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1. An improved 11000g cell-free system for the incorporation of [^{14}C]valine into gramicidin S has been obtained. The cell-free extract used was the supernatant obtained by treating *Bacillus brevis* with ultrasonics for 1 min. followed by centrifugation at 11000g. The optimum pH for the incorporation was 8.2–8.4 and the optimum Mg^{2+} concentration 0.05 M. The presence of ammonium sulphate (0.1 M) and K^+ (0.01 M) increased the incorporation. 2. Cell-free extracts prepared from cells harvested in the early phase of growth (extinction value 0.1) incorporated negligible amounts of [^{14}C]valine into gramicidin S compared with that incorporated by cell-free extracts prepared from cells harvested in the late phase of growth (extinction value 0.5). This was not due to the presence of inhibitors in the cell-free extracts prepared from cells harvested early, since there was no marked decrease in gramicidin S synthesis in a mixture of extracts prepared from cells harvested early and late in the growth phase. 3. The small incorporation of [^{14}C]valine into protein, which took place in cell-free extracts from cells harvested in the late growth phase, was not inhibited by puromycin, chloramphenicol and ribonuclease. However, the substantial incorporation that took place in cell-free extracts prepared from cells harvested in the early phase of growth was completely inhibited by puromycin, chloramphenicol and ribonuclease. On mixing cell-free extracts prepared from cells harvested early and late in the growth phase, it appeared that the small incorporation that occurs in extracts from cells harvested in the late phase of growth was not due to cellular inhibitors.

A cell-free system prepared from *Bacillus brevis* that incorporates the five constituent amino acids in labelled form into gramicidin S has been described (Berg, Frøholm & Laland, 1965). To improve the performance of the cell-free system, several methods for producing cell-free extracts as well as changes in the composition of the incubation mixture have been investigated. The amino acid chosen for measuring the synthesis of gramicidin S was [^{14}C]valine. Compared with that described by Berg *et al.* (1965), a considerably improved cell-free system has been obtained.

Berg *et al.* (1965) briefly mentioned that incubation mixtures with cell-free extracts prepared from cells harvested late in the growth phase, and which synthesized gramicidin S, gave a small incorporation of labelled amino acid into protein that was not affected by puromycin, chloramphenicol and ribonuclease. However, cell-free extracts prepared from cells harvested early in the growth phase carried out incorporation into protein that was prevented by the above inhibitors (Spæren,

Frøholm & Laland, 1965; Berg *et al.* 1965). These extracts gave negligible incorporation into gramicidin S. A further investigation of these findings is reported in the present work. By combining extracts prepared from cells harvested early and late in the growth phase, it was found that the negligible gramicidin S synthesis that occurs in cell-free extracts from cells harvested in the early growth phase was not due to the presence of inhibitory substances. Further, the lack of normal incorporation of amino acids into protein, in cell-free extracts from cells harvested late in the growth phase, is not caused by the presence of inhibitory substances.

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, Refsvik & Laland (1963) at 38–40°. The density of the culture was measured at 650 m μ in 1 cm. cells in a Coleman Junior spectrophotometer.

Disintegration of cells. The Vibrogen Zellmühle used was that described by Berg *et al.* (1965). The ultrasonic apparatus was a MSE-Mullard Ultrasonic Disintegrator (60w and 20kcy./sec.). The bacterial press was that described by Hughes (1951).

The aluminium oxide was levigated alumina purchased from Norton Co., Worcester, Mass., U.S.A.

Preparation of cell-free extracts. Cultures were harvested at an extinction value 0.48–0.52, placed in ice-water for 30 min. and the cells subsequently isolated by centrifugation at 0–5°. Cells from 1000 ml. of medium were washed with cold 5 mM-MgCl₂ solution (300 ml.) and recentrifuged at 0–5°. This procedure was repeated once. The cells were then suspended in a solution of 5 mM-MgCl₂ containing 10 μ moles of GSH/ml. (pH of the solution was adjusted to 7.5 with N-KOH). Each ml. of cell suspension corresponded to cells from 30 ml. of the original culture. In a few experiments, cultures with an extinction value 0.1 were used. In such cases 1 ml. of the final cell suspension corresponded to 150 ml. of the original culture. All cell suspensions were submitted to ultrasonic treatment in 5 ml. portions at 0° for 1 min. and centrifuged at 3000g for 15 min. The supernatant was recentrifuged under identical conditions and the supernatant so obtained was finally centrifuged at 11000g for 15 min., yielding the 11000g supernatant.

Incubation mixtures. A survey of the incubation mixtures used is given in Table 1. For preparing incubation mixture D (Table 1) a volume (0.1 ml.) of a solution (pH adjusted to 9.2 with NaOH) containing glycine (100 μ moles), KCl (5 μ moles) and mercaptoethanol (6 μ moles), 0.05 ml. of a

solution of magnesium acetate (50 μ moles, pH adjusted to 8.4), 0.05 ml. of a solution of ammonium sulphate (100 μ moles, pH adjusted to 7.5 with NaOH), 0.05 ml. of a solution of ATP (2.5 μ moles, pH adjusted to 7.5 with NaOH), 0.05 ml. of a solution of phosphoenolpyruvate (2.5 μ moles), pyruvate kinase (20 μ g.), 0.1 ml. of a solution adjusted to pH 7.5 with N-NaOH and containing 0.1 μ mole of DL-phenylalanine, DL-leucine, DL-serine, DL-threonine, DL-isoleucine, DL-tyrosine and 0.05 μ mole of 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, glycine, L-glutamine and L-glutamic acid and 0.5 ml. of the 11000g supernatant were mixed and the total volume was adjusted to 0.99 ml. The mixture was preincubated at 37° for 1 min. and 0.01 ml. of a solution of 0.5 μ C of DL-[¹⁴C]valine (0.1 μ mole) added. The final pH of the incubation mixture was 8.4.

Extraction of gramicidin S and protein. The incubation mixture (1 ml.) was added to 1 ml. of ice-cold 10% (w/v) trichloroacetic acid containing 0.5% of sodium tungstate (Griffin, Ward, Canning & Holland, 1964) and 0.2 M-DL-[¹²C]valine. The precipitate was centrifuged down for 20 min. at 0° and resuspended in 3 ml. of ice-cold 3% (w/v) trichloroacetic acid containing 0.5% of sodium tungstate and subsequently centrifuged. The washing procedure was repeated once. Determination of the radioactivity of the supernatants showed that two washings were sufficient. The residue was then extracted with 3 ml. of 5% (w/v) trichloroacetic acid containing 0.25% of sodium tungstate at 95° for 30 min. After centrifugation, the solid was

Table 1. *Composition of incubation mixtures A, B, C and D*

	A	B	C	D
KH ₂ PO ₄	5 μ moles			
Tris		100 μ moles	100 μ moles	
Glycine				100 μ moles
(NH ₄) ₂ SO ₄			100 μ moles	100 μ moles
KCl		50 μ moles	5 μ moles	5 μ moles
MgCl ₂	2.5 μ moles			
Magnesium acetate		10 μ moles	10 μ moles	50 μ moles
Glutathione	5 μ moles			
Mercaptoethanol		6 μ moles	6 μ moles	6 μ moles
ATP	2.5 μ moles	2.5 μ moles	2.5 μ moles	2.5 μ moles
Phosphoenolpyruvate	2.5 μ moles	2.5 μ moles	2.5 μ moles	2.5 μ moles
DL-Amino acids*		0.1 μ mole	0.1 μ mole	0.1 μ mole
L-Amino acids†		0.05 μ mole	0.05 μ mole	0.05 μ mole
DL-Phenylalanine	1 μ mole			
DL-Leucine	1 μ mole			
L-Ornithine	0.5 μ mole			
L-Proline	0.5 μ mole			
Pyruvate kinase	10 μ g.	20 μ g.	20 μ g.	20 μ g.
11000g Extract‡	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
Total volume	1 ml.	1 ml.	1 ml.	1 ml.
Final pH	7.5	7.8	7.8	8.4
DL-[¹⁴ C]Valine§	0.5 μ C	0.5 μ C	0.5 μ C	0.5 μ C

* DL-Phenylalanine, DL-leucine, DL-serine, DL-threonine, DL-isoleucine, DL-tyrosine.

† L-Ornithine, L-proline, L-lysine, L-methionine, L-histidine, L-cysteine, L-cystine, L-aspartic acid, L-asparagine, L-alanine, L-tryptophan, glycine, L-glutamic acid, L-glutamine, L-arginine.

‡ 1 ml. of 11000g extract contains 4–9 mg. of protein.

§ The incubation mixture was preincubated at 37° for 1 min. before addition of the radioactive amino acid.

washed with 3ml. of aq. 2% (w/v) Na_2SO_4 solution. Gramicidin S was then extracted with 3ml. of ethanol-0.2N-HCl (9:1, v/v) at room temperature overnight. The residue after gramicidin S extraction was washed with ethanol (3ml.), subsequently with boiling methanol (2-3ml.) for 30 min., ethanol-ether (1:1, v/v) and finally dried with ether. The remaining solid was dissolved in 0.5ml. of N-NaOH and the solution used for counting and estimation of protein.

Methods used for purifying gramicidin S. The extracted 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. To check the purity of labelled gramicidin S isolated in this way, the following experiment was carried out. Labelled gramicidin S (3800 counts/min.) was further purified by two-dimensional thin-layer chromatography on silica (medium H according to Stahl; E. Merck A.-G., Darmstadt, Germany) in ethyl acetate-pyridine-acetic acid-water (60:20:6:11, by vol.) followed by the upper phase of butan-1-ol-acetic acid-water (25:6:25, by vol.).

The gramicidin S area was eluted with ethanol-0.2N-HCl (9:1, v/v). The eluate contained 90% of the original activity. Thus purification of gramicidin S on paper in the butan-1-ol-acetic acid-water solvent system was considered adequate for the present purposes.

Measurement of radioactivity. Radioactivity was measured with a Tracerlab SC-16 windowless flow counter. Radioautography of thin-layer plates was carried out as described by Holm, Frøholm & Laland (1966).

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., and Sigma Chemical Co., St Louis, Mo., U.S.A. GSH, GTP, ATP, UTP, phosphoenolpyruvate, pyruvate kinase (EC 2.7.1.40) in a solution of 2.1M-(NH_4) $_2\text{SO}_4$ (10mg./ml.) and ribonuclease (EC 2.7.7.16) (bovine pancreas, 5 $\times$ crystallized, salt-free and protease-free) were obtained from Sigma Chemical Co.

Radioactive amino acid. DL-[carboxy- ^{14}C]valine (5.0mc/m-mole) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A.

RESULTS

Since the method used for preparing a cell-free extract has a profound influence on the activity of the extract, several methods for producing cell-free extracts from *B. brevis* were compared. The cells were homogenized in the Vibrogen Zellmühle used by Berg *et al.* (1965), disrupted in the bacterial press, ground in an ice-cold mortar with aluminium oxide and disintegrated ultrasonically. Extracts prepared by these methods were examined for their ability to incorporate ^{14}C -labelled amino acids into gramicidin S. Ultrasonic treatment for a 5min. period yielded extracts with incorporating activity very close to that found with the Vibrogen Zellmühle, whereas the other two methods gave less active extracts. Further, it was found that

Table 2. Incorporation of [^{14}C]valine into protein and gramicidin S in 11000g extracts prepared from cells isolated early and late in the growth phase

Incubation mixtures A and B are those described in Table 1. The '0.1' and '0.5' extracts were 11000g cell-free extracts prepared from cells harvested from cultures with extinction values 0.1 and 0.5 respectively. The '0.1' and '0.5' extracts contained 4.8mg. and 4.2mg. of protein respectively per 0.5ml. of extract. The incubation period was 30min. For each experiment duplicate values are given.

Composition of incubation mixture	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
'0.1 cell-free extract' and incubation mixture A	90 270	120 80
'0.1 cell-free extract' and incubation mixture B	50 40	7090 7020
'0.5 cell-free extract' and incubation mixture A	3540 3520	650 600
'0.5 cell-free extract' and incubation mixture B	4110 4230	710 690

the incorporating ability decreased with the duration of the ultrasonic treatment. Additional experiments showed that cell-free extracts with the highest incorporating activity (per mg. of protein) were obtained when the cells were exposed to ultrasonic treatment for as little as 1min., and this procedure has therefore been used throughout this work (see the Materials and Methods section).

The incubation mixture A (Table 1) described by Berg *et al.* (1965) and an incubation mixture B (Table 1) similar to the one described by Matthaei & Nirenberg (1961) for protein synthesis in cell-free extracts from *Escherichia coli* were both used. Table 2 shows that extracts prepared from cells harvested from cultures with an extinction value 0.1 exhibit negligible activity to incorporate [^{14}C]valine into gramicidin S. This was also the case when the incubation mixture of Matthaei & Nirenberg (1961) was used. In contrast, extracts prepared from cells harvested from cultures with an extinction value 0.5 gave substantial incorporation into gramicidin S with either incubation mixture. Thus for the study of the biosynthesis of gramicidin S it is advantageous to harvest the cells late in the growth phase. It is also seen that the incubation mixture of Matthaei & Nirenberg (1961) incorporates more activity into gramicidin S than does the other incubation mixture. Further, it appears that the incorporation into protein is about 10 times as great when the cells were harvested at an extinction value 0.1 as at 0.5.

Effect of altering the composition of the incubation mixture on the incorporation of [^{14}C]valine into gramicidin S. The incubation mixture of Matthaei & Nirenberg (1961) (B, see Table 1) was used as a basis for obtaining a more effective incubation mixture for the synthesis of gramicidin S. Table 3 shows that a tenfold increase of the constituent amino acids somewhat decreased the incorporation. Further, the addition of 100 μmoles of ammonium sulphate increases the incorporation of [^{14}C]valine into gramicidin S, whereas the addition of Mn^{2+} ions decreased the incorporation.

Table 4 shows that increased concentrations of ATP inhibit the incorporation and that the concentrations of ATP and phosphoenolpyruvate used

in incubation mixture B seem to give higher incorporation. Further, it is advantageous to reduce the content of potassium chloride to 5 μmoles . The beneficial effect of mercaptoethanol is also seen.

In the next experiment the effect of the addition of ammonium sulphate together with a reduced potassium chloride content was tested. Table 5 shows that in the presence of 5 μmoles of potassium chloride and 100 μmoles of ammonium sulphate the incorporation into gramicidin S increased more than twofold. It is also seen that when the concentration of ammonium sulphate is decreased the degree of incorporation is decreased.

In studying the effect of pH, tris-hydrochloric acid and glycine-sodium hydroxide were used as buffers. Fig. 1 shows that the pH maximum for the incorporation of [^{14}C]valine in two separate experiments was 8.2–8.4. It is also evident that the presence of glycine gives better incorporation of [^{14}C]valine than does tris-hydrochloric acid. Three different commercial products of tris (from L. Light and Co. Ltd., Colnbrook, Bucks., Sigma Chemical Co. and British Drug Houses Ltd., Poole, Dorset) were used and compared with glycine. It appeared that glycine was superior to tris irrespective of the source of the latter.

The effect of Mg^{2+} ions on the incorporation of [^{14}C]valine into gramicidin S is shown in Fig. 2. It appears that maximum incorporation occurs at a concentration of 0.05M.

As a result of the above experiments, incubation mixture D (Table 1) was designed. After 30 min. incubation the isolated gramicidin S gave 19400 counts/min. Compared with previous incubation mixtures (Berg *et al.* 1965) incubation mixture D incorporates 6–7 times more [^{14}C]valine into gramicidin S.

Table 3. *Incorporation of [^{14}C]valine into gramicidin S by the 11000g extract under varying conditions*

Apart from the changes given below, the incubation mixture B (Table 1) was used. The time of incubation was 30 min. The values given for no addition are duplicates.

Additions	Radioactivity of gramicidin S (counts/min.)
None	3670
10 μmoles of MnSO_4	3700
10 μmoles of $(\text{NH}_4)_2\text{SO}_4$	3020
100 μmoles of $(\text{NH}_4)_2\text{SO}_4$	3880
0.5 μmole of L-proline and L-ornithine	4790
+ 1 μmole of DL-phenylalanine and DL-leucine	3120

Table 4. *Incorporation of [^{14}C]valine into gramicidin S by the 11000g cell-free extract in the presence of varying amounts of ATP, phosphoenolpyruvate and potassium chloride*

Apart from the changes below, the incubation mixture B (see Table 1) was used. The time of incubation was 30 min. The experiments separated by the horizontal line were carried out with different batches of extracts.

Changes	Radioactivity of gramicidin S (counts/min.)
None	5960
0.5 μmole of ATP added	530
10 μmoles of ATP added	4700
40 μmoles of ATP added	1880
0.5 μmole of phosphoenolpyruvate added	4100
10 μmoles of phosphoenolpyruvate added	5880
None	3880
5 μmoles of KCl added	4160
100 μmoles of KCl added	3360
Mercaptoethanol omitted	1710

Table 5. *Combined effect of potassium chloride and ammonium sulphate on the incorporation of [^{14}C]valine into gramicidin S by the 11000g cell-free extract*

The composition of incubation mixtures B and C is given in Table 1. The incubation period was 30 min.

Incubation mixture	Radioactivity in gramicidin S (counts/min.)
B [contains no $(\text{NH}_4)_2\text{SO}_4$ and 50 μmoles of KCl]	4880
C [contains 100 μmoles of $(\text{NH}_4)_2\text{SO}_4$ and 5 μmoles of KCl]	12760
C except that 50 μmoles of $(\text{NH}_4)_2\text{SO}_4$ were used	7890
C except that 200 μmoles of $(\text{NH}_4)_2\text{SO}_4$ were used	10240

Fig. 1. Effect of pH on the incorporation of [¹⁴C]valine into gramicidin S by the 11000g extract. The buffers used were tris-HCl (○) and glycine-NaOH (△). With tris-HCl the composition of the cell incubation mixtures was identical with incubation mixture C (see Table 1) except that pH varied. When the glycine buffer was used, incubation mixtures were identical with C (see Table 1) except that tris was replaced by 100 μmoles of glycine and the pH adjusted with NaOH. All incubations were carried out for 30 min. The pH given is that measured at 20° in the final incubation mixture.

Fig. 2. Effect of Mg²⁺ ions on the incorporation of [¹⁴C]valine into gramicidin S by the 11000g extract. Centrifuged cells were washed three times with 0.05M-tris-HCl buffer, pH 7.5. For producing cell-free extracts, samples of the cells were then suspended in solutions containing 10 μmoles of GSH/ml. and varying amounts of magnesium acetate and submitted to ultrasonic treatment and centrifugation as described (see the Materials and Methods section). The concentration of Mg²⁺ in the 11000g extracts obtained was the same as that in the final incubation mixtures. The incubation mixture was identical with incubation mixture D (see Table 1) except that the concentration of magnesium acetate varied in each experiment. All incubations were carried out for 30 min.

Effect of puromycin, chloramphenicol and ribonuclease on the incorporation of [¹⁴C]valine into gramicidin S and protein. Table 6 shows that puromycin and chloramphenicol have insignificant effect on the incorporation of [¹⁴C]valine into gramicidin S in extracts obtained from cells harvested at an extinction value 0.5. Treatment with ribonuclease lowered the incorporation only slightly. Further, these inhibitors had no effect on the incorporation into protein. However, protein incorporation in extracts from cells harvested early in the exponential phase of growth was very sensitive to these inhibitors (Table 7). Thus 'normal' protein synthesis only takes place in extracts prepared from early exponential-phase cells. As before (see Table 2), the incorporation into gramicidin S, although small, was not affected by the inhibitors.

Mixtures of 11000g extracts prepared from cells harvested from cultures with extinction values 0.1 and 0.5 respectively. The effect on the incorporation of [¹⁴C]valine into gramicidin S and protein when mixing '0.1' and '0.5' extracts is shown in Table 8. The mixed extract exhibits considerable incorporation both into protein and into gramicidin S. The incorporation into protein by the '0.1' extract is hardly affected by the presence of the '0.5' extracts

Table 6. *Effect of protein-synthesis inhibitors on [¹⁴C]valine incorporation into gramicidin S by the 11000g extract from cells harvested from a culture of extinction value 0.5*

Apart from the changes indicated below, incubation mixture D was used (see Table 1). The incubation period was 30 min.

Changes	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
None	19340	530
None	17350	530
10 μg. of puromycin added	18410	520
200 μg. of puromycin added	16920	640
10 μg. of chloramphenicol added	21160	480
100 μg. of chloramphenicol added	20940	560
1 μg. of ribonuclease added	16450	610
10 μg. of ribonuclease added	15530	570
ATP, phosphoenolpyruvate and pyruvate kinase omitted	50	30

Table 7. *Effect of protein-synthesis inhibitors on the incorporation of [14 C]valine into protein and gramicidin S in extracts from cells harvested at extinction value 0.1*

Apart from the changes indicated below incubation mixture B was used (see Table 1). The incubation period was 30 min.

Additions	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
None	260	4810
None	240	5030
10 μ g. of puromycin	250	230
200 μ g. of puromycin	260	80
10 μ g. of chloramphenicol	330	880
100 μ g. of chloramphenicol	270	150
1 μ g. of ribonuclease	380	30
10 μ g. of ribonuclease	300	30

Table 8. *Incorporation of [14 C]valine into protein and gramicidin S by a combination of extracts obtained from cells harvested from cultures with extinction values 0.1 and 0.5*

The incubation mixture contained either 1 ml. of the '0.1' or the '0.5' extract or 0.5 ml. of each of the '0.1' and '0.5' extracts. Incubation mixture B (see Table 1) was used. Total incubation volume was 2.0 ml. and the incubation period 30 min. The '0.1' and '0.5' extracts contained 9.7 and 8.7 mg. of protein/ml. respectively.

Extracts used	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
'0.1' extract	280	9070
	240	8780
'0.5' extract	12580	960
	12490	890
'0.1' extract + '0.5' extract	4540	5060
	4620	5150

whereas the incorporation into gramicidin S by the '0.5' extract is moderately reduced by the addition of the '0.1' extract. The effect of protein synthesis inhibitors on the incorporation into gramicidin S and protein in such mixtures was also studied. The incorporation into gramicidin S is not affected, whereas protein incorporation is drastically reduced (Table 9). The residual incorporation (about 10%) corresponds to that shown by the '0.5' extract alone and is unaffected by these inhibitors.

DISCUSSION

The cell-free system (11000g supernatant) described by Berg *et al.* (1965) incorporated all the

Table 9. *Effect of inhibitors of protein synthesis on the incorporation of [14 C]valine into protein and gramicidin S in a mixture of extracts from cells harvested from cultures with extinction values 0.1 and 0.5 respectively*

Each incubation mixture contained equal amounts (0.25 ml.) of the '0.1' and '0.5' extracts; except for the additions, the incubation mixture was identical with incubation mixture B (see Table 1). Total volume of each incubation mixtures was 1 ml. The incubation period was 30 min. The protein content of the extracts was as in Table 8.

Additions	Radioactivity of gramicidin S (counts/min.)	Radioactivity of protein (counts/min.)
None	1900	2800
None	1990	2820
10 μ g. of puromycin	1990	290
200 μ g. of puromycin	1850	190
10 μ g. of chloramphenicol	2150	540
100 μ g. of chloramphenicol	1910	270
1 μ g. of ribonuclease	2150	180
10 μ g. of ribonuclease	2290	170

five constituent amino acids into gramicidin S. In the present attempt to improve the performance of this system, the incorporation of [14 C]valine was chosen as a measure of gramicidin S synthesis. The reason for this was the presence in the extract of only small amounts of [14 C]valine-containing substances other than [14 C]valine and gramicidin S (H. Holm, L. O. Frøholm & S. G. Laland, unpublished work).

The basic features of the improved 11000g cell-free system were as follows. First, the use of brief ultrasonic treatment improved the activity of the extracts. Secondly, the addition of ammonium sulphate stimulated the incorporation. Further, the pH value (8.2–8.4) used and the Mg^{2+} ion concentration (0.05 M) in the system were optimum for [14 C]valine incorporation.

The beneficial effect of ammonium sulphate on the incorporation of [14 C]valine into gramicidin S is not understood. It is worth noting that NH_4^+ ions have a specific effect on the binding of aminoacyl-transfer RNA to ribosomes (Conway, 1964; Spyrides, 1964). The mechanism of action might, however, be different in the present case and no conclusion can be drawn.

The pH optimum for [14 C]valine incorporation is considerably higher than that described for leucine (7.0) by Berg *et al.* (1965). Different pH optima for the incorporation of some of the amino acids into gramicidin S may indicate that gramicidin S is being synthesized by a mechanism that

is different from that of proteins. It is noteworthy that the optimum concentration for Mg^{2+} ions is tenfold than that found for the optimum stability of 70S ribosomes (*E. coli*) (Tissières, Watson, Schlessinger & Hollingworth, 1959).

An 11000g extract from cells harvested early in the exponential growth phase (extinction value 0.1) exhibited a poor incorporation of [^{14}C]valine into gramicidin S. This is in accordance with the finding that there is very poor synthesis of gramicidin S in a young growing culture of *B. brevis* (extinction value 0.1) (Eikhom *et al.* 1963). The absence of any substantial gramicidin S synthesis was probably not due to the presence in the cell-free extract of inhibitors since there was no marked reduction in gramicidin S synthesis with a mixture of extracts prepared from cells harvested from cultures with extinction values 0.1 and 0.5 respectively (Table 8).

The incorporation of labelled amino acid into protein, which takes place in the 11000g extracts prepared from cells harvested at an extinction value 0.1 is, as would be expected, inhibited by puromycin, chloramphenicol and ribonuclease. However, in 11000g extracts prepared from cells harvested at an extinction value 0.5 there was only small incorporation compared with that occurring in cell-free extracts prepared from cells harvested at an extinction value 0.1. The small incorporation was not inhibited by puromycin, chloramphenicol and ribonuclease. This is a little surprising as studies *in vivo* (Eikhom *et al.* 1963) have shown that protein synthesis in cultures with an extinction value 0.5 is inhibited by puromycin and chloramphenicol. The absence of substantial protein synthesis in the cell-free extracts prepared from cells harvested at an extinction value 0.5 was not caused by the presence of inhibitory substances as normal protein incorporation was not decreased or abolished in a mixture of extracts from cells harvested at extinction values 0.1 and 0.5 respectively.

Thus the difference is probably caused by differ-

ences in the ribosomes. In *E. coli* it has been found by several groups of workers (Lamborg & Zamecnik, 1960; Doerfler, Zillig, Fuchs & Albers, 1962; Tissières, Schlessinger & Gros, 1960) that cells harvested in the early phase of growth yielded ribosomes with higher incorporating activity. A similar phenomenon was also observed (Dietz, Rei & Simpson, 1965) for yeast ribosomes. These workers introduced the term 'active' and 'sluggish' ribosomes. It seems quite likely that a similar situation exists in *B. brevis*.

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