Biosynthesis of Nucleic Acids in *Bacillus megaterium*

4. ROLES OF THE 'NUCLEAR', CYTOPLASMIC AND CYTOPLASMIC-MEMBRANE COMPONENTS OF THE CELL IN THE BIOSYNTHESIS OF RIBONUCLEIC ACID*

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Previous papers in this series (Butler & Godson, 1963; Godson & Butler, 1963) have described the fractionation of protoplasts of *Bacillus megaterium* into: (a) the DNA-containing 'nuclear' material; (b) the cytoplasmic membranes; (c) the 'nuclear' ribosomes (15% of the total ribosomes, previously described as 'nuclear residue'), which sediment with the 'nuclear' material and cytoplasmic membranes; (d) the cytoplasmic ribosomes that are left in the supernatant after fractions (a), (b) and (c) have been removed; (e) the cytoplasmic 'soluble' fraction left after the removal of the cytoplasmic ribosomes from the supernatant. When protoplasts were incubated with a radioactive RNA precursor ($[^3\text{H}]\text{uridine}$) for short periods, RNA with the highest specific radioactivity was present in the 'nuclear' material (the cytoplasmic membranes were not examined in these experiments). This RNA was closely bound to the DNA in the form of a DNA-RNA hybrid and could be displaced into other RNA fractions by 'chasing' with an unlabelled RNA precursor. The so-called 'nuclear' ribosomes were characterized by a specific radioactivity several times higher than that of the cytoplasmic ribosomes. In the present paper a more detailed study has been made of the differences between the two types of ribosomes and other features of RNA biosynthesis. Also, the sequence of labelling of protein of the various fractions with ^{14}C -labelled amino acids has been studied, and finally all of the observations of protein and RNA biosynthesis made in this Laboratory on protoplasts of *B. megaterium* have been drawn together. The roles of the 'nuclear', cytoplasmic and cytoplasmic-membrane components of the cell in macromolecular syntheses are discussed. A preliminary account of part of this work has been given (Butler & Godson, 1964).

METHODS

Growth of cells and preparation of protoplasts. The methods described by Butler & Godson (1963) were used.

Experiments with protoplasts. Cells were harvested after they had been growing in logarithmic phase for 2-3 hr. and were converted into protoplasts, as described by Butler & Godson (1963). For the experiments described in the present paper, the protoplasts were suspended at a concentration of approx. 20 mg. dry wt./ml. in 0.3M-sucrose containing MgCl_2 (5 mM) and KH_2PO_4 (0.05M) adjusted to pH 7.3 with NaOH (sucrose-magnesium-phosphate medium) and equilibrated by gently shaking at 30° for 10-30 min.

To study the incorporation of $[^3\text{H}]\text{uridine}$ into RNA, the labelled metabolite ('uridine-5-6-T' from The Radiochemical Centre, Amersham, Bucks.) (62 mc/m-mole) was added to the incubation mixture and samples were taken after various times by pouring the suspension into a marked cylinder and then quickly pouring this into a flask containing 3 vol. of the same buffered medium (sucrose-magnesium-phosphate), chilled to 0-4° and containing 0.01-0.03% of Lubrol W (Imperial Chemical Industries Ltd.), the higher concentration being used for older cultures. The flask was immediately plunged into an ice-salt bath and the suspension gently swirled until the temperature had dropped to 0-2°.

When the protoplasts were treated with actinomycin D (either before or during incubation with $[^3\text{H}]\text{uridine}$), the actinomycin was added to give a final concentration of 15 $\mu\text{g.}/\text{ml.}$ The same procedure was used when unlabelled uridine was added after a period of incubation with $[^3\text{H}]\text{uridine}$.

In the experiment on the incorporation of ^{14}C -labelled amino acids into protein of the subcellular fractions, the methods employed for labelling and fractionating the cells were the same as those used for studying the incorporation of $[^3\text{H}]\text{uridine}$ into RNA.

Fractionation of protoplasts. When complete lysis had taken place (2-5 min.) and no whole protoplasts could be detected with an interference microscope ($\times 1000$), the lysate was centrifuged at 78000g_{av.} for 40 min. and then fractionated by method B as described by Butler & Godson (1963). This centrifugation yields a gelatinous pad containing all of the cytoplasmic membranes of the cell, all of the DNA-containing 'nuclear' material and the 'nuclear' ribosomes (15% of the total ribosomes). The cytoplasmic membranes could be separated from the other fractions by homogenizing the pellet in 5-10 ml. of 0.01M-tris buffer adjusted to pH 7.3 with HCl (tris-HCl buffer), containing MgCl_2 (10 mM), with a Potter homogenizer for 1-2 min. and then centrifuging the homogenate for 15 min. at 105000g_{av.} The membrane fraction collects as a sediment, leaving the 'nuclear' ribosomes and other 'nuclear' material in the

* Part 3: Godson & Butler (1963).

supernatant. The 'nuclear' ribosomes were separated from the rest of the 'nuclear' material by centrifuging for 2½ hr. at 105000g_{av.}. The cytoplasmic ribosomes in the supernatant were then recovered by centrifuging at 78000g_{av.} for several hours (often overnight); the final supernatant was termed the 'cytoplasmic soluble fraction'. All of the operations were carried out in the cold. Further details of these preparative methods and the chemical constitution and properties of the cell fraction are given by Butler & Godson (1963).

Extraction of ribonucleic acid. The RNA was extracted from ribosome pellets by a modification of the Kirby (1956) procedure. Ribosomes (5–10 mg.) were resuspended in 3–5 ml. of 0.01 M-tris-HCl buffer, pH 7.3, containing MgCl₂ (0.1 mM). Sodium dodecyl sulphate (final concn. 0.5%) was added to the solution and then an equal volume of 90% (w/v) phenol containing 8-hydroxyquinoline (0.1%) was also added, and the mixture was shaken at room temperature for 15 min. The aqueous phase was separated by centrifugation, potassium acetate added (final concn. 2%, w/v) and the RNA precipitated by adding 2 vol. of ethanol and leaving at –10° overnight. The RNA precipitate was washed three times with 75% (w/w) ethanol and then with ethanol, before it was dissolved in 0.01 M-potassium acetate buffer, pH 5.2, ready for analysis on a sucrose density gradient.

RNA was extracted from whole protoplasts by a similar method. The protoplasts were harvested by centrifugation in the cold and immediately shaken with a small volume (20 ml. to approx. 1 g. dry wt. of cells) of 0.01 M-tris-HCl buffer, pH 7.3, containing *p*-aminosalicylic acid (5%, w/v) and sodium dodecyl sulphate (1%, w/v) at room temperature for 30 min. The aqueous phase was separated by centrifugation and adjusted to 2% (w/v) with respect to potassium acetate. The DNA and RNA were partially separated by adding 1 vol. of ethoxyethanol and removing the fibrous DNA, then adding 2 vol. of ethanol and leaving the RNA to sediment overnight at –10°. The RNA was redissolved in 2% potassium acetate and reprecipitated with ethoxyethanol and ethanol three times. The RNA obtained contained very little DNA.

Sucrose-density-gradient separations. A 5–20% (w/w) sucrose density gradient (McQuillen, Roberts & Britten, 1959) was constructed by layering in a Spinco SW 25 rotor, polypropylene tube, 3.5 ml. each of 20%, 17.5%, 15%, 12.5%, 10%, 7.5% and 5% sucrose solution and equilibrating the tube overnight in the cold. The sucrose solutions contained different concentrations of buffer and MgCl₂, depending on the experiment.

To separate ribosomes sedimenting at 70s and above, 1.0 ml. of a solution of the ribosomes (1–3 mg.) in 0.01 M-tris-HCl buffer, pH 7.3, containing MgCl₂ (10 mM) was layered on a 5–20% sucrose density gradient (made up in the same buffer-MgCl₂ solution) and then centrifuged in a Spinco SW 25 swing-out rotor for approx. 2½ hr. at 63000g_{av.}. To separate 30s and 50s ribosome sub-units, the same method was used, except that the MgCl₂ concentration was 0.1 mM throughout and the sucrose density gradient was centrifuged at 63000g_{av.} for 5–6 hr. The ribosome solutions were made by resuspending a pellet of sedimented ribosomes in the cold; for the 'nuclear' ribosomes, the small amount of DNA present (up to 10% of the total DNA sediments with this fraction) was usually degraded by incubating with deoxyribonuclease (Worthing-

ton Biochemical Corp., Freehold, N.J., U.S.A.) (0.3 µg./ml.) for 1 min. at 30° and then immediately cooling the tube to 0–4°.

To fractionate the RNA, 1.0 ml. of a cold solution of RNA, made up in 0.01 M-citrate buffer, pH 5.2, and containing approx. 1.0 mg. of RNA (estimated by extinction at 260 mµ in a Unicam SP. 600 spectrophotometer) was layered on a 5–20% sucrose density gradient (made up in the same buffer solution) and then centrifuged at 51000g_{av.} for 16–17 hr.

After centrifugation, the sucrose density gradient was fractionated by piercing the bottom of the tube with a syringe needle and collecting ten drops in each of a series of tubes (McQuillen, *et al.* 1959). To each tube, 1.0 ml. of 0.01 M-tris-HCl buffer, pH 7.3, was added and the extinction at 260 mµ was measured in a Unicam SP. 600 spectrophotometer (0.5 cm. cell). After this, the RNA was extracted from each tube by a modification (B. Wilkinson, unpublished work) of the method of Kidson, Kirby & Ralph (1963), and the radioactivity was measured by adding 10 ml. of a scintillation fluid [toluene-dioxan-ethanol (5:5:3, by vol.) containing naphthalene (80 g./l.), 2,5-diphenyloxazole (5 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (50 g./l.)] and counting on a Tri-Carb model 500 counter (Packard Instrument Co. Ltd.).

Assay of the incorporation of [³H]uridine into ribonucleic acid. The cell fractions were precipitated by the addition of cold 10% (w/v) trichloroacetic acid and allowing them to stand for several hours. After the precipitate had been washed three times with cold 5% (w/v) trichloroacetic acid and three times with 75% (w/w) ethanol, the RNA was extracted by heating with 5% trichloroacetic acid in a water bath at 90° for 20 min. The radioactivity of 0.1 ml. of the acid extract was measured by scintillation counting and RNA was determined by the orcinol method (Ceriotti, 1955). It was previously shown that this method gave results that compared well with those obtained by hydrolysing the RNA with KOH, separating the UMP obtained by paper chromatography and estimating its specific radioactivity (Godson & Butler, 1963).

Assay of the incorporation of ¹⁴C-labelled amino acids into protein. The cell fractions were precipitated with cold 10% trichloroacetic acid, washed as described above and then dried in a desiccator. Radioactivity was determined by end-window counting as described by Butler, Crathorn & Hunter (1958).

Sedimentation in the ultracentrifuge. Sedimentation velocity was measured, by Mr P. Edwards, in a Spinco model E ultracentrifuge at 35000 rev./min., the observed sedimentation coefficients being corrected to water at 20°.

Calculation of specific radioactivities from sucrose-density-gradient patterns. The specific radioactivity curves were obtained by dividing the counts/min. in each tube by the extinction. The average specific radioactivities of the various peaks were determined by summing the counts/min. present in all the tubes of the band and dividing by the summation of the extinctions in the same tubes.

RESULTS

Effect of actinomycin D on the pattern of incorporation of [³H]uridine into the ribonucleic acid of fractions of protoplasts of Bacillus megaterium. In

this experiment, protoplasts suspended in sucrose-magnesium-phosphate medium were labelled in two batches. One half of the suspension was pre-incubated with actinomycin D (15 $\mu\text{g./ml.}$) for 1½ min. before the addition of 100 μC of [^3H]uridine; in the other half of the suspension, the actinomycin D was omitted. Samples of both suspensions were taken after 1, 3, 8 and 20 min., and the protoplasts were fractionated and the radioactivity of the RNA of the fractions was determined as described in the Methods section. The results are given in Table 1. Without actinomycin the distribution of labelled RNA among the fractions was similar to that observed by Godson & Butler (1963), except that the results for the RNA attached to the cytoplasmic membrane are now given. The specific radioactivity of this RNA associated with the cytoplasmic membranes (1–3 % of the total RNA) was, in this experiment, only a little higher than that of the RNA closely bound to the 'nuclear' material. In some experiments, however, the specific radioactivity of the membrane RNA has been much higher than that of the

'nuclear' RNA and has had by far the highest specific radioactivity of any species of cellular RNA (Table 2). Table 1 also shows that the specific radioactivity of the RNA of the 'nuclear' ribosomes was higher than that of the RNA of the cytoplasmic ribosomes, but this difference decreased with time. The ratios of the specific radioactivity of the 'nuclear' to that of the cytoplasmic ribosomes at 1, 3, 8 and 20 min. were 8.5, 7.7, 5.5 and 4.7 respectively. In the cells treated with actinomycin D the amount of labelling was considerably decreased in all fractions, except the cytoplasmic soluble fraction (probably due to end-labelling of the transfer RNA). In this experiment the degree of inhibition by actinomycin D increased as the incubation proceeded, which suggests that the preincubation period of 1½ min. was insufficient for the antibiotic to become fully active. The delay in the depression of the synthesis of cytoplasmic-membrane RNA by actinomycin D has often been noted in these experiments.

Differences in labelling between the nuclear and cytoplasmic ribosomes. To determine the nature of

Table 1. *Effect of actinomycin D on the incorporation of [^3H]uridine into the ribonucleic acid of cell fractions of protoplasts of Bacillus megaterium*

Protoplasts derived from 2.9 g. dry wt. of cells, suspended in 320 ml. of sucrose-magnesium-phosphate medium at a concentration of approx. 9 mg. dry wt./ml., were equilibrated at 30° for 10 min. and then split into two equal batches. To one batch, actinomycin D (15 $\mu\text{g./ml.}$) was added, and then after 1½ min. the protoplasts were treated with 100 μC of [^3H]uridine. The second batch was treated in the same way but without actinomycin D. Samples (40 ml.) taken after 1, 3, 8 and 20 min. from both batches were lysed by pouring into an equal volume of ice-cold sucrose-magnesium-phosphate medium containing Lubrol W (0.01%) and were fractionated as described in the Methods section. The fractions were precipitated with cold trichloroacetic acid (10%, w/v), and the nucleic acids were extracted with 10% (w/v) trichloroacetic acid at 90° for 20 min. and the specific radioactivities determined. The specific radioactivities of the samples treated with actinomycin D are also given (in parentheses) as percentages of the control values.

Time of labelling (min.)	...	Sp. radioactivity (counts/min./mg. of RNA)							
		Control				Actinomycin D-treated			
		1	3	8	20	1	3	8	20
'Nuclear' material		550	1800	5500	—	— (—)	176 (10)	760 (14)	4250 (—)
'Nuclear' ribosomes		268	1020	2700	7900	16 (6)	57 (5.5)	95 (3.5)	200 (2.5)
Cytoplasmic ribosomes		32	132	485	1670	5 (15)	14 (10.5)	24 (5)	53 (3)
Cytoplasmic 'soluble' fraction		170	—	835	2680	168 (100)	330 (—)	770 (92)	790 (30)
Membrane fraction		640	1930	6500	12400	300 (48)	525 (27)	408 (6)	720 (6)

Table 2. *Labelling of the ribonucleic acid of the cytoplasmic membrane and the 'nuclear' material*

In each experiment protoplasts were labelled and fractionated and the specific radioactivity of the RNA was estimated as described in Table 1 (1 $\mu\text{mc} \equiv 200$ counts/min.).

Time of labelling (min.)	Sp. radioactivity of RNA ($\mu\text{mc/mg.}$)					
	Expt. 1		Expt. 2		Expt. 3	
	Membrane	'Nuclear' material	Membrane	'Nuclear' material	Membrane	'Nuclear' material
1	0.86	0.57	—	—	3.2	1.4
3	—	—	8.7	0.8	4.5	2.3
8	4.1	2.4	14.7	1.7	7.9	5.5
15	—	—	34.8	4.4	11.5	9.5
20	9.1	4.0	—	—	18.5	17.5

the labelling of the 'nuclear' and cytoplasmic ribosomes, the ribosomal pellets were resuspended in a tris-hydrochloric acid buffer containing magnesium chloride and analysed for ribosome size and distribution of radioactivity by centrifugation through a 5–20% linear sucrose density gradient (McQuillen *et al.* 1959).

In the first experiment, the protoplasts were labelled for 1, 3, 8 and 15 min. with [^3H]uridine, and samples of the 'nuclear' and cytoplasmic ribosomes were prepared and resuspended in 0.01M-tris-hydrochloric acid buffer, pH 7.3, containing magnesium chloride (either 10 or 0.1 mM).

(a) Sedimentation in 10 mM-magnesium chloride. Samples (1.0 ml.) of the ribosome solutions made up in 10 mM-magnesium chloride were layered on cold sucrose density gradients containing the same magnesium chloride concentration and centrifuged for $2\frac{1}{2}$ hr. at $63000g_{av.}$ The results are shown in Fig. 1. At this magnesium chloride concentration the ribosomes sedimented at 100–70s and the extinction profiles of both types of ribosomes appeared to be identical in shape and sedimentation characteristics. For the nuclear ribosomes, the extinction peak remaining at the top of the tube was due to residual DNA that had been degraded with deoxyribonuclease before layering. The peak of radioactivity, in both types of ribosomes, ran ahead of the main extinction band in the position of 'active' 70s ribosomes (Riseborough, Tissières & Watson, 1962) or polyribosomes (Warner, Knopf & Rich, 1963). This labelling pattern is typical of both types of ribosomes between 1 and 30 min.

(b) Sedimentation in 0.1 mM-magnesium chloride. Samples (1.0 ml.) of a ribosome solution made up in 0.1 mM-magnesium chloride were layered on sucrose density gradients containing the same magnesium chloride concentration, and centrifuged at $63000g_{av.}$ for 6 hr. A typical example of the results is given in Fig. 2. At this magnesium chloride concentration, both the 'nuclear' and cytoplasmic ribosomes split into 50s and 30s sub-units (Tissières, Watson, Schlessinger & Hollingworth, 1959). Analysis of the material in the ultracentrifuge verified the sedimentation constants as 49 ± 1 and 30 ± 0.5 s. In both cases there was a peak of radioactivity associated with the 30s ribosomes, very little radioactivity associated with the 50s ribosomes and a spread of radioactivity at low *S* values. In the cytoplasmic ribosome sample labelled for 8 min., illustrated in Fig. 2, there was also a peak of radioactive RNA between the 30s and 50s peaks; however, this peak was sometimes not clearly distinguished but appeared as a shoulder of the 30s peak. This pattern of labelling was normal for short labelling times, but at longer times (the time depending on the experiment) the 50s peak became labelled.

Fig. 3 illustrates this point. Fig. 3(a) is a pattern for nuclear ribosomes labelled for 8 min. and Fig. 3(b) is for the ribosomes from the same experiment labelled for 30 min. At 8 min. there was

Fig. 1. Sucrose-density-gradient patterns of 'nuclear' and cytoplasmic ribosomes, under conditions of high (10 mM) Mg^{2+} ion concentration. Protoplasts from 1.3 g. dry wt. of cells, suspended in 60 ml. of sucrose-magnesium-phosphate medium at a concentration of approx. 20 mg. dry wt./ml., were equilibrated at 30° for 15 min. and then labelled with $200 \mu\text{C}$ of [^3H]uridine. Samples (20 ml.) were taken after 3, 8 and 30 min. and immediately poured into 70 ml. of ice-cold sucrose-magnesium-phosphate medium containing Lubrol W (0.025%). The cell fractions were prepared as described in the Methods section. Half of the 'nuclear' ribosomes were resuspended in 1.0 ml. of 0.01M-tris-HCl buffer, pH 7.3, containing MgCl_2 (10 mM), and then incubated with $0.5 \mu\text{g}$. of deoxyribonuclease at 30° for 1 min. Half of the cytoplasmic ribosomes were resuspended in 3.0 ml. of the same tris-HCl buffer- MgCl_2 solution. Then 1.0 ml. of each solution was layered on a cold 5–20% sucrose density gradient (made up with the same tris-HCl buffer- MgCl_2 solution) and centrifuged at $63000g_{av.}$ for $2\frac{1}{2}$ hr. The gradients were fractionated, and the extinction at $260 \text{ m}\mu$ and the radioactivity of each fraction were measured as described in the Methods section. (a) 'Nuclear' ribosomes labelled for 8 min.; (b) cytoplasmic ribosomes labelled for 8 min. ●, Radioactivity (counts/min.); ○, $E_{260 \text{ m}\mu}$.

clearly a peak of radioactivity associated with the 30s ribosomes with a shoulder on the heavy side sedimenting at approx. 40s. The relative high specific radioactivity of this 40s peak is shown in the plot of specific radioactivity. Very little radioactivity appeared to be associated with the 50s ribosomes, and such labelling as occurs at early times was probably due to a shoulder of the 40s peak. At 30 min. there was an appreciable amount of label attached to the 50s ribosomes, although the specific radioactivity of the 30s ribosomes was still higher. It should be noted that the 8 min. sample was treated with deoxyribonuclease and the 30 min. sample was not: in the untreated sample the DNA sedimented as a peak that from analysis with the ultracentrifuge had an *S* value of approx. 23s.

Stability of the radioactive ribonucleic acid associated with the 30s ribosomes. Cytoplasmic ribosomes from the first experiment were treated by various methods in an attempt to remove the radioactivity of the 30s peak, without destroying the ribosomes.

(a) Dialysis against 0.01 M-magnesium chloride. It has been suggested (Gros *et al.* 1961*a*) that in low Mg^{2+} ion concentration (0.1 mM) the messenger RNA is almost completely dissociated from the ribosomes. To check whether the radioactive RNA associated with the 30s ribosomes was due to a residual messenger RNA that was not dissociated under the experimental conditions used as a routine

in these experiments, a sample of cytoplasmic ribosomes labelled for 8 min. from the second experiment described above was dialysed for 6 hr. against 0.01 M-magnesium chloride and then analysed on a sucrose density gradient. The result

Fig. 2. Sucrose-density-gradient pattern of the cytoplasmic ribosomes, under conditions of low (0.1 mM) Mg^{2+} ion concentration. Half of the cytoplasmic ribosomes labelled for 8 min. from the experiment described in Fig. 1 were resuspended in 3.0 ml. of 0.01 M-tris-HCl buffer, pH 7.3, containing $MgCl_2$ (0.1 mM). Then 1.0 ml. of this solution was layered on a cold 5–20% sucrose density gradient (made up with the same tris-HCl buffer- $MgCl_2$ solution) and centrifuged at $63000g_{av.}$ for 6 hr. The gradients were fractionated and analysed as described in Fig. 1. ●, Radioactivity (counts/min.); ○, $E_{260 m\mu}$.

Fig. 3. Sucrose-density-gradient patterns of 'nuclear' ribosomes labelled for short and long periods with $[^3H]$ -uridine and sedimented under conditions of low (0.1 mM) Mg^{2+} ion concentration. Protoplasts from 1.8 g. dry wt. of cells, suspended in 75 ml. of sucrose-magnesium-phosphate medium, were equilibrated at 30° for 20 min. and then labelled with $200 \mu C$ of $[^3H]$ -uridine. Samples (15 ml.) were taken after 1, 3, 8, 15 and 30 min. and immediately poured into 45 ml. of ice-cold sucrose-magnesium-phosphate medium containing Lubrol W (0.03%). The cell fractions were prepared as described in the Methods section. The 'nuclear' ribosomes were resuspended in 2.0 ml. of 0.01 M-tris-HCl buffer, pH 7.3, containing $MgCl_2$ (0.1 mM), and, for the sample labelled for 8 min., treated with $0.5 \mu g.$ of deoxyribonuclease for 1 min. at 30° . Then 1.0 ml. of each solution was layered on a cold 5–20% sucrose density gradient (made up with the same tris-HCl buffer- $MgCl_2$ solution) and centrifuged at $63000g_{av.}$ for 6 hr. The gradients were fractionated and analysed as described in Fig. 1. (a) 'Nuclear' ribosomes labelled for 8 min.; (b) 'nuclear' ribosomes labelled for 30 min. ●, Radioactivity (counts/min.); ○, $E_{260 m\mu}$; ----, specific radioactivity.

is shown in Fig. 4(a): the dialysis did not change the characteristics of the diagram (cf. Fig. 2), except that the amount of material at low S values was much smaller (probably owing to degradation and loss through the dialysis bag). The dialysis did not affect the size of the particles.

(b) Washing with sodium dodecyl sulphate and sodium deoxycholate. It has been reported by Gros *et al.* (1961*b*) that 0.1% sodium dodecyl sulphate solution removes all messenger RNA and extraneous RNA from ribosomes. Thus cytoplasmic ribosomes labelled for 3 min. in the second experiment were thoroughly washed in tris-hydrochloric acid buffer containing sodium dodecyl sulphate

Fig. 4. Stability of the radioactive RNA associated with the 30S ribosome peak. Solutions of cytoplasmic ribosomes resuspended in 0.01M-tris-HCl buffer, pH 7.3, containing MgCl_2 (0.1 mM), from the experiment described in Fig. 1 were used in this experiment. (a) A 1.5 ml. sample of the cytoplasmic ribosomes labelled for 8 min. was dialysed against 500 ml. of cold 0.01M-tris-HCl buffer, pH 7.3, containing MgCl_2 (0.01 mM), for 6 hr., with a change of the external solution after each $1\frac{1}{2}$ hr. The ribosome solution was then layered on a cold 5–20% sucrose gradient (made up with the same tris-HCl buffer-MgCl₂ solution) and centrifuged at $63000g_{av}$ for 6 hr. (b) Sodium dodecyl sulphate (final concn. 0.1%) was added to 2.0 ml. of a solution of cytoplasmic ribosomes labelled for 3 min. and the mixture was shaken in the cold for 10 min. Then 1.0 ml. of the ribosome solution was layered on a 5–20% sucrose density gradient [made up with 0.01M-tris-HCl buffer, pH 7.3, containing MgCl_2 (0.1 mM)] and centrifuged at $260m\mu$ and the radioactivity of each fraction were measured as described in the Methods section. ●, Radioactivity (counts/min.); ○, $E_{260m\mu}$; ----, $E_{260m\mu}$ of control sample.

(0.1%) and then analysed on a sucrose density gradient. The result is shown in Fig. 4(b). The broken line shows the position of the 30S and 50S ribosomes in an untreated sample. It appears that sodium dodecyl sulphate selectively degrades the 30S ribosomes and leaves the 50S ribosomes untouched. The radioactivity, however, still showed a peak symmetrical with the '30S' ribosomes. The label in the 50S ribosomes at 3 min. was almost zero.

An attempt to remove the radioactivity from the 30S ribosomes was also made with sodium deoxycholate. Cytoplasmic ribosomes labelled for 8 min. from the second experiment were washed with 0.1% sodium deoxycholate as described above. This treatment did not alter the distribution of labelled RNA, and the sucrose-density-gradient pattern was almost identical with the control pattern (Fig. 2).

Differences in the labelling of ribonucleic acid in cytoplasmic ribosomes at different times. In this experiment, protoplasts were labelled for 8 and 15 min. and the cytoplasmic ribosomes were prepared as above. The RNA was extracted from the ribosomes and analysed on a sucrose density gradient as described in the Methods section. The results are shown in Fig. 5.

The RNA from ribosomes labelled for 8 min. gave a large 23S peak, a smaller 16S peak and a small hump at low values of S , probably due to transfer RNA (Fig. 5a). The radioactivity was present in two peaks, one closely associated with the 16S RNA and the other, with the higher specific radioactivity, sedimenting at approx. 12S in the position expected for partially degraded messenger RNA (Gros *et al.* 1961*a*). In this preparation it is very likely that the only label in the 23S RNA was represented by the small shoulder at the base of the 16S RNA radioactive curve. At later times, the 23S RNA became labelled as shown in Fig. 5(b) and 5(c). The RNA in Fig. 5(b) was degraded during the preparation and the relative sizes of the 16S and 23S peaks were reversed, although the total amount of material had not changed and there was no accumulation of degraded RNA at low S values.

Labelling of the total cellular ribonucleic acid. In this experiment, protoplasts were labelled with [³H]uridine, and the RNA was extracted immediately after the samples were taken and then analysed on a sucrose density gradient. Fig. 6 illustrates the sort of result obtained. In samples taken after short incubation times there was very little label in the 23S RNA, but a considerable amount in the 16S and 4S RNA peaks. At later times, the label in the 23S RNA approached that of the 16S RNA. The sample illustrated in Fig. 6 represents an intermediate stage.

Metabolic stability of the radioactive ribonucleic acid associated with the 30S ribosomes. To test the

metabolic stability of the RNA of the 30s and 50s peaks in the sucrose density gradient, an experiment similar to that of Levinthal, Keynan & Higa (1962) was carried out to see if the specific radioactivities in the cytoplasmic peaks remained constant when the synthesis of new RNA was interrupted. A suspension of new protoplasts was labelled with [^3H]uridine for 10 min. and then split into several batches. To one was added actinomycin D ($15\text{ }\mu\text{g./ml.}$), which has been shown (Table 1) to decrease RNA synthesis greatly; to another was added an excess (2 mg./ml.) of unlabelled uridine, which would also be expected to decrease the synthesis of labelled RNA owing to dilution of the label; and to a third both actinomycin D and uridine at the same amounts were added. These suspensions were incubated for a further 10 min. and control samples were also taken before and after the treatments, i.e. at 10 and 20 min. The

cytoplasmic ribosomes obtained were analysed on sucrose density gradients and the average specific radioactivities of the 30s and 50s bands are given in Table 3. The specific radioactivity in the 30s band was increased somewhat by both the actinomycin D and the uridine treatment, but to a much smaller extent than when the incubation with [^3H]uridine was continued for the full 20 min. The amount of increase was not greater than would be expected if the incorporation of radioactive precursor continued for a minute or two after the additions, which may well be due to the time required to establish the new condition, as suggested by Godson & Butler (1963). Unlabelled uridine and actinomycin D together were more effective in stopping further synthesis, and the specific radioactivities remained at the 10 min. values. These experiments suggest, though not conclusively, that the newly synthesized RNA in the 30s and 50s

Fig. 5. Sucrose-density-gradient patterns of RNA extracted from cytoplasmic ribosomes. Protoplasts from 1.75 g. dry wt. of cells, suspended in 80 ml. of sucrose-magnesium-phosphate medium, were equilibrated at 30° for 20 min. and then labelled with $200\text{ }\mu\text{C}$ of [^3H]uridine. Samples (20 ml.) were taken after 1, 3, 8 and 15 min. and immediately poured into 40 ml. of sucrose-magnesium-phosphate medium containing Lubrol W (0.02%). The cell fractions were prepared and the RNA was extracted from the cytoplasmic ribosomes as described in the Methods section. Approx. 1 mg. of RNA was dissolved in 1.0 ml. of 0.01 M-potassium acetate buffer, pH 5.2, and then layered on a cold 5–20% sucrose density gradient (made up with the same buffer solution) and centrifuged at $51000g_{av.}$ for 17 hr. The gradients were fractionated and the fractions analysed as described in the Methods section. (a) RNA from cytoplasmic ribosomes labelled for 8 min.; (b) RNA from cytoplasmic ribosomes labelled for 15 min.; (c) RNA from cytoplasmic ribosomes labelled for 15 min. in a different experiment from (a) and (b), but under the same conditions. ●, Radioactivity (counts/min.); ○, $E_{260}\text{ m}\mu$.

Fig. 6. Sucrose-density-gradient pattern of RNA extracted from whole protoplasts. Protoplasts from 1.3 g. dry wt. of cells, suspended in 90 ml. of sucrose-magnesium-phosphate medium, were equilibrated by gently shaking at 30° for 15 min. and then labelled with 200 μ C of [3 H]uridine. Samples (30 ml.), taken after 3, 10 and 20 min., were immediately cooled to 0–4° and the protoplasts harvested by centrifugation in the cold. The RNA was extracted as described in the Methods section, and approx. 1 mg. was dissolved in 1.0 ml. of 0.01 M-potassium acetate buffer, pH 5.2, and analysed on a sucrose density gradient, as described in Fig. 5. The sample labelled for 10 min. is shown in this Figure. ●, Radioactivity (counts/min.); ○, $E_{260 \text{ m}\mu}$.

bands is stable, after interference with RNA synthesis, up to about 10 min.

Relationship between the nuclear and cytoplasmic ribosomes. It has proved possible to obtain consistent results by calculating the total and specific radioactivities of the various peaks of the sucrose-density-gradient patterns. Fig. 7 shows the change in the average specific radioactivities of the 'nuclear' and cytoplasmic ribosomes with time, calculated for the 70–100s ribosomes from the sucrose-density-gradient patterns in 10 mM-magnesium chloride of the second experiment described in the present paper.

This is a typical result and suggests that the difference in the specific radioactivity of the 'nuclear' and cytoplasmic ribosomes decreases with time. This is corroborated by Fig. 9, which correlates the results of several experiments and plots the ratio of the specific radioactivity of the 'nuclear' and cytoplasmic ribosomes against time: in all experiments this ratio decreased steadily. When the sucrose-density-gradient patterns in 0.1 mM-magnesium chloride are examined it is found that the differences of the average specific radioactivity in the 30s bands of cytoplasmic and 'nuclear' ribosomes are appreciably less (Fig. 8). The specific radioactivities in the 50s bands do not differ significantly at shorter times.

Incorporation of 14 C-labelled amino acids into protein of the various cell fractions. Protoplasts can actively incorporate 14 C-labelled amino acids into protein (Butler *et al.* 1958). In this experiment, protoplasts were labelled with 14 C-labelled protein

Table 3. *Effect of actinomycin D and unlabelled uridine on the distribution of labelled ribonucleic acid associated with cytoplasmic ribosomes of labelled protoplasts*

Protoplasts from 2.0 g. dry wt. of cells, suspended in 100 ml. of sucrose-magnesium-phosphate medium, were equilibrated at 30° for 15 min. and labelled with 200 μ C of [3 H]uridine. After 10 min. incubation, 20 ml. samples were poured into flasks containing (1) 40 ml. of ice-cold sucrose-magnesium-phosphate medium containing Lubrol W (0.02%); (2) 300 μ g. of actinomycin D (final concn. 15 μ g./ml.); (3) 40 mg. of unlabelled uridine (final concn. 2 mg./ml.); (4) 300 μ g. of actinomycin D plus 40 mg. of unlabelled uridine. After a further 10 min. incubation, the contents of flasks (2), (3) and (4) and the remaining 20 ml. of the incubation mixture were poured into 40 ml. each of the ice-cold sucrose-magnesium-phosphate medium containing Lubrol W (0.02%). The cell fractions were prepared as described in the Methods section. The cytoplasmic ribosomes were resuspended in 8 ml. of 0.01 M-tris-HCl buffer, pH 7.3, containing MgCl_2 (0.1 mM). A 1 ml. sample of each solution was layered on a cold 5–20% sucrose density gradient made up in the same buffer and MgCl_2 concentrations and centrifuged at 63 000 g_{av} for 5 hr. The gradients were fractionated into a series of tubes, and the extinction at 260 $\text{m}\mu$ and the radioactivity of each fraction were measured as described in the Methods section. The average specific radioactivities in the 30s and 50s bands were determined by dividing the total counts/min. by the total extinction.

Treatment	$10^{-3} \times \text{Sp. radioactivity of RNA (counts/min./} E_{260 \text{ m}\mu})$	
	50s band	30s band
Control incubation for 10 min.	0.18	0.35
Control incubation for 10 min. + actinomycin D for 10 min. more	0.18	0.51
Control incubation for 10 min. + uridine for 10 min. more	0.35	0.54
Control incubation for 10 min. + actinomycin D and uridine for 10 min. more	0.18	0.35
Control incubation for 20 min.	0.63	1.36

hydrolysate and fractionated as above. The results are shown in Fig. 10. At early times of labelling the specific radioactivity of the protein of the cytoplasmic membrane exceeded that found in the soluble protein and ribosome fractions. Also, at early times of labelling, the specific radioactivity of the nuclear ribosomes exceeded that of the cytoplasmic ribosomes, but at later times this was reversed.

DISCUSSION

The sucrose-density-gradient patterns of the 'nuclear' and cytoplasmic ribosomes are very similar at both high and low magnesium chloride concentrations (Figs. 1, 2 and 3), and, further, no significant differences of sedimentation constant are found in the ultracentrifuge. The only difference

Fig. 7. Average specific radioactivities of the 'nuclear' and cytoplasmic ribosomes of the experiment described in Fig. 1, calculated from the sucrose-density-gradient patterns in 10 mM-Mg²⁺ ion concentration as described in the Methods section. ○, 'Nuclear' ribosomes; ●, cytoplasmic ribosomes.

Fig. 8. Average specific radioactivities of 30s bands of 'nuclear' and cytoplasmic ribosomes, obtained from experiments described in Figs. 2 and 3, calculated from sucrose-density-gradient patterns in 0.1 mM-Mg²⁺ ion concentration. ○, 'Nuclear' ribosomes; ●, cytoplasmic ribosomes.

observed is the high degree of labelling of the 'nuclear' ribosomes when protoplasts are incubated with an RNA precursor. The difference is greater with short times of incubation and decreases as the incubation proceeds (Figs. 7 and 9). The difference of degree of labelling could be due either to the nuclear ribosomes carrying a greater amount of attached messenger RNA, or to a higher degree of labelling of their ribosomal RNA.

The studies of the distribution of radioactivity in sucrose density gradients has enabled these possibilities to be distinguished to some extent. Similar distributions were obtained in both cases, both in high and low Mg²⁺ ion concentrations. In high (10 mM) Mg²⁺ ion concentrations the radioactivity sediments as a broad band in front of the main 100–70s ribosomal peak, in the position of 'active' 70s ribosomes of *Escherichia coli* (Riseborough *et al.* 1962) or polyribosomes (Warner *et al.* 1963; Gierer, 1963). In low (0.1 mM) Mg²⁺ ion concen-

Fig. 9. Changes in the difference of specific radioactivity of the 'nuclear' and cytoplasmic ribosomes with time. □, Calculated from the sucrose-density-gradient patterns described in Fig. 1; △, calculated from Table 1; ○, calculated from sucrose density gradients; ●, calculated from estimations of the radioactivity of the total pellet of ribosomes, after precipitation with trichloroacetic acid; ■, calculated from Table 1 of Godson & Butler (1963).

Fig. 10. Incorporation of ¹⁴C-labelled protein hydrolysate into the protein of the protoplast cell fractions. Protoplasts derived from 2.1 g. dry wt. of cells, suspended in 120 ml. of sucrose-magnesium-phosphate medium, were equilibrated by gently shaking at 30° for 35 min. and then labelled with 20 μc of ¹⁴C-labelled *Chlorella vulgaris* protein hydrolysate (200 μc/mg.). Samples (30 ml.) were taken after 1, 3, 8 and 15 min. and immediately poured into 60 ml. of ice-cold sucrose-magnesium-phosphate medium containing Lubrol W (0.025%). The cell fractions were prepared and the specific radioactivity of the proteins was measured as described in the Methods section. ■, Cytoplasmic-membrane fraction; ○, cytoplasmic soluble fraction; ●, nuclear ribosomes; □, cytoplasmic ribosomes.

trations the ribosomes separate into their 50s and 30s sub-units (Tissières *et al.* 1959), and after short periods of labelling (usually up to 10 min.) the radioactivity is found in three regions: (a) at low values of *S* between 4 and 20s, which has been ascribed to messenger RNA (Gros *et al.* 1961a) or precursors of the RNA of the 30s ribosomes ('eosomal RNA'; McCarthy, Britten & Roberts, 1962); (b) a peak associated with the 30s ribosomes; (c) between the 30s and 50s peaks (Figs. 2 and 3a). This is also clearly shown in the curves of specific radioactivity (Fig. 3a).

The label of the 30s peak is not removed by dialysis against 0.01 M-magnesium chloride, or by washing with sodium dodecyl sulphate or sodium deoxycholate (Figs. 4a and 4b). This radioactive RNA is also metabolically stable, as it remains when the labelled protoplasts are further incubated with unlabelled uridine or actinomycin D. It is thus likely that the labelling of the 30s peak is due to newly formed ribosomal RNA, and moreover the amount of this label is greater in the 'nuclear' than in the cytoplasmic ribosomes.

The 43s 'neosomal' peak of radioactivity appears to spread into the 50s ribosomal region (Figs. 2 and 3a), and it is doubtful if any real labelling of the 50s peak itself occurs with short times of labelling (less than 10 min.); but, with longer times, labelling of this peak becomes apparent (Fig. 3b).

This sequence of labelling is confirmed by examining the ribosomal RNA. The 30s ribosomes contain exclusively 16s RNA, and 50s ribosomes exclusively 23s RNA (Kurland, 1960), and when the RNA is extracted from 100–70s ribosomes the 16s RNA is found to be labelled before the 23s RNA (Fig. 5). This is also seen in RNA extracts of whole protoplasts (Fig. 6). Thus either the 16s ribosomal RNA is synthesized at a far higher rate than the 23s RNA, or the 16s RNA is a precursor of the 23s RNA, but the experiments do not distinguish between these possibilities. However, in samples of degraded RNA the relative size of the 16s and 23s peaks is reversed, but with no apparent loss of total material or accumulation of degraded RNA at low *S* values (Figs. 5a and 5b).

It is concluded therefore that the higher specific radioactivity of the 'nuclear' ribosomes is due, not only to the attachment of more messenger RNA, but in part to the presence of more newly synthesized ribosomal RNA and ribosomal precursor RNA. Also, as the difference in the specific radioactivity diminishes with time (Fig. 9), it is suggested that the 'nuclear' ribosomes contain a greater proportion of newly synthesized ribosomes and later the 'nuclear' ribosomes become 'cytoplasmic' ribosomes.

Finally, the high degree of labelling found in the RNA of the membrane fraction is to be noted (Tables 1 and 2). This means that the membrane is

the recipient of at least part of the newly synthesized RNA; the small amount present (1–2 % of total; Godson & Butler, 1962) has prevented a full investigation of its nature. It was also observed (Godson, Hunter & Butler, 1961; Godson, 1962) that the membrane fraction from osmotically lysed protoplasts contains a small proportion of the ribosomes tightly bound to the cytoplasmic membrane, which is highly active in protein synthesis; it could well be that the ribosomes designated 'nuclear' ribosomes are similar to these and are associated *in vivo* with the cytoplasmic membrane rather than with the 'nuclear' material. It has been noted (Schlessinger, 1963) that polyribosomes are present in a membrane fraction of *B. megaterium*, and also (Suit, 1963) that a DNA-like RNA is closely bound to the cytoplasmic membrane of *E. coli*. However, the membranes that received newly synthesized RNA of high specific radioactivity must be closely associated with the 'nuclear' material, because if this RNA had to pass through the cytoplasm to reach the membranes its distinctively high radioactivity would be lost. This may mean either that the cytoplasmic membrane is in contact with the 'nuclear' material in some places or that the cells contain an internal reticulum.

The sequence of labelling of the protein with ¹⁴C-labelled amino acids, in the protoplast fractions produced by lysis with Lubrol (Fig. 10), almost exactly confirms the features of labelling found in similar fractions produced by osmotic lysis (Fig. 3 of Godson *et al.* 1961), i.e. a high labelling of the cytoplasmic membrane at early times, which is later surpassed by the labelling of the soluble free protein and a fairly low labelling of the ribosomal protein at all times.

As membranes prepared by this method are less contaminated by other constituents than those obtained by osmotic lysis, the experiment provides more conclusive evidence than previously given (Hunter, Brookes, Crathorn & Butler, 1959; Godson *et al.* 1961) of the importance of the membranes as an initial receptor of newly synthesized protein, and supports the suggestion of Hunter & Godson (1961) that the membranes, with their associated RNA, are an important site of polymerization of amino acids into proteins.

SUMMARY

1. When protoplasts of *Bacillus megaterium* are incubated for short periods with [³H]uridine, RNA with the highest specific radioactivity is present in the 'nuclear' material and cytoplasmic membrane. The 'nuclear' ribosomes (possibly attached to the cytoplasmic membrane) have a higher specific radioactivity than the cytoplasmic ribosomes, but

this difference diminishes with time, suggesting a precursor relationship.

2. Labelling of the RNA in all cell fractions, except the cytoplasmic fraction, is quickly inhibited by actinomycin D. The inhibition of the labelling of the membrane RNA occurs rather more slowly than that of the 'nuclear' fractions.

3. There is no apparent physical difference between the 'nuclear' and cytoplasmic ribosomes. The higher specific radioactivity of the 'nuclear' ribosomes is due to the presence of more labelled ribosomal and messenger RNA. It is therefore suggested that the 'nuclear' ribosomes contain the initial products of nuclear RNA synthesis.

4. At early times of labelling, only the 16s RNA of the 30s ribosome sub-unit is labelled, but at later times the 23s RNA of the 50s ribosome sub-unit becomes labelled.

5. Studies of the incorporation of ^{14}C -labelled amino acids into protein confirmed previous observations that the cytoplasmic membrane is an important site of protein synthesis in the cell.

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REFERENCES

- Butler, J. A. V., Crathorn, A. R. & Hunter, G. D. (1958). *Biochem. J.* **69**, 544.
Butler, J. A. V. & Godson, G. N. (1963). *Biochem. J.* **88**, 176.

- Butler, J. A. V. & Godson, G. N. (1964). *Nature, Lond.*, **201**, 876.
Ceriotti, G. (1955). *J. biol. Chem.* **214**, 59.
Gierer, A. (1963). *J. molec. Biol.* **6**, 148.
Godson, G. N. (1962). Ph.D. Thesis: University of London.
Godson, G. N. & Butler, J. A. V. (1962). *Nature, Lond.*, **193**, 655.
Godson, G. N. & Butler, J. A. V. (1963). *Biochem. J.* **88**, 259.
Godson, G. N., Hunter, G. D. & Butler, J. A. V. (1961). *Biochem. J.* **81**, 59.
Gros, F., Gilbert, W., Hiatt, H., Attardi, G., Spahr, P. F. & Watson, J. D. (1961b). *Cold Spr. Harb. Symp. quant. Biol.* **25**, 111.
Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Riseborough, R. W. & Watson, J. D. (1961a). *Nature, Lond.*, **190**, 581.
Hunter, G. D., Brookes, P., Crathorn, A. R. & Butler, J. A. V. (1959). *Biochem. J.* **73**, 369.
Hunter, G. D. & Godson, G. N. (1961). *Nature, Lond.*, **189**, 140.
Kidson, C., Kirby, K. S. & Ralph, R. K. (1963). *J. molec. Biol.* **7**, 312.
Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
Kurland, C. G. (1960). *J. molec. Biol.* **2**, 83.
Levinthal, C., Keynan, A. & Higa, A. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1631.
McCarthy, B. J., Britten, R. J. & Roberts, R. B. (1962). *Biophys. J.* **2**, 27.
McQuillen, K., Roberts, R. B. & Britten, R. J. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 1437.
Riseborough, R. W., Tissières, A. & Watson, J. D. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 430.
Schlessinger, D. (1963). *J. molec. Biol.* **7**, 569.
Suit, J. (1963). *Biochim. biophys. Acta*, **72**, 488.
Tissières, A., Watson, J. D., Schlessinger, D. & Hollingworth, B. R. (1959). *J. molec. Biol.* **1**, 221.
Warner, J. R., Knopf, P. M. & Rich, A. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 122.

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A Study of the Steroid Metabolism of an Interstitial-Cell Tumour of the Testis

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Although there are many reports on the clinical aspects of virilizing interstitial-cell tumours of the testis, little information is available on the biochemical aspects of this disorder. Thus Savard *et al.* (1960) carried out clinical, morphological and bio-

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chemical studies on a boy with an interstitial-cell tumour by using radioactive substrates. However, the blood concentrations of steroids were not reported in their extensive investigation. Dominguez (1961) reported a case of bilateral testicular tumour, of uncertain histological type, complicating congenital adrenocortical hyperplasia. Results