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Biosynthesis of Nucleic Acids in *Bacillus megaterium*

2. THE FORMATION OF RIBONUCLEIC ACID BY NUCLEAR MATERIAL *IN VITRO**

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Butler & Godson (1963) have described the preparation of nuclear material, from *Bacillus megaterium*, containing nearly all the DNA of the cell and a small proportion of the RNA, which had been shown (Godson & Butler, 1962) to be highly active metabolically and to a great extent the site of the initial synthesis of RNA when protoplasts are incubated with the appropriate radioactive precursors. In the experiments reported below the various cell fractions were incubated *in vitro* with a radioactive RNA precursor, and the conditions under which the synthesis of RNA takes place and the nature of the product formed are examined in detail.

EXPERIMENTAL

Materials. ATP, GTP, CTP, UTP, UDP and barium 3-phosphoglycerate were obtained from the Sigma Chemical Co., [³H]uridine of specific radioactivity 3c/m-mole from The Radiochemical Centre, Amersham, Bucks., and deoxyribonuclease from the Worthington Biochemical Corp. Streptomycin sulphate was obtained from The Distillers Co. (Biochemicals) Ltd., and protamine sulphate (from salmon milt) from British Drug Houses Ltd. All other materials were of AnalaR grade or were the purest available.

Growth and fractionation of cells. *Bacillus megaterium* was grown, harvested in logarithmic-growth phase and converted into protoplasts as described by Butler & Godson (1963). The fractionations were also done as described by Butler & Godson (1963), with the following modifications: lysis by Lubrol was carried out in a medium consisting of 0.1M-tris-HCl buffer, pH 7.6, containing sucrose (0.3M) and MgCl₂ (5 mM), and in the presence of 0.03% of Lubrol W; nuclear material was sedimented by centrifuging at 100 000 g for 1 hr. As prepared in this way, nuclear material contained ribosomes (see Butler & Godson, 1963), which could be removed by centrifuging at 100 000 g for 2 hr.

Purification of ribonucleic acid polymerase. The method of Chamberlin & Berg (1962) was used: portions (10 ml.) of nuclear material containing 50 mg. of protein were made 0.1 mM with respect to EDTA and 10 mM with respect to 2-mercaptoethanol. Deoxyribonuclease (10 µg.) was added and the material digested at 37° for 30 min. A portion (0.5 ml.) of a 10% (w/v) solution of streptomycin sulphate was added with stirring and the solution set aside for 15 min. The solution was centrifuged at 30 000 g for 15 min. and 1 ml. of a 1% (w/v) solution of protamine sulphate was added to the supernatant. The precipitate was collected by centrifuging at 30 000 g for 15 min. and washed with buffer solution A [5 mM-tris-HCl, pH 7.6, containing MgCl₂ (10 mM) and 2-mercaptoethanol (10 mM)]. The precipitate was then extracted with 1 ml. of 0.1M-ammonium sulphate in buffer A and the residue removed by centrifuging at 30 000 g for 15 min. The supernatant was removed and stored at -25°. Storage for 3 days resulted in a 30% loss of RNA-polymerase activity.

Preparation of [³H]uridine triphosphate. Spleen (0.5 g.) obtained from Aki mice bearing a virus-induced leukaemia (Bianchi, Butler, Crathorn & Shooter, 1961) was homogenized in 1 ml. of a medium consisting of 10 mM-tris-HCl buffer, pH 8, containing sucrose (0.23M) and KCl (5 mM), and centrifuged at 100 000 g for 1 hr. The supernatant was used to phosphorylate [³H]uridine to [³H]UTP and showed no loss of activity on storing for 6 months at -25°, even after repeated freezing and thawing. The incubation mixture contained, in a volume of 1 ml.: 0.6 ml. of mouse-spleen supernatant fraction, 53 µmoles of ATP, 64 µmoles of sodium 3-phosphoglycerate, 53 µmoles of MgCl₂, 180 µmoles of KCl, 3.8 m-moles of tris-HCl, pH 8, 83 m-moles of sucrose and 200 µC of [³H]uridine. The time of incubation to produce the maximum yield of [³H]UTP was determined for each batch of enzyme and was between 15 and 30 min. at 37°. The incubation mixture was made 5% (w/v) with respect to trichloroacetic acid and the acid-soluble nucleotides were separated by chromatography. The overall yield of [³H]UTP from [³H]uridine was 40-50%, and batches of 100 µC in a volume of 1 ml. were easily isolated by washing the chromatograms by downward flow of ethanol and eluting the appropriate band into a tube by downward

* Part 1: Butler & Godson (1963).

flow of water and collection of the drops. The radioactive band was located by cutting a strip 4 cm. wide from one edge of the chromatogram and scanning it with a gas-flow windowless chromatogram scanner (Nuclear-Chicago Aetigraph II). [^3H]CTP and [^3H]ATP were also prepared by this method from cytidine and adenosine respectively.

Determination of incorporation of [^3H]UTP into RNA. Incubation mixtures were cooled to 0° and 2 mg. of rat-liver RNA was added as carrier. They were then either treated immediately or stored at -25° overnight and then thawed out at 0°. Mixtures were made 5% (w/v) with respect to trichloroacetic acid and the precipitates washed four times with cold 5% (w/v) trichloroacetic acid, twice with ethanol and twice with ether. The residues were dried at room temperature and were always hydrolysed in 0.2 ml. of 0.3N-KOH for 1 hr. at 37° (Hutchison & Munro, 1961). *n*-Perchloric acid (0.1 ml.) was added to the digest and samples (0.1 ml.) of the supernatants were counted on the Tri-Carb liquid-scintillation counter with a scintillator suitable for aqueous solutions. The overall efficiency of counting, after allowance for quenching, was about 10%. Quenching was estimated by adding to the samples an internal standard, [^3H]hexadecane (The Radiochemical Centre).

For isolation of the nucleotides and nucleosides from the acid-washed trichloroacetic acid-insoluble material, digestion was carried out in 0.3N-KOH at 37° for 18 hr. (Davidson & Smellie, 1952).

Determination of deoxyribonucleic acid, ribonucleic acid and protein. Acid-washed trichloroacetic acid-insoluble material was extracted with 5% (w/v) trichloroacetic acid for 15 min. at 90° and deoxyribose was determined with diphenylamine as described by Burton (1956). RNA was determined according to the methods recommended by Hutchison & Munro (1961). Acid-washed trichloroacetic acid-insoluble material was hydrolysed in 0.3N-KOH at 37° for 1 hr. and the extinction was measured at 260 μm on the acid-soluble material thus released. A check was made that this treatment did not hydrolyse protein and that digestion of RNA was complete. Purified rat-liver RNA was used as a standard to relate the extinction to the amount of RNA phosphorus. Protein was determined by the method described by Lowry, Rosebrough, Farr & Randall (1951).

Chromatography. (a) UTP, UDP, UMP and uridine were separated from one another on sheets of Whatman no. 1 paper or on 4 cm. strips of Whatman no. 541 paper in isobutyric acid-aq. *N*-NH₃ soln.-0.1M-EDTA (disodium salt) (125:75:2, by vol.). Radioactivity associated with these substances was measured by scanning the strips with the chromatogram scanner.

(b) 3'-Cytidylic acid, 3'-uridylic acid, uridine and cytidine were separated from one another in alkaline digests of RNA by running chromatograms successively in the same direction in butanol-water (43:7, v/v) for 24 hr. and in isobutyric acid-aq. *N*-NH₃-0.1M-EDTA (disodium salt) (125:75:2, by vol.) for 18 hr. (Burdon & Smellie, 1961). Carriers of all four substances to be separated were added and the radioactivity associated with each was measured as follows: the appropriate spots were cut out and the material was eluted by downward flow of water and collection of 3 drops in the vials used for liquid-scintillation counting; further series of 3 drops were collected until no more radioactivity could be eluted.

Sucrose-gradient centrifuging. The technique described

by Britten & Roberts (1960) was used. Sucrose gradients (5-20%) were formed by layering in a Spinco SW25 tube portions (3.4 ml.) of solutions containing 20.0, 17.5, 15.0, 12.5, 10.0, 7.5 and 5.0% of sucrose together with 5 mM-tris-HCl, pH 7.4, and Mg²⁺ ions. The tube was set aside at 4° for 24 hr. before use and 1 ml. of ribosome suspension in appropriate medium was carefully layered on top of the gradient. The tubes were centrifuged under the conditions described below and a hypodermic needle was inserted through the bottom of the tube. Fractions of 10 drops were collected. A portion (0.15 ml.) of each fraction was withdrawn and made up to 1.5 ml. with buffer for measurement of the extinction at 260 μm in 5 mm. silica cells. To the remainder of each fraction 1 mg. of rat-liver RNA was added with an equal volume of 10% (w/v) trichloroacetic acid. The precipitates were washed once with 5% (w/v) trichloroacetic acid, once with ethanol and once with ether. The dry pellets were hydrolysed with 0.2 ml. of 0.3N-NaOH at 37° for 1 hr. and the hydrolysate was transferred quantitatively to scintillation vials for measurement of the radioactivity of each fraction.

RESULTS

Incorporation of [^3H]uridine triphosphate into ribonucleic acid by disrupted protoplasts and the isolated nuclear material. First, a comparison was made of the incorporation of [^3H]UTP into RNA by lysed protoplasts and isolated nuclear material. The incubation medium contained the complete incorporation mixture, including all four ribonucleoside triphosphates. Table 1 shows that nuclear material is considerably more active under these conditions than lysed protoplasts. The time-course of the incorporation by nuclear material is shown in Fig. 1, which shows a maximum incorporation at 37° after incubation for 15 min.

To determine whether the incorporation into RNA is terminal or not, an analysis was made of

Table 1. *Incorporation of [^3H]uridine triphosphate into ribonucleic acid by disrupted protoplasts and isolated nuclear material*

The incubation mixtures contained, in 0.5 ml.: 120 μmoles of sucrose, 48 μmoles of tris-HCl, pH 8.0, 4 μmoles of MgCl₂, 2 μmoles of ATP, 2.4 μmoles of 3-phosphoglycerate, 0.17 μmole of CTP, 0.4 μC of [^3H]UTP (125 μmoles) and 0.17 μmole of GTP. The mixtures each contained about 1 mg. of protein. Incubation was at 37° for 60 min.

Type of incubation	Total radioactivity in RNA nucleotides (disintegrations/ min.)	UTP incorporated into RNA (μmoles)
Lysed protoplasts	10 500	1.57
Boiled lysed protoplasts	2 600	0.39
Isolated nuclear material	57 000	8.55
Boiled nuclear material	4 600	0.69

the nucleotides and nucleosides obtained by alkaline hydrolysis of a sample of RNA obtained from nuclear material that had been labelled for 15 min. *in vitro* as in Table 1. Table 2 shows that about 90% of the radioactivity was recovered as 3'-uridylic acid, showing that most of the [^3H]UTP had been incorporated into non-terminal positions of the RNA molecule.

Table 3 shows experiments in which various components of the complete incorporation system were omitted. Although maximum incorporation is obtained when all four ribonucleoside triphosphates are present, a considerable amount occurs when cytidine triphosphate is omitted. This may be due to the limited ability of the system, as shown in Table 2, to convert uridine triphosphate into cytidine triphosphate, or to a small amount of CTP in the commercial samples of ATP and GTP. No incorporation is observed when [^3H]UTP alone is present.

Nuclear material easily breaks down [^3H]UTP into UDP and UMP. For example, after incubation of nuclear material for 1 hr. with [^3H]UTP, 74% of the acid-soluble radioactive material was recovered as UMP, 8% as UDP and 18% as UTP. It was thought that it might be possible to distinguish between UTP and UDP as the actual RNA precursor by including unlabelled UDP or UTP in the incorporation medium together with [^3H]UTP. However, UTP was only slightly better than UDP in preventing the incorporation of [^3H]UTP into RNA (Fig. 2). It is possible that unlabelled UDP added is effectively phosphorylated to UTP. It has been possible to show only that UTP is more likely than UDP to be the direct precursor of RNA in this system.

Fig. 1. Incorporation of [^3H]UTP into RNA by nuclear material. The conditions of incubation were as described in Table 1. O, Expt. 1; Δ, Expt. 2.

Table 2. Position in ribonucleic acid into which [^3H]uridine triphosphate is incorporated by nuclear material

Four samples of nuclear material were labelled for 15 min. under the conditions shown in Table 1 and the radioactivity was determined in the nucleotides and nucleosides.

	Mean radioactivity observed (counts/min.)	Percentage of total recovered radioactivity
2'(3')-Uridylic acid	6300	90
Uridine	300	5
2'(3')-Cytidylic acid	225	3
Cytidine	120	2

Table 3. Effect of triphosphates of adenosine, guanosine and cytidine on the incorporation of [^3H]uridine triphosphate into ribonucleic acid by nuclear material

The incubation mixtures contained the materials indicated in the amounts shown in Table 1. Incubation was at 37° for 15 min. and the control consisted of a boiled preparation of nuclear material.

Triphosphates present in incubation mixture	Radioactivity in nucleotides from RNA digest (disintegrations/min.)	UTP incorporated into RNA (μmoles)
[^3H]UTP	600	0.09
[^3H]UTP + ATP	7 100	1.07
[^3H]UTP + ATP + GTP	14 400	2.16
[^3H]UTP + ATP + GTP + CTP	29 500	4.43
Control	600	0.09

Fig. 2. Incorporation of [^3H]UTP into RNA in the presence of unlabelled UDP or UTP. Nuclear material was incubated for 15 min. under the conditions described in Table 1 with the additions of UDP or UTP as indicated: Δ, UDP; O, UTP.

Dependence of ribonucleic acid synthesis on deoxyribonucleic acid. Digestion of nuclear material with 10 μ g. of deoxyribonuclease/ml. at 37° for 30 min. destroyed its subsequent ability to synthesize RNA (Table 4). Under these conditions no ribonuclease activity was detected in the deoxyribonuclease. Hence the severe decrease in RNA synthesis cannot be attributed to the presence of additional ribonuclease. When DNA was added to a preparation of nuclear material digested with deoxyribonuclease, a small but consistent recovery of RNA synthetic ability was observed (Table 4) despite the continued presence of deoxyribonuclease. A similar addition to intact nuclear material produced no stimulation of RNA synthesis. RNA polymerase was partially purified according to the methods described above. The

Table 4. *Effect of deoxyribonuclease on the incorporation of [³H]uridine triphosphate into ribonucleic acid by nuclear material*

The incubation mixtures were as described in Table 1. Incubation was carried out at 37° for 15 min. Each result represents the mean of two incubations. Where appropriate, 90 μ g. of native calf-thymus DNA was added to the incubation mixture. Where appropriate, nuclear material was treated with a concentration of 10 μ g. of deoxyribonuclease/ml. at 37° for 30 min.

Preparation of nuclear material used for incubation	Radioactivity in RNA nucleotides (disintegrations/min.)		
	Expt. 1	Expt. 2	Expt. 3
Stored at 0° for 30 min.	101 800	—	—
Stored at 37° for 30 min.	77 900	68 700	88 900
Treated with deoxyribonuclease for 30 min.	1 300	2 000	1 400
Treated with deoxyribonuclease and DNA added	3 500	3 000	4 300
DNA added	—	64 200	—
Boiled for 5 min.	700	1 000	1 000

Table 5. *Synthesis of ribonucleic acid by partially purified ribonucleic acid polymerase*

Incubation mixtures contained, in 0.25 ml., where appropriate: 60 μ moles of sucrose, 24 μ moles of tris-HCl, pH 8.0, 2 μ moles of MgCl₂, 1 μ mole of ATP, 0.08 μ mole of GTP, 0.08 μ mole of CTP, 1.2 μ moles of 3-phosphoglycerate, 0.2 μ c of [³H]UTP (63 μ moles), 100 μ g. of calf-thymus DNA, and 0.1 ml. of enzyme solution containing 0.1 mg. of protein. The control contained 0.1 ml. of enzyme that had been heated at 100° for 5 min. and 100 μ g. of native calf-thymus DNA. Each result is the mean of two incubations.

Primer added	Radioactivity in RNA nucleotides (disintegrations/min.)
Native DNA	82 400
Heat-denatured DNA	82 500
None	1 000
Control	1 000

object was to separate polymerase as far as possible from deoxyribonuclease. The preparation obtained caused incorporation of [³H]UTP into RNA only when DNA was added (Table 5). DNA dissolved at a concentration of 2 mg./ml. in 5 mm-tris-HCl, pH 7.4, containing Mg²⁺ ions (0.1 mm) was boiled for 5 min. and rapidly cooled. DNA denatured in this way was as effective a primer as native DNA. Of the added DNA, 10 % was degraded to acid-soluble material during the incubation. It is concluded that the synthesis of RNA by nuclear material *in vitro* is directed by DNA.

Comparison of nuclear material and cytoplasm. Table 6 shows that the cytoplasmic fraction is not active in incorporating [³H]UTP under those conditions in which nuclear material is active. It was thought that this might be due to the absence of DNA to direct RNA synthesis. However, the addition of native or denatured DNA did not cause any detectable incorporation. Nor did the addition of rat-liver RNA cause any incorporation. The possibility that Lubrol present in the cytoplasmic fraction might be inhibiting RNA synthesis was excluded by showing that the addition of Lubrol to actively incorporating nuclear material in the amounts present in the cytoplasmic fraction had no effect on RNA synthesis (Table 6). Hence it is concluded that the enzyme system necessary for RNA synthesis from ribonucleoside triphosphates is localized in the nuclear material.

Nature of the ribonucleic acid formed by nuclear material in vitro. Nuclear material as employed in the experiments described above contained ribosomes. These could be separated from nuclear

Table 6. *Comparison of the ability of nuclear material and cytoplasm to incorporate [³H]uridine triphosphate*

Conditions of incubation were as described in Table 1. Incubation was at 37° for 15 min. Incubations involving cytoplasmic fraction contained 0.85 mg. of protein in Expt. 1 and 0.90 mg. in Expt. 2. Those involving nuclear material contained respectively 0.81 and 0.83 mg. of protein. Calf-thymus DNA (90 μ g.) and 90 μ g. of rat-liver RNA were added where appropriate.

Type of incubation	Radioactivity recovered in RNA nucleotides (disintegrations/min.)	
	Expt. 1	Expt. 2
Cytoplasm	2 000	1 900
Cytoplasm + native DNA	1 900	2 000
Cytoplasm + heated DNA	2100	2 000
Cytoplasm + RNA	—	1 800
Boiled cytoplasm	2 000	2 000
Nuclear material	60 300	63 200
Nuclear material + 0.03 % of Lubrol	—	64 000
Boiled nuclear material	800	700

material by centrifuging at 100 000 *g* for 2 hr., when 80 % of the total RNA was sedimented. Nuclear material containing ribosomes was incubated for various times with [³H]UTP and the complete incorporation medium. Ribosomes were then isolated. The total radioactivity of RNA in ribosomes and in the remaining part of the nuclear material was measured, and Table 7 shows that only a small proportion of the newly formed RNA is associated with the ribosomes, notwithstanding the fact that the ribosomes in this experiment contain four times as much RNA as the remainder of the nuclear fraction. Therefore the non-ribosomal RNA present in the nuclear material has a relatively much higher specific radioactivity.

In another experiment, nuclear material containing ribosomes was labelled for 8 min. with [³H]UTP and then a large excess of unlabelled UTP was added. Incubation was continued and samples were taken at various times. In each sample, ribosomes and the remaining nuclear material were separated and the total amount of radioactive RNA associated with each fraction was determined (Fig. 3). The amount of radioactive RNA associated with ribosomes continues to rise, despite the dilution of the isotope, and the amount of non-ribosomal RNA falls. The loss of radioactivity of the remaining nuclear material is somewhat greater than the gain of radioactivity in the ribosomes. This must be ascribed to partial degradation of RNA under these conditions. These findings are consistent with the hypothesis that RNA is first synthesized in close association with DNA and that part of it is then transferred to ribosomes. Further support for this hypothesis comes from an experiment in which ribosomes and nuclear material were first separated from one another and then supplied

with the necessary precursors (Table 8). Nuclear material was much more effective in RNA synthesis, the small amount observed during the incubation of ribosomes being due, probably, to contamination with nuclear material, since the ribosomes were not resuspended and resedimented to wash them, but were only rinsed.

The nature of the radioactive RNA associated with ribosomes was further clarified by the use of the sucrose-gradient technique in various concentrations of Mg²⁺ ions. The sedimentation coefficients of the ribosome components in solutions of 5 mM-tris-hydrochloric acid, pH 7.4, containing Mg²⁺ ions (10 mM or 0.1 mM) were determined with a Spinco analytical centrifuge. In the presence of 10 mM-Mg²⁺ ions two major components were observed with sedimentation coefficients 64s and 80s: with 0.1 mM-Mg²⁺ ions, three major components were found with sedimentation coefficients 16s, 27s and 34s. A simple calculation was then made (Nomura, Hall & Spiegelman, 1960) to determine in which fractions from the sucrose-gradient

Table 7. *Incorporation of [³H]uridine triphosphate into ribonucleic acid in nuclear material and ribosomes*

Portions (2.5 ml.) of nuclear material containing ribosomes were incubated for the times indicated and under the conditions described in Table 1. Ribosomes were removed from nuclear material by centrifuging at 100 000 *g* for 2 hr. and radioactivity in RNA and the RNA phosphorus content were determined for each fraction.

Time of incubation (min.) ...	5	10	20
Total radioactivity in RNA nucleotides from ribosomes (disintegrations/min.)	9 500	19 400	37 100
Total radioactivity in RNA nucleotides from nuclear material (disintegrations/min.)	39 800	76 000	112 700
Sp. radioactivity of ribosomal RNA (disintegrations/min./μg. of RNA P)	57	108	197
Sp. radioactivity of nuclear material RNA (disintegrations/min./μg. of RNA P)	976	1 526	2 387

Fig. 3. Incorporation of [³H]UTP into RNA of ribosomes and nuclear material. A portion (15 ml.) of nuclear material containing ribosomes was incubated for 8 min. with 2.2 μmoles of [³H]UTP under conditions corresponding to those described in Table 1. At the time indicated by the arrow 6 μmoles of UTP were added and two samples of 2.5 ml. removed from the incubation mixture. Incubation was continued and 2.5 ml. samples were removed at the times indicated. The total radioactivity associated with ribosomes and nuclear material was determined: Δ, ribosomes; ○, nuclear material.

experiments components with these sedimentation coefficients should appear. The maxima in the sucrose gradients were found to occur within one fraction from the position expected. Ribosomes, incubated with RNA precursors and nuclear material and then isolated by centrifuging at 100 000 g for 2 hr., were rinsed and resuspended in either (1) 5 mM-tris-hydrochloric acid, pH 7.4, containing 10 mM-Mg²⁺ ions or (2) 5 mM-tris-hydrochloric acid, pH 7.4, containing 0.1 mM-Mg²⁺ ions. The results of various sucrose-gradient analyses are shown in Figs. 4–6. With 10 mM-Mg²⁺ ions (Fig. 4) the radioactive RNA sediments to the same posi-

tions as ‘64s and 80s ribosomes’. When ribosomes were resuspended in 0.1 mM-Mg²⁺ ions they were observed to sediment in two bands, one containing material sedimenting with a sedimentation coefficient of 16s and the other containing material sedimenting at 27s and 34s. However, the radioactive RNA was associated with neither of these (Figs. 5 and 6). The majority of it sedimented as a rather heterogeneous band of material with sedimentation coefficients between 8s and 16s. In addition there was a small peak of radioactive RNA, which, from its position on the sucrose gradient (Fig. 6), had a sedimentation coefficient of about 25s.

Table 8. Incorporation of [³H]uridine triphosphate into ribonucleic acid by nuclear material and ribosomes

Nuclear material (8 ml.) was centrifuged at 100 000 g for 2 hr. and the rinsed ribosomal pellet was resuspended in 1 ml. of incubation medium. Portions (0.4 ml.) of each were incubated with [³H]UTP for 15 min. under the conditions shown in Table 1. Ribosome suspensions contained 185 µg. of RNA P and 23 µg. of DNA in 0.4 ml.; 0.4 ml. of nuclear material contained 46 µg. of RNA P and 475 µg. of DNA.

	UTP incorporated into RNA (µmoles)
Incorporation by 0.4 ml. of nuclear material	22.7
Incorporation by boiled nuclear material	0.3
Incorporation by 0.4 ml. of resuspended ribosomes	1.6
Incorporation by boiled ribosomes	0.3
Total incorporation by nuclear material	454.0
Total incorporation by ribosomes	4.0

Fig. 4. Sucrose-density-gradient analysis of ribosomes labelled with [³H]UTP for 15 min. *in vitro* in the presence of nuclear material. The 5–20% linear sucrose gradient contained 5 mM-tris-HCl, pH 7.4, and 10 mM-MgCl₂. It was centrifuged at 25 000 rev./min. for 3 hr. in the Spinco SW 25 rotor. —, Extinction of the fractions; - - -, corresponding radioactivity of the fractions. Arrows indicate calculated positions for the s values shown.

Fig. 5. Sucrose-density-gradient analysis of ribosomes labelled with [³H]UTP. The conditions were as described in Fig. 4 except that the concentration of MgCl₂ was 0.1 mM.

Fig. 6. Sucrose-density-gradient analysis of ribosomes labelled with [³H]UTP. The conditions were as described in Fig. 5, except that the centrifuging was continued for 11 hr.

DISCUSSION

These experiments have demonstrated that the nuclear fraction obtained from *B. megaterium* is capable of RNA synthesis when incubated with the four ribonucleoside triphosphates and Mg^{2+} ions. This synthesis is directed by DNA, as shown by the fact that deoxyribonuclease digestion of the nuclear fraction destroys its ability to form RNA, and by the fact that DNA is required by the purified enzyme to synthesize RNA. Thus a RNA polymerase is present in this fraction having similar properties to that obtained from *Escherichia coli* by Stevens (1960, 1961), Chamberlin & Berg (1962) and Furth, Hurwitz & Anders (1962), from *Azotobacter vinelandii* by Ochoa, Burma, Kröger & Weill (1961) and Burma, Kröger, Ochoa, Warner & Weill (1961), and from *Micrococcus lysodeikticus* by Weiss & Nakamoto (1961). The enzyme cannot be detected in cytoplasmic fractions even when DNA is added as a primer, and it was found that ribosomes were capable of forming only a minute amount of RNA (Table 8). Since nuclear material contains only 12 % of the total protein (see Butler & Godson, 1963) the enzyme is therefore apparently closely associated with DNA and is presumably bound to it in the form of a complex.

There is no possibility in these experiments that the incorporation of precursor into RNA *in vitro* is caused by the presence of a small number of whole bacteria or whole protoplasts in the nuclear material. The reasons for this contention are as follows: during the process of removing cell membranes, nuclear material is layered on to sucrose and subjected to a centrifugal force of 35 000 g for 30 min., which leaves nuclear material as a layer on top of the sucrose. This would be expected to sediment any whole organisms present. Moreover, when nuclear material is centrifuged for 2 hr. at 100 000 g (Table 8), the ability to incorporate precursors into RNA resides in the supernatant fraction and very little incorporation is associated with the pellet. The low radioactivity observed in this fraction is probably due to small amounts of contaminating supernatant fraction.

Investigation of the nature of the RNA formed indicated that new molecules of RNA are being synthesized. Table 2 indicates that most of the RNA precursor, [3H]UTP, is recovered as [3H]-3'-uridylic acid after alkaline hydrolysis of labelled RNA. Therefore most of the precursor is incorporated into non-terminal positions of the molecules. However, if a number of molecules of UTP were added to the ends of already existing RNA molecules, then labelling would still be recovered as 3'-uridylic acid. But with at least one class of RNA it has been shown that this was not the case. In fact, ribosomal RNA as such never became labelled in

these experiments (Fig. 6). Such labelled RNA as was found associated with ribosomes (e.g. Fig. 4) was removable by decreasing the concentration of Mg^{2+} ions of the ribosomal suspension.

The size of the newly synthesized RNA attached to ribosomes during 15 min. of incubation in the presence of nuclear material and precursors was estimated from the sucrose-gradient experiments. When labelled ribosomes were analysed in a high concentration (10 mM) of Mg^{2+} ions the radioactive RNA sedimented to the same position as 64s and 80s ribosomes. But when they were analysed in media of a low concentration (0.1 mM) of Mg^{2+} ions most of the radioactive RNA behaved as heterogeneous material of high specific radioactivity sedimenting with sedimentation coefficients between 8s and 16s. In addition, a small amount of labelled RNA with a sedimentation coefficient of 25s was detected. It represented 8 % of the total labelled RNA originally attached to ribosomes in the experiment described in Fig. 6. No precautions were taken in these experiments against the action of ribonuclease, except to handle materials at less than 4°. It is possible that much of the slowly sedimenting radioactive RNA represents breakdown products of the larger molecules. Ishihama, Mizuno, Takai, Otaka & Osawa (1962) have shown that only rapidly sedimenting 'messenger RNA' is capable of being attached to ribosomes *in vitro*. If a comparable situation exists in *B. megaterium* it is possible that the sedimentation pattern shown in Fig. 6 represents essentially degraded RNA that was originally of large size when attached to ribosomes. Indeed, radioactive RNA analysed for only 3 hr. in the sucrose gradient (Fig. 5) shows more radioactive RNA in the region of the 25s RNA than in the region of the smaller RNA. Further evidence for instability of the newly formed RNA is derived from the observation that there is a rapid decline of incorporation of [3H]UTP into RNA after 15 min. of incubation with nuclear material and the complete incorporation medium (Fig. 1).

The origin of RNA that becomes associated with ribosomes during incubation has been shown (Fig. 3) to be nuclear material. This is known to be the site of all of the DNA of the cells (Butler & Godson, 1963) and all of the RNA polymerase (Table 6). Hence RNA is formed in the nuclear material under the influence of RNA polymerase and DNA, and is then transferred and attached to ribosomes. It is uncertain whether the whole of this process has any functional meaning; but this attachment to ribosomes may be compared with results of Gros *et al.* (1961), Monier, Naono, Hayes, Hayes & Gros (1962) and Risebrough, Tissières & Watson (1962), who have shown that 'messenger RNA' becomes associated with ribosomes under

certain circumstances. These authors, however, note that their 'messenger RNA' fraction loses its radioactivity during a 'chase' incubation in the presence of unlabelled RNA precursor and that the radioactivity then appears in ribosomal RNA itself. In the present experiments incorporation of RNA precursors into ribosomal RNA itself was never observed: the newly formed RNA found attached to ribosomes is easily removed at low concentrations of Mg^{2+} ions.

SUMMARY

1. Nuclear material isolated from *Bacillus megaterium* is capable of synthesis of ribonucleic acid in the presence of the appropriate precursors.

2. The conditions in which this synthesis occurs have been investigated.

3. The ribonucleic acid polymerase is clearly associated with deoxyribonucleic acid in nuclear material, but it has been isolated from it in a relatively pure form.

4. The main product of the polymerase is a slowly sedimenting ribonucleic acid of a heterogeneous nature. Under certain conditions it becomes attached to the ribosomes present in the nuclear fraction.

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Biosynthesis of Nucleic Acids in *Bacillus megaterium*

3. BIOSYNTHESIS OF RIBONUCLEIC ACID *IN VIVO*

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This paper reports the results of experiments designed to study the role of the nuclear material, prepared as described by Butler & Godson (1963), in the synthesis of RNA by intact protoplasts of *Bacillus megaterium*. Preliminary accounts of part of the work have been given (Godson & Butler, 1962*a*, *b*).

METHODS

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

Experiments with protoplasts. Cells were usually harvested after they had been growing in logarithmic phase for 2-3 hr. (from the beginning of logarithmic phase to stationary