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

phase takes 6–7 hr.). The protoplasts were suspended at a concentration of approx. 10 mg. dry wt./ml. in either 0.5M-phosphate-salts medium (see Butler & Godson, 1963) or 0.3M-sucrose containing MgCl_2 (10 mM) and tris (10 mM) brought to pH 7.2 with HCl, depending on the fractionation procedure to be employed, and equilibrated by gently shaking at 30° for 15 min.

In experiments to study the incorporation of [^3H]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 an equal volume of the same buffered medium (0.5M-phosphate-salts medium or sucrose- MgCl_2 -tris medium), chilled to 0° and containing 0.02% of Lubrol W (final concn. 0.01%). The flask was immediately plunged into an ice-salt bath and the suspension gently swirled until the temperature had dropped to 0–4°, when it was placed in a refrigerator until complete lysis had taken place (2–5 min.). When no whole protoplasts could be detected with an interference microscope ($\times 1000$), the lysate was centrifuged at 78 000 g_{av} for 30 min. and fractionated by one of the methods described by Butler & Godson (1963).

In the 'chasing' experiments, the suspension was incubated with [^3H]uridine for a few minutes, and then cooled to 0–4° as rapidly as possible in an ice bath. The protoplasts were then harvested in the cold by centrifuging, resuspended at a concentration of approx. 10 mg./ml. in either 0.5M-phosphate-salts medium or sucrose- MgCl_2 -ions medium at 30° and 'starved' of uridine by gently shaking for 1 hr. at 30°. A sample of the suspension was taken before and after the 'starvation' period to assay the radioactivity of the RNA before the 'chasing'. The unlabelled uridine or uridine metabolite used for the 'chasing' was then added to the incubation mixture and samples were taken after various times. The samples were poured into an equal volume of ice-cold buffer plus Lubrol W as above.

Fractionation of the protoplasts. The preparation of the subcellular fractions from the protoplasts after lysis with Lubrol W (nuclear fraction, ribosomes, soluble cytoplasmic fraction and membrane fraction) is described by Butler & Godson (1963). In some experiments the residue of ribosomes present in the nuclear fraction was separated by 'method B₂' of Butler & Godson (1963).

Assay of the incorporation of [^3H]uridine into ribonucleic acid. The cell fraction was precipitated with 10% (w/v) trichloroacetic acid and the RNA extracted by a modification of the procedure of Schmidt & Thannhauser (1945). The acid precipitate was washed three times with cold 5% (w/v) trichloroacetic acid, three times with 75% (v/v) ethanol and once with ether, and dried in a desiccator. The dry residue was then dissolved in a small volume of 0.3N-KOH for 18 hr. at 37°, as recommended by Davidson & Smellie (1952a, b). The DNA and protein were precipitated in the cold by acidification with 60% (v/v) perchloric acid and removed by centrifuging. The pH of the supernatant was brought back to pH 7 with N-KOH and the digest left in the cold for a few hours to precipitate as much as possible of the KClO_4 . The radioactivity of the RNA was assayed in two ways:

(a) Directly by scintillation counting. A sample (0.1 ml.) of the hydrolysate was added to 10 ml. of scintillation fluid [toluene-dioxan-ethanol (5:5:3, by vol.) containing naph-

thalene (80 g./l.), 2,5-diphenyloxazole (5 g./l.) and 1,4-bis-2-(5-phenyloxazolyl)benzene (50 g./l.)], and the radioactivity was counted in a Tri-Carb model 500 counter (Packard Instrument Co. Ltd.). Internal standards were added to each sample and the radioactivity was recounted. The RNA present in 0.1 ml. of the KOH digest was determined by the orcinol method (Ceriotti, 1955).

This method, however, records the radioactivity present in every compound in the digest and some of these might not be derived from the RNA. Also, end labelling of the RNA molecules with cytidine or uridine is recorded as RNA synthesis. This scintillation method, therefore, was used to check that the experiment was successful before a rigorous determination of RNA synthesis was performed.

(b) To measure the synthesis of new RNA molecules, portions of the KOH digest were spotted on Whatman no. 1 paper and descending chromatography was carried out with propan-2-ol-HCl-water (170:41:39, by vol.). This solvent effectively separated UMP, CMP and a mixture of AMP and GMP. Any bases derived from the DNA remained near the origin. The nucleotide spots were located on the chromatogram with an ultraviolet lamp (2537 Å) (Hanovia Chromatolite), cut out and each eluted in 5 ml. of 0.1N-HCl. The amount of nucleotide was determined by its extinction at its λ_{max} . The solution was freeze-dried, plated on an aluminium planchet and counted at zero self-absorption with a windowless gas-flow counter, corrections being applied for thickness when necessary by using an empirically derived factor. Alternatively, samples were eluted from the paper by cutting out each spot and standing it upright in dilute ammonia solution so that the nucleotides concentrated along the top edge. The paper was wrapped in aluminium foil, centrifuged, washed with 0.1 ml. of water and centrifuged again. This method rapidly and quantitatively removed all of the nucleotide from the paper. The amount of nucleotide was determined by its extinction and its radioactivity counted as above.

This method was used to measure the amount of uridine incorporated non-terminally into the RNA. The results usually agreed well with the results obtained by direct scintillation counting of the KOH digest.

Determination of the terminal labelling of the ribonucleic acid. The terminal nucleotide moieties of the RNA were removed with dilute alkali (Burdon & Smellie, 1961). The trichloroacetic acid precipitate after extraction of lipid was digested with 0.3N-KOH and freed from DNA and protein, as described above. Portions of the digest, containing 2',3'-nucleotides and the nucleosides derived from the terminal moieties of the RNA, were spotted on Whatman no. 1 paper, together with 50 μg . each of uridine and cytidine to act as carriers. The paper was run in butanol-water (43:7, v/v) for 24 hr. This separated the uridine from the cytidine and left all the nucleotides at the origin. The nucleotides were eluted, spotted on fresh Whatman no. 1 paper and run in propan-2-ol-HCl for 24 hr. This effectively separated UMP, CMP and a mixture of AMP and GMP. The spots were located, cut out and eluted by standing in a dilute solution of ammonia and centrifuging as described above. The eluted nucleotides and nucleosides were plated on aluminium planchets and counted in a Nuclear-Chicago windowless gas-flow counter.

In some experiments the nucleic acids were extracted from the washed acid precipitate of the cell fraction by heating with 10% (w/v) trichloroacetic acid for 20 min. at 90°. The amount of RNA present in the extract was assayed

by the orcinol method (Ceriotti, 1955) and the radioactivity determined by scintillation-counting as described above.

Caesium chloride experiments. Nuclear material, labelled with [^3H]uridine and free from cytoplasmic membranes, prepared as described above, was homogenized with CsCl and the volume adjusted (with CsCl) so that the final solution contained 2–4 mg. of nuclear material in 2.5 ml. and had a refractive index of 1.404. The solution was centrifuged in a Spinco SW39 swinging-bucket rotor at 35 000 rev./min. for 24–48 hr. Under these conditions the DNA formed a band near the middle of the tube, the protein floated to the top and the RNA collected at the bottom of the tube. After centrifuging, the gradient was fractionated by piercing the bottom of the tube with a syringe needle and collecting the emergent droplets in a series of tubes. The CsCl was removed by dialysis against 10 mM-tris buffer, pH 7.8, containing MgCl_2 (10 mM). Samples of each fraction were diluted with water and the extinctions at 260 μ measured in a Unicam SP. 600 spectrophotometer. The radioactivity of each fraction was measured by adding a sample to a vial containing 50 μg . of deoxyribonuclease, incubating the mixture at 37° for 1 hr., and then adding 10 ml. of scintillation fluid and counting as described above.

RESULTS

Incorporation of [^3H]uridine into the ribonucleic acid of protoplasts. When protoplasts of *B. megaterium*, suspended in 0.5 M-phosphate-salts medium or sucrose-magnesium chloride-tris medium, were

incubated with [^3H]uridine, the cellular RNA rapidly became labelled. The rate of incorporation of the [^3H]uridine into the RNA of the various sub-cellular fractions of the protoplasts (nuclear, ribosomal and soluble) is shown in Fig. 1.

In this experiment, the protoplasts suspended in sucrose-magnesium chloride-tris medium, were labelled with 20 μC of [^3H]uridine and samples were taken after 1, 3, 5, 10 and 20 min. The protoplasts were lysed, and the pellet containing the nuclear material and cytoplasmic membranes was collected by centrifuging. The specific radioactivities of the RNA contained in this pellet and in the ribosomal and low-molecular-weight RNA of the cytoplasmic fraction are given in Fig. 1. The specific radioactivity of the nuclear RNA is higher than that of the ribosomal RNA or soluble RNA and increases very rapidly for the first few minutes, falls between 3 and 5 min. and then increases again at a slower rate. This temporary fall in the specific radioactivity of the nuclear RNA has been a constant feature of labelling experiments and always occurs between 3 and 10 min. The specific radioactivity of the soluble RNA is slightly higher than that of the ribosomal RNA. The kinetics of labelling of the soluble RNA and that of ribosomal RNA, however, are different. The radioactivity of the ribosomal RNA increases linearly from zero time, whereas that of the soluble RNA rises rapidly for a few minutes, falls and then increases slowly again, in a similar manner to that of the nuclear RNA.

The nuclear material assayed in this experiment (obtained by 'method B' of Butler & Godson, 1963) contains 12–15 % of the total cellular RNA and probably contains RNA sedimented from the cytoplasmic fraction during the preparation (see Butler & Godson, 1963). In the next experiment, protoplasts were labelled for 2, 6, 10 and 20 min. as above, but the original nuclear material was treated with ultrasonic radiation ('method B₂' of Godson & Butler, 1963) and centrifuged for 2 hr. at 105 000 g. This divided the original nuclear fraction into a DNA-containing supernatant fraction (nuclear material) and a 'nuclear residue' sediment which has been described as ribosomal. The supernatant fraction normally contains approx. 4 % of the total RNA and the sediment approx. 10–15 % (Table 5 of Butler & Godson, 1963), but in this experiment the supernatant contained 7–8 % and the sediment 9 % of the total RNA. This could be due to a breakdown in this experiment of some of the sedimentable RNA by the ultrasonic treatment. The specific and the total radioactivities of the RNA in the various fractions are given in Table 1. The specific and total radioactivities of the RNA of the nuclear material do not increase greatly after the first 2 min., whereas those in the nuclear residue continue to increase. The radioactivities of the cytoplasmic ribosomal

Fig. 1. Incorporation of [^3H]uridine into the RNA of the nuclear, ribosomal and soluble fractions of protoplasts derived from logarithmic-phase cells of *Bacillus megaterium*. The protoplasts, suspended in 180 ml. of sucrose- MgCl_2 -tris medium at a concentration of 10 mg. dry wt./ml., were equilibrated by gently shaking at 30° for 15 min. and then labelled with 20 μC (80 μg .) of [^3H]uridine. Samples (35 ml.) were taken after various times and the cell fractions prepared by 'method B' of Butler & Godson (1963). The radioactivity present in the RNA was measured as described in the Methods section. Δ , Nuclear material; $\blacktriangle$, ribosomes; $\circ$, soluble fraction.

RNA and of the soluble RNA follow a similar course to those in the preceding experiment. If the specific radioactivity of the original nuclear fraction is calculated from the values for nuclear material and nuclear residue, a similar pattern of labelling is obtained as in the first experiment.

It is unlikely that any depletion of radioactive precursor occurs in the longer times as after 20 min. only about 2% of the added radioactivity is incorporated into the RNA.

Fate of the incorporated uridine. The next experiment was designed to investigate how much of the [^3H]uridine is incorporated into terminal and non-terminal positions in the RNA, both as uridine and cytidine moieties.

Protoplasts were incubated with $20\text{ }\mu\text{C}$ of [^3H]uridine and the suspension was sampled after 1, 2, 5, 10 and 15 min. These times were chosen so that

samples of the RNA fractions were taken during the three phases of labelling of the nuclear RNA, i.e. the early rapid rise, the temporary fall and the subsequent slow increase in its specific radioactivity. The results are shown in Table 2.

At early times there was a considerable amount of terminal labelling in all of the cell fractions, but as the incubation proceeded the proportion decreased, until after 15 min. the terminal labelling was only 20–30% of the amount after 1 min. of incubation. Also, in all of the RNA fractions, most of the terminal label was present as radioactive cytidine. In the nuclear RNA and soluble RNA, the proportion of the non-terminal label that was contributed by cytidine was low at first but increased during the incubation, until after 15 min. 30–40% of the total non-terminal label was accounted for by cytidine. In the ribosomal RNA, the amount of non-terminal

Table 1. *Incorporation of [^3H]uridine into the ribonucleic acid of nuclear, nuclear residue, ribosome and soluble fraction of protoplasts of Bacillus megaterium*

Protoplasts derived from 5.0 g. of logarithmic-phase cells, suspended in 400 ml. of sucrose-MgCl₂-tris medium at a concentration of approx. 9 mg. dry wt./ml., were equilibrated for 20 min. at 30° and then labelled with $50\text{ }\mu\text{C}$ of [^3H]uridine. Samples of 35 ml., taken after 2, 6, 10 and 20 min. of incubation, were lysed as described in the Methods section, and fractionated by 'method B₃' of Butler & Godson (1963). The fraction was precipitated with cold 10% (w/v) trichloroacetic acid and the nucleic acids were extracted with 10% (w/v) trichloroacetic acid at 90° for 20 min. The total RNA was measured by the orcinol method and the radioactivity measured by scintillation counting, as described in the Methods section.

Time of labelling (min.)	Nuclear material		Nuclear residue		Cytoplasmic ribosome		Soluble RNA	
	Sp. radio-activity (counts/min./mg.)	Total radio-activity (counts/min.)	Sp. radio-activity (counts/min./mg.)	Total radio-activity (counts/min.)	Sp. radio-activity (counts/min./mg.)	Total radio-activity (counts/min.)	Sp. radio-activity (counts/min./mg.)	Total radio-activity (counts/min.)
2	502	904	1051	1780	148	1850	358	1270
6	578	876	1022	2080	153	1910	297	1035
10	488	905	1150	2070	198	2480	321	920
20	714	1222	2287	7390	536	6760	871	2890

Table 2. *Fate of [^3H]uridine incorporated into the ribonucleic acid of cell fractions of protoplasts of Bacillus megaterium*

Protoplasts derived from 2.2 g. of logarithmic-phase cells, suspended in sucrose-MgCl₂-tris medium at a concentration of 11 mg. dry wt./ml., were labelled with $20\text{ }\mu\text{C}$ ($80\text{ }\mu\text{g.}$) of [^3H]uridine. Samples were taken after various times, as described in the text, and the cell fractions prepared by 'method B₁' of Butler & Godson (1963). The amount of radioactivity present in the cytidine and uridine moieties of the RNA, incorporated both terminally and non-terminally, was determined as described in the Methods section. NM, Nuclear material; S, soluble fraction; R, ribosomal fraction.

Time of labelling (min.)	Sp. radioactivity of RNA (counts/min./mg.)			Fraction of radioactivity incorporated terminally (%)			Fraction of non-terminal radio-activity present as cytidine (%)			Fraction of terminal radioactivity present as cytidine (%)		
	NM	S	R	NM	S	R	NM	S	R	NM	S	R
1	710	290	40	19.5	10	17	10	12	—	68	56	86
2	1650	520	40	17.5	9.5	8	10	33	54	70	49	42
5	1140	1010	60	12.5	7	7	24	23	33	76	—	45
10	2110	1850	70	9.5	3.5	7	31	31	35	82	78	50
15	2410	2280	130	6.5	2.5	2.5	42	35	36	79	81	38

labelling contributed by cytidine was high at all times and remained constant. There was no label in the guanine and adenine moieties of the RNA or DNA fractions.

Transfer of labelled ribonucleic acid from the nuclear fraction to the cytoplasmic fraction. Attempts were made to 'chase' the labelled RNA from the nuclear material to the cytoplasmic fraction by incubating the protoplasts with [^3H]uridine for a short period and then adding a large amount of unlabelled uridine or of uridine plus ATP to the incubation mixture. These experiments were not successful. A typical result is shown in Table 3.

In this experiment, protoplasts were incubated with $10\text{ }\mu\text{C}$ of [^3H]uridine, and after 5 and 20 min. of labelling samples were removed and treated with 10 mg. of unlabelled uridine. The rest of the incubation mixture was used to follow the normal course of labelling in the nuclear RNA and cytoplasmic RNA (soluble RNA and ribosomal RNA). The specific radioactivity of both the nuclear RNA and the cytoplasmic RNA continues to increase in the presence of unlabelled uridine.

There are, however, objections to this type of experiment, which are discussed below. In the next experiment, protoplasts were labelled with [^3H]uridine for a short period, harvested, resuspended

and then 'starved' to deplete their endogenous nucleotide pool by gently shaking for 1 hr. at 30° before treatment with a relatively small amount of unlabelled uridine metabolite. The results are shown in Fig. 2.

The specific radioactivities of all the RNA fractions increase during the period of 'starvation'. Immediately after the addition of the unlabelled reagents, the specific radioactivity of the nuclear RNA fell sharply and after 15–30 min. almost no radioactivity was present in this fraction. The changes of specific radioactivity in the other fractions were somewhat complex. In the first 3 min. after adding the unlabelled compounds there was a marked rise in radioactivity in the residual ribosomes of the nuclear fraction and in the soluble fraction, followed by a fall. After 7 min. there was a slow increase in the radioactivity of the cytoplasmic fractions.

Another experiment was carried out under similar conditions with 30 min. of 'starvation' and in this case the total radioactivities present in the RNA are given (Table 4). The total radioactivity of the nuclear fraction diminished rapidly after the

Table 3. *Incorporation of [^3H]uridine into ribonucleic acid of cell fractions of protoplasts of Bacillus megaterium and attempts to displace labelled ribonucleic acid from the nuclear material into the cytoplasmic fraction*

Protoplasts derived from 2.0 g. of stationary-phase cells, suspended in 0.5 M-phosphate-salts medium at a concentration of 9 mg. dry wt./ml., were equilibrated for 15 min. at 30° and labelled with $10\text{ }\mu\text{C}$ of [^3H]uridine. Samples, taken after 2, 5, 10 and 20 min. of incubation, were lysed as described in the Methods section and fractionated by 'method A' of Butler & Godson (1963). Additional samples, taken after 5 and 20 min., were treated with 10 mg. of unlabelled uridine and incubated for a further 15 and 40 min. respectively before they were lysed and treated in the same way as the other samples. The radioactivity present in the RNA was measured by scintillation counting of an alkaline digest as described in the Methods section.

Time of labelling (min.)	Sp. radioactivity ($\mu\text{mc}/\text{mg. of RNA}$)	
	Nuclear material	Ribosomes and soluble fractions
2	17.8	3.6
5	21.5	5.6
10	24.0	11.7
20	23.6	18.5
5*	31.0	9.4
20†	30.0	16.8

* Plus unlabelled uridine to 20 min.

† Plus unlabelled uridine to 60 min.

Fig. 2. Transfer of labelled RNA from the nuclear material to the ribosomal and soluble fractions (Expt. 1). Protoplasts derived from 1.6 g. of logarithmic-phase cells were suspended in 200 ml. of sucrose- MgCl_2 -tris medium at a concentration of 6 mg. dry wt./ml. and labelled with $15\text{ }\mu\text{C}$ ($60\text{ }\mu\text{g.}$) of [^3H]uridine for 5 min. The incorporation was stopped by cooling, the protoplasts were recovered, resuspended in sucrose- MgCl_2 -tris medium (30°) at a concentration of 10 mg. dry wt./ml. and 'starved' for 1 hr. as described in the Methods section. Unlabelled material (2 mg. of uridine, 2 mg. of ATP and 2 mg. of UTP) was added to the incubation mixture and samples were taken after various times. The protoplasts were fractionated by 'method B₁' of Godson & Butler (1963) and the radioactivity present in the RNA was determined by separation of the UMP moiety as described in the text. Δ , Nuclear material; $\circ$, soluble fraction; $\blacktriangle$, ribosomes; $\bullet$, 'nuclear residue'.

Table 4. *Fate of labelled ribonucleic acid in the nuclear material*

Details of the experiment (Expt. 1) are given in the caption of Fig. 2. The total radioactivities present in the RNA (bottom line) are given after 'starvation' for 30 min. and after incubation with unlabelled compounds for the times stated.

Time after addition of unlabelled compounds (min.) ...	Radioactivity (counts/min.)				
	0	3	7	15	30
Nuclear material	440	290	290	200	140
Cytoplasmic ribosomes	2340	2540	2320	2280	2510
Nuclear 'residue' (ribosomes)	1210	1650	1330	1090	720
Soluble fraction	370	410	340	370	440
Total RNA	4360	4920	4280	3940	3810

Table 5. *Loss of labelled ribonucleic acid from the nuclear material on standing*

Samples of nuclear material, prepared from protoplasts labelled with [^3H]uridine for 3–5 min. by 'method B₁' of Butler & Godson (1963) were diluted with 10 mM-tris buffer, pH 7.3, containing MgCl_2 (10 mM) to give a concentration of approx. 7 mg. dry wt./ml. and kept at 30°. Duplicate samples were taken after various times and precipitated with 10% (w/v) trichloroacetic acid; the total radioactivity of each precipitate was determined, after thorough washing, by extracting the RNA with 0.5 ml. of 10% (w/v) trichloroacetic acid at 90° for 20 min. and measuring the radioactivity of a sample of the supernatant.

Time of sampling (min.)	Sp. radioactivity ($\mu\text{mc}/\text{mg. of RNA}$)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
0	1.71	1.88	0.355	2.69
15	—	1.01	—	2.47
30	1.33	0.55	—	2.37
60	1.27	0.09	0.320	2.08
200	—	—	0.190	—

Fig. 3. Location of labelled RNA in the nuclear material. Nuclear material derived from 0.5 g. dry wt. of protoplasts labelled for 6 min. with [^3H]uridine by 'method B' of Godson & Butler (1963) was homogenized in CsCl and the solution made up to contain 4 mg. dry wt. of nuclear material with a refractive index of 1.404 and then centrifuged at 35 000 rev./min. in a Spinco SW 39 rotor for 24 hr. The gradient fractions were collected and their extinctions (○) and radioactivities (●) measured as described in the text.

addition of the unlabelled compounds. Again an increase in the total radioactivities of the ribosomal soluble fractions and nuclear residue occurred in the first 3 min. after the addition of the unlabelled compounds, followed by a fall during the next few minutes. In this experiment a marked decrease in the radioactivity present in the nuclear ribosomes was observed after 3 min., which was largely responsible for the overall decrease of labelled RNA in the system after 3 min.

Loss of radioactive ribonucleic acid from the nuclear material in vitro. Experiments were performed on the loss of radioactivity in the trichloroacetic acid-precipitable RNA from the nuclear material when it was kept at 30° in the presence of magnesium chloride-tris buffer for various times. The results (Table 5) were variable: in three experiments little change occurred in the total amount of labelled RNA over a period of 1 hr. In another experiment (2), considerable loss of radioactivity occurred.

Location of the labelled ribonucleic acid in the nuclear material. When the nuclear material from labelled protoplasts was homogenized with caesium chloride and centrifuged, as shown in Fig. 3, most of the radioactivity was associated with the DNA peak. In this experiment, the caesium chloride gradient was not centrifuged quite long enough for complete equilibrium conditions to develop, and the DNA band was more diffuse than normal. The peak sedimenting at the bottom of the tube was free RNA.

DISCUSSION

Fig. 1 shows that the initial rate of rise of specific radioactivity of the RNA in the nuclear fraction is much greater than that in the other fractions. This suggests that the nuclear fraction is the primary site of synthesis of RNA in the protoplast. When the nuclear fraction is broken down into the DNA-containing nuclear material and the nuclear residue, it is evident that both of these subfractions become highly labelled at short times, but whereas the radioactivity in the nuclear material does not increase greatly during the later times that of the

residue continues to rise. When the total radioactivities are considered the total amount appearing in the cytoplasmic ribosomes at the shortest time, although the specific radioactivity is low (Table 1), is difficult to reconcile with an origin in the nuclear material, unless the turnover rate of the latter is extremely high. However, it is not unlikely that the initial rate of labelling of the nuclear material is so rapid that the earliest times of sampling may be too late to observe the most significant events. On the other hand, an independent origin of the RNA of the cytoplasmic fractions is clearly possible.

The high specific radioactivity of the nuclear residue (largely ribosomal) as compared with that of the cytoplasmic ribosomes shows that it is not composed of ribosomes that were merely entangled with the nuclear material. They are either highly labelled or contain a very highly labelled component. Evidence has been given from experiments *in vitro* (Barr & Butler, 1963) that ribosomes that are associated with the nuclear material receive at least a part of the newly synthesized RNA of the nuclear material.

In view of the high specific radioactivity of the nuclear residue it could be argued that the radioactivity in the nuclear material itself is also due to a highly radioactive contaminant of the same kind, but the caesium chloride experiment shows that most of this radioactivity is intimately associated with the DNA. The caesium chloride sedimentation pattern is similar to that obtained by centrifuging phenol extracts of whole cells of *Escherichia coli* in caesium chloride (Hayashi & Spiegelman, 1961).

The peculiar fall in the specific and total radioactivities of the nuclear RNA and of the soluble RNA between 3 and 10 min. is not due to unspecific terminal labelling and is so far unexplained.

The importance of the nuclear material in RNA biosynthesis in micro-organisms can be inferred from the caesium chloride-gradient studies of DNA-RNA complexes in *E. coli* (Hayashi & Spiegelman, 1961; Gros *et al.* 1961) and from the existence of DNA-dependent RNA-synthesizing enzyme systems in *E. coli* (Weiss, 1960; Steven, 1960; Furth, Hurwitz, King & Alexander, 1961; Chamberlin & Berg, 1962; Hurwitz & Bresler, 1961) and *Micrococcus lysodeikticus* (Weiss & Nakamoto, 1961*a, b, c*). Ezekiel (1961), using *B. megaterium*, has shown that a cell fraction containing most of the DNA, cytoplasmic membranes, some ribosomes and other cellular material contained highly labelled RNA. The combined radioautographic and electron-microscope studies of *E. coli* (Caro & Forro, 1961) have directly demonstrated that the first synthesized RNA is present in the chromatin areas of the cell and that it appears later in the non-chromatin areas.

In the experiments designed to displace the nuclear RNA with unlabelled precursor, the fate of the RNA leaving the nuclear fraction cannot be determined with certainty, owing to complicating factors. It is evident (Fig. 2) that the radioactivity of the nuclear RNA (nuclear fraction plus nuclear residue) increases during the 'starvation' period and that further radioactive RNA is formed (Table 4) for 3 min. after the addition of the unlabelled compounds. There is therefore within the cells a pool of labelled precursors which is not exhausted during the 'starvation' period. The fall in the radioactivity of the RNA of the nuclear material is solely an effect of adding unlabelled precursors and is clearly due to the displacement of radioactive material by unlabelled material.

Since the amount of radioactivity in the 'nuclear residue' increases, and that in the 'nuclear material' falls off, it is difficult to avoid the conclusion that transfer both of radioactive RNA present initially and possibly also of some RNA newly synthesized from a pool of radioactive precursors occurs during this period. That the loss of radioactivity of the nuclear material itself is due to enzymic breakdown is rendered unlikely by the fact that little loss of radioactivity from the isolated nuclear material usually occurs in a non-metabolic medium (Table 4). Although the results are somewhat variable, in no case has a loss of radioactivity as rapid as that in Fig. 2 been observed. Evidence from experiments *in vitro* of transfer from nuclear material to nuclear ribosomes has been given by Barr & Butler (1963).

However, there is certainly an overall loss of radioactivity of RNA of the combined nuclear fractions during the later stages of the displacement experiment (Table 4) which is not accounted for by any increase in the radioactivity of the cytoplasmic fractions. This is perhaps similar to the loss of radioactivity of nuclear RNA observed by Harris, Fisher, Rodgers, Spencer & Watts (1963) in HeLa cells, which is not due to transfer to the cytoplasm and is regarded as the result of enzymic breakdown within the nucleus.

During the initial period after the addition of unlabelled compounds the radioactivities of RNA of the cytoplasmic fractions also rise somewhat (Table 4). These are followed by rather complicated changes that cannot be interpreted fully on the basis of the present evidence.

The results therefore indicate a rapid synthesis in the nuclear material and a rapid rise of the radioactivity in the nuclear ribosomes, probably derived from the former.

These experiments do not give clear evidence of the transfer of RNA from the nuclear material to cytoplasmic RNA; but the existence of RNA with terminal cytidine in the nuclear fraction indicates a

possible origin of the soluble RNA here. More complete investigations are required to establish the origin of the RNA of the cytoplasmic fractions.

SUMMARY

1. When protoplasts of *Bacillus megaterium* are incubated with [³H]uridine, RNA with the highest specific radioactivity is found after short periods in the two components of the nuclear fraction. Whereas the radioactivity of the DNA-containing component soon reaches a stage of slow increase, that in the nuclear residue (ribosomal) continues to increase.

2. The specific radioactivity in cytoplasmic ribosomes increases slowly and approximately linearly with time, but the total amount present soon exceeds that in the nuclear material.

3. In all RNA fractions, part of the radioactivity is incorporated terminally, the amount being high during the first few minutes of labelling but falling progressively with time.

4. When the nuclear material is analysed on a caesium chloride gradient, most of the radioactivity is associated with the DNA band.

5. Experiments are given suggesting that the labelled RNA can be displaced from the nuclear material to other cell fractions.

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Physicochemical Studies on Cytochrome *b*₂

SOME PROPERTIES OF MODIFIED FORMS OF THE ENZYME AND OF THE DEOXYRIBONUCLEIC ACID COMPONENT

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Cytochrome *b*₂, the L(+)-lactate dehydrogenase of baker's yeast, was first isolated as a crystalline

deoxyribonucleoprotein (Appleby & Morton, 1954, 1959*a*, 1960; Morton, 1955) containing equimolecular proportions of flavin monophosphate and protohaem (Appleby & Morton, 1954, 1959*b*). This material behaves as an apparently homogeneous protein on sedimentation and on electrophoresis (Armstrong, Coates & Morton, 1963). The

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