The Conformation of Ribonucleic Acids in *Escherichia coli* Ribosomes

INFERENCES FROM THE MODE OF ACTION OF RIBONUCLEASE II

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1. Ribonuclease II of *Escherichia coli* degrades pulse-labelled RNA associated with ribosomes and polyuridylic acid on ribosomes and in solution to mononucleotides. 2. Ribosomal and pulse-labelled RNA in solution and ribosomal RNA in chloramphenicol particles (protein-deficient ribosomes) are degraded to oligonucleotides. 3. Ribosomal RNA in mature ribosomes is not attacked by the enzyme. 4. From the mode of action of ribonuclease II, which is specific for single-stranded polyribonucleotides and does not attack helical forms, it is inferred that pulse-labelled RNA associated with ribosomes of *E. coli* exists as a single-stranded structure and that ribosomal RNA in chloramphenicol particles has a pronounced helical character. 5. The different behaviour of ribonuclease II towards newly synthesized RNA, ribosomal RNA and chloramphenicol-particle RNA in *E. coli* ribosomes is discussed.

As Spahr (1964) has shown, ribonuclease II of *Escherichia coli* acts on RNA both as exonuclease and endonuclease. It behaves as exonuclease towards polyU.* Evidence for the endonuclease activity was supported by the finding of oligonucleotides in a partial digest of ribosomal RNA and by the collapse of the structure of R-17-phage RNA when only a small amount of nucleotides was liberated. It has been suggested that an increase in the secondary structure of polynucleotides is accompanied by a greater resistance to the enzyme. Singer & Tolbert (1965) confirmed the findings of Spahr (1964) and showed that ribonuclease II is specific for single-stranded polyribonucleotides. Helical forms were not attacked, nor did they inhibit the hydrolysis of single-stranded polyribonucleotides. It has been suggested that purified ribonuclease II may serve as tool for the study of polyribonucleotide secondary structure.

In this paper we describe studies on the activity of ribonuclease II towards pulse-labelled RNA, ribosomal RNA, RNA of CM particles and polyU on ribosomes and in solution. From the mode of action of ribonuclease II the conformation of pulse-labelled RNA on ribosomes and in solution and of RNA of CM particles was inferred.

* Abbreviations: polyU, polyuridylic acid; CM particles, chloramphenicol particles (protein-deficient ribosomes).

MATERIALS AND METHODS

Cells. *E. coli* B/u (a uracil-requiring strain) and *E. coli* Q₁₃ (a ribonuclease I-defective mutant) were used throughout the work. *E. coli* B/u was grown at 37° with aeration in the synthetic medium of Davis & Mignoli (1950) supplemented with glucose (0.2%) and uracil (20 µg./ml.). *E. coli* Q₁₃ was grown under similar conditions in 1% tryptone broth supplemented with NaCl (0.5%). Both media supported exponential growth with a generation time of approx. 75 min. Stock cultures were maintained on nutrient Difco agar slopes and were transferred every 3 weeks.

Labelling experiments. The cells were grown as described above. [2-¹⁴C]Uracil (2 µC/100 ml. of medium) was added to exponentially growing cultures. After 15–45 sec. of exposure to [2-¹⁴C]uracil the cells were poured on to crushed frozen 5 mm-tris-HCl buffer, pH 7.4, containing magnesium acetate (10 mM) (tris-Mg²⁺ buffer), and collected by centrifugation in a refrigerated Serrvall RC-2 centrifuge. These cells are referred to as 'pulse-labelled' cells. Ribosomes and RNA were prepared as described below.

For experiments with labelled ribosomal RNA, the bacteria after exposure to [2-¹⁴C]uracil for 45 sec. were allowed to grow for an additional 35 min. (approx. one-half of the generation time) with a 100-fold excess of non-radioactive uridine. The cells were collected as above and used for the preparation of ribosomes or RNA or both. These cells are referred to as 'chased' cells.

Preparation of spheroplasts. The bacteria were grown on a rapid reciprocal shaker at 37° in a modified 3XD broth (Guthrie & Sinsheimer, 1960) supplemented with uracil (20 µg./ml.). Exponentially growing cells were centrifuged

in the cold and suspended in precooled 20% (w/v) sucrose–30mm-tris–HCl buffer, pH 8.2, to give a cell concentration of E_{550} 3.0. Lysozyme (20 μ g./ml.) and sodium EDTA, pH 8.0 (4 μ moles/ml.), were added to the cell suspension. The mixture was gently agitated in a water bath at 4° for 10 min. and the formation of spheroplasts was determined by lysability (decrease in E_{550}) of samples diluted tenfold in water. The EDTA spheroplasts were sedimented by centrifugation in the cold and resuspended in tris–Mg²⁺ buffer.

Preparation of [2-¹⁴C]uracil-labelled chloramphenicol particles. CM particles with labelled RNA were obtained by the following procedure. A 50 ml. culture of exponentially growing cells (E_{550} 0.2) was exposed to chloramphenicol (20 μ g./ml. of medium). Growth was immediately arrested and protein synthesis inhibited by more than 98%. Then 10 min. after the addition of chloramphenicol 2 μ C of [2-¹⁴C]uracil was added and the cells were incubated with shaking for an additional 30 min. At the end of incubation the cells were harvested by centrifugation in the cold and washed in tris–Mg²⁺ buffer. CM particles were prepared from ultrasonically treated cells, alumina-ground cells or EDTA spheroplasts by centrifuging the S30 supernatant (i.e. the supernatant after centrifuging at 30000g) in the Spinco model L ultracentrifuge (no. 50 rotor) at 30000 rev./min. for 18 hr.

Preparation of ribosomes. All operations were carried out at 0–4°. Ribosomes were prepared from: (a) cells disrupted in a 20 kcyc./sec. MSE ultrasonic disintegrator for 3 min.; (b) cells ground with twice their weight of alumina; (c) EDTA spheroplasts lysed in 5mm-tris–HCl buffer, pH 7.4, containing magnesium acetate (10mm), KCl (60mm) and Triton X-100 (0.5%). The resultant preparations were clarified by centrifugation at 30000g for 20 min. The pellet, consisting of cell debris and unbroken or unlysed cells, was discarded and the ribosomes were obtained by centrifuging the S30 supernatant in the Spinco model L ultracentrifuge (no. 50 rotor) at 49000 rev./min. for 60 min.

Extraction of RNA. To 1 ml. of EDTA spheroplasts or ribosomes in tris–Mg²⁺ buffer was added 1 ml. of 5mm-tris–HCl buffer, pH 7.4, containing sodium dodecyl sulphate (1%, w/v) and NaCl (0.1M), followed by 2 ml. of freshly distilled 90% (w/v) phenol. The phenol mixture was centrifuged at 10000g for 10 min. in the cold. The upper aqueous layer was carefully collected and freed from phenol by five ether extractions. The dissolved ether was removed by air-blowing and the nucleic acids were precipitated with 2 vol. of ice-cold 95% (v/v) ethanol in the presence of 0.1M-NaCl. Nucleic acids were collected by centrifugation and dissolved in 5mm-tris–HCl buffer, pH 7.4. In some experiments the solution of RNA was made 1mM with respect to Mg²⁺ ions, and deoxyribonuclease (10 μ g./ml.) was added. After 15 min. of incubation at 4° the RNA was reisolated by ethanol precipitation. The product was stored under ethanol at –15°.

Treatment of RNA with dimethyl sulphoxide. RNA precipitated by 95% ethanol was dissolved in 1 ml. of dimethyl sulphoxide, incubated for 15 min. at room temperature and recovered by adding 2 vol. of ether and 2 vol. of 95% ethanol.

Sedimentation of ribosomes and RNA on linear sucrose gradients. Linear sucrose gradients (5–20% or 15–30%, w/v) in suitable buffers and ionic conditions were used throughout this work (the buffers and salts present in gradients are

listed in the Figure legends). For analytical purposes gradients of volume 4.4 ml. were run in the Spinco model L or L.2 ultracentrifuge (SW 39 rotor). Ribosomal samples of suitable buffer and ionic composition in a volume of 0.2–0.4 ml. were layered on top of sucrose gradients and centrifuged for 70 to 120 min. as listed in the Figure legends. Samples (0.2–0.4 ml.) of RNA in 5mm-tris–HCl buffer, pH 7.4, containing NaCl (0.1M) and EDTA (1mM) (unless stated otherwise) were centrifuged in sucrose gradients for 4–5 hr. at 4°.

From each gradient were collected 27–35 fractions. Each fraction was made up to 1 ml. with 0.1M-NaCl or water and the E_{260} value determined. Thereafter to each fraction 2 drops of 1% albumin solution and 1 ml. of ice-cold 10% (w/v) trichloroacetic acid were added. The trichloroacetic acid-insoluble material was collected on Millipore filters and after drying placed in vials for the determination of radioactivity.

Scintillation counting. All samples were counted in a Packard Tri-Carb scintillation counter. Samples on filter-paper disks or Millipore filters were counted in 10 ml. of a scintillation fluid containing 0.4% 2,5-diphenyloxazole and 0.01% bis-1,4-(5-phenyloxazol-2-yl)benzene in toluene.

Breakdown of RNA on ribosomes. The reaction mixture contained, in 1.0 ml. of tris–Mg²⁺ buffer, 30.0 E_{260} units of CM particles, ribosomes from pulse-labelled cells or ribosomes from cells chased with non-radioactive uridine. After various times of incubation at 37° the breakdown of RNA was ascertained by analysing the ribosomes or the RNA extracted from them in sucrose gradients as described above. In other experiments the release of acid-soluble degradation products was determined. For this purpose at zero time and after various times of incubation the samples were mixed with an equal volume of cold 10% trichloroacetic acid. After 20 min. in the cold the precipitate was removed by centrifugation and the E_{260} value of the ether-extracted trichloroacetic acid-soluble fraction was measured; radioactivity was determined as described above.

In experiments where the bacterial strains are not mentioned the results were identical whichever strain of *E. coli* was used, the only difference being that the rate of RNA breakdown on ribosomes of *E. coli* B/u was higher than that on ribosomes of *E. coli* Q13.

Identification of acid-soluble degradation products. In experiments designed to identify the products of degradation of pulse labelled RNA on ribosomes, the latter (400 mg. of ribosomes) were incubated in 5 ml. of tris–Mg²⁺ buffer at 37° for 90 min. At the end of the incubation period the reaction mixture was mixed with an equal volume of 1.0N-HClO₄. After 20 min. in the cold the mixture was centrifuged at 10000g for 10 min. and the E_{260} value of the material soluble in HClO₄ was measured in a Beckman model DU spectrophotometer after suitable dilution (to determine the amount of RNA degraded to acid-soluble products). The HClO₄-soluble degradation products were freed from ClO₄[–] ions by neutralization with KOH and removal of KClO₄ by centrifugation. This partially desalted solution was adjusted to pH 6.0 and passed through activated charcoal (British Drug Houses Ltd., Poole, Dorset) supported on a Millipore filter or sintered-glass column. Activated charcoal (2mg./ E_{260} unit of nucleotides) was washed on the filter with 20 ml. of distilled water. The nucleotide solution was passed through followed by 20 ml. of water. The nucleotides were eluted with 2% (w/v) NH₃

in 50% (v/v) ethanol, and recovered as solid by evaporating under reduced pressure or in vacuum desiccator at room temperature. The dry substance containing the degradation products was taken up in a minimum of water and used for fractionation by paper electrophoresis or paper chromatography or both.

Paper chromatography. This was done by the descending technique. The substances were applied to Whatman no. 3 filter paper and dried under a cold-air blower. The following solvents were used. Solvent I was propan-2-ol-water (7:3, v/v) with NH_3 in the vapour phase [0.35 ml. of aq. NH_3 (sp. gr. 0.88)/l. volume of the Chromatocarb]. This solvent separates GMP from a mixture of AMP, CMP and UMP (R_F 0.16 and 0.30 respectively) and 2',3'-(cyclic)-GMP from a mixture of 2',3'-(cyclic)-AMP, 2',3'-(cyclic)-CMP and 2',3'-(cyclic)-UMP (R_F 0.40 and 0.60 respectively). Solvent II was 96% (v/v) ethanol-1.0 M-ammonium acetate (15:6, v/v) saturated with borax and the pH adjusted to 10.0 with aq. NH_3 . This solvent permits the separation of nucleoside 3'-phosphates from their 5'-nucleotide isomers. Borate-ribonucleoside 5'-phosphate complexes move much more slowly in this solvent than the corresponding ribonucleoside 2'- and 3'-phosphates. Solvent III was ethanol-1.0 M-ammonium acetate (4:3, v/v) for the separation of UMP from UDP.

Paper electrophoresis. High-voltage electrophoresis was performed with a field strength of 40 v/cm. in 50 mM-ammonium formate buffer, pH 3.5, for 4 hr. After electrophoresis or chromatography the papers were dried at room temperature, the spots viewed under ultraviolet light and the contours outlined with pencil. For quantitative determination of nucleotides the spots were excised, placed in vials and their radioactivities determined as described above, or eluted with water or 0.1 M-NaCl until no further 260 μ -absorbing material was eluted, concentrated under reduced pressure at room temperature and the E_{260} values measured in a Beckman model DU spectrophotometer against blanks prepared by eluting appropriate strips of paper.

Breakdown of [^3H]polyuridylic acid on ribosomes. This was determined in different reaction mixtures for the detection of the activities of ribonuclease I, ribonuclease II and polynucleotide phosphorylase. All reaction mixtures contained, in a final volume of 0.6 ml., ribosomes (0.8 mg.), [^3H]polyU (200 μ g.; 7000 counts/min.) and tris-HCl buffer, pH 7.4 (2.5 mM). To this, the following additions were made: to reaction mixture I, magnesium acetate (10 mM); to reaction mixture II, KCl (75 mM) and magnesium acetate (2.5 mM); to reaction mixture III, sodium phosphate buffer, pH 7.4 (20 mM), and EDTA (20 mM); to reaction mixture IV, sodium phosphate buffer, pH 7.4 (50 mM), and magnesium acetate (2.5 mM).

At zero time and after various times of incubation samples were directly applied to Whatman no. 3 filter paper, dried and developed in solvents I, II and III. Undegraded polyU in all three solvents remained at the starting line. The spots were viewed under ultraviolet light, excised, placed in vials and their radioactivities determined as described above.

Partial purification of ribonuclease II. This was done essentially as described by Spahr (1964). Ribosomes were prepared from the uracil-requiring strain of *E. coli* by alumina grinding as described above. Ribosomes (20 E_{260} units) were layered on top of a linear sucrose gradient (5–20%, w/v) in tris- Mg^{2+} buffer and centrifuged in the

Spinco model L ultracentrifuge (SW 39 rotor) at 37 000 rev./min. for 2 hr. at 4°. Twenty 5-drop fractions were collected. In this procedure a large percentage of ribonuclease II activity previously associated with ribosomes was recovered free of ribosomes in the last four fractions at the top of the gradient. The enzyme was used immediately for the experiments.

Action of ribonuclease II on RNA in solution. The reaction mixture contained, in a final volume of 0.6 ml., magnesium acetate (10 mM), KCl (75 mM), enzyme from the top of the sucrose gradient (0.4 ml.) and ribosomal RNA (250 μ g.) with pulse-labelled RNA extracted from EDTA spheroplasts. At the end of the incubation period Mg^{2+} ions were removed by addition of equivalent amount of sodium EDTA, pH 7.5, and samples were layered on top of sucrose gradients (5–20%, w/v) prepared in 5 mM-tris-HCl buffer, pH 7.4, containing NaCl (0.1 M) and EDTA (1 mM). After centrifugation 27 fractions were collected and their E_{260} values and radioactivities determined as described above.

In experiments with [^3H]polyU the last addition consisted of non-labelled ribosomal RNA (500 μ g.) and [^3H]polyU (20 μ g.; 7000 counts/min.). At zero time and after various times of incubation the samples were layered on top of 5–20% (w/v) sucrose gradients prepared in tris- Mg^{2+} buffer, centrifuged and analysed as described above.

RESULTS

Breakdown of pulse-labelled RNA on *E. coli* ribosomes. When ribosomes prepared from exponentially growing cells of *E. coli* are incubated at 37° in tris- Mg^{2+} buffer a release of acid-soluble 260 μ -absorbing material is observed. The rate of release of the acid-soluble material is extremely slow. Under the specified conditions 2.8–2.9% of RNA became soluble within 90 min. of incubation (Table 1). To observe RNA breakdown on *E. coli* ribosomes under these conditions relatively large amounts of ribosomes ought to be used (at least 20–40 E_{260} units of ribosomes/ml. of the reaction mixture), as otherwise an apparent stability of ribosomal preparations would be inferred. The kinetics of the release of acid-soluble RNA degradation products tells us nothing about the species of RNA degraded. It could equally well be assumed that the release of acid-soluble compounds indicates slow breakdown of ribosomal RNA, degradation of transfer RNA attached to ribosomes or degradation of pulse-labelled RNA (newly synthesized RNA), which according to the current views is composed of messenger RNA and neosomes. It is possible to distinguish between the first and last possibilities by using ribosomes from cells pulse-labelled with radioactive precursor of RNA ([2- ^{14}C]uracil) and from cells that after a short exposure to the radioactive precursor were allowed to grow in the presence of a large excess of non-radioactive uridine. In the first instance the bulk of the RNA of mature ribosomes will be non-labelled and the entire label will be confined to the newly synthesized (pulse-labelled) RNA. In the second instance RNA of

Table 1. *Breakdown of pulse-labelled RNA on E. coli ribosomes*

Bacteria were pulse-labelled with [2-¹⁴C]uracil or pulse-labelled and chased with non-radioactive uridine as described in the Materials and Methods section. Ribosomes were prepared from cells disintegrated by ultrasonic treatment or grinding with alumina and incubated in tris-Mg²⁺ buffer. The release of acid-soluble degradation products was followed by determining E_{260} and radioactivity. Specific radioactivity is expressed in terms of counts/min./ E_{260} unit of ribosomes.

Ribosomes	Incubation time (min.)	Breakdown of RNA (E_{260})	Breakdown of RNA (%)	Breakdown of labelled RNA (counts/min.)	Breakdown of labelled RNA (%)	Radioactivity remaining on ribosomes (counts/min.)	Sp. radio-activity of degradation products	Sp. radio-activity of ribosomal RNA
Ribosomes from pulse-labelled cells	0	—	—	—	—	16514	—	535
	30	0.47	1.57	7610	45.8	8900	16000	290
	60	0.67	2.16	9000	54.5	7400	13500	242
	90	0.87	2.80	12280	74.5	4500	14100	150
Ribosomes from cells chased with non-radioactive uridine	0	—	—	—	—	13000	—	420
	30	0.50	1.61	464	3.57	12500	928	415
	60	0.70	2.26	549	4.22	12300	785	410
	90	0.89	2.88	600	4.60	12300	675	410

mature ribosomes will be labelled and the newly synthesized RNA will be devoid of radioactivity. The results of experiments aimed at the elucidation of this problem (Table 1) show that the amount of labelled RNA degraded on ribosomes from pulse-labelled cells was 45.8, 54.5 and 74.5% of the total radioactive RNA after 30, 60 and 90 min. of incubation respectively. The corresponding values for labelled RNA degraded on ribosomes from cells chased with non-radioactive uridine were 3.6, 4.2 and 4.6%. The specific radioactivity of the acid-soluble degradation products released from ribosomes of pulse-labelled cells was high, 15 000 counts/min./ E_{260} unit on average, and constant throughout the incubation. The specific radioactivity of the RNA that remained undegraded was low and decreased with the incubation from 535 counts/min./ E_{260} unit at zero time to 150 counts/min./ E_{260} unit after 90 min. of incubation. The specific radioactivity of the acid-soluble degradation products released from ribosomes of cells chased with non-radioactive uridine was low and decreased with the incubation from 928 counts/min./ E_{260} unit after 30 min. of incubation to 675 counts/min./ E_{260} unit after 90 min. of incubation, approaching the specific radioactivity of undegraded RNA. The specific radioactivity of undegraded RNA remained constant throughout the incubation. The above results indicate that under the specified conditions there is a selective breakdown of newly synthesized (pulse-labelled) RNA to acid-soluble compounds and that RNA of mature ribosomes is stable under these conditions. However, the method used reveals only breakdown of RNA to acid-soluble compounds, and cleavage of RNA to acid-insoluble oligonucleotide chains would remain undetected. To ascertain

whether the RNA of mature ribonucleoprotein particles is stable or broken down to oligonucleotides under conditions when pulse-labelled RNA is degraded to acid-soluble compounds the following experiment was carried out. Ribosomes prepared from pulse-labelled cells were suspended in tris-Mg²⁺ buffer and divided into two portions. One portion was left in the cold and the other incubated at 37° for 90 min. At the end of the incubation period the ribosomal preparations were analysed by the sucrose-density-gradient technique. Fig. 1 summarizes sucrose-density-gradient studies from several representative experiments. Figs. 1(a) and 1(b) show that after incubation at 37° the ribosomes appeared to be intact whereas about 90% of the radioactivity representing rapidly labelled RNA became degraded. Spirin (1963) has pointed out that short exposure to exogenous ribonuclease may bring about scissions in polynucleotide chains while the RNA is still on ribosomes, and that the sedimentation pattern of such particles remains unaltered. To test whether such scissions in the continuous polynucleotide chains occurred during the incubation of ribosomes in tris-Mg²⁺ buffer, RNA was extracted from ribosomes at zero time and after 90 min. incubation at 37° and analysed in sucrose gradients. The features of RNA pattern extracted from ribosomes incubated at 37° for 90 min. were similar to those of RNA extracted from ribosomes at zero time (Figs. 2a and 2b). There were two peaks corresponding to 23s and 16s RNA. The only difference was that the ratio of 16s to 23s RNA extracted from incubated ribosomes was higher than that of RNA extracted from ribosomes before incubation. This feature was more marked in some experiments than in others. Treatment of RNA

Fig. 1. Effect of incubation in tris- Mg^{2+} buffer on sedimentation of ribosomes from pulse-labelled cells. Ribosomes were prepared from cells pulse-labelled with $[2-^{14}C]$ uracil for one-hundredth of the generation time and incubated in tris- Mg^{2+} buffer at 4° or 37° for 90 min. as described in the Materials and Methods section. Samples (0.2 ml.) were sedimented in a sucrose gradient (15–30%, w/v) in 5 mM-tris-HCl buffer, pH 7.4, containing KCl (60 mM) and magnesium acetate (10 mM), at 4° for 70 min. at 37000 rev./min. in the Spinco model L ultracentrifuge (SW 39 rotor). The gradient was fractionated, and E_{260} values and acid-insoluble radioactivity were determined as described in the Materials and Methods section. (a) Sedimentation pattern after incubation at 4° . (b) Sedimentation pattern after incubation at 37° . ●, E_{260} ; ○, acid-insoluble radioactivity.

Fig. 2. Sedimentation analyses of ribosomal RNA extracted from ribosomes incubated in tris- Mg^{2+} buffer. *E. coli* ribosomes were incubated in tris- Mg^{2+} buffer. At zero time and after 90 min. of incubation at 37° 2 ml. samples were removed, the nucleic acids extracted with sodium dodecyl sulphate-phenol, recovered by ethanolic precipitation and dissolved in 2 ml. of 5 mM-tris-HCl buffer containing NaCl (0.1 M), as described in the Materials and Methods section. Nucleic acid samples (0.25 ml.) were sedimented in a sucrose gradient (5–20%, w/v) in 5 mM-tris-HCl buffer containing NaCl (0.1 M), EDTA (1 mM) and sodium dodecyl sulphate (0.5%) at 20° for 4 hr. at 37000 rev./min. in the Spinco model L.2 centrifuge (SW 39 rotor). The gradient was fractionated and the E_{260} values were determined. (a) Sedimentation of ribosomal RNA extracted from ribosomes before incubation. (b) Sedimentation of ribosomal RNA extracted from ribosomes after 90 min. of incubation. ●, E_{260} .

with dimethyl sulphoxide also failed to reveal any breaks in the polynucleotide chain after incubation of ribosomes in tris- Mg^{2+} buffer.

Identification of acid-soluble degradation products.

Ribosomes (400 mg.) from the uracil-requiring mutant of *E. coli*, suspended in 5 ml. of tris- Mg^{2+} buffer, were incubated at 37° for 90 min. and the acid-soluble degradation products were desalted

and concentrated as described in the Materials and Methods section. Samples (0.05 ml.) and appropriate markers were chromatographed in solvent I. Chromatography revealed two strong and four faint spots. The two strong spots were identified as GMP and a mixture of mononucleotides other than GMP. The two faint spots were identified as 2',3'-(cyclic)-GMP and a mixture of cyclic mononucleotides other than 2',3'-(cyclic)-GMP. The two remaining spots were unidentified, but, from their mobility in solvent I (which was close to that of cyclic mononucleotides) and from their mobility towards the cathode in electrophoretic runs, they were probably nucleosides.

The spots of the separated cyclic and non-cyclic nucleotides were excised, eluted and the E_{280}/E_{260} ratios measured in each eluent. Approx. 5–7% of the nucleotides released from *E. coli* ribosomes incubated in tris- Mg^{2+} buffer were shown to be in the form of nucleoside 2',3'-(cyclic)-phosphates.

The desalted and concentrated degradation products were subjected to paper electrophoresis at pH 3.5 as described in the Materials and Methods section. Four spots were detected and identified as CMP, AMP, GMP and UMP (nucleosides were not detected because samples were applied close to the cathode). The nucleotides were eluted and treated with 0.1N-hydrochloric acid for 4 hr. at 20° to convert cyclic nucleotides quantitatively into their corresponding 2'- or 3'-phosphates. Each of the nucleotides so treated was chromatographed in solvent II. The corresponding 2'- or 3'-nucleotides and 5'-nucleotides were run as markers. In all experiments each nucleotide was resolved in solvent II into two spots. One strong spot close to the starting line was identified as nucleoside 5'-phosphate and a faint spot with greater mobility was identified as nucleoside 2'- or 3'-phosphate. All spots were excised and eluted, and the E_{280} values of the eluents were measured. The results of determinations of several experiments showed that 5'-nucleotides comprised 85–90% of all degradation products.

Breakdown of [3H]polyuridylic acid on *E. coli* ribosomes. The results of experiments on the breakdown of polyU on ribosomes of *E. coli* B/u and Q₁₃ are illustrated in Table 2 and Fig. 3. It is evident that under the conditions used in the experiments on the breakdown of pulse-labelled RNA (reaction mixtures I and II) polyU is rapidly and quantitatively degraded to UMP. Under these conditions the breakdown of RNA is catalysed by ribonuclease II and not by polynucleotide phosphorylase, which requires phosphate ions (reaction mixture IV), or ribonuclease I (reaction mixture III), which is lacking in *E. coli* Q₁₃ and requires EDTA in *E. coli* B/u.

Degradation of RNA on protein-deficient ribo-

Table 2. Breakdown of [3H]polyuridylic acid on *E. coli* B/u ribosomes

Ribosomes and [3H]polyU were incubated at 37°. After 30 min. of incubation the samples were directly applied to Whatman no. 3 paper and developed with solvents II and III. The degradation products were identified and determined as described in the Materials and Methods section. For the composition of the reaction mixture see the Materials and Methods section.

Reaction mixture	Degradation products (%)			
	UMP	3'-UMP	UDP	Uridine
I	76	6	—	18
II	82	3	—	15
III	—	100	—	—
IV	8	—	92	—

Fig. 3. Breakdown of [3H]polyU on *E. coli* Q₁₃ ribosomes. Ribosomes and [3H]polyU were incubated at 37°. At intervals samples were directly applied to Whatman no. 3 paper and developed in solvents II and III, and the undegraded polyU and degradation products were identified and determined as described in the Materials and Methods section. (a) Incubation in reaction mixture II (see the Materials and Methods section). (b) Incubation in reaction mixture III (see the Materials and Methods section). ●, PolyU; ○, UMP; ■, uridine; ▲, 2',3'-(cyclic)-UMP; △, UDP.

somes. Next, we attempted to ascertain whether the stability of ribosomal RNA was due to protection of RNA against nuclease action afforded by binding of ribosomal RNA to ribosomal proteins. For this purpose we studied the stability of ribosomal RNA in protein-deficient ribosomes (CM particles) after incubation in tris- Mg^{2+} buffer under conditions where ribosomal RNA in normal 'mature' ribosomes was stable. CM particles were labelled with [2- ^{14}C]-uracil and incubated in tris- Mg^{2+} buffer as described above. Sucrose-density-gradient centrifugation profiles of CM particles before and after incubation

Fig. 4. Effect of incubation in tris- Mg^{2+} buffer on CM particles. Experimental details for the preparation of $[2-^{14}C]$ uracil-labelled CM particles and incubation in tris- Mg^{2+} buffer are given in the Materials and Methods section. (a) Sedimentation pattern of ribosomes and $[2-^{14}C]$ uracil-labelled CM particles before incubation centrifuged in a sucrose gradient (15–30%, w/v) in 5 mm-tris-HCl buffer containing KCl (60 mM) and magnesium acetate (10 mM) at 4° for 2 hr. at 37 000 rev./min. in the Spinco model L ultracentrifuge (SW 39 rotor). (b) Sedimentation pattern of ribosomes and CM particles after incubation in tris- Mg^{2+} buffer at 37° for 90 min. Sedimentation analysis was carried out as in (a). (c) Sedimentation pattern of total nucleic acid extracts from cells with $[2-^{14}C]$ uracil-labelled CM particles. RNA was extracted from intact cells after conversion into EDTA spheroplasts (see the Materials and Methods section). RNA samples were sedimented in a sucrose gradient (5–20%, w/v) in 5 mm-tris-HCl buffer containing NaCl (0.1 M) and EDTA (1 mM) at 4° for 4 hr. at 37 000 rev./min. in the Spinco model L ultracentrifuge (SW 39 rotor). (d) Sedimentation pattern of RNA extracted from ribosomes and $[2-^{14}C]$ uracil-labelled CM particles after incubation in tris- Mg^{2+} buffer at 37° for 90 min. Sedimentation analysis was carried out as in (c). All gradients were fractionated and the E_{260} values and radioactivity determined as described in the Materials and Methods section. ●, E_{260} ; ○, acid-insoluble radioactivity.

in tris- Mg^{2+} buffer at 37° for 90 min. are shown in Figs. 4(a) and 4(b). As shown in Fig. 4(a), RNA synthesized in the presence of chloramphenicol sedimented more slowly than the 70s ribosome, in the region expected for CM particles (Nomura & Watson, 1959; Kurland, Nomura & Watson, 1962). After incubation (Fig. 4b) the entire radioactivity that represents RNA of CM particles remained acid-

insoluble. As a result of the incubation, however, the sedimentation of CM particles changed drastically: they sedimented extremely slowly, appearing almost at the top of the gradient. The sucrose-density-gradient sedimentation patterns in Figs. 4(c) and 4(d) show that the slowly sedimenting material appearing after incubation in tris- Mg^{2+} buffer represents the RNA moiety of CM particles

Fig. 5. Effect of ribonuclease II on isolated ribosomal and pulse-labelled RNA. Nucleic acids were extracted from pulse-labelled cells after conversion into EDTA spheroplasts. Ribonuclease II was partially purified from *E. coli* ribosomes and incubated with the nucleic acids as described in the Materials and Methods section. At time-intervals 0.3 ml. samples were removed and sedimented in a sucrose gradient (5–20%, w/v) in 5 mM-tris-HCl buffer containing NaCl (0.1 M) and EDTA (1 mM) at 4° for 4 hr. at 37 000 rev./min. in the Spinco model L ultracentrifuge (SW 39 rotor). The gradients were fractionated and the E_{260} values and acid-insoluble radioactivity determined as described in the Materials and Methods section. (a) RNA at zero time. (b) RNA after 2 hr. of incubation with ribonuclease II. (c) RNA after 4 hr. of incubation with ribonuclease II. ●, E_{260} ; ○, acid-insoluble radioactivity.

that became degraded to acid-insoluble low-molecular-weight oligonucleotides. Fig. 4(c) shows the sedimentation pattern of total RNA extracted from spheroplasts containing CM particles. There are three main components of RNA, sedimenting at 23s, 16s (ribosomal RNA of mature ribosomes and CM particles) and 4s (transfer RNA). Fig. 4(d) shows sedimentation properties of RNA extracted from CM particles after incubation in tris- Mg^{2+} buffer. After incubation, the 260 m μ -absorbing peak of the 23s and 16s RNA markedly decreased and a rather broad peak appeared near the top of the gradient. At the same time the entire radioactivity that represents the RNA of CM particles disappeared from the region of high-polymer ribosomal RNA and appeared in the region of 4–8s. The remaining 23s and 16s peaks are clearly those of RNA from normal ribosomes, which are stable under these conditions.

Activity of ribonuclease II on isolated RNA. The effect of partially purified ribonuclease II on isolated pulse-labelled and ribosomal RNA is illustrated in Figs. 5(a), 5(b) and 5(c). In Fig. 5(a) a typical profile of ribosomal and pulse-labelled RNA is shown. In Fig. 5(b) a profile of the nucleic acid preparation is shown after 2 hr. of incubation with ribonuclease II. It is evident that the 23s peak (fractions 12 and 13 in Fig. 5a) and the 16s peak (fractions 15 and 16 in Fig. 5a) disappeared. The ribosomal RNA now sedimented in a broad and diffuse region extending from fraction 17 to the top of the gradient. The radioactivity of pulse-labelled RNA remained acid-insoluble and became slightly

displaced towards regions of lower sedimentation coefficient. After 4 hr. of incubation (Fig. 5c) ribosomal RNA and the bulk of the pulse-labelled RNA were degraded to acid-insoluble low-molecular-weight oligonucleotides sedimenting in the region of 4–8s.

In Figs. 6(a) and 6(b) a similar experiment with [3H]polyU is presented. Ribosomal RNA was mixed with [3H]polyU and incubated with ribonuclease II. Since in this case the appearance of acid-insoluble degradation products of polyU could not be ascertained in samples run in sucrose gradients prepared in 0.1 M-sodium chloride–1 mM EDTA (under these conditions undegraded polyU sediments free of ribosomal RNA at 4–6s) the reaction mixture with polyU was centrifuged in sucrose gradients containing magnesium acetate (10 mM), where undegraded polyU is in the form of complexes with ribosomal RNA. The sedimentation profile of ribosomal RNA and [3H]polyU before incubation with ribonuclease II is shown in Fig. 6(a). It shows, in agreement with Möller & Boedtker (1961) and Marcot-Queiroz & Monier (1965), complex-formation between 16s and 23s ribosomal RNA, as manifested by the appearance of 39s and 28s peaks in addition to the 16s peak. Under these conditions polyU also formed complexes with ribosomal RNA. After 2 hr. of incubation (Fig. 6b) more than 90% of the [3H]polyU became degraded to acid-soluble products, whereas the 260 m μ -absorbing material of ribosomal RNA remained acid-insoluble and sedimented as a broad diffuse band from about 16s to 4s. Under these conditions

Fig. 6. Effect of ribonuclease II on polyU in solution. Nucleic acids were extracted as described in the legend to Fig. 5. [^3H]PolyU ($20\text{ }\mu\text{g.}$; 7000 counts/min.) mixed with RNA ($500\text{ }\mu\text{g.}$) was incubated with partially purified ribonuclease as described in the Materials and Methods section. At zero time and after 2 hr. of incubation at 37° , 0.3 ml. samples were layered on top of a sucrose gradient ($5\text{--}20\%$, w/v) in 5 mm-tris-HCl buffer containing KCl (60 mm) and magnesium acetate (10 mm) and sedimented at 4° for 4 hr. at 37000 rev./min. in the Spinco model L ultracentrifuge (SW 39 rotor). The gradients were fractionated and the E_{260} values and acid-insoluble radioactivity determined as described in the Materials and Methods section. (a) Sedimentation at zero time. (b) Sedimentation after 2 hr. incubation at 37° . $\bullet$, E_{260} ; $\circ$, acid-insoluble radioactivity.

polyU was degraded to UMP, identified by paper chromatography in solvent II.

DISCUSSION

The major findings of this work are that under the specified conditions ribonuclease II of *E. coli*: (1) produces mononucleotides from pulse-labelled RNA associated with ribosomes and from polyU on ribosomes and in solution; (2) produces oligonucleotides from ribosomal RNA in CM particles and from pulse-labelled and ribosomal RNA in solution; (3) does not attack ribosomal RNA in mature normal ribosomes.

Interpretation of these results is based on the finding that ribonuclease II is specific for single-stranded polyribonucleotides and does not attack helical forms (Singer & Tolbert, 1965).

Ribonuclease II behaves towards polyU, which exists as a single-stranded structure on ribosomes and in solution, as exonuclease, UMP being the only degradation product. Pulse-labelled RNA associated with ribosomes has also apparently little double-helical character, being degraded by ribonuclease II to acid-soluble products that were identified as nucleoside $5'$ -phosphates. Pulse-labelled RNA isolated from ribosomes assumes helical conformation in solution, like all natural RNA and synthetic heteropolymers, and becomes relatively resistant to ribonuclease II. In this case ribonuclease II acts as endonuclease, attacking susceptible sites in the polynucleotide chain,

presumably single-stranded segments between helical regions that remain undegraded. This is also the case for ribosomal and CM-particle RNA in solution.

The different behaviour of ribonuclease II towards ribosomal RNA in mature ribosomes and in protein-deficient ribosomes (CM particles) deserves special comment. The stability of ribosomal RNA in mature ribosomes may be a consequence of protection of RNA against nuclease action afforded by the binding of ribosomal RNA to ribosomal proteins. Nuclease-resistance induced by interaction of RNA with polylysine has been reported by Sober, Schlossman, Yaron, Latt & Rushizky (1966). CM particles with abnormally low protein content do not afford protection to the RNA in the particles and the former is attacked by ribonuclease II. From the mode of action of the enzyme on RNA in CM particles it could be inferred that it has an appreciable helical structure.

A recent study on the conformation of RNA in mature ribosomes of *E. coli* (Furano, Bradley & Childers, 1966) provided strong evidence that ribosomal RNA in *E. coli* ribosomes exists essentially as a single-stranded structure. It would therefore appear that the conformation of ribosomal RNA in mature ribosomes is like that of pulse-labelled RNA and differs from the conformation of ribosomal RNA in CM particles. Ribosomal RNA in protein-deficient ribosomes behaves as it does in solution: it assumes helical structure and becomes relatively resistant to ribonuclease II, which acts on it as

endonuclease, attacking vulnerable sites that probably represent single-stranded segments linking helical regions.

These data indicate that a full complement of ribosomal proteins is required for the protection of RNA in ribosomes against nuclease action and for holding the RNA in single-stranded conformation.

The finding that pulse-labelled and ribosomal RNA in *E. coli* ribosomes exist as single-stranded chains though the former is extremely sensitive and the latter completely resistant to ribonuclease II poses a question that at our present state of knowledge on ribosomal maturation and on the nature of pulse-labelled RNA cannot be unequivocally answered. There is experimental evidence that an important percentage of renewable (pulse-labelled) RNA moiety is equivalent to ribosomal RNA precursors (Bolton & McCarthy, 1962; Midgley, 1962; Artman, Silman & Engelberg, 1967). There is no need for elaborate experiments to prove that RNA synthesized during any period of exponential growth, no matter how short, should contain ribosomal RNA. Thus cells growing exponentially with a generation time of 75 min. will synthesize, during 45 sec., one-hundredth of the generation time (our experimental conditions), one-hundredth or 1% of its total RNA content (ribosomal and transfer RNA) and about 18% of its total messenger RNA, which also comprises 1% of total cell RNA content (this is based on the currently held view that messenger RNA comprises about 5% of total cell RNA content and has a half-life of about 2 min.). Thus under our experimental conditions ribosomal RNA should comprise about 50% of the total pulse-labelled RNA. In our experiments on the breakdown of pulse-labelled RNA on ribosomes, we have shown that more than 90% of the newly synthesized RNA was broken down to mononucleotides, which indicates that, in contrast with ribosomal RNA in

mature ribosomes, newly synthesized ribosomal RNA is sensitive to ribonuclease II. It would be idle at present to speculate on the causes of the different behaviour of ribonuclease II towards newly synthesized and 'old' ribosomal RNA in *E. coli* ribosomes. This may be due to different methyl content of newly synthesized RNA and ribosomal RNA (Moore, 1966) or to some reactions involved in ribosomal maturation, a process the knowledge of which is still at best rudimentary.

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