Purification and Mechanism of Action of Ribonuclease from Escherichia coli Ribosomes

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The ribosomes from Escherichia coli contain an enzyme that degrades ribonucleic acid (1-3). Its activity appears only after separation of the ribonucleic acid from the protein moiety by means of an agent such as urea.

Although the properties of this enzyme resemble those of pancreatic ribonuclease, its identity has not yet been rigorously established (2). Thus, the study of the mechanism of action of the isolated enzyme seemed of interest as a step in its characterization, and in elucidating the possible reason for its location in the ribosome.

We report here the purification of this enzyme from E. coli ribosomes prepared in this laboratory by the procedure of Tissières et al. (4), together with some of its properties. From the specific activity of the purest preparation, the enzyme in the ribosomes has been estimated on the assumption that its molecular weight is the same as that of pancreatic ribonuclease. The study of its mode of action on ribonucleic acid shows that it is a ribonuclease of the same type as that isolated from rye grass by Shuster (5) and studied by Shuster et al. (6).

In the course of this investigation, an acid phosphatase has been found in the ribosomes. It has been partially purified and some of its properties are reported. This brings to four the number of enzymes found in the ribosomes, as a latent deoxyribonuclease has been found by Elson (2) and a leucine aminopeptidase by Bolton et al. (3).

A preliminary account of this work has appeared (7).

EXPERIMENTAL PROCEDURE

Materials

Ribosomes from E. coli with sedimentation constants of 30 S, 50 S, and 70 S were prepared by Dr. A. Tissières and Mr. D. Schlessinger, following the procedure described (4). RNA from 70 S ribosomes was isolated in this laboratory by Mr. C. Kurland according to his procedure (8); its nucleotide composition has been determined (9). Crystalline pancreatic RNase and o-carboxyphenyl phosphate were obtained from Worthington Biochemical Corporation. Dowex 50-X4 (200 to 400 mesh), and P-glycerophosphate were obtained from Worthington Biochemical Corporation. Domes l-X8 (minus 400 mesh), Los Angeles, California. Prostatic phosphatase and a sample of Crotalus adamanteus venom from Ross Allen's Reptile Institute, Silver Springs, Florida, were kindly supplied by Dr. G-Schmidt. Yeast RNA, purine and pyrimidine bases, and nucleotides were obtained from Schwarz Laboratories, Inc. The yeast RNA was purified further according to the procedure described by Frisch-Niggemeyer and Reddi (10). Cyclic 2',3'-guanosine, adenosine, cytidine, and uridine phosphates were gifts from Dr. L. Heppel. The barium salts were converted to the sodium form by treatment with a small amount of Dowex 50-X4, sodium form. The cyclic adenylic and guanylic acids, which are unstable on storage, were purified before use by paper chromatography in Solvent I (see below). Glucose 6-phosphate, fructose-1,6-diphosphate, AMP, ATP, p-nitrophenyl phosphate, and calcium (di-p-nitrophenyl phosphate), were products from Sigma Chemical Company. Chlorobutanol (β,β,β-trichloro-tert-butyl alcohol), Amberlite CG-50, Type II (200 to 400 mesh) and Type III (400 to 600 mesh), were purchased from Fisher Scientific Company. The Amberlite was treated before use according to Hirs et al. (11). Sodium dodecyl sulfate was obtained from K and K Laboratories.

Methods

The activity of E. coli RNase was determined by a slight modification of the procedure of Anfinsen et al. (12). The reaction mixture, unless otherwise stated, contained: 1 ml of a 1% solution of purified yeast RNA in 0.1 M Tris buffer, pH 8.1; 0.5 ml of the same buffer; and 1 ml of enzyme in H2O. After incubation at 25° for 25 minutes, 0.5 ml of 0.75% uranyl acetate in 25% perchloric acid was added and the precipitate immediately centrifuged; 0.2 ml of the clear supernatant was immediately added to 6 ml of H2O, and the absorbancy of this solution was read at 260 mμ. A unit of E. coli RNase was defined as the amount of enzyme which, under the above conditions, gave an absorbancy of 0.1. The assay was linear up to 0.6 absorbancy. Under these conditions, 0.1 μg of pancreatic RNase gave an absorbancy of 0.215. The specific activity is expressed in units per milligram of protein.

Acid phosphatase was assayed by a modification of the procedure of Brandenberg and Hanson (13). The reaction mixture contained: 2 ml of 0.3 M acetate buffer, pH 5; 0.5 ml of enzyme solution; and 0.5 ml of substrate (o-carboxyphenyl phosphate solution in H2O, 1 mg per ml), added at zero time. Readings were taken at 298 mμ at 1-minute intervals. Alkaline phosphatase activity was measured under the same conditions except that 0.3 M glycine-NaOH buffer, pH 9.1, containing 0.003 M Mg++, was substituted for the acetate buffer. A unit of phosphatase is defined as the amount of enzyme that produces an...
increase in absorbancy at 298 m\(\mu\) of 0.001 in 1 minute at 25\(^\circ\). The activity of phosphatase on substrates other than \(\alpha\)-carboxyphenyl phosphate was determined by estimating the \(P_1\) liberated from the substrate and expressed as micromoles of \(P_1\) per hour at 25\(^\circ\). \(P_1\) in the presence of phosphate esters was estimated by the Fiske-SubbaRow (14) procedure under the conditions described by Bartlett (15). Toluene was added to any reaction mixture with an incubation time longer than 2 hours.

Phosphodiesterase activity was assayed on calcium di-\(p\)-nitrophenyl phosphate according to Frisch-Niggemeyer and Reddi (10). DNase activity was assayed either by the method of Kunitz (16) or by estimating the acid-soluble material liberated from DNA, as described by Shuster (5). Protein was estimated by the biuret method according to Gornall et al. (17) and by the phenol procedure of Lowry et al. (18). RNA was estimated by the orcinol method as described by Dische (19). The concentration of ribosomes in solution was calculated with use of the extinction coefficients given by Tissières et al. (4).

Centrifugations were carried out at 0\(^\circ\) and 2000 \(x\) for 10 minutes, unless otherwise stated. Ultracentrifugal analysis of 50 S and 30 S preparations was performed in the Spinco model E centrifuge. The relative amounts of each kind of ribosome in a single preparation were estimated by analyzing two different dilutions and measuring the areas under the peaks on plates taken with the schlieren optical system.

Paper chromatography was done by the descending technique. The substances were applied to the paper and dried under a cold air blower. The following solvents were used: Solvent I, isopropanol-water, 70:30 (volume for volume) with ammonia in the vapor phase (21); Solvent II, \(n\)-propanol-water, concentrated ammonium hydroxide 60:10:30 (volume for volume) (6); Solvent III, ammonium sulfate saturated in water-isopropanol-0.5 M acetate, 80:2:18 (volume for volume) (21); and Solvent IV, isopropanol-concentrated HCl-water, 170:44:36 (volume for volume) (22).

Paper electrophoresis was performed at low voltage in an E. C. apparatus (E. C. Apparatus Company, New York) and at 0 \(x\) voltage on a cooling plate. The buffers used were 0.05 M ammonium formate, pH 3.5, and 0.05 M sodium phosphate, pH 7.4.

After either electrophoresis or chromatography, the papers were dried at 45\(^\circ\) in an air-circulating oven, the spots viewed under ultraviolet light, and their contours outlined lightly with pencil. They were then excised, together with blanks, eluted with water, and the eluates taken to dryness under vacuum at room temperature.

When both methods were used in combination, paper chromatography was performed before electrophoresis. An aliquot, usually 0.1 ml, was applied as a narrow band 1 inch long at a corner of a sheet of Whatman No. 3 MM filter paper (18 \(x\) 22\(\frac{1}{2}\) in.). Successive applications of the 0.1 ml aliquot were dried under a cold air blower. The sheet was chromatographed allowing the solvent to run along the longer direction of the paper. After development and drying at 45\(^\circ\), the sheet was turned at right angles and wetted cautiously with the buffer used in the subsequent electrophoresis, starting from both ends of the paper. Care was taken that the two fronts of the moistening buffer joined exactly at the region where the spots, developed by chromatography, were located. This was found necessary in order to obtain compact spots after electrophoresis. The sheet was then blotted and subjected to electrophoresis.

**Results**

**Isolation and Purification of E. coli RNase**

Preliminary experiments showed that 70 S ribosomes in 0.005 M Tris buffer, pH 7.4, and 0.01 M Mg\(^{++}\) at 4\(^\circ\) did not, after precipitation of a sample with 0.15 M HClO\(_4\), release any acid-soluble substances absorbing at 260 m\(\mu\) over a period of several days. Ethylenediaminetetraacetic acid (0.01 M), NaCl (0.3 m), or urea (4 m) all initiated the breakdown of the ribosomes at 4\(^\circ\) with release of RNase. However, the rate of degradation was much faster in the urea solutions, about half of the ribosomal RNA appearing as acid-soluble material after 2 hours. The reaction then slowed down and reached completion only after several hours. When the degradation products were continuously removed by dialysis against the same buffer containing urea, the reaction was complete in a much shorter time, indicating that the accumulation of breakdown products inhibited the enzyme. At the end of the reaction, less than 1\% of the RNA was left inside the dialysis sac. The total absorbancy of the dialyzable fragments at 260 m\(\mu\) was 40 to 50\% higher than that of the initial ribosome solution in agreement with the finding of Schlesinger (20). Dialysis of the ribosomes against a buffer containing urea was therefore chosen as the first step of the isolation procedure described below.

When the release of ultraviolet-absorbing material in the dialysate ceased, the reaction mixture was exhaustively dialyzed against water. A large precipitate, which represented about half of the ribosomal protein, appeared inside the dialysis sac. It was removed by centrifugation. The clear supernatant was then fractionated with ammonium sulfate at pH 4.5. The material precipitating between 0.5 and 0.8 saturation was dissolved in water and dialyzed exhaustively; any precipitate that formed at this stage was removed by centrifugation and the supernatant was lyophilized. Additional fractionation by ammonium sulfate achieved no further purification. Apart from RNase, this material showed a considerable amount of phosphotase activity.

The lyophilized material was then chromatographed on Amberlite CG-50 (Type II or III). A systematic study of the distribution coefficient of the RNase between the solution phase and the resin, at different pH values and ionic strengths, was carried out by the test tube equilibration technique described by Hirs et al. (11). Reversible adsorption of the E. coli RNase with a finite distribution coefficient was found in the pH range of 6.4 to 7.8. In 0.2 M phosphate buffer, pH 7.18, at room temperature, the distribution coefficient of E. coli RNase is about 3.

Fig. 1 shows a typical chromatogram of E. coli RNase. The large peak of RNase is preceded by a small one, the position of which is comparable to peak B of the pancreatic enzyme (11). This peak was, however, not observed in some ribosome preparations. After 1 hold-up volume of buffer has passed through...
A typical purification experiment is described in detail below. Table I gives the activity and protein content for each step of the procedure.

**Step I**—A solution of ribosomes, 15 ml, (29.3 mg per ml) in 0.005 M Tris buffer, pH 7.4, and 0.01 M Mg++ were added to an equal volume of the same buffer that also contained 8 M urea and 0.1 M NaCl. The resulting mixture was dialyzed in the cold against three changes of 1 liter of buffer (0.005 M Tris, pH 7.4, 0.05 M NaCl, and 4 M urea), over a period of 24 hours. The solution, which was slightly turbid, was then extensively dialyzed against water (four changes each of 3 liters) in the cold to remove urea. A large precipitate appeared inside the sac and was centrifuged off. The supernatant was brought to pH 4.5 by addition of 5 ml of 0.2 M acetic acid buffer.

**Step II**—The solution was brought to 0.5 saturation with 11.2 g of ammonium sulfate, added in the cold with stirring. After standing for 10 minutes, the precipitate was centrifuged off and the supernatant (40 ml) brought to 0.8 saturation by adding 8.3 g of ammonium sulfate. The suspension was stirred in the cold for 10 minutes and then centrifuged. The supernatant was discarded and the precipitate dissolved in 10 ml of water and dialyzed against three changes of 3 liters of water in the cold. A small precipitate which formed at this stage was removed by centrifugation and the clear supernatant lyophilized.

**Step III**—A column (1 × 30 cm) of Amberlite CG-50 (Type II or III), buffered at pH 7.18 with 0.2 M phosphate buffer, was prepared according to Hirs et al. (11). Since the Amberlite CG-50 is a fine resin, a small plug of glass wool was placed at the bottom of the column resting on the sintered glass disk to prevent clogging of the latter. Several hold-up volumes of buffer were run through at room temperature before use. The lyophilized material from Step II (25 mg) was dissolved in 1.2 ml of 0.2 M phosphate buffer, pH 7.18, any insoluble material removed by centrifugation, aliquots withdrawn for activity and protein estimations, and the remainder loaded on the column.

After adsorption of the sample, the walls of the chromatographic tube were rinsed twice with 0.5 ml of buffer and the column connected with a reservoir containing the same buffer to which a few crystals of chlorobutanol were added to prevent bacterial contamination. Elution was performed at room temperature at the rate of 1 to 1.5 ml per hour, and fractions of 1 ml size were collected automatically. A 4% solution of bovine serum mercaptalbumin in water, 25 μl, was added to each receiving tube, starting from Fraction 30 on. Aliquots of 0.05 to 0.1 ml were

### Table I

<table>
<thead>
<tr>
<th>Purification step</th>
<th>RNase</th>
<th>Protein</th>
<th>% of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomes</td>
<td>450</td>
<td>163</td>
<td>2.8</td>
</tr>
<tr>
<td>Step I: Urea treatment and dialysis</td>
<td>450</td>
<td>90</td>
<td>5.0</td>
</tr>
<tr>
<td>Step II: Ammonium sulfate fractionation</td>
<td>370</td>
<td>25</td>
<td>14.8</td>
</tr>
<tr>
<td>Step III: Chromatography on Amberlite CG-50</td>
<td>290</td>
<td>0.27*</td>
<td>1072</td>
</tr>
</tbody>
</table>

* Estimated in a parallel chromatogram with no bovine serum mercaptalbumin added to the receiving tubes.
Fraction Number

FIG. 2. Chromatography of RNase from E. coli ribosomes on Amberlite CG-50 Type III (400 to 600 mesh). Conditions are the same as in Fig. 1, except that the column was run in the cold (4°C) and eluted with 0.1 M phosphate buffer, pH 7.14, containing 0.12 M NaCl. Phosphatase was not estimated in this chromatogram.

withdrawn from the tubes and assayed for RNase and phosphatase activity. The fractions containing the RNase were pooled, dialyzed in the cold against three changes of 2 liters of water (each change being left 3 hours), lyophilized, and stored at -20°C.

Purity of Enzyme Preparation—The purified E. coli RNase is free of acid and alkaline phosphatase and of DNase. Thus, the DNase reported by Elson (2) to be present in the ribosomes is separated from the RNase during the purification procedure.

The purified E. coli RNase has no activity towards calcium di-p-nitrophenyl phosphate, either at pH 5.1 or at pH 8.5. It does not release P_i from AMP or ATP.

Some Properties of E. coli RNase

Specific Activity—Table I shows that the specific activity of the purified RNase is 1070 units per mg of protein. However, under the conditions of chromatography (see Fig. 1) the RNase peak was still contaminated by some other ribosomal proteins. The enzyme was therefore purified further to estimate its specific activity. It was found that the RNase peak could be considerably retarded on the column and freed from impurities when chromatographed at 4°C on a column of Amberlite CG-50, Type III, buffered at pH 7.14 with a 0.1 M phosphate buffer containing 0.12 M NaCl (μ = 0.25). Under these conditions, the RNase appears in the effluent after about 7 hold-up volumes have passed through the column. The chromatogram is represented in Fig. 2. When the RNase peak appeared, the tubes containing the activity were immediately pooled, the total RNase activity estimated on an aliquot, and the remainder dialyzed rapidly against water to remove most of the phosphate ions, and lyophilized. Estimation of the protein content was carried out on the residue. The specific activity of such a preparation was found to be 2040 units per mg of protein. This is a minimal figure inasmuch as the yield of activity in such a chromatogram as represented in Fig. 2 is only 62% (no protein being added to stabilize the enzyme) as compared to 80% in the chromatogram of Fig. 1.

Optimal pH—The activity of E. coli RNase at different pH values was measured in Tris buffers ranging from pH 7.5 to 8.8, all of which were of constant ionic strength (μ = 0.1). The optimal pH was found to be 8.1; at pH 7.5 and 8.6, the activity is approximately 85% of that at 8.1. When phosphate buffers of the same ionic strength were substituted for Tris buffers, the activity of E. coli RNase was enhanced; at pH 8.1, the activity is 25% higher in phosphate than in Tris. Veronal buffers over the same pH range do not enhance the activity. At pH 5.0, in ammonium acetate buffer, only 8% of the activity assayed at pH 8.1 in Tris was observed.

Heat Stability—This was tested at pH 3.1 (ammonium formate, μ = 0.1) and pH 8.8 (Tris, μ = 0.1). A solution of E. coli RNase in water, 0.5 ml (stabilized by mercaptoalbumin), was added to 0.25 ml of buffer. An aliquot was taken from each solution and activity assayed at pH 8.1. E. coli RNase retained 53% of its activity after heating at pH 3.1, but all activity was lost after heating at pH 8.8. This behavior is similar to that of pancreatic RNase (24).

Stability to pH—A water solution of E. coli RNase (stabilized by mercaptoalbumin), 1 ml, was added to 0.3 ml of the following 0.1 M buffers: ammonium formate, pH 3.9; ammonium acetate, pH 6.2; Tris, pH 8.0; glycine-NaOH, pH 9.8. The solutions were left at 4°C and after 0, 23, 71, and 99 hours, aliquots of 0.25 ml were withdrawn and assayed at pH 8.1. E. coli RNase was found to be stable between pH 3.9 and 8.0, no activity being lost after 4 days. At pH 9.8, 8% of the activity was lost after the same time.

Effect of NaCl, Mg++, and Sodium Dodecyl Sulfate—Various amounts of NaCl, magnesium acetate, and sodium dodecyl sulfate were added to the assay mixture (3 to 4 RNase units) to give the final concentration indicated by the points in Fig. 3.

In the study of Mg++ effects, RNA blanks were run at each Mg++ concentration, inasmuch as their values decrease slightly

![Graph](http://www.jbc.org/)

Fig. 3. Effect of NaCl, Mg++, and sodium dodecyl sulfate (SDS) on the activity of RNase from E. coli ribosomes. A/A_0 = ratio of activity in the presence of the various agents to the activity assayed in their absence, as described in the text. On the abscissa, the concentration is expressed in molarity for NaCl and Mg++ and in percentage (weight per volume) for SDS.)
ions on the activity of pancreatic RNase, Dickman (25) observed with increasing concentrations of this cation. This effect was because of the effect of Mg\(^{++}\) ion concentration on the precipitability of acid by the poly nucleotide mixture, resulting from the action of the enzyme on RNA. This effect was found to be much smaller for E. coli RNase assays in the presence of Mg\(^{++}\) ions and could not account for the inhibition found with this cation. That this effect is larger with the pancreatic than with the E. coli enzyme might be due to the fact that the latter produces largely mononucleotides from RNA. The precipitability of mononucleotides by acid would be less affected by Mg\(^{++}\) ions than that of the poly nucleotide mixture liberated by the pancreatic enzyme. The data shown in Fig. 3 have been corrected for this nonenzymatic effect of Mg\(^{++}\) ions on the assay.

KCl and NaF enhance the activity of E. coli RNase to the same extent as NaCl.

**Mechanism of Action**

**Study of Total Digest of RNA by E. coli RNase**—The reaction mixture contained 6 mg of RNA from 70 S ribosomes, 75 units of E. coli RNase in 0.7 ml of 0.1 m Tris buffer, pH 7.4, and 0.1 m NaCl. The RNA was already in solution when the enzyme was added. The incubation was carried out at 37° for 24 hours in the presence of a few drops of toluene. A control containing RNA alone was incubated under the same conditions. At the end of incubation, the reaction mixture was diluted with 5 volumes of water, the pH adjusted to 8.0 with dilute NaOH, and the resulting solution analyzed on a column of Dowex 1-X8, as described under “Methods.” The chromatogram showed four peaks; three of them were identified as the 3'-isomers of cytidylic, adenylic, and guanylic acids. The fourth was uridylic acid and although, under the chromatographic conditions employed, the two isomers are not resolved from each other, the ratio of absorbance at 260 μm to that at 260 μm measured in each fraction of the peak gave consistently the characteristic value for the 3'-isomer (0.32 compared to 0.28 for the 2'-isomer). The nucleotide composition of the RNA estimated in this way was in close agreement with that obtained from alkaline hydrolysates (9).

These results were confirmed by submitting a total digest of RNA by RNase to paper chromatography in Solvent I and to subsequent electrophoresis at pH 3.5 (2 hours, 30 volts per cm) as described under “Methods.” Only four spots were found; these were identified as the 2'-isomers of adenylic and guanylic acids and as the 2'-isomer, 3'-isomer, or both, of cytidylic and uridylic acids by the procedures described under “Methods.” They were subjected to chromatography in Solvent II and subsequent electrophoresis at pH 3.5 (3 hours, 45 volts per cm). Thirteen spots and in addition a strong band which remained at the origin probably represented some undigested or partially degraded RNA.

**Hydrolysis of Cyclic Nucleotides by E. coli RNase**—The incubation mixtures contained 3 moles of the cyclic nucleotide to be studied in 0.05 ml of water, and 0.25 ml of a solution of E. coli RNase (1.5 units) in 0.12 m Tris buffer, pH 7.4, and 0.06 m NaCl. Controls containing the cyclic nucleotide in 0.3 ml of 0.1 m Tris buffer and 0.05 m NaCl were run in parallel. Toluene was added and the mixtures incubated at 37°. After 18 and 36 hours, aliquots of 0.1 ml were subjected to high voltage electrophoresis that Whatman No. 3 MM paper, (pH 7.4, 2 hours, 35 volts per cm). The spots were excised from the sheet, cut into small pieces, introduced into centrifuge tubes, and 6 ml of 0.1 m HCl added to each tube. After thorough mixing, the tubes were left overnight. They were then centrifuged, and the absorbancy of the clear supernatant read at 260 μm. The results are given in Table II.

**Nucleotide Composition of Dialyzable Products Released During Successive Stages of Digestion**—A solution of RNA from 70 S ribosomes in 0.1 m ammonium bicarbonate (12 mg/0.7 ml) was mixed with 0.2 ml of a solution of E. coli RNase in water (7 units) and dialyzed against 7 ml of 0.1 m ammonium bicarbonate at 25°. Aliquots (0.1 ml) of the dialysate were removed at intervals, diluted to 2.6 ml with 0.1 m ammonium bicarbonate, and their absorbancies read at 260 μm. The outer liquid was replaced by fresh ammonium bicarbonate when its absorbancy reached a value corresponding to the percentage of degradation of RNA shown in Table III. The five dialysates so obtained were lyophilized and the dry residue hydrolyzed in 0.05 ml of 70% HClO₄ at 100° for 1 hour. The base composition of each hydrolysate was estimated by the technique described by Wyatt (22). The results given in Table III indicate that the first fragments released from RNA by the enzyme (Dialysate I, Table III) are rich in adenine and poor in guanine, whereas the part of RNA which is more resistant to E. coli RNase has a high content of guanine. The ratio (A + U) / (G + C) is high at the

<table>
<thead>
<tr>
<th>Cyclic nucleotide</th>
<th>18 hrs</th>
<th>36 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine 2',3'-cyclic-P</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>Guanosine 2',3'-cyclic-P</td>
<td>9.3%</td>
<td>18%</td>
</tr>
<tr>
<td>Cytidine 2',3'-cyclic-P</td>
<td>50.3%</td>
<td>99%</td>
</tr>
<tr>
<td>Uridine 2',3'-cyclic-P</td>
<td>8.9%</td>
<td>17.5%</td>
</tr>
</tbody>
</table>

* The enzyme was prepared according to the procedure outlined in Fig. 2.

1 Alternatively, aliquots were chromatographed in Solvents I and II.
beginning and decreases continuously as the digestion proceeds further.

These results show that *E. coli* RNase cleaves all internucleotide linkages in RNA, giving rise to nucleoside 2',3'-cyclic phosphates. These in turn are converted slowly to the nucleoside 3'-phosphates exclusively, the 6-amino 2',3'-cyclic nucleoside phosphates being cleaved about 5 times faster than the 6-keto.

In the initial stage of RNA digestion, the enzyme releases more adenylc and uridylic than guanylic and cytidylc acids.

**Amount of RNase in *E. coli* Ribosomes**

The RNase activity per 100 mg of 70 S ribosomes was estimated in six different preparations, and the values obtained ranged from 95 to 105 units. Four of the preparations were assayed after Step I of the purification procedure. In the fifth preparation, portions of the 4 M urea digest were assayed before and after removal of the urea by dialysis. The same activity was found in both cases, thus excluding the possibility of inactivation or removal by precipitation of some RNase during the dialysis of the urea digest against water. The last preparation had been purified by centrifugation in a CsCl solution of 1.46 density (27). Under these conditions, the ribosomes alone sedimented to the bottom of the tube, whereas all material of lower densities floated to the top where it formed a solid layer. The RNase content of this preparation was assayed after Step I of the purification procedure.

The specific activity of the purest preparation of *E. coli* RNase was found to be 2050 units per mg of protein. Its molecular weight could not be determined in view of the small amount of enzyme isolated. To calculate the amount of enzyme per mole of ribosomes, its molecular weight was assumed to be the same as that of pancreatic RNase. A value of 0.1 mole of RNase per mole of ribosome (molecular weight, 2.8 x 10^6 (4)) was obtained. Since it is likely that the specific activity of pure *E. coli* RNase is higher than the value reported above it would appear that this figure is a maximum.

If it were assumed that the specific activity and the molecular weight of *E. coli* RNase are both the same as those of the pancreatic enzyme, our value would become 0.01 mole per mole of 70 S ribosome. Bolton et al. (3) have reported a value ranging from 0.1 to 1 mole per mole of ribosomes and one can calculate from the data of Elson (2) a value of about 0.08 mole per mole.

**Is *E. coli* RNase Integral Component of Ribosomes?**

Since there is less than 1 molecule of RNase per ribosome, it is important to determine whether this enzyme is an integral part of the ribosomes in which it occurs, or merely a contaminant. The procedure described by Tissières et al. (4) separates a cell-free extract from *E. coli* into three different fractions: (a) cell debris consisting mostly of pieces of cell walls and cell membranes; (b) crude extract or supernatant after centrifugation at 6000 X g for 15 minutes; and (c) supernatant obtained after centrifuging the crude extract for 2 hours at 100,000 X g to sediment the ribosomes. This last fraction was further submitted to two cycles of centrifugation for 6 hours at 100,000 X g in order to remove completely any ribosomes still present. It was then dialyzed against H_2O and lyophilized. RNase activity was assayed on a portion dissolved in water. Another portion was treated with 4 M urea as described in the purification procedure and its activity assayed after Step I. Both assays were negative. Although the cell debris fraction might still contain some activity, it would appear that all the RNase of a cell-free extract is associated with the ribosomes. Similar results have been obtained by Bolton et al. (3) and Elson (2).

Ribosome preparations purified by centrifugation in CsCl (27) were then investigated. RNase activity of the solid layer and of the ribosome pellet (see above) was assayed both before and after treatment with 4 M urea. No RNase activity could be found in the solid layer, although the assay was sensitive enough to detect less than 0.3% of the activity present in the untreated preparation. All RNase activity was present in a latent form in the purified pellet of ribosomes.

These results, together with the finding of a constant average amount of RNase activity per mg in all the ribosome preparations studied, support the view that *E. coli* RNase is so tightly bound to the ribosomes that it cannot be dissociated without the use of reagents that cause the breakdown of the nucleoprotein.

**Acid Phosphatase in *E. coli* Ribosomes**

During the purification of *E. coli* RNase, phosphatase activity was detected in the material after Step II. The activity belongs

<table>
<thead>
<tr>
<th>Bromides</th>
<th>Final absorbancy*</th>
<th>A</th>
<th>G</th>
<th>C</th>
<th>U</th>
<th>A + U</th>
<th>G + C</th>
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<tr>
<td>I</td>
<td>22.5</td>
<td>22</td>
<td>25</td>
<td>10</td>
<td>24</td>
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<td></td>
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<tr>
<td>II</td>
<td>24.0</td>
<td>26</td>
<td>21</td>
<td>22</td>
<td>22</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>25.0</td>
<td>25</td>
<td>23</td>
<td>19</td>
<td>19</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>26.0</td>
<td>22</td>
<td>36</td>
<td>24</td>
<td>18</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>26.0</td>
<td>21</td>
<td>40</td>
<td>25</td>
<td>14</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Combined dialysates</td>
<td>100.0</td>
<td>26</td>
<td>32</td>
<td>22</td>
<td>20</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Composition of RNA from 70 S ribosome (9) . . .</td>
<td>25.0 ± 0.5</td>
<td>31.5 ± 0.6</td>
<td>22.1 ± 0.6</td>
<td>21.4 ± 0.6</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Final absorbance is 1.4 times the initial absorbancy of the RNA solution.
† A, G, C, U, stand for adenylic, guanylic, cytidylic, and uridylic acid, respectively.
to a protein which is apparently less basic than most of the proteins of the ribosomes, since it is not retained by Amberlite CG-50 in the chromatographic Step III (see Fig. 1). It can thus be separated from the bulk of proteins and freed from RNase. The chromatographic fraction containing the phosphatase activity can be dialyzed and lyophilized to yield a stable preparation.

Preliminary experiments have shown that this phosphatase is active at pH 3 to 5 but inactive at pH 7. It is completely inhibited by 0.01 M sodium fluoride, and phosphate ions have a strong inhibitory effect, about 20% of the activity remaining in 0.007 M phosphate. It cleaves α-carboxyphenyl phosphate, p-nitrophenyl phosphate, β-glycerophosphate, and fructose 1,6-diphosphate in that order of decreasing affinity; it shows low affinity for nucleotide monophosphates.

Since both acid and alkaline phosphatase activities exist in cell suspensions or cell-free extracts (28) it is important to know whether the phosphatase found in the ribosomes is a contaminant or not. Experiments with ribosomes purified by CsCl (similar to the experiments described in the preceding paragraph for RNase) cannot be performed since phosphatase activity is destroyed at the salt concentration used in this procedure. However, when ribosomes purified by centrifugation in 50% sucrose or not. Experiments with ribosomes purified by CsCl (similar to the experiments described in the preceding paragraph for RNase) cannot be performed since phosphatase activity is destroyed at the salt concentration used in this procedure. However, when ribosomes purified by centrifugation in 50% sucrose were used, a procedure yielding results similar to those obtained by the CsCl treatment, all activity was found associated with the pure ribosomes. This suggests that the phosphatase is tightly bound to the ribosomes.

Inasmuch as phosphatase activity can be demonstrated only in the pH range 3 to 6, in which the ribosomes are known to be unstable, it is difficult to determine whether the enzyme is present in a latent form. When ribosomes are assayed for phosphatase activity at pH 5 in 0.05 M acetate, no activity is found during the first 25 minutes of incubation at 25°C. Activity then begins to appear. It may be that phosphatase activity is released only when the ribosomes break down, but further experiments are required to decide whether this enzyme is latent or not.

Location of Ribonuclease and Phosphatase in E. coli Ribosomes

The ribosomes isolated from E. coli are formed of two subunits with sedimentation constants of 50 S and 30 S. The two units combine in the presence of sufficient magnesium to yield a ribosome of sedimentation constant of 70 S (4). Elson and Tal (29) have reported that virtually all the RNase activity is associated with the 30 S component.

Three different preparations of ribosomes, two of 50 S and one of 30 S, were analyzed in the ultracentrifuge as described under “Methods.” These preparations were then treated with urea as described in Step 1 of the purification procedure and assayed for RNase and phosphatase activity. The results are presented in Table IV. The low activity found in the two 50 S preparations can be accounted for by the presence of small amounts of the 30 S component as a contaminant. Indeed, by assuming that in the 70 S ribosome (for which the constant RNase content is given in Table IV, Column 2) the weight ratio of 50 S to 30 S is 1:2.5 (4) and that all RNase activity is present in the 30 S component exclusively, one can calculate what would be the percentage of 30 S in all three preparations. The result of such a calculation is given in Table IV, Column 6. The agreement between the values obtained both by ultracentrifugation and activity estimations is reasonably good and thus confirms Elson’s and Tal’s finding.

In the case of phosphatase, however, the distribution of activity between the two kinds of ribosomes indicates that they both contain the enzyme. The amount of phosphatase per milligram of ribosome is about twice as great in the 30 S as in the 50 S ribosome.

TABLE IV

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>Activity* of ribosomes</th>
<th>50 S and 30 S measured by ultracentrifugation†</th>
<th>30 S calculated from activity measurements (see text)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphatase</td>
<td>RNase</td>
<td>30 S</td>
</tr>
<tr>
<td>50 S</td>
<td>40</td>
<td>88.0</td>
<td>12.0</td>
</tr>
<tr>
<td>60 S</td>
<td>97</td>
<td>84.6</td>
<td>15.4</td>
</tr>
<tr>
<td>30 S</td>
<td>319</td>
<td>7.4</td>
<td>92.6</td>
</tr>
<tr>
<td>70 S</td>
<td>100†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are the mean of four estimations.
† Mean value for six different preparations.
‡ Values are the mean of measurements of two frames of the picture taken with the schlieren optical system.

DISCUSSION

The association of a ribonuclease with a ribonucleoprotein particle isolated from tobacco leaves was first reported by Pirie (30, 31). Attempts to dissociate the enzyme from the nucleoprotein resulted in destruction of the latter. Following the discovery of a latent RNase in E. coli ribonucleoprotein particles by Elson (1), RNase was found in ribosomes from yeast (32), pea seedlings (33), guinea pig pancreas (34), liver (35), and in microsomes from the mouse pancreas (36). In those cases, however, with the exception of E. coli, RNase was also present in other fractions of the cell, such as mitochondria and cell supernatant. It was therefore difficult to demonstrate that the association of RNase with the nucleoprotein was not due to contamination, because this is known to occur (37). The results obtained here indicate that in a crude extract from E. coli, all RNase activity is located in the ribosomes, thus confirming earlier observations (2, 3). Furthermore, in agreement with Elson and Tal (29), we found all the RNase of the ribosomes associated exclusively with the 30 S component. This renders unlikely the possibility that RNase became bound to ribosomes during the isolation procedure.

The significance of this enzyme in the ribosomes is not known. As discussed by Elson (2) RNase might be one of the proteins of the cell synthesized by the ribosomes or it might play some role in ribosome metabolism. In the former case, the ribosomes should also contain other enzymes. Indeed, a leucine aminopeptidase (3) and a latent DNase (2) have been found, and we report here the presence of a fourth enzyme, an acid phosphatase. The fact that there is less than 1 molecule of RNase per ribosome could mean either that only a fraction of the ribosomes synthesize this particular enzyme, or that only a fraction of the enzyme molecules are in a finished, fully active form. In any case, however, on this hypothesis one would expect to find some RNase released from the ribosomes in the cell, but this was not observed. If the alternative explanation were correct, RNase might act in both degradation and synthesis. As a degradative enzyme, E. coli RNase produces mainly cyclic nucleotides and, more slowly, nucleoside 3'-phosphates, both of which are useless for the pool of nucleoside 5'-phosphates. However, the cyclic nucleotides could act as substrates for limited synthesis and transnucleotidation of
oligonucleotides, inasmuch as it has been shown that some RNases (38, 39) catalyze these reactions. That RNase might be concerned with synthesis as well as breakdown of RNA has been recently suggested (40).

Through the study of its mode of action, the enzyme present in the ribosomes from E. coli and responsible for RNA degradation, was identified definitely as an RNase. Its mechanism of action resembles most closely that of the RNase from rye grass (6). Both enzymes cleave all internucleotide linkages in RNA, giving rise to nucleoside 2',3' cyclic phosphates that, in turn, are converted to the nucleoside 3'-phosphates exclusively. However, whereas RNase from rye grass hydrolyzes cyclic adenylic and cyclic uridylic acids at about the same rate, E. coli RNase hydrolyzes cyclic adenylic and cytidylic acids (6-aminonucleotides) 5 times faster than cyclic guanylic and uridylic acids (6-keto nucleotides). It must be pointed out, however, that the results of the action of E. coli RNase on the cyclic nucleotides were observed with an enzyme prepared by the chromatographic procedure outlined in Fig. 2. When the enzyme was purified by the procedure given in Fig. 1, the activity on cyclic cytidylic acid, as compared to that on RNA, was lower. This can be explained in two ways: either there are two enzymes with similar chromatographic behavior, or there is only one enzyme which performs two functions, an inhibitor of one function (41) being present in one preparation but not in the other. It is interesting to note in this connection that the examination of the data available in the literature on the specificity of RNases from very different origins suggests that RNases with an alkaline pH optimum display a narrower specificity than those with an acid pH optimum. If this is true, then the broad specificity of E. coli RNase, the optimal pH of which is alkaline, suggests that there may be some contamination by another enzyme, possibly an RNase of the acid type.

E. coli RNase could be used, as Shuster et al. (6) have demonstrated for the rye grass RNase, as a tool for structural studies of oligonucleotides, inasmuch as both enzymes possess the same specificity. It was used as an hydrolyzing agent for the determination of the nucleotide composition of E. coli soluble RNA (42), as all bonds are broken by the enzyme, whereas the alkaline hydrolysis of RNA always leaves a small fraction of resistant oligonucleotides (43). Furthermore, as RNA hydrolysis by E. coli RNase is carried out in dilute buffer at a neutral pH, there is no need of removing excess ions, such as potassium in KOH hydrolysates, and the digest, after suitable dilution, can be immediately loaded on an anion exchanger for chromatographic analysis.

Although the properties of E. coli RNase considerably resemble those of pancreatic RNase, for instance, pH optimum, heat stability at acid pH, heat lability at alkaline pH, and salt effects on activity, its chromatographic behavior is quite different. In 0.2 M phosphate buffer, pH 6.47, the distribution coefficient of E. coli RNase between resin phase and solution is about 100 as compared to 3 for the pancreatic enzyme; hence, the former is a more basic protein. Another difference was found in the effect of magnesium, which is an activator of pancreatic RNase (25) but an inhibitor of E. coli RNase.

The phosphatase found in the ribosomes from E. coli is of the acid type. No alkaline phosphatase activity could be detected but it must be pointed out that in E. coli grown on normal medium there is much less alkaline than acid phosphatase (28); if this proportion is maintained in the ribosome, such a low activity would not have been detected.

**Summary**

An enzyme responsible for ribonucleic acid degradation, which has been reported to exist in a latent form in Escherichia coli ribosomes, has been purified and identified as a ribonuclease by its mode of action.

After a 730-fold purification, the specific activity per milligram of protein is about 10 times lower than that of pancreatic ribonuclease. The preparation is free of phosphatase and deoxyribonuclease. It shows maximal activity at pH 8.1 and retains half of its activity when heated 10 minutes at 100° at pH 3.1. It is stable in the cold between pH 4 and 8; its activity is enhanced by sodium chloride, potassium chloride, and sodium fluoride, but inhibited by magnesium salts and by sodium dodecyl sulfate. From its chromatographic behavior, the molecule appears to be more basic than pancreatic ribonuclease.

E. coli ribonuclease cleaves all internucleotide bonds in ribonucleic acid, giving rise to nucleoside 2',3' cyclic phosphates, which in turn are more slowly hydrolyzed to the corresponding nucleoside 3'-phosphates exclusively. The enzyme hydrolyzes the cyclic adenylic and cytidylic acids (6-aminonucleotides) about 5 times faster than the cyclic guanylic and uridylic acids (6-keto nucleotides). In the initial stages of ribonucleic acid digestion the enzyme releases more adenylic and uridylic acids than guanylic and cytidylic acids.

The ribonuclease activity of E. coli appears to be associated exclusively with the ribosomes, with the possible exception of the cell wall and the cell membrane, which were not examined. The results confirm the finding that ribonuclease is present only in the ribosomes that sediment at 30 S (30 S ribosomes).

The ribonuclease content of the ribosomes, based on the specific activity of the purest preparation, is 0.1 molecule per ribosome; in this calculation the molecular weight of the enzyme is taken to be the same as that of pancreatic ribonuclease. If the specific activity of E. coli ribonuclease were comparable to that of the pancreatic enzyme, this figure would become 0.01 molecule per ribosome.

An acid phosphatase is also present in the ribosomes. It has been separated from the ribonuclease and some of its properties are reported. The 30 S ribosomes contain about twice as much phosphatase as the 50 S ribosomes.

**Acknowledgments**—We wish to express our appreciation to Dr. J. T. Bolton for his constant advice and interest, to Dr. A. Tissières and J. D. Watson for discussions and for reading the manuscript, and to Dr. A. Tissières and Mr. D. Schlessinger for supplying us with the ribosome preparations.

**REFERENCES**

Purification and Mechanism of Action of Ribonuclease from *Escherichia coli* Ribosomes

P. F. Spahr and B. R. Hollingworth