Partial Purification and Properties of a Ribonuclease from Rabbit Reticulocytes*

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SUMMARY

A ribonuclease has been partially purified from the soluble fraction of rabbit reticulocytes. The enzyme degraded RNA predominantly to oligonucleotides with an average chain length of six. It degraded polyctidylic acid and polyuridylic acid but not polyadenylic acid, polyguanylic acid, or polyinosinic acid.

When natural RNA was the substrate, the oligonucleotide product had mainly uridine, guanosine, and some cytidine residues at the 3' end. The 3' terminus had a cyclic 2',3'-phosphodiester bond.

The enzyme was capable of degrading polyribosomes. Hemin inhibited reticulocyte RNase. Hemin did not inhibit pancreatic RNase or RNase T1, but did inhibit pea leaf RNase. Both reticulocytes and pea leaves contain metalloporphyrin. An hypothesis is presented for the role of reticulocyte RNase in control of protein synthesis in these cells.

EXPERIMENTAL PROCEDURE

Preparation of Erythroid Cells

Reticulocytosis was induced in rabbits by daily injection of phenylhydrazine, 7.0 mg per kg of body weight, for 4 days. On the 7th day the animals were bled by cardiac puncture. The blood was heparinized and centrifuged at 500 x g for 10 min to collect the cells.

As assay of RNase

One unit of reticulocyte RNase activity was defined as the amount of enzyme which under the conditions described below rendered an increased optical density of 0.1 unit per hour in the perchloric acid supernatant fluid. The reaction mixture contained 1.0 mg of *Escherichia coli* tRNA, 100 μmoles of sodium phosphate buffer, pH 6.5, and 3 to 15 units of the RNase in a final volume of 1.8 ml. Suitable controls, without added RNase,

in reticulocytes. There have been reports which concluded that reticulocyte ribosomes had virtually no RNase activity (4-6). On the other hand, preliminary studies in this laboratory (7) indicated that reticulocyte ribosomes and supernatant fractions did contain RNase. Adachi et al. (8) reported on studies of a RNase purified from rabbit blood cells which were lysed by freezing, thawing and disruption in a French press, a method that disrupts leukocytes, platelets, and erythrocytes.

In this paper a method for obtaining an approximate 2100-fold purification of a RNase from rabbit reticulocyte soluble fraction which is free of acid and alkaline phosphatase and deoxyribonuclease is reported. This enzyme is an endonuclease which produces predominantly oligonucleotides (of an average size of six nucleotides) with 2',3'-cyclic phosphodiester bonds at the 3' end, is inhibited by relatively low concentrations of Mg2+ and K+, and is able to degrade ribosomal RNA and tRNA to acid-soluble fragments. The acid-soluble product contained uridine, guanosine, and cytidine but no adenosine residues at the 3' terminus.


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were performed for every experiment to establish that the reagents were not contaminated by RNase. Incubation was at 37° for 2 hours. In experiments in which long time incubations were employed (greater than 2 hours), toluene or chloroform was added to the reaction mixture to prevent microbial growth. The reaction was stopped by addition of 0.50 ml of the reaction mixture to 0.70 ml of ice cold 1.0 M perchloric acid. The resulting suspension, after mixing, was kept at 0° for 10 min and then centrifuged at 2000 × g for 10 min. The optical density at 260 nm of the supernatant fluid was determined. It has been shown that oligonucleotides which are normally acid-soluble can be co-precipitated by RNA (9). This would result in a negative error in the RNase assay. Therefore, in experiments in which the substrate concentration was varied, additional RNA was added just prior to the addition of perchloric acid so that each tube had the same concentration of RNA. In these experiments where the effects of inhibitors were studied, e.g., hemin, the inhibitor was always added to tubes incubated in the absence of the inhibitor to correct for the effects on the solubility of the oligonucleotides formed by the enzyme. These effects were less than 5%. In experiments in which poly C degradation was studied, 280 nm was the wave length employed. When poly I or poly G was studied, 250 nm was employed. Poly U degradation could not be assayed by measuring the formation of acid-soluble fragments since poly U is itself acid-soluble. Alcohol was used to remove un degraded poly U (10). The product of digestion of poly U, poly A, and poly G was also measured by sucrose density centrifugation (11). Protein was determined by the method of Lowry et al. (12).

**Solvent Systems for Chromatography**

The solvents used for paper chromatography had the following compositions: Solvent I, 95% ethanol-1 M ammonium acetate, 60:15 (10); Solvent II, 95% ethanol-1 M ammonium acetate, 70:30; Solvent III, isopropyl alcohol-concentrated HCl (180:42) brought to 250 ml with water (13).

**Degradation of Cyclic Mononucleotides**

The activity of the reticulocyte RNase preparations in degrading nucleoside 2',3'-cyclic phosphates was assayed as follows. The reaction mixture contained 40 μmoles of Tris HCl buffer, pH 7.4, 2.0 units of reticulocyte RNase, and 0.26 μmole of nucleoside 2',3'-cyclic phosphate in a final volume of 0.45 ml. Incubation was at 37°. At 30-min intervals for up to 4 hours, aliquots were removed from the reaction mixture. The formation of noncyclc nucleoside phosphates was assayed by two methods. (a) Aliquots (0.05 ml each) of the reaction mixture were added to a solution containing 50 μg of E. coli, alkaline phosphomonoesterase (chromatographically purified), and 20 μmoles of glycine buffer, pH 9.5, in a final volume of 0.2 ml. This reaction mixture was incubated at 37° for 1 hour. The reaction was terminated by addition of 0.1 ml of cold 20% trichloroacetic acid. After centrifugation, the supernatant fluid was assayed for inorganic phosphate by the method of Ames and Dubin (14) and (b) 0.01 ml aliquots of the reaction mixture were applied to Whatman No. 1 paper and the chromatograms were developed with Solvent I in a descending system. Under these conditions, nucleoside 2',3'-cyclic phosphates have \( R_f \) values of 0.85 while nucleosides bearing a phosphononoester group have \( R_f \) values of 0.50.

**Analysis of Product of Reticulocyte RNase Activity**

The acid-soluble products of the reticulocyte RNase were characterized in the following manner.

**Experiment Showing That Reticulocyte RNase Forms a Product Phosphorylated at the 3' End**—Undegraded polynucleotide was removed as described above with perchloric acid. The supernatant was neutralized with KOH, maintained at 0° for 30 min, and the precipitated KClO₄ was removed by centrifugation at 0°. The supernatant solution was recovered, concentrated by lyophilization, and then digested with 0.2 n KOH for 18 hours at 37°. The solution was then neutralized with Dowex 50 (H⁺). Paper chromatography to separate nucleosides, nucleotides, and nucleoside diphosphate was carried out in Solvent I. The chromatograms were analyzed by elution of areas containing ultraviolet-absorbing material. In experiments where radioactive polynucleotides were used as substrates, the paper was cut into 1-cm strips, introduced into vials, and a solution containing 5.0 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis(4-methyl-5-phenyloxazolyl)benzene in 1 liter of toluene was added. The samples were counted in a liquid scintillation spectrometer.

**Experiments Showing That Oligonucleotides with Cyclic Phosphodiester Termini Were Formed**—RNA was degraded by the enzyme as described above in 0.14 M ammonium acetate, pH 6.5, for 105 min. In order to hydrolyze the diester bond of nucleoside 2',3'-cyclic phosphate, the pH of the solution was adjusted to 1.0 with 0.45 M perchloric acid and the solution was maintained at 4° for 1 hour. Exposure to pH 1 brought about complete hydrolysis of 2',3'-cyclic UMP. The solution was then adjusted to pH 8.5 with 3 N NH₄OH and alkaline phosphatase was added to give 75 μg per ml. The solution was incubated for 20 min at 37° and assayed for formation of inorganic phosphate.

**Average Chain Length of Product**—An aliquot of the perchloric acid-soluble material was neutralized with KOH and treated with alkaline phosphatase. Another aliquot of the neutralized product was ashed. The ratio of total ashed phosphate to phosphatase-labile (terminal) phosphate was determined. Phosphatase determination and ashing were performed according to the method of Ames and Dubin (14).

**Identification of Nucleotide at 5' Terminus**—The neutralized perchloric acid supernatant was concentrated at 37° in a rotary flash evaporator. Then 0.2 mg of product were incubated at 37° for 15 min with 2.0 mmoles of ammonium acetate, pH 8.5, and 600 μg of alkaline phosphatase in a volume of 30 ml. It was previously shown that the KClO₄ remaining in the product did not interfere with the reaction. The phosphatase reaction was monitored by removing aliquots and assaying for inorganic phosphate. Alkaline phosphatase hydrolyzes phosphonomonoester groups attached to long polynucleotides (15).

The reaction mixture was immersed in a 100° water bath for 4 min and cooled in an ice bath. It was then adjusted to 0.3 N KOH and maintained at 37° for 18 hours. The hydrolysate was neutralized with Dowex 50-H⁺. The resin was removed by filtration and washed with several volumes of 3.0 N ammonium hydroxide and the filtrates were combined. The pooled filtrates were taken to dryness at 37°, redissolved in a minimal volume, and spotted on Whatman No. 3MM filter paper and subjected to high voltage electrophoresis as described by Ingram and Sjoquist (16). The areas containing ultraviolet absorption in the nucleoside region were eluted with water and analyzed by descending chromatography in Solvent II and in Solvent III to separate...
and identify the nucleosides. The spectra of the nucleosides were determined with a Zeiss PMQ II spectrophotometer and the spectral data were compared with the spectra published by Pabst laboratories.\(^6\)

**Materials**

Poly U, poly C, poly A, poly G, and poly I were purchased from Miles Chemical Company. The values of the sedimentation coefficients as provided by the manufacturer were 7.2 for poly U, 6.1 for poly C, 8.3 for poly A, and 11.8 for poly G. E. coli RNA was obtained from General Biochemicals. \(^32\)P-Labeled DNA (1.2 \(\times\) 10\(^{6}\) cpmp per pmole of nucleotide) was a gift of Dr. Jerard Hurwitz. Poly AC (1:1) and poly AU (5:1) were gifts of Dr. A. Wahba. E. coli alkaline phosphatase was obtained from Worthington Biochemical Corporation and contained 2200 units per ml. \(^32\)P-Poly C had a specific activity of 5.7 \(\times\) 10\(^{6}\) cpmp per mg. RNase T\(_1\) was obtained from Calbiochem. Pea leaf RNase, prepared according to the method of Holden and Pirie (17), was a gift of Dr. Paul W. Wigler.

**Preparation of Ribosomal RNA**

Rabbit liver ribosomal RNA was prepared by the following modification of the method of Kirby (18). The liver was removed and immediately frozen in Dry Ice to the consistency of hard butter. It was then added to 5 volumes of 0.1 M acetate buffer, pH 5.0, 0.1 M NaCl, 0.01 M EDTA (Solution I). Two volumes of water-saturated phenol and bentonite were added. The concentration of bentonite was 0.5\%. The mixture was homogenized for 1 min in a Waring Blender. The aqueous layer was collected and extracted twice with 3 volumes of diethyl ether. Two volumes of 95\% ethanol were added and the suspension was stored for 2 hours at 15\%. The RNA was collected by centrifugation and dissolved in a minimal volume of Solution I. The precipitation with ethanol was repeated. The DNA was further purified by sedimentation through a 5 to 20% sucrose density gradient prepared in Solution I. Then 22 mg of DNA in 2 ml were layered per gradient. Sedimentation was performed in a Spinco SW 25.2 rotor at 25,000 rpm for 12 hours at 4\%. The fractions of the gradients containing 23 S and 18 S RN, were pooled and the RNA was precipitated by addition of 2 volumes of 95\% ethanol. The precipitated RNA was dissolved in water and stored frozen at -15\%.

Reticulocyte RNA (19) was prepared from sedimented ribosomes of the initial 105,000 \(\times\) g centrifugation of the reticulocyte supernatant described below. The sedimented ribosome pellet, washed twice with a solution of 1.0 M Tris HCl buffer, pH 7.4, and 1.5 mM MgCl\(_2\), was resuspended in a small volume of 1.0 M Tris HCl buffer, pH 7.4. This ribosome suspension was extracted with 2 volumes of water-saturated phenol for 5 min at 4\%. The aqueous phase was recovered and 1.0 M NaCl was added to a final concentration of 0.1 M NaCl. The RNA was precipitated with 2 volumes of 95\% ethanol at -15\%. The precipitated RNA was dissolved in water and stored frozen at -15\%.

*E. coli* ribosomal RNA, prepared according to the method of Monier et al. (20), was further purified on a sucrose density gradient as described above. For the isolation of uniformly labeled \(^14\)C-RNA. *E. coli* B cells were grown in 300 ml of minimal medium (21) supplemented with 1 mg of glucose per ml and 0.5 mC of uniformly labeled \(\alpha\)-glucose-\(^14\)C. The bacteria were grown under forced aeration at 37\% with three 10 mM sodium hydroxide traps attached in series to the outlet tube. At the end of logarithmic growth the cells were poured onto crushed ice, harvested, washed three times with 0.01 M Tris HCl, pH 7.8, and the total RNA was extracted (20).

**RESULTS**

**Purification of Reticulocyte Ribonuclease**

All of the procedures described below were carried out at 4\% unless otherwise noted.

**Preparation of Reticulocyte Supernatant Solution and Ribosomes**

**Fractions (Stage A)**—The erythrocyte cells were washed five times with 5 volumes of 0.15 M NaCl. Repeated washing of the cells was found necessary to remove plasma which contained RNase activity. In a typical preparation the plasma contained 1510 units per ml of ribonuclease activity, the second wash, 167 units per ml, the third wash 56 units per ml, the fourth wash 2 units per ml, and the fifth wash had no detectable ribonuclease activity. The final wash solution was always assayed to be certain that it contained no RNase activity.

The washed, packed cells were lysed by shock by a short exposure to a hypotonic solution (19). Four volumes of 1 mM Tris HCl buffer, pH 7.4 and 1.5 mM MgCl\(_2\) were added to the packed cells. The suspension was gently shaken for 1 min, and after this time 0.1 volume of 10% NaCl was added. This method has been shown to lyse only erythroid cells (22). The unlysed cells and stroma were removed by centrifugation at 25,000 \(\times\) g for 20 min. The sedimented material was discarded and the supernatant fluid recovered and centrifuged at 105,000 \(\times\) g for 2 hours. This sedimented pellet contained ribosomes which were used for the preparation of RNA. The supernatant fluid was recovered and again centrifuged for 2 hours at 105,000 \(\times\) g. A second, smaller pellet was obtained which was discarded and the supernatant fraction was recovered. This fraction is designated as Stage A. Stage A preparation had a specific activity of 0.065 unit per mg of protein and was taken as 100\% for purposes of calculation of yield of subsequent stages in the purification procedure. The specific activity and yield of the enzyme preparation at each stage in the purification is illustrated in Table I.

**Ammonium Sulfate Precipitation (Stage B)**—Stage A was brought to 40\% of saturation by addition of a saturated solution

<table>
<thead>
<tr>
<th>Stage and procedure</th>
<th>Total RNase</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Hemolysate, 105,000 (\times) g supernatant ...</td>
<td>2,100</td>
<td>54.0</td>
<td>3.5</td>
<td>0.065</td>
</tr>
<tr>
<td>B. 40 to 60% ammonium sulfate ...</td>
<td>1,045</td>
<td>214</td>
<td>52.3</td>
<td>2.2</td>
</tr>
<tr>
<td>C. Acid and heat precipitate ...</td>
<td>8,130</td>
<td>0.61</td>
<td>214</td>
<td>351</td>
</tr>
<tr>
<td>D. Ca(_3)(PO(_4))(_2) gel ...</td>
<td>2,220</td>
<td>0.11</td>
<td>117</td>
<td>1,060</td>
</tr>
</tbody>
</table>

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\(^6\)Pabst Laboratories Circular OR-17, Pabst Laboratories, Milwaukee, Wisconsin.
of ammonium sulfate adjusted to pH 7.5 with solid Tris. The resulting suspension was stirred mechanically for 1 hour. The precipitate was sedimented by centrifugation at 10,000 x g and discarded. The supernatant fluid was recovered and adjusted to 50% of saturation by addition of the saturated solution of ammonium sulfate. This suspension was stirred for 1 hour; the precipitate was recovered by centrifugation and dissolved in a minimum amount of 0.01 M Tris buffer, pH 7.8. This solution was dialyzed against 200 volumes of 0.01 M Tris buffer, pH 7.8, for 12 hours with two changes of the dialyzing fluid. This solution designated as Stage B was assayed after dialysis (Table I).

**Combine Acid Heat Treatment (Stage C)—** The pH of the dialysate was adjusted to 3.0 with 5% acetic acid. The solution was placed in a water bath and maintained at 85° for 7 min and then rapidly cooled in an ice bath. The pH of the solution was re-adjusted to 7.4 by addition of 3 M Tris, centrifuged at 10,000 x g to remove the precipitate, and the clear supernatant fluid was recovered. The precipitate was washed with 0.01 M Tris HCl buffer, pH 7.8, and the wash fluid was combined with the first supernatant fluid. The solution was dialyzed against 200 volumes of 0.01 M Tris HCl buffer, pH 7.8, for 12 hours with two changes. Stage C was assayed after dialysis (Table I).

**Adsorption and Elution from Ca₃(PO₄)₂ Gel (Stage D)—** Ca₃(PO₄)₂ gel was prepared according to the method of Keilin and Hartree (23). The enzyme preparation from (Step C) was equilibrated with 5 mM phosphate buffer, pH 6.2, by passing it through a column of Sephadex G-25 which had been equilibrated with this buffer. Then 16 mg of Ca₃(PO₄)₂ were added per 1.0 mg of protein. The Ca₃(PO₄)₂ gel was added from an aqueous suspension that had 28 mg of gel per ml. The suspension was stirred mechanically for 5 min. The gel was then collected by centrifugation at 10,000 X g for 10 min. The gel with the adsorbed RNase was washed with 0.04 M phosphate buffer, pH 6.5. The volume of buffer used was equal to twice the volume of the enzyme solution. The gel was collected by centrifugation and the supernatant fluid, which contained up to 10% of the total RNase activity, was discarded. The gel was washed two more times with 0.4 M phosphate buffer, pH 6.5. These clutes containing most of the activity were pooled. The enzyme at Stage D (Table I) was unstable (see below) and all experiments unless otherwise specified were performed with Stage C. Attempts to further purify Stage C or Stage D by chromatography on DEAE-cellulose or carboxymethyl cellulose led to loss of activity.

**Properties of Enzyme**

**Effect of pH—** The activity of reticulocyte RNase at different pH values was measured in 0.09 M sodium phosphate buffer (pH 5.8 to 8.0), 0.09 M acetate buffer (pH 3.0 to 5.8), and 0.09 M Tris HCl buffer (pH 7.2 to 8.8). The substrate for these assays was *Escherichia coli* tRNA, 0.55 mg per ml of assay mixture. The optimal pH was at pH 6.5. At pH 5.8 in phosphate buffer the enzyme was 76% as active as at the optimum. In acetate buffer, it was 22% as active at pH 5.8 and inactive at 5.0. At pH 7.0 and 7.8 the enzyme was 59% and 13% as active as at pH 6.5. Similar results were obtained when the buffer concentrations were 0.2 M.

**Effect of Ions—** Reticulocyte ribonuclease did not require the presence of either potassium or magnesium ions for its activity. When the purified enzyme was dialyzed against 0.01 M EDTA for 16 hours, no requirement for any cations could be demonstrated. Mg²⁺ was inhibitory at 1.1 mM and completely in-
TABLE II
Inhibitors and activators

The reaction mixture contained 160 pmol of Tris HCl buffer, pH 7.4. Otherwise the conditions were as described in the text. The figures given in the table are relative to the amount of perchloric acid-soluble material formed by the enzyme in the absence of inhibitor or activator. In each experiment, appropriate controls were run to make certain that the substance tested was not contaminated with nuclease activity.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Additions</th>
<th>Final molarity in assay</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CoSO₄</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>CoSO₄</td>
<td>0.5</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>CaSO₄</td>
<td>0.5</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>NiSO₄</td>
<td>0.5</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td>CaCl₂</td>
<td>0.5</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>MnCl₂</td>
<td>0.5</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>FeSO₄</td>
<td>0.5</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>50</td>
<td>28.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Activators</th>
<th>Additions</th>
<th>Final molarity in assay</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mercaptoethanol</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Mercaptoethanol</td>
<td>10</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Mercaptoethanol</td>
<td>100</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>p-Hydroxymercuribenzoate</td>
<td>0.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Fig. 3. Dependence of rate of degradation of RNA on enzyme concentration. The assay was described in the text. The amount of protein (Stage C) added to assay is indicated on the figure.

amount of enzyme added in the range of 6.1 to 18.3 μg of protein, which was the range employed in the assay for the activity of the reticulocyte RNase (Fig. 3). The rate did not remain linear, however, but decreased after 150 min. A concentration of 0.72 mg per ml, saturated the reticulocyte RNase when Stage D was used at a protein concentration of 15 μg per ml (Fig. 4).

Activity of Reticulocyte RNase with Different Polynucleotides—The reticulocyte ribonuclease was tested with RNA of several different types. The enzyme degraded all the natural RNA preparations assayed, which included reticulocyte and liver ribosomal RNA and E. coli tRNA (Table III). Of the RNA preparations tested, the reticulocyte RNase formed acid-soluble material more rapidly from E. coli tRNA than from ribosomal RNA. The RNA was present in a concentration that saturated the enzyme.

The activity of the enzyme was tested with synthetic ribonucleotide homopolymers (Table III). Poly C was readily degraded but the enzyme had essentially no effect on poly A, poly I, or poly G. Neither E. coli DNA or calf thymus DNA were attacked by the enzyme. Poly U degradation could not be measured by our standard assay since poly U is itself acid-soluble. Ethanol rather than perchloric acid was utilized to determine if poly U was attacked by reticulocyte RNase (10, 24). The nuclease did not degrade poly U to alcohol-soluble fragments. The lack of activity of the reticulocyte RNase with poly A or poly U was in contrast to the susceptibility of the synthetic copolymers, poly AU (5:1) and A:C, to degradation by the enzymes. The relative rates of degradation of the copolymers presented in Table III is taken as a qualitative indication of the susceptibility of these substrates to the action of the reticulocyte RNase, but not as a quantitative estimate of the extent of breakdown since the total nucleotidic material after alkaline hydrolysis of the acid-soluble products was not determined.

![Fig. 4](http://www.jbc.org/) Dependence of rate of degradation of RNA on substrate concentration. The rate was determined at different concentrations of tRNA. The conditions for the reaction are described in the text.

TABLE III
Reticulocyte RNase activity with different polynucleotides

The assay procedure is described in the text (see “Experimental Procedure”). E. coli tRNA is given the arbitrary value at 1.00 and the rate at which the other polynucleotides were degraded is indicated relative to that of E. coli tRNA.

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>Relative rate of degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli tRNA</td>
<td>1.00</td>
</tr>
<tr>
<td>Rabbit liver ribosomal RNA</td>
<td>0.59</td>
</tr>
<tr>
<td>Rabbit reticulocyte ribosomal RNA</td>
<td>0.59</td>
</tr>
<tr>
<td>Poly A</td>
<td>0.02</td>
</tr>
<tr>
<td>Poly C</td>
<td>0.59</td>
</tr>
<tr>
<td>Poly G</td>
<td>0</td>
</tr>
<tr>
<td>Poly I</td>
<td>0</td>
</tr>
<tr>
<td>Poly AC (1:1)</td>
<td>3.50</td>
</tr>
<tr>
<td>Poly AU (5:1)</td>
<td>1.11</td>
</tr>
<tr>
<td>DNA</td>
<td>0</td>
</tr>
</tbody>
</table>
The above results suggested that poly U was being attacked but the products were oligonucleotides that were so large that they were precipitated by ethanol. The susceptibility of poly U to the reticulocyte RNase was tested by determining the change in rate of sedimentation of poly U during the course of degradation (Fig. 5). The size of the poly U was decreased by the enzyme and the degree of degradation was dependent on the amount of enzyme added. The ability of the enzyme to degrade poly A and poly G was also examined by analysis of the polynucleotides on sucrose density gradients. The sedimentation rate of these polynucleotides was not altered by reticulocyte RNase.

Activity with Cyclic Phosphodiesters and Phosphomonoesters - The activity of reticulocyte RNase with cytidine 2',3'-cyclic phosphate, uridine 2',3'-cyclic phosphate, guanosine 2',3'-cyclic phosphate, and adenosine 3',5'-cyclic phosphate was assayed. No nucleosides bearing a phosphomonoester product could be detected with any of these substrates when the reaction mixture was analyzed by paper chromatography in Solvent I. The more sensitive assay, which measures the extent of hydrolysis of the cyclic phosphodiesters by reacting the product with alkaline phosphatase and measuring the inorganic phosphate formed, revealed that only 0.006 μmole of CMP, 0.001 μmole of UMP, and 0.01 μmole of GMP were formed in 10 hours by 2.0 units of the enzyme. This represented less than 2% of the amount of cyclic nucleotide available.

The reticulocyte RNase preparations at Stage C and at Stage D were assayed for activity in dephosphorylating 2',(3')-AMP, CMP, and UMP. No phosphomonoesterase activity (25) could be detected with either of these enzyme preparations with any of these substrates in 0.04 M sodium maleate, pH 6.5, 0.04 M Tris, pH 7.4, or 0.04 M Tris HCl, pH 8.1.

Characterization of Product of Reticulocyte RNase Activity, Terminal Phosphate—In a typical experiment, 2.9 mg of liver ribosomal RNA were incubated with 28 units of the enzyme in 8.8 ml of phosphate buffer for 8 hours at 37°C. The reaction was stopped by addition of perchloric acid. Of the added RNA, 38% was degraded to acid-soluble material. The acid-soluble products were degraded to mononucleotides by alkaline hydrolysis. By this procedure, oligonucleotides phosphorylated at the 3' end yield 2',(3')-mononucleotides exclusively, 5'-phosphate ended oligonucleotides yield mostly 2',(3')-mononucleotides, and 1 mole each of nucleoside and nucleoside 2',(3') 5'-diphosphate per mole of oligonucleotide. The alkaline hydrolysis of the reticulocyte RNase product yielded only 2',(3')-mononucleotides indicating that the product retained the phosphate at the 3' end. 3',5'-Nucleoside diphosphates and the nucleosides would have been detected by this procedure had they been present at about 15% of the total nucleotide residues.

To further confirm that the product of reticulocyte RNase retained phosphate at the 3' end, poly C labeled with 14C was utilized as substrate. The entire reaction mixture was applied to chromatography paper. Examination of the chromatogram after 20 hours of development in Solvent I revealed that some of the product was small enough to migrate in this solvent. The product formed a band stretching 4 cm from the origin. In a control tube which did not contain enzyme, all of the ultraviolet-absorbing material and all of the radioactivity remained at the origin. The degraded material that had migrated at least 1 cm from the origin was eluted, concentrated by lyophilization, and degraded to mononucleotides by alkaline hydrolysis. The alkali digest was examined by paper chromatography after neutralization. 2',
Formation of phosphomonooesterase-labile phosphate at pH 1.0 from reticulocyte RNase product

| Reticulocyte RNase followed by pH 1.0 | 0.074 |
| Reticulocyte RNase followed by pH 6.5 | 0.005 |
|Minus reticulocyte RNase, pH 6.5         | 0.004 |
|Minus reticulocyte RNase, pH 1.0         | 0.008 |

Identity of Bases at 3'-Phosphorylated Term. of Product—

These experiments were performed with total E. coli RNA and rabbit reticulocyte RNA in separate experiments. The digestion of the polynucleotides was carried out as described above in 0.08 m ammonium acetate, pH 6.5. The incubation mixture was assayed and when 20% of the RNA was acid-soluble the reaction was stopped by addition of cold perchloric acid. The identity of the nucleotides at the phosphorylated terminus was determined by reacting the acid-soluble product with alkaline phosphatase followed by alkaline hydrolysis. This procedure gives a mixture of nucleoside 2', 3'-monophosphates (all nucleotides other than phosphate terminal) and nucleosides (phosphate terminal). After removal of the nucleotides the product was analyzed by paper electrophoresis.

The only region of the electropherogram that had ultraviolet-absorbing material was that occupied by guanosine and uridine. Since the conditions used resolves these nucleosides poorly (16), they were eluted and analyzed by paper chromatography in Solvents II and III. These systems resolve guanosine and uridine. The spots were eluted from the chromatograms and guanosine and uridine were identified by their ultraviolet spectra. The same experiment was carried out with 3H-labeled total E. coli RNA. In this experiment some cytidine was detected amounting to 9% of the material present at the 3' terminus of the perchloric acid-soluble oligonucleotides. In one experiment with tRNA, the addition of perchloric acid was eliminated and the digest was spotted onto filter paper and analyzed in a two dimensional system (16). A substance having the mobility and spectral characteristics of 3',5'-GDP was obtained.

Effect of Reticulocyte RNase on Polyribosomes—

During the course of maturation of reticulocytes, the number of ribosomes present as aggregates relative to the total ribosome content decreases (2, 26). In order to test the effect of the reticulocyte RNase on these particles, a ribosome suspension was layered onto a sucrose density gradient (Fig. S1). After incubation for 60 min with 12 μg of RNase, there were no detectable polyribosomes (Fig. S2). Incubation of the ribosome suspension without addition of exogenous RNase was associated with some degradation of polyribosomes. This observation is consistent with previous reports that there is RNase activity in the reticulocyte ribosomes fraction (4, 5, 7). This RNase activity was associated with the ribosomes even after passage through a Sephadex G-200 column.
as well as after sedimentation in the ultracentrifuge. This RNase activity could be removed from the ribosomes by lowering the pH of the ribosomal suspension to 5.7 with a 0.2 M sodium phosphate buffer, pH 5.7. This released enzyme preparation had

\[
\text{absorbance} = 2.80, 2.00, 1.20, 0.40, 0.00, \text{Volume (ml)}
\]

![Graph A](image)

**Fig. 8.** Effect of reticulocyte RNase on polyribosomes. To a solution containing 8 moles of Tris HCl buffer, pH 7.4, and 0.9 \( \mu \) mole of MgCl₂ in a total volume of 0.6 ml, 7.8 O.D₄₅₀ units of ribosomes were added. **A**, without further additions; this sample was layered onto a 15 to 30% sucrose gradient and sedimented for 3½ hours in a Spinco SW 25.3 rotor. Ribosomes corresponding to a coefficient of sedimentation of approximately 80 S were at 20 ml volume. **B**, this sample was incubated with 12 \( \mu \)g of reticulocyte RNase at 37° for 60 min before sedimentation analysis. Ribosomes corresponding to a coefficient of sedimentation of approximately 80 S were at 18 ml volume. **C**, this sample was incubated at 37° without addition of reticulocyte RNase and then layered on a sucrose gradient. Ribosomes corresponding to a coefficient of sedimentation of approximately 80 S were at 18 ml volume.

![Graph B](image)

![Graph C](image)

**Fig. 9.** Effect of temperature on depolymerization of RNA by reticulocyte RNase. Solutions containing RNA were brought to the appropriate temperature and reticulocyte RNase (Stage C) was added. The solutions were incubated for 10 min at the indicated temperatures, cooled, and the ultraviolet absorption in the perchloric acid-soluble fraction was determined. Controls without enzyme were run simultaneously in order to correct for nonenzymatic depolymerization of RNA.

**Table V**

<table>
<thead>
<tr>
<th>Nuclease</th>
<th>Concentration of hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Reticulocyte RNase</td>
<td>0.017</td>
</tr>
<tr>
<td>Pancreatic RNase (bovine)</td>
<td>0.039</td>
</tr>
<tr>
<td>RNase T₁</td>
<td>0.007</td>
</tr>
<tr>
<td>Pea leaf RNase, pH 5.2</td>
<td>0.047</td>
</tr>
<tr>
<td>Pea leaf RNase</td>
<td>0.072</td>
</tr>
</tbody>
</table>

3% of the activity of the hemolysate and was not purified further. It was activated and inhibited by K⁺ and Mg²⁺ in the same manner as the supernatant enzyme (Figs. 1 and 2). The RNase associated with the ribosomes degraded poly C to perchloric acid-soluble fragments but could not degrade poly A, nor did it degrade poly U to alcohol soluble nucleotides.

**Effect of Temperature**—The effect of varying the temperature on the depolymerization of RNA by the reticulocyte RNase is shown in Fig. 9.

**Effect of Hemin**—The addition of hemin to suspensions of reticulocytes causes an increase in polyribosome content and in rate of protein synthesis (27); therefore, we have investigated the effect of hemin on reticulocyte RNase.

Reticulocyte RNase is inhibited by hemin at 37° (Table V). The effect of hemin on other RNase was also examined and it is seen that bovine pancreatic RNase and RNase T₁ are not inhibited. RNase extracted from pea leaves was inhibited at pH 6.5 but not at pH 5.2. At pH 5.2, hemin actually activated.

The effect of hemoglobin on Stage C was studied and it was found that at 0.9 mg per ml it inhibited RNA degradation by 6%.
Ribonuclease from Rabbit Reticulocytes

The rate at which poly C is degraded by the enzyme is markedly decreased by the presence of poly I which is not a substrate. The results of the high degree of secondary structure in poly G. The enzyme, only polymeric substrate poly C was exhaustively incubated with the enzyme. The products of the action of the enzyme are oligonucleotides with an average chain length of six nucleotides. When the homopolymer substrate poly C was exhaustively incubated with the enzyme, only 6% of the product was converted to mononucleotides. Endonuclease activity with formation of refractory oligonucleotide units is not limited to those enzymes which form 3'-phosphorylated end groups. The RNase of Azotobacter agilis described by Stevens and Hilmo (28) also forms oligonucleotides with an average chain length of six, but these oligonucleotides are phosphorylated at the 5' end. This enzyme also hydrolyzes DNA (29). The presence of guanosine residues at the 3'-terminus of the oligonucleotides formed by depolymerization of RNA and the inability of reticulocyte RNase to degrade poly C can be the result of the high degree of secondary structure in poly G. The rate at which poly C is degraded by the enzyme is markedly decreased by the presence of poly I which is not a substrate. The effects of Mg²⁺ and monovalent cations suggest that the secondary structure of the RNA affects the activity of this enzyme. Mg²⁺ and other cations have been shown to increase secondary interactions of polynucleotides (30).

Hemoglobin comprises more than 95% of the protein in reticulocytes. The high degree of purification of this enzyme is due, in part, to removal of hemoglobin. The combined heating and acidification (Stage C) Table I) was largely responsible for the removal of hemoglobin. This stage not only accounted for a large purification of the enzyme but also resulted in an almost 8-fold activation of the RNase. Since it was shown that hemoglobin inhibits the RNase this could explain the marked activation by Stage C. In stating that the enzyme was 2085-fold purified, a correction was made for this activation at Stage C by dividing the specific activity by the degree of activation. This reticulocyte RNase could not be due to circulating pancreatic RNase since the latter enzyme is not inhibited by hemin, readily forms mononucleotides, and the blood cells used for preparation of the enzyme were washed until there was no contaminating extracellular RNase activity. The enzyme described in this report could not be a contaminant from other cells that are present along with the reticulocytes since the procedure used by us for lysing the reticulocytes has been shown to lyse only erythroid cells (19, 22).

The reticulocyte RNase is capable of degrading polyribosomes and may be responsible for the loss of polyribosomes that has been observed during the course of reticulocyte maturation (2, 26). The reticulocyte RNase is inhibited by hemin a compound that has been shown to stimulate globin synthesis and to increase the proportion of ribosomes present as polyribosomes in reticulocytes (27, 31). It has also been shown that heme can act as a feedback inhibitor of its own biosynthesis (32). A new control mechanism of protein synthesis in erythroid cells can be postulated which could also account for the loss of ribosomes, RNA, and protein-synthesizing capacity as these cells mature to erythrocyes.

(a) Newly synthesized globin chains combine with heme and lower the intracellular concentration of heme to a level at which it is no longer inhibitory to reticulocyte RNase. (b) The reticulocyte RNase depolymerizes the cellular RNA. (c) As the heme concentration is lowered heme biosynthesis proceeds until the concentration of free heme is high enough for feedback inhibition to begin. (d) The free heme inhibits reticulocyte RNase until the heme concentration is again lowered due to hemoglobin synthesis. (e) During early stages of erythroid cell development, synthesis of new RNA provides the components necessary for protein synthesis (19, 33). (f) In later stages of erythroid cell development, there is no RNA synthesis (19, 34). As erythroid cells mature from late polychromatophilic and orthochromic normoblast stages to reticulocytes and then to mature red blood cells, the RNase action causes a loss of polyribosomes associated with a loss in capacity to synthesize protein (2, 26).

It can be seen in Table V that at least two RNases from sources other than reticulocytes were not similarly inhibited by hemin. However, the RNase of pea leaves was inhibited by hemin. Like reticulocytes, pea leaves contain a large amount of metalloporphyrin and it is possible that these compounds may play a role in regulating RNA and protein metabolism in cells other than erythroid cells.

Zucker and Schultman (35) have recently reported that addition of hemin to a cell-free system prepared from reticulocyte-stabilized polyribosomes. They stated that this was not due to inhibition of RNase since heme did not protect the polyribosomes against exogenous RNase. However, in this report we show that pancreatic RNase is not inhibited by hemin. It is possible that stabilization of polyribosomes in the system described by these authors is due to inhibition of reticulocyte RNase.

Fe⁺⁺ and Cu⁺⁺ stimulate globin synthesis by reticulocytes (36) and also inhibit reticulocyte RNase (Table II) but the concentration at which these substances exert a hematopoietic effect is lower than that at which they inhibit reticulocyte RNase.

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