A New Endoribonuclease from *Escherichia coli*

**RIBONUCLEASE N**

TAPAN K. MISRA, SUZANNE RHEE, AND DAVID APIRION

From the Department of Microbiology and Immunology, Washington University School of Medicine, Division of Biological and Biomedical Sciences, St. Louis, Missouri 63110

A new ribonuclease called RNase N was isolated from *Escherichia coli*. It is a nonspecific endoribonuclease that can cleave rRNA, poly(U), and poly(C) to small oligonucleotides and 5'-mononucleotides. It requires monovalent cations and is inhibited by divalent cations. It is suggested that this enzyme plays a role in the decay of rRNA, under various starvation conditions and perhaps in the decay of mRNA.

In a series of studies on the turnover of ribosomes in *Escherichia coli* during deprivation of various nutrients essential for growth, it was concluded by Kaplan and Apirion (3, 4) that a common mechanism operates during the turnover of rRNA under the various starvation conditions. Their *in vivo* studies implicated that the turnover process is initiated by an endonucleolytic attack on rRNA in ribosomal subunits. From some detailed analyses of the process (3) and a review of current literature about ribonucleases in *E. coli* (5, 6) it could have been considered that the enzyme, RNase III, which degrades double stranded RNA (7), could have been this enzyme. However, when RNase III and RNase III' strains were compared, it was found that degradation of rRNA during carbon starvation was not reduced in RNase III- strains (8, 9), thus, eliminating this possibility. To understand the lacuna of the process, we started to search for a new ribonuclease(s) in cell extracts which could endonucleolytically attack rRNA, preferentially in ribosomes. We shall report here that at least one and perhaps two such enzymes are present in the *E. coli* cell.

One cuts rRNA in a restricted number of sites and the other degrades rRNA to very short oligonucleotides. We call the latter RNase N. RNase N activity has been purified and freed of RNase I and a modified polynucleotide phosphorylase was used throughout the following studies.

EXPERIMENTAL PROCEDURES

Materials

**Biological** - *Escherichia coli* strain N7060 (10) lacking RNase I and having a thermolabile RNase II and a modified polynucleotide phosphorylase was used throughout the following studies.

**Chemicals** - Carrier-free H$_2$PO$_4$ (6 to 8 mCi/ml) was purchased from Amersham/Searle, [3H]uracil (59 mCi/mmole) from New England Nuclear, poly(3H)cytidylic acid/potassium salt (8.2 mCi/mmol of polynucleotide phosphorus), and poly(3H)uridylic acid/potassium salt (7.76 mCi/mmol of polynucleotide phosphorus) from Schwarz Bio-Research, Inc. DNase I (RNase-free) was purchased from Worthington Biochemical Corp., sucrose from Schwarz/Mann, 5'-UMP, 3'-UMP, and 2',3' cyclic UMP from Sigma Chemical Co.

**Buffers** - Buffer A: 0.01 M Tris/HCl, pH 7.5, 0.001 M magnesium acetate, 0.01 M KH$_2$PO$_4$, 0.01 M KH, 0.2 M NH$_4$Cl. Buffer B: 0.01 M Tris/HCl, pH 7.5, 0.001 M magnesium acetate, 0.01 M KC1, 1 M NH$_4$Cl. Buffer BB: 0.01 M Tris/HCl, pH 7.5, 0.01 M magnesium acetate, 1 M NH$_4$Cl. Buffer C: 0.01 M potassium phosphate, pH 7.5, 0.001 M magnesium acetate, 0.01 M KC1. Buffer D: 0.1 M Tris/HCl, pH 7.5, 0.001 M magnesium acetate, 0.01 M KC1, Buffer E: 0.05 M Tris/HCl, pH 7.5, 0.01 M magnesium acetate, 0.01 M KC1. Buffer F: 0.05 M Tris/HCl, pH 7.5, 0.1 M dithioerythritol, 5% glycerol (v/v). Buffer G: 0.01 M potassium phosphate buffer, pH 7.5, 0.1 M dithioerythritol, 0.1 M KC1, 5% glycerol (v/v).

**Growth of Cells** - Cells were grown at 37° in gestation medium (11) in 60 liters in a Biogen fermenter to A$_{600}$ 1.6, dumped in ice, harvested by centrifugation in a Sharples centrifuge, washed twice with Buffer E, and stored at 70° until used for extraction of enzymes.

For the preparation of [32P]labeled substrates, cells were grown for 12 h in 6-liter Bismeyer flasks containing 600 ml of low phosphate medium (3) supplemented with H$_2$PO$_4$ (0.6 to 1.2 mCi) at 37° with shaking. After growth the culture was quickly chilled by adding ice, harvested by centrifugation in the cold, washed twice with Buffer A, and processed for the preparation of substrates. For the preparation of [3H]uracil-labeled substrate, cells were grown essentially by following the above method except that minimal medium (12) supplemented with 0.2% sucrose and 0.2% casamino acids was used and [3H]uracil (10 μCi/600 ml of growth medium) was added instead of H$_2$PO$_4$. Cells for the preparation of cold substrate were grown in the above medium devoid of any radioactive precursor compound.

Preparation of Ribosome Substrate - All steps in the preparation of ribosome substrate were performed at 0-4°. Cells were grown with alumina (13) and extracted with Buffer AA. The clarified extract (30,000 x g) was centrifuged in a fixed angle rotor for 2 h at 200,000 x g. The pellet was resuspended in Buffer B, centrifuged at 20,000 x g for 20 min, and the clear supernatant was reconstituted at
200,000 × g for 3 h. The pellet was resuspended in Buffer BB and centrifuged at 20,000 × g for 20 min. The supernatant was layered on the top of Buffer C containing 10%, enzyme grade, sucrose (2-ml sample layered on 3 ml of Buffer C) and centrifuged for 4 h at 200,000 × g in a fixed angle rotor. The pellet was resuspended in Buffer BB and centrifuged through Buffer C containing sucrose twice, as above. Finally, the ribosome pellet was suspended either in Buffer C or D and preserved in small aliquots at −70° before use. The amount of RNA in the ribosome substrate suspension was determined spectrophotometrically. One unit of absorbance at 260 nm corresponds with 40 μg of RNA.

Preparation of RNA Substrate—Purified ribosomes from the substrate preparation were suspended in sodium acetate buffer, pH 5 (final concentration 100 mg/ml), 0.25, extracted three times with an equal volume of water-saturated phenol, dissolved in Buffer C or D, and precipitated twice with 2 volumes of ethanol at −20° (overnight). RNA was collected by centrifugation at 20,000 × g for 10 min and dried under vacuum.

Enzyme Purification

Preparation of Cell Extract—All subsequent operations for enzyme purification were carried out at 0–4° unless otherwise stated. Frozen E. coli (N70601 cells (80 g wet weight) were ground with alumina (2.5 g of alumina/g wet weight of cells). DNase (RNase-free) solution, 0.8 ml/l 100 g, was added and the uniform paste was extracted with 200 ml of Buffer E. The mixture was centrifuged at 20,000 × g for 20 min to remove alumina, cells, and cell debris. This fraction will be referred to as cell extract. The cell extract was then centrifuged at 30,000 × g for 30 min. The supernatant remaining after centrifugation is referred to as S-30.

Ultra centrifugation of S-30—Ribosomes were removed from the S-30 fraction by centrifugation at 200,000 × g for 2 h in a fixed angle rotor. The supernatant obtained is referred to as S-200 or “high-speed supernatant fraction.”

Extraction of Enzyme from Ribosomes—The ribosome pellet from the previous step was suspended in Buffer B, centrifuged at 20,000 × g for 20 min, and the clear supernatant was recentrifuged at 200,000 × g for 2.5 h. The supernatant after removing ribosomes was again centrifuged at 20,000 × g as above and the clear ribosome wash was dialyzed overnight against 100 volumes of Buffer E with two changes. A white precipitate appeared in the dialysis bag and was removed by centrifugation.

Ammonium Sulfate Fractionation—Solid ammonium sulfate (24.3 g/100 ml of S-200 or ribosome wash) was added slowly with constant stirring. The pH was prevented from dropping below 7.0 by occasionally adding 10% NH₄OH. The mixture was then stirred for an additional 30 min and centrifuged at 20,000 × g for 20 min to remove the precipitate (40% saturation). The supernatant was further fractionated by adding 13.2 g of solid ammonium sulfate/100 ml and treated as above (60% saturation). The remaining supernatant was precipitated at 80% saturation by adding 14.3 g of ammonium sulfate/100 ml. Precipitates obtained after ammonium sulfate cuts were dissolved separately in Buffer F and dialyzed overnight against 100 volumes of the same buffer with two changes. Each fraction was tested for the degrading activity of RNA in the ribosome.

DEAE-Sephadex Chromatography—DEAE-Sephadex A-50 was packed in a column (45 × 2.5 cm) at a flow rate of 35 ml/h and equilibrated with Buffer F by washing the bed at the same flow rate for 18 h. The stabilized bed height measured 32 cm. Protein solution (45.5 ml, 500 mg) from the 40 to 60% ammonium cut of S-200 (dialyzed against the column buffer) was loaded on the column at a flow rate of 32 ml/h and eluted with 700 ml of a 0 to 1 M KC1 gradient, in the same buffer system at the same flow rate. Five-milliliter fractions were collected and the absorbance of the fractions was monitored at 280 nm.

Phosphocellulose Chromatography—A phosphocellulose column (20.2 × 1.5 cm) was made following the Burgess procedure (14) and equilibrated with Buffer G. DEAE-Sephadex fractions showing RNase N activity were pooled, dialyzed against Buffer G, applied to the column, and eluted by a 0 to 0.5 M potassium phosphate gradient, pH 7.5, 250 ml at a flow rate of 15 ml/h, collecting 4-ml fractions.

Gel Filtration—A Sephadex G-150 gel bed (53 × 1.5 cm) was equilibrated with Buffer G at a flow rate of 8 ml/h. Phosphocellulose column fractions showing RNase N activity were pooled and concentrated. The concentrated sample was dialyzed against Buffer G and applied (1 ml) to the gel. Elution was performed with the same buffer at the same flow rate (8 ml/h) and 2.5-ml fractions were collected.

Storage of Enzyme—The enzyme from the last step of purification could be stored frozen at −20° without any detectable loss of activity for over 4 months. For detailed studies of the enzyme characteristics samples were frozen in small aliquots and thawed at approximately 9°, dialyzed against assay buffer, and kept in ice for a period of 4 to 6 weeks.

Assay of RNase Activities—All assays essentially consisted of a Tris/HCl or potassium phosphate buffer (pH 7.5), enzyme from different sources, labeled substrates (in a volume of 5 μl in either Buffer C or D), and salt solution at the desired final concentration. The mixture (in 50 μl volume) was incubated at the desired temperature for an appropriate length of time with 20 μg of RNA in ribosomes, except if otherwise stated. For determining blank counts no enzyme control incubations were performed. Reaction was terminated by adding 1 ml volume of 0.5% SDS, 1.19 μM RNA, 50% sucrose, and 0.1% bromphenol blue mixture. Sucrose helped to layer samples on polyacrylamide gels and bromphenol blue served the purpose of a marker in gel electrophoresis. For assays with ribosomes as substrate, samples were incubated at room temperature for 30 min, prior to application on the gel, to dissociate RNA from protein.

Gel Electrophoresis—RNA samples were analyzed on a 5 to 12% tandem polyacrylamide gel (15) or a 5 to 15% tandem polyacrylamide gel. For most studies the bottom 4 cm of the gel contained 15% polyacrylamide gel, 0.4% methyl biocrylamide, and the top layer contained 5% polyacrylamide, 0.133% methyl biocrylamide in a slab gel system (100 × 145 × 1.5 mm) (15). Gels were prerun at 80 V for 0.5 h, then run with samples for 0.5 h at the same voltage, and another 2.5 to 3 h at 150 V. Other conditions of electrophoresis were the same as described previously (3, 15). Quantitation of RNA (large or smaller fragments) was done as described before (9). This gel system could retain large RNA at the origin, while pieces smaller than 5 S entered into the 15% gel. The interphase and the 5% gel contained various intermediate species. For quantitation, each region of the gel was cut and counted, values were corrected for background (no enzyme), and the total normalized. Also for some experiment samples were analyzed in tube gels (10 × 0.5 cm). After electrophoresis, gels were cut into 0.5-cm pieces, digested with H₂O and radioactivity was determined (3). The products of RNase N digest of 32P-labeled ribosomes were analyzed by a two-dimension polyacrylamide gel electrophoresis after Varriochio and Ernst (16). Samples were run in the first dimension, 15% gel with 7 M urea at pH 8.3, followed by an identical second dimension gel without urea. The bottom region of the first dimension gel, showing apparently a single distinct spot on the autoradiogram, was cut out and placed on the second dimension gel. Electrophoresis conditions were the same for the first and second dimension. Gel was dried after electrophoresis and an autoradiogram prepared as usual. This gel system has been used successfully by others (16) and by us to separate low molecular weight RNA (rRNA) from E. coli to a large number of species.

Separation of Mononucleotides by Paper Chromatography—Samples (5 to 40 μl) containing mononucleotides or reaction mixture or both were applied on Whatman No. 3 filter paper (45 × 45 cm). The chromatogram was developed for 24 h by ascending chromatography in a solvent system consisting of 1 M ammonium acetate/50% ethanol (1/1) saturated with Na₂B₄O₇. Five micrograms of each of the mononucleotides, 5'-UMP, 2':3'-cyclic UMP, 2'-UMP, and 3'-UMP, were applied individually or in a mixture, with and without an enzyme reaction mixture, which was incubated for 2 h at 40°. In this system the Rf of 5'-UMP is 0.327 and 2':3'-cyclic UMP is 0.700. Both 2'-UMP and 2':3'-UMP have the same Rf of 0.100. The mobilities of the standard samples were not affected by the presence of the components from the enzymatic digestion. 14C Uracil-labeled ribosomes were incubated with RNase N under conditions which gave approximately 65% acid-soluble products and applied on the chromatography paper (a no enzyme control was also included). Both reaction and control were applied in the same way to the paper and the chromatogram was developed as described. Spots containing nucleotides were identified under ultraviolet light after drying the chromatography paper at 45° for 30 min. Pieces of 1 cm each were cut and counted in toluene-based scintillation fluid for determining radioactivity.

RESULTS

Isolation of Enzymes

We could detect rRNA degradative activities in the S-200 as well as in the ribosome wash. In Fig. 1 we can see that one of

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
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FIG. 1 (top left). RNase activities. $^{32}$P-labeled ribosome substrate, in Buffer C, was incubated by itself (left side) or with protein fractions isolated from a ribosome wash for 2 h at 40°. Samples were analyzed on a 5 to 12% tandem polyacrylamide gel. A, no enzyme; B, 40 to 60% ammonium sulfate cut; C, 60 to 80% ammonium sulfate cut.

FIG. 2 (top right). Temperature optimum of RNase activity in the 40 to 60% ammonium sulfate cut. $^{32}$P-labeled ribosome substrate (20 pg of RNA) was incubated by itself or with 40 pg of 40 to 60% ammonium sulfate cut from S-200 in 0.01 M potassium phosphate buffer, pH 7.5, containing 0.1 M KCl, 1 mM magnesium acetate. Reaction was terminated after 90 min and samples analyzed on a 5 to 12% tandem gel.

FIG. 3 (bottom left). Temperature optimum of RNase N. Incubations were carried out at indicated temperatures for 15 min with 20 pg of rRNA in ribosomes and 2 pg of RNase N in 0.1 M Tris/HCl buffer, pH 7.5. Samples were analyzed on a 5 to 15% tandem gel and quantitated as described in the text. Empty bars measure large molecules disappearing from the origin; shaded bars, material appearing in 5% gel and also in the interphase between the 5 and 15% gels; and closed bars, small molecules appearing in the 15% part of the gel.

FIG. 4 (bottom right). Enzyme activity with different levels of ribosome substrate. Assays were carried out in 0.1 M Tris/HCl buffer, pH 7.5, for 120 min at 45° and samples analyzed on a 5 to 15% tandem gel. In all of the assays the substrate contained 9600 cpm of $^{32}$P-labeled ribosomes. For further details see the text.

We tested the temperature optimum of the RNase N activity in the 40 to 60% ammonium sulfate cut. As can be seen in Fig. 2 there is increased rRNA (in ribosomes) degrading activity at 45° and 50° as compared to 22° and 37°.

Protein from the 40 to 60% ammonium sulfate fractionation was eluted from a DEAE-Sephadex column into two major distinct peaks. One was eluted with 0.35 to 0.50 M KCl and the other with 0.55 to 0.82 M KCl. RNase N-like activity could be detected in a broad region of the column fractions corresponding to the first major peak. Proteins in these fractions were estimated to be 12% of the total loaded on the column. Proteins from these fractions were further separated by phosphocellulose column chromatography.

Proteins from the phosphocellulose column were eluted in three peaks. The major one also contained extensive RNase N-
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like activity. Sixteen per cent of the total protein charged on the column was eluted with the RNase N activity. Another activity which degrades RNA in the ribosomes to a limited extent was detected in later fractions which contained relatively small amounts of protein. This activity could be similar to the one detected in the 60 to 80% ammonium sulfate fraction from the ribosome wash.

Fractions showing RNase N activity on the phosphocellulose column were pooled, concentrated, dialyzed, and filtered through a Sephadex G-100 column. While most of the protein was eluted in a large peak, fractions eluted between 40 and 53 ml contained relatively little protein but showed very intensive RNase N activity. Those fractions were combined and used as the enzyme in all of the subsequent studies reported here. Determination of the specific activity of RNase N is rather complex with unpurified material because of the additive effect of other enzymes like RNase II and polynucleotide phosphorylase etc.

Purity of the protein material from the various fractions was determined by analysis in polyacrylamide gels according to the procedure of Davis (17). The last step in the purification decreased greatly the complexity of the proteins in the enzyme fraction. The enzyme preparation contained only three proteins, two major and one minor.

Properties of RNase N

Requirements of Ions and Inhibition by Divalent Cations—Since the substrate (ribosomes) is rather complex, it did not seem desirable to remove all of the ions from the assay mixtures. Thus, in all of the assays there was 0.1 mM magnesium acetate. As shown in Table I, monovalent cations enhance the reaction at 0.1 M. All monovalent cations tested seemed to be equally effective, Na+, K+, NH4+, and Tris. At higher salt concentration (0.2 M and above) the enzyme activity is reduced.

Mg2+ and Mn2+ inhibited the reaction almost completely at 20 mM. The presence of 2 mM Mg2+ or 8 mM Mg2+ in the assay mixture caused 80% inhibition of the reaction.

pH Optimum—The pH optimum was tested by using a variety of buffers at a pH range of 4 to 10. The enzyme is active at a broad range of pH values (5 to 9) and its maximal activity is at pH 8 to 9.

Temperature Optimum—The temperature optimum of the reaction was retested with the more purified preparation of RNase N (Fig. 3). Consistent with the less purified enzyme preparation (see Fig. 2) higher temperatures, 40-50°, were more effective.

Heat Inactivation—The enzyme is rather heat-stable but it can be irreversibly inactivated by heating at relatively high temperatures. After being heated at 70° for 30 min the enzyme activity was completely lost, while after heating at 55° for 30 min it retained 25% of its activity.

Kinetics—The kinetics of the reaction was tested at 45°. It was seen that the large molecules disappeared rather rapidly: in 15 min 60% of the substrate became smaller, while during the same time only 20% of the substrate entered the 15% gel, i.e. was degraded to small oligonucleotides. Within 1 h most of the substrate was degraded to smaller pieces but only 60% of the substrate was in small oligonucleotides and nucleotides.

Substrate Specificity—The specificity of the enzyme was tested. An assay was carried out at different temperatures using rRNA as the substrate. At higher temperatures the activity increased. However, in these assays the increase of activity with temperature was found to be more monotonic and there was no discontinuity as observed when ribosomes were used as a substrate (see Fig. 3). This suggests that particularly at low temperatures (37° and lower) there is some protection of the RNA by the ribosomal proteins—i.e. less accessibility of sensitive sites to RNase N.

Synthetic single stranded RNAs like poly(U) and poly(C) are also substrates for RNase N. At 40°, 2.2 pmol of 23 S and 16 S rRNA were hydrolyzed/h by 1 µg of RNase N. Under the same conditions of assay 0.5 pmol of poly(U), Mw = 1 x 105, or 0.66 pmol of poly(C), Mw = 3 x 104, was hydrolyzed/h by 1 µg of RNase N. Hydrolysis of 23 S or 16 S RNA from the ribosomes or purified rRNA was almost at the same rate at 40°. (Measurements were made for the diminution in the size of the substrates.) The above figures are first approximations. It is rather difficult to carry out precise quantitation because of the complexity of the reaction and also the complex nature of the substrates themselves. Molecular weight of the synthetic RNA homopolymers was determined from their relative mobilities on the gel using rRNA as internal standards, but the homopolymers were not uniform in size and an accurate quantitative reading was impossible because more substrate is created for the enzyme. 32P-labeled E. coli DNA when tested as a substrate for RNase N was not degraded to any detectable extent.

Mode of Action—The level of small molecules formed at different temperatures (40°, 45°, and 50°; see Fig. 3) is proportional to the rate of disappearance of large molecules when substrate is not limiting, but the amount of small molecules accumulating is small as compared to the amount of large RNA disappearing. Also, the appearance of intermediate molecules is not proportional to the rate of disappearance of large molecules. These phenomena could be explained by assuming that RNase N is either an endonuclease or a nonprocessive exonuclease but not a processive exonuclease (18, 19).

To clearly distinguish a nonprocessive exonuclease from an endonuclease, the following experiment was carried out. The level of enzyme was left constant while the level of substrate was increased (keeping the same input counts in all assays by using a labeled substrate preparation and diluting it with an appropriate amount of unlabeled substrate) and the assay was carried out for a constant length of time (120 min). As can be seen in Fig. 4, when higher levels of nonlabeled substrate competed with a constant amount of labeled substrate, although most of the large molecules disappeared from the origin of the gel the level of material accumulating in the 15% part of the gel decreased (some of the material was nucleotides, see below). These results indicate that the enzyme is not an exonuclease. Should it be an exonuclease, the level of labeled nucleotides accumulated should have been proportional to the level of large molecules disappearing. Since 32P-labeled E. coli DNA was not a substrate for RNase N, these

| Salts          | 0.01 M | 0.1 M | 0.2 M | 0.4 M |
|----------------|--------|-------|-------|-------|
| Tris/HCl, pH 7.5| 497    | 2326  | 1231  | 585   |
| KCl in 0.01 M Tris/HCl, pH 7.5 | 774    | 2380  | 1670  | 25    |
| Potassium phosphate, pH 7.5 | 1440   | 2855  | 2351  | 81    |
| NaCl in 0.01 M Tris/HCl, pH 7.5 | 2401   |       |       |       |
| NH4Cl in 0.01 M Tris/HCl, pH 7.5 |       |       | 2364  |       |

* Not tested.
experiments suggest that the enzyme is a nonspecific endoribonuclease.

**Products** – In order to define the terminal products of the reaction, an exhaustive digestion of $^{32}$P-labeled rRNA was run on a 5 to 15% polyacrylamide tandem gel with 7 M urea. The bottom of the 15% part of the gel was cut out and rerun on a similar gel system without urea (see "Methods"). Again all of the material migrated to a single spot (Fig. 5), indicating that the final products are rather homogenous and very likely to be small oligonucleotides or nucleotides. The product(s) which runs as a single spot at the bottom of the gel in the first dimension was found to be soluble in 5% trichloroacetic acid.

In order to characterize the product further, $[^{14}C]$uracil-labeled ribosomes were used as substrate and after exhaustive digestion with RNase N the products were subjected to paper chromatography under conditions which clearly separate 2'-3'-cyclic nucleotides from 5'- and 3'-mononucleotides. The results of one such experiment are summarized in Fig. 6. While even after prolonged incubation only a small percentage of the material migrated to a single spot (most remained in the origin), almost all of the product which left the origin clearly co-migrated with 5'-UMP and not at all with the 3'-mononucleotide or the 2'3'-cyclic nucleotide. Thus, it is evident that the enzyme preparation is capable of degrading rRNA, at least partially, to 5'-mononucleotide or 2',3'-cyclic nucleotide. Hence, all of the counts found to be acid soluble (5% trichloroacetic acid). This suggests that small oligonucleotides were among the reaction products. This again indicates that the enzyme is an endonuclease. The fact that the mononucleotides produced after exhaustive digestion with this enzyme are 5'-mononucleotides suggests that the oligonucleotides produced by this enzyme contain phosphates at their 5' end and hydroxyl groups at their 3' end. The inset in Fig. 6 shows an analysis of a identical sample (after exhaustive digestion with the enzyme) analyzed on a 5 to 15% tandem polyacrylamide gel, as described previously. Again, we can see that most of the digestion products migrate as oligonucleotides (see Fig. 5).

**DISCUSSION**

The studies presented in this paper show that Escherichia coli contains a nonspecific endoribonuclease, RNase N. Another known enzyme of E. coli, RNase I (20), resembles RNase N to some extent, especially with respect to substrate nonspecificity and endonucleolytic activity. This enzyme is plasmidic (21) and its physiological function has not yet been established. The enzyme RNase N is distinguished from RNase I in at least two respects, it does require monovalent cations for its activity, while RNase I does not, and its final products are 5'-mononucleotides, while the products of RNase I are cyclic mononucleotides.

There are certain features which distinguish RNase N from other known E. coli ribonucleases. RNase III is an endonuclease specific for double stranded RNA. Moreover, it has been demonstrated that RNase III cannot attack rRNA (22) or single stranded RNA, and its final products are oligonucleotides (23, 24). RNase II in strain N7060 (from where RNase N was isolated) is thermostable and it has been previously demonstrated (10) that it could be completely inactivated by heating the enzyme solution at 50° for 10 min. RNase N is stable under these conditions, and the optimal temperature for its activity against rRNA is 45–50°. Both RNase III and RNase II activities require Mg$^{2+}$, while it is inhibitory for RNase N activity.

RNase N is probably an intracellular enzyme, but further studies are necessary to establish this point. The fact that it can be found, after cells are opened, in the ribosome pellet or

**Fig. 5.** Analysis of product by two-dimensional gel electrophoresis. Lanes 1, sample after digestion of 20 $\mu$g of $^{32}$P-labeled RNA in ribosomes with 4 $\mu$g of RNase N preparation, at 45° for 3 h, was run in a 5 to 15% urea gel (16). Lane 2, the bottom region of the first dimension gel was cut from the wet gel and rerun in the second dimension without urea. Lane 3, ribosome substrate incubated without enzyme on the second gel. For details see the text. The above figure is a composite presentation of two different autoradiograms from first and second dimension gels. The autoradiograms were developed after exposing gels to the films for different lengths of time.

**Fig. 6.** Analysis of products by paper chromatography. $[^{14}C]$Uracil RNA (20 $\mu$g) in ribosomes was incubated with 4 $\mu$g of RNase N preparation in 0.1 M Tris/HCl, pH 7.5, at 45° for 120 min and products analyzed by paper chromatography as described in the text. One-centimeter pieces, along each lane in the paper, were cut and counted in toluene-based scintillation fluid. Counts in the first 1 cm in the plot include also a region 1 cm below the base-line, where samples were applied on the paper. A time zero incubation sample was run on the same paper and counted. Its values (30 to 40 cpm) were used to assess the background counts. The plot was corrected for these background counts. The inset in the figure shows an identical sample (after digestion with RNase N) on a 5 to 15% tandem gel, as described previously. Mononucleotides migrate at the very bottom region, between 8.5 and 10 cm.
in the supernatant does not suggest a precise physiological location, since association with the ribosome pellet or with the supernatant is not necessarily its in situ location (25). However, this distribution between ribosome and supernatant could be meaningful and would place this enzyme in a similar physiological category as elongation factors involved in protein synthesis. Such a location would fit perfectly with its putative role in turnover of rRNA and a possible role in decay of mRNA.

Since it is a nonspecific endoribonuclease, it could be easily envisaged that RNase N would start to decay mRNA (see for instance Refs. 5, 26, 27, and below). Thus far no intracellular endoribonuclease has been detected in E. coli which could cleave nonspecifically single-stranded RNA in the cell (see Refs. 5, 6, and 26). RNase N seems to fill this gap. Since it can attack a variety of RNAs one must envisage protection of the various RNAs from such an activity (26). Evidence is accumulating for a role for ribosomes as protectors for mRNA (26), and, as discussed by Kaplan and Apirion (3), the rRNA is not accessible to ribonucleases in the cell in polyribosomes or in monosomes. The ribosome substrate used in these studies is most likely in the subunit form due to the extensive washes with high salt concentrations. Moreover, the enzyme is more active in the absence of divalent cations, an ideal condition to keep ribosomes in subunits. RNase N participates in the process of turnover of ribosomes (obviously mutant analyses should be very useful in this respect) then the requirements for RNase II and polynucleotide phosphorylase in the process, as postulated from in vitro studies (3, 29), could be well understood, since the production of nucleotides by RNase N alone is rather slow. Moreover, the few per cent of material co-migrating with 5'-UMP (Fig. 6) could be due to a contaminating exonuclease. (Further analysis with a more purified preparation of RNase N would be necessary to settle this point.)

An activity which degrades rRNA only to a limited extent was detected in ammonium sulfate fractions from the ribosomal wash (Fig. 1) and in protein fractions eluted from the phosphocellulose column (see "Results"). That this activity is different from RNase N is rather clear from the fact that their mode of action is dissimilar. When about the same level of large substrate disappears from the origin of the gel, the picture as revealed from the autoradiogram of the gel is very different for both activities (Fig. 1). Moreover, even after exhaustive digestion it failed to convert most of the substrate (rRNA in ribosomes) to small oligonucleotides and nucleotides. In addition, the presence of 1 to 2 mM Mg	extsuperscript{2+} and Mn	extsuperscript{2+} inhibited the decay of the substrate. Thus, it is unlikely that this activity is RNase III which requires Mg	extsuperscript{2+} for its activity. Moreover, RNase III was reported to be incapable of degrading rRNA (22). Further characterization of this activity is obviously in order.

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