Purification and Properties of a Potassium-activated Phosphodiesterase (RNAase II) from *Escherichia coli**

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ABSTRACT: A potassium-activated phosphodiesterase (RNAase II) that hydrolyzes polyribonucleotides to 5′-nucleoside monophosphates has been purified approximately 600-fold from extracts of *Escherichia coli* B. RNAase II is distinct from the latent ribosomal ribonuclease (RNAase I), although RNAase II does bind to ribosomes to some extent. The action of RNAase II on polyribonucleotides appears to be primarily, if not exclusively, exonucleolytic. Short-chain oligonucleotides are not hydrolyzed. In addition to K^+, a divalent cation (e.g., Mg^{2+}) is required for activity. The enzyme is specific for single-stranded polyribonucleotides; helical forms are not hydrolyzed, nor do they inhibit the hydrolysis of single-stranded chains. Thus the purified enzyme is an excellent tool for the study of polyribonucleotide secondary structure. Several examples of such use are reported.

In comparison with polynucleotide phosphorylase and RNAase I, RNAase II activity is high in *E. coli*. In view of the characteristics of the enzyme the possibility that RNAase II is concerned with the breakdown of messenger RNA is discussed.

Three enzymes that catalyze the breakdown of RNA have been described in *Escherichia coli*. These enzymes, polynucleotide phosphorylase which yields nucleoside diphosphates, the so-called ribosomal ribonuclease (RNAase I), which is an endonuclease and yields, ultimately, nucleoside 3′-monophosphates, and the potassium-activated phosphodiesterase (RNAase II) which yields nucleoside 5′-monophosphates, are discussed in a recent review article (Lehman, 1963). The terminology RNAase I and II was introduced by Spahr (1964) and will be used throughout this paper. Spahr (1964) reported a 30-fold purification of RNAase II starting from *E. coli* ribosomes. In addition, various properties of crude and partly purified enzyme preparations have been described (Spahr and Schlessinger, 1963; Spahr, 1964). In an earlier report (Singer and Tolbert, 1964) we briefly described a potassium-activated phosphodiesterase from *E. coli* and demonstrated the specificity of that enzyme for polyribonucleotides that lack secondary structure. Helical forms are resistant to hydrolysis. At that time we mentioned that the enzyme had been purified approximately 600-fold and, further, that the properties of the purified enzyme indicated it was identical with the RNAase II described by Spahr and Schlessinger (1963).

In this paper we will describe in detail the 600-fold purification of RNAase II, as well as some of the properties of the purified enzyme. Methods for the stabilization of the purified enzyme are presented. Additional evidence concerning substrate specificity and sensitivity to secondary structure is included. Data concerning the distribution and levels of this enzyme as well as polynucleotide phosphorylase and RNAase I in wild type cells and in mutants reportedly lacking RNAase I are also recorded.

Materials and Methods

Materials. Unless specifically noted all the polymers used in these studies were synthesized in this laboratory, using polynucleotide phosphorylase from *Micrococcus lysodeikticus* (Singer and Guss, 1962; Singer, 1958). All these polymers were dialyzed extensively against 1 mM EDTA and then distilled water before use. Throughout the paper the concentrations of polyribonucleotides are expressed as μmoles of polymer phosphate per ml. The homopolymers of 4-*N*-methylcytidylic acid and 4-*N*,*N*-dimethylcytidylic acid were the generous gift of Dr. Colin Reese. Poly-UG[1] (43%G) was the gift of Dr. Philip Leder. *E. coli* K12λ [^{3}H]DNA was kindly supplied by Dr. Arthur Weissbach. Salmon sperm DNA was obtained from Worthington Biochemical Corporation. DNA was denatured by the procedure of Von Hippel and Felsenfeld (1964). The ^{14}C-labeled yeast RNA was isolated by the procedure of Crestfield *et al.* (1955) from yeast grown on [^{14}C]orotic acid and was purified further by phenol extraction. The unlabeled carrier RNA used in the assay system was obtained from Schwarz BioResearch, Inc., and purified in the following way. A 1% aqueous solution of the RNA was adjusted

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[1] The following abbreviations are used: A, U, G, C, and I represent the residues of adenylic, uridylic, guanylic, cytidylic, and inosinic acids, respectively, in polyribonucleotide chains. For example, poly-A is polyadenylic acid and poly-UG is a copolymer of uridylic and guanylic acids. DAPR is 2,6-diaminopurine ribonucleoside; 5′-AMP is adenosine 5′-monophosphate; 5′-GMP is guanosine 5′-monophosphate.

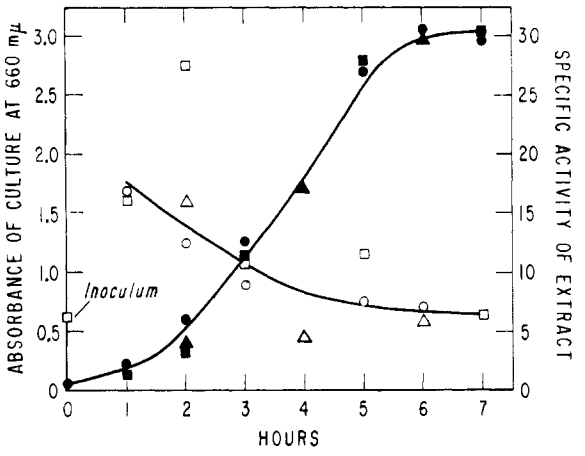

FIGURE 1: Activity of RNAase II as a function of cell growth. Cells were grown and washed as described in text. Absorbance of the culture was determined at 660 mμ. Experiment 1 (circles), 0.2 g of cells suspended per ml of buffer B; experiment 2 (triangles), 0.47 g of cells suspended per ml of buffer A; experiment 3 (squares), cells were suspended as for experiment 2. After disruption of the cells with the sonifier, as described in the text, the suspensions were centrifuged at 20,000 × *g* for 1 hour. The supernatants were assayed for activity and protein by the standard procedures described in the methods section. The closed symbols indicate absorbance; the open symbols specific activity.

to *p*H 4.2 with 1 N NaOH and filtered. The solution was made 0.06 M in NaCl and cooled, and the RNA was precipitated with 2 volumes of ethanol. The precipitate was collected, washed with 67% ethanol containing 0.02 M NaCl, and dissolved in one-third the original volume of H_2O. The solution was dialyzed for 3 to 5 days against H_2O and lyophilized. A further purification is also useful to decrease the blank values in the enzyme assay. A 0.5% solution of the RNA in 0.05 M Tris, *p*H 7.5, 0.05 M KCl, 0.1 M NaCl, and 0.5 mM $MgCl_2$ was prepared and the RNA was precipitated with 3 volumes of ethanol. This precipitation was repeated two more times and the RNA was finally dissolved in 0.2 N NaCl at a concentration of about 20 μmoles/ml.

The mutants MRE 600 and A-19, which are reported to lack ribonuclease I, were gifts from Dr. H. E. Wade and Dr. N. Zinder, respectively. Mutant A-19 is a strain developed in the laboratory of Dr. J. D. Watson.

The 5′-nucleotidase of snake venom was prepared by a published procedure (Lehman, *et al.*, 1962). *E. coli* alkaline phosphatase was obtained from Worthington Biochemical Corp. The particular preparation used was devoid of significant diesterase contamination. Bovine serum albumin was obtained from Pentex Inc., Kankakee, Ill. The albumin was dialyzed for several days against 1 mM EDTA and then against distilled H_2O.

DEAE-cellulose, Type 20, was the product of Brown and Co.; hydroxylapatite (Hypatite C) was obtained from the Clarkson Chemical Company, Inc., Williamsport, Pa. Biogel P-10, a polyacrylamide used for gel filtration, was the product of BioRad Laboratories.

Two buffer mixtures were used routinely. Buffer A contains 0.01 M Tris, *p*H 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM β-mercaptoethanol. Buffer B contains 0.01 M Tris, *p*H 7.5, 5 mM $MgCl_2$, and 1 mM β-mercaptoethanol.

Assays. The standard assay mixture for ribonuclease II contained (in 0.1 ml) 0.1 M Tris, *p*H 7.5, 0.1 M KCl, 1.5 mM $MgCl_2$, 0.2 μmole of ^{14}C-labeled poly-A (specific activity approximately 30,000 cpm per μmole), and enzyme. After 20 minutes at 37° the tubes were placed in ice and treated in one of two ways. (A) (Spahr and Schlessinger, 1963) A cold solution (0.1 ml) containing 10 mg/ml of unlabeled carrier RNA in 0.2 M NaCl was added followed by 0.4 ml of cold absolute ethanol. (B) Cold 2.5% perchloric acid (0.4 ml) was added. With either procedure A or B the mixtures were allowed to stand for 10 minutes at 4° and then centrifuged, and 0.3 ml of the supernatant solution was taken for counting. The μmoles of soluble nucleotide produced per 0.1 ml of reaction mixture was calculated. One unit is equal to the production of 1 μmole of 5′-AMP per hour, and specific activity is units per mg protein. Under the conditions given above the extent of polymer hydrolysis is proportional to time of incubation until at least 50% of the polymer is hydrolyzed (see, for example, Figures 5 and 8). Hydrolysis is not strictly dependent on enzyme concentration with the crudest fractions; it is so after the protamine step. Thus, with a protamine supernatant fraction, 3.2 and 9.6 μg of protein gave 3.7 and 12 mμmoles of 5′-AMP, respectively, in 20 minutes. Similarly, with an hydroxylapatite fraction, 0.16 and 0.32 μg of protein hydrolyzed 97 and 210 mμmoles of poly-A, respectively. In certain cases hydrolysis of unlabeled polyribonucleotides was assayed by measuring the formation of acid-soluble, ultraviolet absorbing materials. In these cases unreacted polymer was precipitated, in the cold, by addition of an equal volume of 0.25% uranium acetate in 4% perchloric acid. The precipitate was removed by centrifugation and the optical density of a suitably diluted portion of the supernatant fluid was determined at an appropriate wavelength.

Purified preparations of RNAase II were tested for contamination by various other known enzymes. The several assays used are described below. In each case the results were expressed as μmoles of product produced per hour per ml of enzyme in order to facilitate comparison with RNAase II activity. DNAase was measured in reaction mixtures (0.2 ml) that contained 20 μmoles of Tris buffer, *p*H 7.6, 0.3 μmole of $MgCl_2$, 20 μmoles of KCl, 15 mμmoles (0.1 optical density unit) of [^3H]DNA (6×10^5 cpm/μmole), and enzyme. The tubes were incubated at 37° for various times. The treatment of the reaction mixtures and the counting techniques were according to Weissbach and Korn (1962). The cobalt-requiring 5′-nucleotidase of *E. coli* was assayed as described by Neu and Heppel (1964a). Polynucleotide phosphorylase was determined by the procedure of Singer and Guss (1962). Phosphomonoesterase was assayed by standard procedures. *E. coli* RNAase I was

TABLE I: Purification of RNAase II of *Escherichia coli*.

Fraction	Description	Units/ml	Specific Activity	Total Units
I	20,000 × *g* supernatant	181	6.9	16,500
II	Streptomycin supernatant	58	6.4	14,500
III	Protamine supernatant	23	3.7	5850
IV	$CdCl_2$ precipitate	39	16.0	4813
V	$(NH_4)_2SO_4$ (40–52)	202	25.2	3230
VI	DEAE (tubes 43–49)	40	572	1920
VII	Hydroxylapatite	212[a]	2120[a]	424

[a] The values for the peak tube of the hydroxylapatite column are given. See the text for a more complete description.

measured by RNAase assay A of Neu and Heppel (1964b). In order to express the results as μmoles of mononucleotide equivalents solubilized, the average molar extinction coefficient of the soluble nucleotide material was taken as equal to 10,000 at 260 mμ. The cyclic phosphodiesterase of *E. coli* recently described by Anraku (1964) was determined by the procedure of Neu and Heppel (1964a).

Protein was determined by the method of Lowry *et al.* (1951). In those cases where small amounts of protein had to be determined, a modification (Layne, 1957) of the Lowry method was used. ^{14}C was counted in a Packard liquid scintillation counter in 10 ml of Bray's solution (Bray, 1960) containing 0.1 ml of 1 N NH_4OH. Internal standards were used to correct for any quenching. Inorganic phosphate was determined as described by Ames and Dubin (1960).

Cell Growth and Preparation of Extracts. E. coli B was grown in a medium containing the following, per liter: 20 ml of 50× concentrated Vogel-Bonner medium (Vogel and Bonner, 1956), 10 ml of 50% glucose, 20 ml of 5% Difco yeast extract, and 1 ml of trace elements solution (Ames *et al.*, 1960). In general, a 2% inoculum of stationary phase cells was used; this inoculum corresponds to an initial absorbancy of 0.07 (at 660 mμ) for the growth medium. After growth, cells were collected by centrifugation and washed once with 1.19% KCl containing 5 mM $MgCl_2$. Cells were either used immediately or stored frozen until required. For preparative work, cells were grown in a 300 liter fermentor and the washed cells stored frozen.[2]

A study of the variation of enzyme activity with the growth of cultures was made. Figure 1 shows the results obtained in three separate experiments. Cells were grown, samples were collected at various times, and the cells were washed. Suspensions of washed cells (see legend to Figure 1 for details) were treated for two 20-second periods with a Branson sonifier set at positon 5. After centrifugation for 1 hour at 20,000 × *g* the specific activity of the resulting supernatant fluid was determined. Cells in the early logarithmic phase of growth appear to be the richest source of enzyme. The high specific enzymic activity early in the growth curve results from an increase in enzyme units/gram of cells, not from a decreased protein concentration in the extract. On the basis of these experiments cells were usually grown to an absorbance of about 0.30 and then collected. In our experiments this corresponds to the beginning of logarithmic growth.

Cell extracts were prepared in one of three ways. All three procedures gave essentially identical results. (1) Cells were ground with three weights of alumina and extracted with buffer. (2) A cell suspension was passed through a French pressure cell at 8000 p.s.i. (3) Cell suspensions were subjected to high-frequency sonic vibration in a Branson Sonifier for a total of 40 seconds. The temperature was maintained below 10° by means of an ethanol–dry ice bath and by limiting any one period of treatment to 20 seconds.

Purification Procedure. A typical preparation is described here and summarized in Table I. Unless noted otherwise, all operations were carried out at 4°.

Frozen cells were allowed to thaw partly and 39.9 g was suspended in 85.4 ml of cold buffer A. Equally satisfactory results are obtained by suspending cells in buffer B and, with either buffer, at a concentration of 1 g of cells/5 ml of buffer. Fifteen-ml portions of the cell suspension were treated by method 3 (sonic vibration). The broken cell suspensions were pooled and centrifuged for 1 hour at 20,000 × *g*. The supernatant fluid (fraction I) was diluted to 13 mg of protein/ml with buffer A. To this, 0.5 volume of a 1% neutral solution of streptomycin sulfate was added dropwise. The suspension was stirred for an additional 20 minutes and then centrifuged 1 hour at 20,000 × *g*. The supernatant fluid (fraction II, 260 ml) was treated with 9.9 ml of a 1% solution of protamine sulfate that was adjusted to *p*H 7. After centrifugation at 20,000 × *g* for 30 minutes, 250 ml of supernatant fluid (fraction III) was collected.

To fraction III, 4.14 ml of 0.1 M $CdCl_2$ (0.26 μmole of $CdCl_2$/mg of protein) was added dropwise; the suspension was stirred for 10 minutes and centrifuged for 20 minutes at 20,000 × *g*. The yellow precipitate was redissolved in 125 ml of buffer B (fraction IV). The por-

[2] We are indebted to Mr. D. Rogerson and Dr. J. C. Keresztesy for the operation of the fermentor.

tion of this solution insoluble between 40 and 52% saturation with $(NH_4)_2SO_4$ was collected as follows: Fraction IV (124 ml) was treated dropwise with 68.5 ml (30% saturation) of a saturated solution of $(NH_4)_2SO_4$ (saturated at room temperature and adjusted to *p*H 7.7 with NH_4OH). After 10 minutes of stirring the suspension was centrifuged for 20 minutes at 13,000 $\times$ *g*. The 196 ml of supernatant fluid was treated with 16.3 ml (40% saturation) of saturated $(NH_4)_2SO_4$, and the supernatant fluid (206 ml) was collected in the same manner. This fluid was brought to 52% saturation by adding 51.5 ml of saturated $(NH_4)_2SO_4$. After centrifugation the precipitate was dissolved in 16 ml of buffer B (fraction V).

A column (16 $\times$ 6.2 cm^2) of DEAE-cellulose was prepared and equilibrated with buffer B. Fifteen ml of fraction V was diluted to 45 ml with buffer B and the solution was passed through the column at a rate of 0.5 ml/min. After washing the column with 30 ml of buffer B it was eluted in stepwise fashion with buffer B containing KCl as follows: 150 ml of 0.1 M KCl, 200 ml of 0.13 M KCl, and 200 ml of 0.15 M KCl. Eight-ml fractions were collected every 5 minutes. Approximately 100% of the enzyme units placed on the column were recovered in tubes 41–62 and the specific activities of these fractions ranged from 100 at the beginning and end of the peak to 630 in the peak tube (tube 48). Tubes 43–49 were pooled (fraction VI).

A column (0.66 cm^2 $\times$ 7.5 cm) of hydroxylapatite was prepared and equilibrated with buffer B. Forty-eight ml of fraction VI was diluted to 130 ml with buffer B, and the solution was passed through the column at a rate of 5 ml/hour. The column was then eluted with a linear gradient: the mixing chamber contained 75 ml of buffer B and the reservoir 75 ml of buffer B containing 0.3 M potassium phosphate buffer, *p*H 7.5. The column was eluted at a rate of 2.8 ml/hour and 2-ml fractions were collected. Approximately 98% of the units placed on the column was recovered in tubes 21 through 30, with specific activities ranging from 256 to 2200. The peak tube, 23 (corresponding to 0.082 M potassium phosphate), had a specific activity of 2120 (fraction VII). Tubes 22 to 26 yielded 70% of the original activity and all had specific activities over 1000.

In order to study certain properties of the purified preparaton it was necessary to free the enzyme from the potassium phosphate and Mg^{2+} used in purification on hydroxylapatite. A solution of fraction VII containing 1 mg/ml of dialyzed bovine serum albumin was passed through a column of Biogel P-10 that had previously been equilibrated with 0.01 M Tris buffer, *p*H 7.5, containing 1 mg/ml of the albumin. Approximately 55% of the activity was recovered. Over 99% of the inorganic phosphate was removed by this procedure.

Results

Contamination with Other Enzymes. The purified enzyme (fraction VII) was tested for a number of contaminating enzymes. DNAase activity was measured using both native and denatured *E. coli* K12λ DNA. Enough enzyme to hydrolyze 2.5 μmoles of poly-A/hour and optimal conditions for ribonuclease II activity were used. There was no detectable hydrolysis of native DNA, even after 6 hours. There was some release of acid-soluble, radioactive material from denatured DNA after 4 hours of incubation, but no further increase after 6 hours. The amount of material released corresponded to 12 $\times$ 10^{-5} μmole. Therefore, under the conditions of ribonuclease II activity, DNAase activity is essentially absent.

Fraction VI was tested for the cobalt-stimulated 5′-nucleotidase recently described by Neu and Heppel (1964a). An amount of ribonuclease II which would have hydrolyzed 1.25 μmoles of poly-A/hour released no detectable P_i from 5′-AMP after 3 hours. Four mμmoles would have been detected.

As described previously, fraction VI preparations showed no phosphatase activity at a variety of *p*H values, nor is polynucleotide phosphorylase detectable in these preparations.

E. coli RNAase I, the so-called ribosomal ribonuclease, was not detectable in our best preparations. However, one fraction VII preparation, which did not have a high specific activity, showed a slight contamination (an amount of enzyme which hydrolyzed 100 μmoles of poly-A in the standard RNAase II assay produced 0.5 μmole of acid-soluble nucleotide in the RNAase I assay).

E. coli cyclic phosphodiesterase activity was less than 1% of the RNAase II activity in fraction VII. A sample of fraction VII that was passed over a column of Biogel P-10 was used for this assay.

Stability. In an earlier publication (Singer and Tolbert, 1964) we reported that highly purified preparations of ribonuclease II are quite unstable, losing 95% of their activity after 2 weeks at 4°, even in the presence of bovine serum albumin. Several stabilizing conditions have now been found. For example, a sample of fraction VII was made 20% in glycerol. This solution was stored at −20° for 3 days and lost 35% of its activity. Thereafter it was stored at −85° and lost no activity after 2 months; another 50% of its activity was lost after a total of 4 months. The enzyme can also be stored (at −85° or less) in solutions that are 1 mg/ml in dialyzed bovine serum albumin. Undialyzed albumin is ineffective. Solutions stored in this manner are stable for at least 6 months. It is advantageous to avoid repeated freezing and thawing by storing the enzyme in many small aliquots.

Although the quantitative results have been variable, solutions of enzyme purified through the hydroxylapatite column step are easily inactivated. After 5 minutes at 40°, 30–70% of the activity is lost. The presence of dialyzed bovine serum albumin affords some protection, as does KCl (Table II). Inactivation corresponding to 90 and 100% is obtained after 5 minutes at 45 and 50°, respectively. At 45°, KCl affords little protection (Table II). As shown in Table II, enzymic activity is markedly stabilized at both 40 and 45° by the presence of denatured DNA. In other experiments denatured DNA also enhanced the stability of the

TABLE II: Effect of Elevated Temperature on RNAase II.[a]

Experiment	Temperature (°C)	Conditions	Original Activity Remaining (%)
1	40	Enzyme alone	30
		Plus 0.33 M KCl	55
		Plus 0.33 mg dDNA/ml	105
2	40	Enzyme in BSA	64
		Plus 0.5 M KCl	84
		Plus 0.5 mg dDNA/ml	106
3	45	Enzyme alone	8
		Plus 0.5 M KCl	10
		Plus 0.5 mg dDNA/ml	63

[a] Incubation mixtures containing fraction VII (in 0.08 M potassium phosphate, *p*H 7.5, 0.01 M Tris, *p*H 7.5, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 0.2 mg protein per ml) and the components given in the table were prepared at 0°. Denatured salmon sperm DNA (dDNA) was used. At zero time and after 10 minutes of incubation samples of the mixtures were tested for activity as described for the standard assay procedure. Results are expressed as the per cent of the initial activity remaining after 10 minutes at the indicated temperature. Experiment 1: 0.1 ml of enzyme was incubated in a final volume of 0.15 ml. Suitable samples were removed and cooled in ice. Reagents for the assay were added and poly-A was used to start the reaction (37°). Experiment 2: 50 μl of enzyme (containing 1 mg/ml dialyzed bovine serum albumin) were incubated in a final volume of 0.1 ml. The procedure was the same as in experiment 1. Experiment 3: same as experiment 2 except no bovine serum albumin was present.

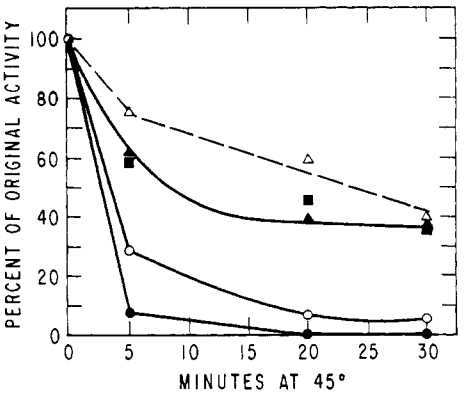

FIGURE 2: Effect of DNA on stability of RNAase II to elevated temperature. Aliquots (0.05 ml) of fraction VII (see legend to Table II) were heated at 45° in a total volume of 0.15 ml containing salmon sperm DNA as indicated. At the indicated times 30-μl samples were removed and added to cold solutions containing all components, except the substrate, for the standard assay. The assay was started by the addition of poly-A, at 37°. The symbols refer to: ●, no addition; ○, 0.33 mg native DNA/ml; ▲, 0.17 mg denatured DNA/ml; △, 0.33 mg denatured DNA/ml; ■, 0.67 mg denatured DNA/ml.

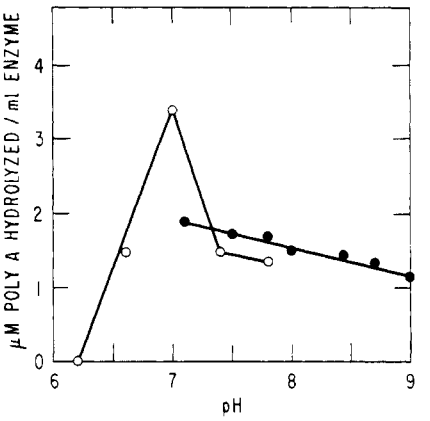

FIGURE 3: The effect of *p*H on RNAase II. The reaction mixtures and procedure were as described for the standard assay. All buffers were 0.1 M. The enzyme was a fraction VII preparation which had been passed through a Biogel P-10 column as described in the text. Symbols: ○, imidazole; ●, Tris.

enzyme at 50°. The activity remaining after heating in the presence of denatured DNA gives the usual product, namely, 5′-AMP (from poly-A). As shown in Figure 2, the stabilization by denatured DNA does not depend markedly on the DNA concentration, at least in the range studied. Native DNA is without effect. It should be pointed out that at the concentrations of DNA studied neither native nor denatured DNA had any effect on the activity of the enzyme.

Substrate does not appear to be effective in stabilizing the enzyme. Thus at 48°, the hydrolysis of poly-A or of poly-U came to a halt within 10 minutes, although less than 5% of the substrate had been hydrolyzed.

Effect of Ions and pH. The dependence of RNAase II activity on *p*H is shown in Figure 3. The results in Tris buffer are similar to those reported earlier (Spahr and Schlessinger, 1963; Spahr, 1964). In imidazole buffer there is a sharp optimum at *p*H 7 as well as a stimulation of the reaction. Spahr has reported in detail the effects of mono- and divalent cations on the hydrolysis of poly-U. Our experiments have been primarily with poly-A and are in qualitative agreement with those reported. Thus, the enzyme shows a requirement for K^+ and Mg^{2+}. Some quantitative differences that appear to

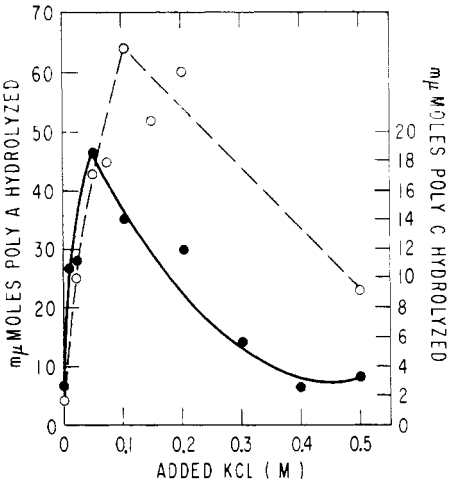

FIGURE 4: Dependence of ribonuclease II on KCl. Reaction mixtures and procedure were as described for the standard assay. The poly-A and poly-C concentrations were 2.7 and 2.9 μmoles P per ml, respectively, and KCl was as indicated. The enzyme was a fraction VII preparation that had been passed through a column of Biogel P-10 as described in text. For the poly-A experiment (○), 0.064 enzyme units and an incubation period of 1 hour were used. For the poly-C experiment (●), 0.17 enzyme units and a 2-hour incubation period were used.

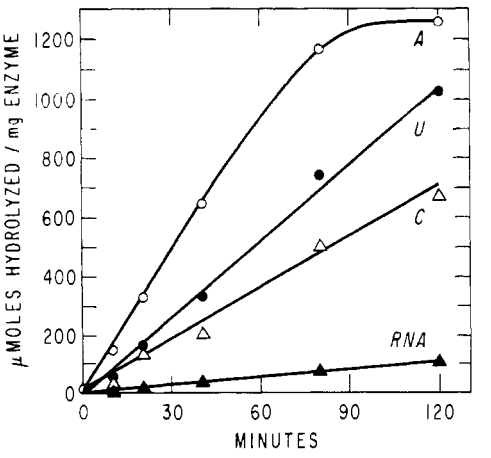

FIGURE 5: Hydrolysis of polyribonucleotides by *E. coli* RNAase II. Reaction mixtures and procedure were as described for the standard assay procedure. A separate tube was set up for each time, and reactions were stopped by the carrier RNA-NaCl procedure. Polymer concentrations (μmoles P per ml) were as follows: [^{14}C]poly-A, 3.9 (38,400 cpm per μmole), [^{14}C]poly-U, 4.1 (44,300 cpm per μmole), [^{14}C]poly-C, 4.4 (38,400 cpm per μmole), and high molecular weight yeast RNA, 6.4 (6380 cpm per μmole). For experiments with the homopolymers 0.32 μg and with RNA 3.2 μg of enzyme (fraction VII, specific activity 940) were used.

TABLE III: Effect of Mg^{2+} on Poly-A Hydrolysis.[a]

Reaction Mixture	μMoles Hydrolyzed
Complete (1.5 mM $MgCl_2$)	5.1
− $MgCl_2$	4.5
+ 1 mM EDTA–$MgCl_2$	0.1
+ 1 mM EDTA + 1 mM $MgCl_2$	5.0
+ 1 mM EDTA + 1.5 mM $MgCl_2$	6.6
+ 1 mM EDTA + 2 mM $MgCl_2$	7.2
+ 1 mM EDTA + 3 mM $MgCl_2$	5.8

[a] The reaction mixtures and procedure were as described for the standard assay. Results are expressed as μmoles hydrolyzed per hour per ml enzyme. The enzyme was a fraction VII preparation that had been passed through a Biogel P-10 column, as described in the text.

depend on the particular polymer used as substrate have been found. For example, the optimum KCl concentrations are 0.05 and 0.1 M for poly-C and poly-A, respectively (Figure 4). The enzyme used for these studies was desalted on a Biogel P-10 column. Nevertheless, some activity was detectable without added KCl (Figure 4). This may result from K^+ ions binding to the enzyme even after the gel filtration. Thus, although the sample of fraction VII passed through the P-10 column lost over 99% of its inorganic phosphate it did retain some Mg^{2+} (Table III). As shown in Table III, omitting Mg^{2+} from the assay resulted in only a small loss of activity, while EDTA gave essentially complete inhibition of RNAase II, and this inhibition was overcome by Mg^{2+}. In addition, nucleoside diphosphates cause inhibition and this, too, can be overcome by Mg^{2+}. In general, 1.5 mM $MgCl_2$ has proved optimal for poly-A hydrolysis.

Substrate Specificity and Products. POLYNUCLEOTIDES AS SUBSTRATES. Various polyribonucleotides serve as substrates for *E. coli* RNAase II. As already indicated, DNA is not degraded by the most highly purified enzyme preparations. Figure 5 shows the relative rates of hydrolysis of poly-A, poly-U, poly-C, and high molecular weight yeast RNA. The conditions used were optimal for poly-A. Experiments reported above and by others (Spahr, 1964) indicate that the difference in rates between the homopolymers may be caused by different ionic requirements for optimal rates. The dependence of the rates of hydrolysis of poly-A and poly-U on polymer concentration is shown in Figure 6. Similar results were obtained with poly-C. The shapes of these curves vary somewhat with different polymer preparations. Experiments with high molecular weight yeast RNA indicated that optimal rates were obtained with

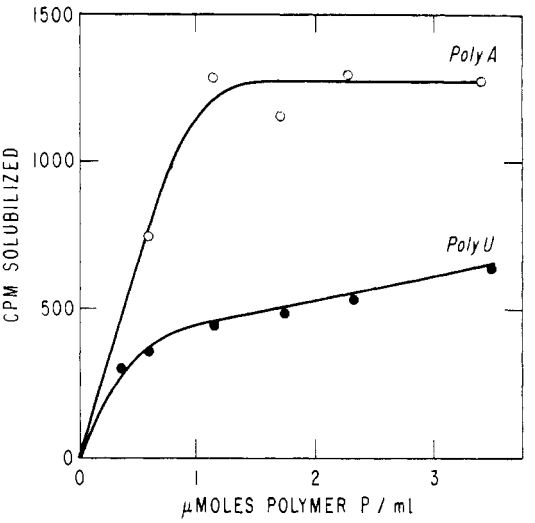

FIGURE 6: The hydrolysis of poly-A and poly-U by *E. coli* RNAase II as a function of polymer concentration. Reaction mixtures were made up as described for the standard assay and at the end of the incubation were treated by the NaCl carrier RNA procedure. Fraction VI enzyme was used.

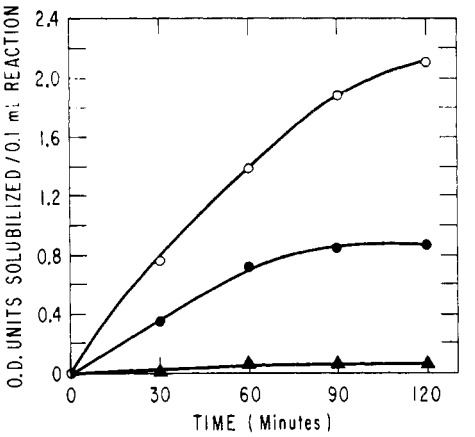

FIGURE 7: Hydrolysis of poly-UG by RNAase II. Incubation mixtures contained 0.1 M Tris, *p*H 7.5, 0.1 M KCl, 1.5 mM $MgCl_2$, 1.4 enzyme units/ml (fraction VI), and polyribonucleotides as follows: poly-A (○), 20 OD units (2 μmoles) per ml; poly-UG (43% G) (●), 20 OD units per ml; poly-UG (63% G) (▲), 17 OD units per ml. Incubation was at 37° and at the indicated times 0.1-ml samples were withdrawn and added to 0.1 ml of uranium acetate–perchloric acid mixture. After 10 minutes the tubes were centrifuged and 0.1-ml portions of the supernatant fluids diluted to 1.1 ml with H_2O. Absorbance at 260 mμ was determined. Controls without enzyme were carried through the incubation: no optical density units were made soluble.

about 5 μmoles P/ml. The general conclusion that the various polymers have similar affinities for the enzyme was confirmed in the experiments described in Table IV. Thus, addition of unlabeled poly-C to a reaction mixture inhibits [^{14}C]poly-A hydrolysis about as much as an equivalent amount of unlabeled poly-A. Similar results are shown for the effect of unlabeled poly-A on the [^{14}C]poly-C hydrolysis.

TABLE IV: Competition between Poly-A and Poly-C as Substrates.[a]

^{14}C Polymer	Unlabeled Polymer	Solubilized in 30 Minutes (cpm)
Poly-A		2442
Poly-A	Poly-A	1362
Poly-A	Poly-C	1698
Poly-C		930
Poly-C	Poly-C	564
Poly-C	Poly-A	628

[a] The reaction mixtures (0.1 ml) and procedures were as described for the standard assay. The polymer concentrations (μmoles P per ml) were as follows: [^{14}C]-poly-A, 2.7; [^{14}C]poly-C, 2.9; poly-A, 2.0; poly-C, 2.0. Fraction VII was used.

Various other polymers were tested as substrates for this reaction. As indicated previously (Singer and Tolbert, 1964) poly-I is not significantly hydrolyzed. We also found that a homopolymer of 4-*N*-methylcytidylic acid and a homopolymer of 4-*N*,*N*-dimethylcytidylic acid are not hydrolyzed under conditions that afforded extensive breakdown of poly-C itself. The rates of hydrolysis of two poly-UG preparations (43 and 63% G) are compared to the rate of poly-A hydrolysis in Figure 7.

The primary products of the hydrolysis of polyribonucleotides are the corresponding 5′-mononucleotides (Spahr and Schlessinger, 1963; Spahr, 1964; Singer and Tolbert, 1964). We have identified these products by means of paper chromatography in a variety of solvents as well as by the dephosphorylation of the mononucleotides to the corresponding nucleosides by 5′-nucleotidase of snake venom. A variety of polymers were investigated in this way: poly-A, poly-U, poly-C, high molecular weight yeast RNA, and s-RNA. For example, in a reaction identical with that shown for poly-A in Figure 5, paper chromatographic investigation of the products after 2 hours of incubation (100% degradation) showed 5′-AMP as the only product. In certain cases, after extensive digestion paper chromatograms indicated the accumulation of some oligonucleotide material. Additional evidence for the accumulation of oligonucleotides late in a digestion is shown in Figure 8. The degradation of [^{14}C]poly-U by RNAase II was followed in the usual manner, i.e., determining

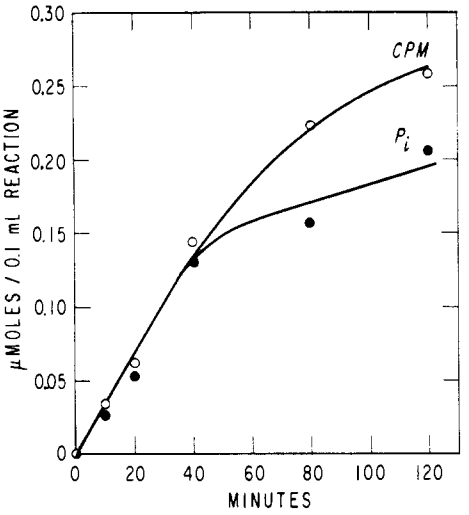

FIGURE 8: The products of the hydrolysis of poly-U by RNAase II. Two 1-ml reaction mixtures containing the components of the standard assay mixture but with 4.1 μmoles of [^{14}C]poly-U as substrate were set up. Enzyme was added to one, the other served as a control. At the indicated times after incubation at 37°, 0.1-ml samples were removed and treated with NaCl-RNA and alcohol, as described in the section on methods, in order to determine alcohol-soluble radioactive material (○). At the same time 50 μl was removed and incubated (in a total volume of 0.1 ml) with an excess of *E. coli* alkaline phosphatase. After 10 minutes at 37°, uranium acetate–perchloric acid reagent was added, the precipitate was removed by centrifugation, and inorganic phosphate was determined on an aliquot of the supernatant fluid (●). In both procedures, blank values obtained from the control tube were subtracted.

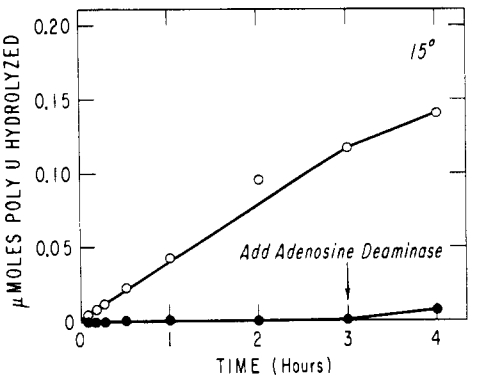

FIGURE 9: The effect of DAPR on poly-U hydrolysis at 15°. Reaction mixtures (1.0 ml) contained 0.05 M Tris, *p*H 7.5, 0.1 M KCl, 1.5 mM $MgCl_2$, 2 μmoles poly-U-P per ml (260,000 cpm per μmole), 5.6 mM DAPR where indicated, and 3.5 enzyme units per ml. Samples (0.1 ml) were removed at the indicated times and treated with NaCl-RNA and alcohol as described in the section on methods. ○, no DAPR; ●, with DAPR. Excess adenosine deaminase was added at 3 hours.

production of alcohol-soluble counts. In addition, the total amount of phosphate sensitive to alkaline phosphatase was also determined. Initially, these values are identical (as expected if 5′-UMP were the sole product), but after about 50% of the poly-U was degraded, more alcohol-soluble than phosphatase-sensitive material was produced. This suggests the formation of resistant oligonucleotides. An identical reaction mixture was set up and allowed to incubate long enough for the reaction to come to a halt. The solution was chromatographed in 1-propanol-NH_4OH-H_2O (55:10:35, v/v/v) and the material remaining at the origin was eluted and reincubated with fresh enzyme under identical conditions. Only 10% or less was degraded further to 5′-UMP; the bulk of the material was resistant to further hydrolysis.

OLIGONUCLEOTIDES AS SUBSTRATES. The implication of the preceding experiments, namely that oligonucleotides are resistant to degradation by RNAase II, was confirmed in a direct way. A series of adenine-containing oligoribonucleotides that terminated in 5′phosphomonoester end groups were tested as substrates for RNAase II. Hydrolysis was measured both by following the increase in alkaline phosphatase sensitive phosphate and by paper chromatography. In each case, the hydrolysis of poly-A was simultaneously measured. Using incubation conditions that ensured complete hydrolysis of the poly-A, no hydrolysis of pApA, pApApA, pApApApA, or pApApApApA was detected. However, when the chain length of the oligonucleotide was seven or higher hydrolysis did occur, yielding 5′-AMP and a family of oligonucleotides as products. Thymidine oligodeoxynucleotides of chain length 2 to 8 were not hydrolyzed.

EFFECT OF SECONDARY STRUCTURE. We have already described experiments which indicate the sensitivity of *E. coli* RNAase II to the secondary structure of polyribonucleotide substrates (Singer and Tolbert, 1964). The experiments with poly-UG that are described above support this interpretation. Polymers rich in guanylic acid were hydrolyzed very poorly, and it was shown earlier that the degree of secondary structure of poly-UG depends on the per cent of guanylic acid units (Singer *et al.*, 1963). Additional experiments that indicate the extent of this sensitivity involved a study of the hydrolysis of poly-U, at different temperatures, in the presence of 2,6-diaminopurine ribonucleoside. The incubation conditions were such as to allow the formation of a triple-stranded helix by the interaction of two strands of poly-U with the nucleoside (Miles and Frazier, 1965; Maxwell and Barnett, 1965; Howard and Singer, 1965). The T_m of this complex would be approximately 30°. At 15° (Figure 9), which is below the melting temperature of the complex, DAPR inhibits the hydrolysis of poly-U completely. In the experiment shown in Figure 9 adenosine deaminase, which is known to deaminate DAPR (Kornberg and Pricer, 1951), was added to the reaction mixture at 3 hours. Hydrolysis of poly-U then took place as the DAPR was removed. A series of similar (though less extensive) experiments

TABLE V: Interaction of Poly-(A + 2I) with Poly-C.[a]

Experiment	First Incubation (components)	Second Incubation (additions)	Third Incubation (cpm Released)
1	[^{14}C]Poly-A + Poly-I		94
2	[^{14}C]Poly-A + Poly-I	Poly-C	1570
3	Poly-A + Poly-I	[^{14}C]Poly-C	242
4	Poly-A	[^{14}C]Poly-C	900
5	[^{14}C]Poly-A	Poly-C	1532

[a] In the first incubation (total volume 40 μl) the reaction mixtures contained 10 μmoles of Tris buffer, *p*H 7.5, 10 μmoles of KCl, 0.15 μmoles of $MgCl_2$, 50 mμmoles of poly-A or [^{14}C]poly-A (38,400 cpm per μmole), and 100 mμmoles of poly-I where indicated. After 1 hour at 23°, 100 mμmoles of poly-C or [^{14}C]poly-C (38,400 cpm per μmole) were added as shown in the table. The second incubation (total volume 60 μl) was then carried out for 2 hours at 23°. At the end of the second incubation, 40 μl of RNAase II (fraction VII, 30 units/ml) were added. The third incubation (total volume, 100 μl) was for 15 minutes at 23°. The reaction was stopped and the mixtures were treated as described in procedure A in the section on methods. The alcohol-soluble radioactivity produced, per reaction mixture, is given in the third column.

were carried out at several temperatures between 15 and 37°. The per cent inhibition of the initial rate of poly-U hydrolysis by DAPR at each temperature was calculated, and Figure 10 shows the resulting data. Figure 10 is therefore an enzymatically determined melting curve for the poly-U-DAPR helix. At 37°, which is above the melting temperature for the 2 poly-U-DAPR complex, rapid hydrolysis of poly-U occurs although some inhibition remains (Figure 10). The reason for this inhibition is obscure,[3] especially since at 30° the inhibition is considerably less (Figure 10). Disregarding the 37° point, the T_m was calculated to be 29.5° under these conditions.

Another example of the sensitivity of ribonuclease II to secondary structure is shown in Table V. Sigler *et al.* (1962) described the displacement reaction [equation (1)] between the triple-stranded helix, poly-(A + 2I) and single-stranded poly-C, to form the double-stranded helix poly-(I + C) and single-stranded poly-A.

$$\text{poly-(A + 2I)} + 2\ \text{poly-C} \rightarrow 2\ \text{poly-(I + C)} + \text{poly-A} \quad (1)$$

In the experiment described in Table V, poly-(A + 2I) was formed in the first incubation by incubating either poly-A or [^{14}C]poly-A with 2 moles of poly-I. Then 2 moles of poly-C (or [^{14}C]poly-C) was added and the mixtures were incubated for 2 hours. Finally *E. coli* RNAase II was added, and after 15 minutes the reaction was stopped and alcohol-soluble counts were determined. The data indicate that [^{14}C]poly-A is unavailable to RNAase II degradation when it is present as poly-(A + 2I) (Table V, compare experiments 1 and 5) (Singer and Tolbert, 1964). However, when cold poly-C is added to form poly-(I + C), the poly-A is degraded (experiment 2, Table V). Similarly, when [^{14}C]poly-C is used, and unlabeled poly-(A + 2I), poly-C is unavailable for RNAase degradation (compare experiments 4 and 3, Table V). Thus the reaction shown in equation (1) is readily demonstrable by this enzymic technique. Under the conditions of our experiments the displacement reaction takes place very rapidly since, if the time of the second incubation is reduced to zero, exactly the same results are obtained.

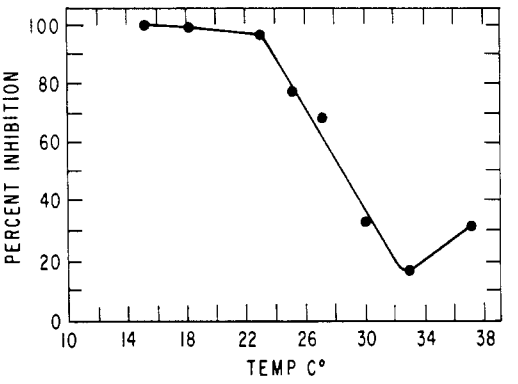

FIGURE 10: The effect of temperature on the 2 poly-U-DAPR helix. Reaction mixtures (0.1 ml) contained 0.05 M Tris, *p*H 7.5, 0.1 M KCl, 1.5 mM $MgCl_2$, 2 μmoles of poly-U-P per ml (280,000 cpm per μmole), 5.6 mM DAPR when indicated, and a suitable amount of fraction VII. Reactions were run for 15 minutes and treated by the RNA-NaCl and alcohol procedure described in the section on methods. The rate of hydrolysis of poly-U in the presence and absence of DAPR was determined at two enzyme concentrations and the results were averaged. The per cent inhibition by DAPR was calculated.

[3] In recent repeats of this experiment, there has been no inhibition of poly-U hydrolysis by DAPR at 37°.

TABLE VI: Distribution of Ribonucleases.[a]

Fraction	*E. coli* B				MRE-600				A-19			
	Protein	PNP	I[b]	II	Protein	PNP	I[b]	II	Protein	PNP	I	II
4,000 × *g* supernatant	11.6	7.8	75	202	12.7	2.5	0.0	128	12.2	2.8	5.4	208
20,000 × *g* supernatant	11.0	6.2	71	249	9.2	2.0	0.0	138	13.7	2.0	4.3	229
100,000 × *g* supernatant	5.7	6.4	11	83	6.6	0.6	0.0	79	7.0	1.4	0.6	56
Ribosomes, resuspended		2.0	70	67		0.9	0.0	34	6.0	0.7	4.1	117

[a] Cell extracts were prepared by grinding with alumina and extracting with 6 volumes of buffer B, as described in Methods. The extracts were centrifuged at 4000 × *g* for 15 minutes, at 20,000 × *g* for 1 hour, and at 100,000 × *g* for 2 hours. The ribosomes were resuspended in a volume of buffer B equal to the volume of 100,000 × *g* supernatant fluid obtained. The results are calculated as follows: protein, mg/ml; polynucleotide phosphorylase (PNP), RNAase I and RNAase II, μmoles mononucleotide produced per hour per ml extract. Each enzyme was assayed under optimal conditions for that enzyme (see section on methods). [b] We are indebted to Dr. Leon A. Heppel for these assays.

Distribution of the Enzyme. As reported previously (Singer and Tolbert, 1964; Spahr, 1964), from 25 to 40% of the RNAase II activity of crude extracts of *E. coli* B is recovered in the ribosome-free supernatant fluid obtained after centrifugation at 100,000 × *g* or after removal of RNA with protamine sulfate. In our experiments with *E. coli* B, varying proportions of the remaining units can be detected upon assay of the resuspended ribosomes (Table VI), but we have not been able to recover these units effectively by washing the ribosomes. These findings are in agreement with those published by Spahr (1964). Furthermore, these findings are typical of cells harvested at various stages during the growth cycle.

The distribution of RNAase II, as well as RNAase I, and polynucleotide phosphorylase was studied in two mutant organisms reported to be missing RNAase I. The results are summarized in Table VI. The mutant A-19 has about 7% the RNAase I activity of *E. coli* B. MRE-600 has no detectable RNAase I activity. In neither case were the levels of RNAase II or polynucleotide phosphorylase greatly different from the levels in *E. coli* B. RNAase II can be purified from MRE-600 by the procedure described in this paper. The results are essentially identical with those reported for *E. coli* B. It is of particular interest that the ratio of the rate of poly-A hydrolysis to the rate of high molecular weight yeast RNA hydrolysis is the same for purified enzyme from both *E. coli* B (see Figure 5) and MRE-600.

Discussion

As shown by Spahr (1964) and confirmed here, separation of the cell components by differential centrifugation leads to a distribution of potassium-activated phosphodiesterase activity between the ribosomes and the supernatant fraction. Spahr (1964) has purified the enzyme from ribosomes approximately 30-fold and we have purified the activity from the supernatant fraction about 600-fold. The various properties of the two preparations indicate that the enzyme being studied in each case is the same. Therefore, adopting Spahr's (1964) terminology we refer to the enzyme as *E. coli* RNAase II. Spahr (1964) reported that the instability of the enzyme in the supernatant fraction made purification difficult. We experienced little trouble with instability during the course of purification. However, as reported before (Singer and Tolbert, 1964), the fractions obtained after hydroxylapatite chromatography were quite unstable. This problem has now been overcome. As described above purified fractions can be stored satisfactorily for at least 6 months, either in dialyzed bovine serum albumin or in glycerol, at very low temperatures. The tendency for dilute protein solutions to lose enzymic activity was frequently observed. Thus, for example, the reproducible success of gel filtration depended on the inclusion of bovine serum albumin in the equilibrating medium. In view of the effect of denatured DNA on the stability of the enzyme to elevated temperatures, DNA may also be useful in storing the enzyme.

The purified enzyme has no apparent specificity for a particular type of purine or pyrimidine base. Thus poly-A, poly-U, and poly-C are all readily degraded. Poly-G has not been tested but would be expected to be resistant as a result of its secondary structure. 5′-GMP has, however, been detected as one of the breakdown products of the high molecular weight yeast RNA. The existence of poly-I as a triple helix, under the conditions of our experiments (Rich, 1958),[4] can explain the resistance of this polymer to enzymic attack (Singer and Tolbert, 1964). Only preliminary data concerning the secondary structure of the homopolymers of 4-*N*-methylcytidylic and 4-*N*,*N*-dimethylcytidylic acids are available.[5] The data suggest that at neutral *p*H the con-

[4] M. F. Singer, unpublished experiments.

[5] R. Brimacombe, personal communication.

figuration of these polymers is similar to that of poly-C. Thus it is not clear why these polymers are resistant to degradation by RNAase II and poly-C is not. It is possible that the methyl groups themselves are responsible. In any case, it is of interest to point out that unmethylated s-RNA is degraded 2–3 times faster than methylated s-RNA by RNAase II (Littauer *et al.*, 1963).

The action of RNAase II on the homopolymers appears to be primarily, if not exclusively, exonucleolytic. Thus, 5′-mononucleotides are the primary and initial products. The accumulation of resistant oligonucleotides late in the degradation of poly-U is consistent with the independently established finding that oligonucleotides are resistant to attack. The per cent of a given polymer that is resistant to degradation will clearly depend on the initial chain length of the polymer. Thus with other polymer preparations (including other poly-U preparations) degradation in certain cases went essentially to completion (for example, the hydrolysis of poly-A in Figure 5). Spar (1964) has also observed that oligonucleotides are resistant to degradation by RNAse II.

The great sensitivity of RNAase II to the macromolecular structure of its substrates is of interest from several points of view. First, the experiments already described indicate the usefulness of the enzyme as a tool for the study of polyribonucleotide secondary structure. This usefulness is enhanced by the discovery of methods for stabilizing the purified enzyme as well as the absence of pertinent contaminating enzymes. The fact that enzymic activity is not inhibited by helical polyribonucleotides (Singer and Tolbert, 1964) is also of importance here.

Second, this type of substrate specificity is pertinent to any discussion of the physiological role of RNAase II. Spahr has discussed the possibility that the RNAase II is responsible for the breakdown of both viral and synthetic messenger RNA *in vitro* that has been noted by various workers (Tissières and Watson, 1962; Barondes and Nirenberg, 1962; Sekiguchi and Cohen, 1963). The conditions used in all these experiments as well as the fact that 5′-mononucleotides (mono-, di-, and triphosphates) accumulated as products of messenger RNA breakdown (Barondes and Nirenberg, 1962; Sekiguchi and Cohen, 1963) are consistent with the action of RNAase II. Polynucleotide phosphorylase was also implicated as the degradative enzyme, especially since the degradation was stimulated by the addition of potassium phosphate (Sekiguchi and Cohen, 1963). As suggested by Spahr (1964), such a stimulation could well reflect the effect of the potassium ions on RNAase II. This proposal is supported by the data in Table VI; in extracts of *E. coli* RNAase II is at least 40 times more active in degrading polynucleotides than is polynucleotide phosphorylase. Recently, Artman and Engleberg (1964) studied the degradation, catalyzed by ribosomes, of ribosome-bound, pulse-labeled *E. coli* messenger RNA in the absence of phosphate. They concluded that RNAase I was responsible for the degradation although RNAase I is known to be latent and inactive with intact ribosomes (Elson, 1959; Spahr and Hollingworth, 1961). As shown in this report and by Spahr (1964), a considerable portion of RNAase II is bound to ribosomes. Thus the results of Artman and Engleberg may also be explained by RNAase II action. The specificity of RNAase II for polyribonucleotides in a random coil configuration is consistent with the suggestion that this enzyme is responsible for messenger RNA degradation. Thus, several lines of evidence indicate that messenger RNA is a single-stranded, random coil polyribonucleotide (Nirenberg and Matthaei, 1961; Singer *et al.*, 1963; Bautz, 1963; Szer and Ochoa, 1964; Hayashi *et al.*, 1963; Tocchini-Valenti *et al.*, 1963; Geiduschek *et al.*, 1964).

Recently, Keir *et al.* (1964) reported the partial purification of a potassium-activated phosphodiesterase from ribosomes of *Lactobacillus casei*. This enzyme is also specific for RNA and yields 5′-nucleoside monophosphates as products. It will be of considerable interest to learn whether similar enzymes exist in other bacteria.

Acknowledgment

We are grateful to Dr. Frank Howard of this laboratory for assistance with the experiments on the poly-U-DAPR helix.

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Catalytic Properties of Polymerized α-Chymotrypsin*

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ABSTRACT: The catalysis of the hydrolysis of acetyl-L-tyrosine *p*-nitroanilide by α-chymotrypsin was investigated as a function of enzyme concentration in the range 7–2500 μM. The rate measurements were carried out under experimental conditions identical (except for the presence of substrate) with those employed by Rao and Kegeles in their ultracentrifugal study of the polymerization of α-chymotrypsin. Comparison of observed rates with rates predicted on the basis of many different sets of assumptions concerning the numbers and properties of the catalytic sites on the polymeric species present was made by means of an IBM 7094 computer.

Although the results do not permit a definitive statement concerning the effect of polymerization on the catalytic properties of the enzyme, it seems necessary to conclude that the polymeric species are not completely inactive. Satisfactory agreement between observed and predicted rates may be obtained with a number of different kinetic models.

The association of α-chymotrypsin has been studied by several investigators (Schwert, 1949; Schwert and Kaufman, 1951; Steiner, 1954; Massey *et al.*, 1955; Tinoco, 1957; Rao and Kegeles, 1958; Winzor and Scheraga, 1963, 1964), using light scattering, depolarization of fluorescence, ultracentrifugation, and gel filtration. Martin and Niemann (1958) determined the activity of the enzyme as a function of enzyme concentration under conditions where they considered that only monomeric and dimeric species were present; they concluded that although both species can combine with the substrates they employed, only the compounds formed with the monomeric enzyme can decompose to give reaction products at a significant rate.

The most extensive study of the polymerization of α-chymotrypsin is that of Rao and Kegeles (1958). These authors employed the Archibald (1947) ultracentrifuge method, and concluded that under the conditions of their experiments dimers and trimers are present in addition to the monomers. In an effort to obtain information concerning the catalytic properties of the polymeric species of the enzyme, we have carried out kinetic measurements over a wide range of enzyme

* From the Department of Molecular Biology and Biophysics, Yale University, New Haven, Conn. *Received February 18, 1965.* This work was supported in part by a grant (GM-04725) from the National Institutes of Health, U.S. Public Health Service.