THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 243, No. 6, Issue of March 25, pp. 1133-1139, 1968
Printed in U.S.A.

The Purification and Properties of a Ribonuclease from *Salmonella typhimurium* Extract*

(Received for publication, January 31, 1967)

KALPANA CHAKRABURTTY‡ AND D. P. BURMA

From the Department of Biochemistry and Biophysics, College of Medical Sciences, Banaras Hindu University, Varanasi-5, U. P., India

SUMMARY

A ribonuclease has been purified about 140-fold from extracts of *Salmonella typhimurium* through ammonium sulfate fractionation, gel filtration, and DEAE-cellulose chromatography. The optimum pH for the hydrolytic breakdown of RNA is 7. The enzyme hydrolyzes polyadenylic acid (poly A), polycytidylic acid (poly C), and polyuridylic acid (poly U) at much faster rates than transfer RNA. Polyinosinic acid (poly I) was not hydrolyzed at all. Higher concentrations of poly A and poly U (above 200 μg per ml) were inhibitory. A mixture of poly A and poly U in the proportion 1:2, which is known to produce maximum secondary interaction, is also inhibitory. These results indicate that the secondary structures of nucleic acids interfere with the action of the nuclease. The first product of hydrolysis is a 2′,3′-cyclic nucleotide which is poorly hydrolyzed to the 3′-nucleotide. The enzyme behaves as an endonuclease. The properties of the *S. typhimurium* nuclease have been compared with those of RNase I of *Escherichia coli*. Although there are some differences, the *S. typhimurium* nuclease is like RNase I in its mode of action.

Two ribonucleases of *Escherichia coli* have been well characterized. One is the RNase I, according to Spahr's terminology (1), and the other is the K^+-activated phosphodiesterase or RNase II. Elson (2) first reported the presence of RNase in the ribosomes of *E. coli*, the properties of which resembled those of pancreatic RNase. Spahr and Hollingworth (3) purified the enzyme about 730-fold and studied some of its properties. Owing to its association with the 30 S component of isolated ribosomes, the enzyme was thought to be involved in the metabolism of RNA (4, 5). It was shown later, however, that when *E. coli* cells are converted to spheroplasts, most of the RNase is released into the medium (6, 7). Furthermore, when partially purified *E. coli* RNase was mixed with the ribosomes, the enzyme was firmly and exclusively absorbed by the 30 S subunit (6). Studies with spheroplasts low in RNase activity (8) and with mutants lacking in RNase I activity (9) ruled out the involvement of this enzyme in the breakdown of messenger RNA. Wade (10) and Wade and Lovett (11) were the first to recognize two ribonucleases in *E. coli* ribosomes. The K^+ activation of the phosphodiesterase or RNase II of *E. coli* was demonstrated by Spahr and Schlessinger (12). The enzyme preparation described by these authors, a crude extract from which ribosomes had been removed by centrifugation, hydrolyzed polyribonucleotides, including poly U[1] and the messenger RNA of bacteriophage T2, to 5′-nucleotides. Thus, the enzyme was distinguishable from the RNase I of *E. coli* which produces 3′-mononucleotides. This enzyme was partially purified by Spahr (1). It was subsequently purified about 600-fold by Singer and Tolbert (13) from extracts of *E. coli* and was found to be specific for the single-stranded polyribonucleotides. Helical forms of polyribonucleotides are not hydrolyzed. They do not, however, inhibit the hydrolysis of the single-stranded chains. This enzyme is found in the supernatant fraction, although it does bind to ribosomes to some extent (14). The action of RNase II on polynucleotides appears to be primarily, if not exclusively, exonucleolytic. In addition to K^+, a divalent cation, Mg^{++}, was required for activity. In view of the characteristics of the enzyme, the possibility that RNase II is concerned with the breakdown of messenger RNA has been implied.

While the polynucleotide phosphorylase was being purified from extracts of *Salmonella typhimurium*, the rapid disappearance of poly A in the absence of phosphate was observed. Subsequently, a ribonuclease has been partially purified from the extracts. The study of the characteristics of this enzyme indicates its close resemblance to the RNase I of *E. coli*. The purification and properties of this nuclease will be described.

EXPERIMENTAL PROCEDURE AND RESULTS

Materials—2′,3′-Cyclic-AMP and 2′,3′-cyclic-CMP were prepared according to the method of Khorana (15). 2′,3′-

* This work was supported by grants received from the Council of Scientific and Industrial Research, New Delhi, India, and from the Department of Atomic Energy, Government of India.

‡ Senior Research Fellow under a project sponsored by the Council of Scientific and Industrial Research, New Delhi, India.

[1] The abbreviations used are: poly A, polyadenylic acid; poly U, polyuridylic acid; poly C, polycytidylic acid, poly I, polyinosinic acid.

Cyclic-GMP and 2′,3′-cyclic-UMP were procured from Schwarz BioResearch. All the cyclic nucleotides were purified by chromatography. Poly A, poly U, poly C, poly I, and Sephadex G-100 were obtained through the courtesy of Dr. Maxine Singer and Dr. Gary Felsenfeld of the National Institutes of Health. tRNA was prepared from yeast by the method described by Monier, Stephenson, and Zamecnik (16). Alumina (A-301) was the product of Aluminium Company of America; DEAE-cellulose was from Carl Schleicher & Schuell Company.

Assay—The following method of assay was adopted to follow the nuclease through various purification steps. The incubation was carried out at 35° for 10 min in 25 μmoles of Tris (pH 7.0), 50 μg of poly A, and the requisite amount of enzyme protein, in a total volume of 0.25 ml. The reaction was stopped by the addition of 0.05 ml of 50% trichloracetic acid containing 0.75% uranyl acetate. Carrier serum albumin (0.2 mg) was added before the addition of trichloracetic acid for complete precipitation of polynucleotides and proteins. After ½ hour the precipitate was removed by centrifugation. The release of acid-soluble nucleotides was measured at 260 mμ in a Zeiss ultraviolet spectrophotometer after suitable dilution. The blank against which the readings were taken contained trichloracetic acid and uranyl acetate in the same proportions as in the diluted reaction mixtures. The amount of nucleotide released was calculated on the basis of the molar extinction coefficient of AMP as 15.4×10^3. One unit of enzyme was defined as that amount which catalyzes the release of 1 μmole of acid-soluble nucleotide under the conditions of incubation as described above. The specific activity was defined as the units per mg of protein. Protein was measured by the Folin reagent, as described by Lowry *et al.* (17).

Purification—All the operations were carried out at 0° unless otherwise stated.

Crude Extract—*S. typhimurium* cells were grown at 37° in minimal medium containing 0.5% glucose and were harvested during the logarithmic phase of growth and stored at −20°. Stored cells were used for the preparation of crude extracts. The cells (5 g, wet weight) were ground with a double amount of alumina (A-301) in a mortar and pestle. The ground material was suspended in 30 ml of 0.05 M Tris, pH 7.4, containing 0.2 μmole of mercaptoethanol per ml. The suspension was centrifuged at 10,000 × *g* for 30 min to separate alumina and unbroken cells. The supernatant fraction (30 ml) contained 9.0 mg of protein per ml.

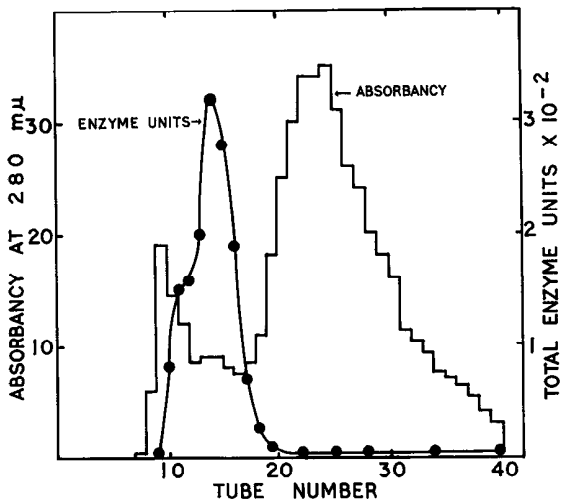

FIG. 1. Elution of *S. typhimurium* nuclease from Sephadex G-100 column.

Ammonium Sulfate Fractionation I—To the crude extract (30 ml) were added 6.8 g of ammonium sulfate to 40% saturation. The addition was done in small portions with constant stirring with the help of a magnetic stirrer. After the addition, the mixture was kept at 0° for 10 min to allow complete precipitation. The protein was removed by centrifugation at 10,000 × *g* for 10 min. To the supernatant (30 ml) were gradually added 4.6 g of ammonium sulfate, with constant stirring as described above, until 65% saturation was attained. The precipitated protein was collected by centrifugation after 10 min and was dissolved in 8.5 ml of the above mentioned Tris buffer containing 2×10^{-4} M mercaptoethanol. The protein concentration of this fraction was 10 mg per ml.

Fractionation on Sephadex G-100 Column—The ammonium sulfate fraction (40 to 65%) was passed through a Sephadex G-100 column. The gel filtration technique was carried out according to the method described by Andrews (18). Sephadex G-100 (grain size, 40 to 120 μ) was allowed to swell in water for 2 to 3 days, and the smallest particles were removed by decantation. After deaeration the gel slurry was poured into a column of 3-cm diameter until a bed of 50-cm height was obtained. The column was then equilibrated with 0.05 M Tris, pH 7.4, containing 0.1 M KCl and 2×10^{-4} M mercaptoethanol. The ammonium sulfate fraction (8.5 ml) was carefully placed on the top of the column under the buffer already present. The density of the solution was increased by the addition of sucrose (10 mg) to facilitate the layering. The eluting buffer reservoir was connected with the column when a very thin layer of the enzyme solution was left behind. Fractions of 10 ml were collected. Each collection took about 30 min. All the fractions were assayed for protein by measuring absorption at 280 mμ, and for enzyme activity by the assay method already described. Fig. 1 shows the elution pattern and also the enzyme activity of the fractions. Two major peaks in terms of absorbance at 280 mμ were obtained. The enzyme activity did not coincide with either of these two peaks but was confined to the tubes between the peaks. To avoid contamination with other proteins, only the tubes numbered 13 to 15 were pooled. This represented most of the recoverable enzyme activity. The amount of protein, however, was about 20% of the total protein applied to the column in terms of absorption at 280 mμ. There was a considerable amount of fractionation of nucleic acids as well. The two peaks absorbing at 280 mμ had $A_{280}:A_{260}$ ratios of 0.4 and 0.55, an indication of the presence of considerable amounts of nucleic acids; whereas the pooled fractions had an $A_{280}:A_{260}$ ratio of 0.9, an indication of small amounts of nucleic acids in these fractions. The total volume of the pooled fractions was 30 ml, with 0.7 mg of protein per ml.

Ammonium Sulfate Fractionation II—To the Sephadex eluate (30 ml) were added 11.9 g of ammonium sulfate to bring the solution to 65% saturation. The precipitated protein was collected by centrifugation and was dissolved in 5 ml of the above mentioned buffer. The protein concentration of the fraction was 4 mg per ml. Although no purification was achieved by this procedure, the step was essential for concentrating the enzyme.

Dialysis of Ammonium Sulfate Fraction II—The enzyme preparation obtained by concentrating the Sephadex eluate with ammonium sulfate was dialyzed for 4 hours with three changes

against 0.01 M Tris, pH 7.4, containing 2×10^{-4} M mercaptoethanol (total volume, 3 liters). During the dialysis some protein was denatured, as indicated by precipitation. The denatured protein with no enzyme activity was removed by centrifugation at 25,000 $\times$ *g* for 20 min. The volume of the supernatant was 6.5 ml, and the protein concentration was 2.2 mg per ml. Although there was a considerable loss of activity due to dialysis (about 40%), this step could not be avoided because ammonium sulfate interfered with the subsequent step (chromatography on DEAE-cellulose), as described below.

DEAE-cellulose Column Chromatography—The dialyzed enzyme was further fractionated through a DEAE-cellulose column. The column was prepared according to the method described by Peterson and Sober (19). DEAE-cellulose (15 g) was allowed to swell in water. The material was washed with 1 M NaOH, water, 1 M HCl, water, alcohol, water, and 1 M NaOH in the sequence shown, and finally with water so as to obtain a neutral suspension in a volume of 1 liter. Part of this suspension (120 ml) was deaerated and poured into a glass chromatographic column (diameter, 1.4 cm) packed with glass wool at the bottom. The column height after packing was 12 cm, and the calculated bed volume was about 19 ml. The packed column, after being washed with water, was equilibrated with 0.02 M Tris, pH 7.4, containing 2×10^{-4} M mercaptoethanol. The dialyzed protein solution (6.5 ml) was loaded slowly onto the column during a period of about 45 min. After the adsorption of the protein solution, about 50 ml of the same buffer were allowed to pass through the column so as to elute the unadsorbed proteins. The eluate was collected in 5-ml fractions. The column was subsequently eluted with the same buffer containing increasing amounts of KCl. About 70% of the total enzyme activity emerged from the column during washing with buffer, which indicated that the enzyme was not adsorbed by DEAE-cellulose. The remaining activity (about 30%) could not be accounted for. The protein concentrations of those fractions containing activity were very low. Only the tubes numbered 3 and 4 were pooled to avoid contamination with other proteins. The total volume of the pooled fractions was 10 ml, and the protein content was about 0.1 mg per ml. About 70% of the total enzyme activity was recovered.

Ammonium Sulfate Fractionation III—To concentrate the DEAE-cellulose column eluate, 6 g of ammonium sulfate were added to obtain 90% saturation. The precipitated protein was collected by centrifugation and was dissolved in 2.0 ml of 0.05 M Tris, pH 7.4, containing 2×10^{-4} M mercaptoethanol. The protein concentration was 0.4 mg per ml. This step led to only a slight increase in specific activity, with practically no loss of total units. The step was used for concentrating the enzyme. About a 140-fold purification of the nuclease was obtained by these procedures, with a total recovery of 40% of the activity present in the crude extract. Table I presents the total units, specific activity, etc., at various purification stages. The purified enzyme had an $A_{280}:A_{260}$ ratio of 0.9.

Proportionality of Activity with Amount of Enzyme Protein—The release of trichloracetic acid-soluble nucleotide from poly A by the action of the partially purified nuclease of *S. typhimurium* was studied with the use of different amounts of enzyme protein. The conditions of incubation were the same as described under "Assay." Varying amounts (0.15 to 0.8 μg) of the Ammonium Sulfate Fraction III (final step) were used. The results are shown in Fig. 2. The rate of the reaction was proportional to the amount of protein up to about 0.4 μg, beyond which there was deviation from linearity.

TABLE I

Purification of ribonuclease from extracts of Salmonella typhimurium

Fraction	Volume	Total units	Specific activity
	ml		*units/mg protein*
Crude	30.0	600	2.2
Ammonium sulfate I (40 to 65%)	8.5	800	9.4
Sephadex eluate	30.0	780	37.0
Ammonium sulfate II (0 to 65%)	5.0	650	32.5
Dialyzed fraction	6.5	390	27.3
DEAE-cellulose eluate	10.0	260	260.0
Ammonium sulfate III (0 to 90%)	2.0	240	300.0

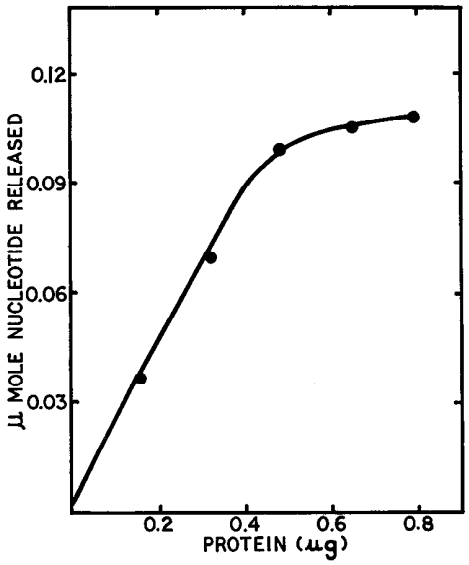

FIG. 2. Variation of the rate of hydrolysis of poly A with th amount of protein added.

Optimum pH of Reaction—The variation with pH of the rate of degradation of poly A by the *S. typhimurium* nuclease is shown in Fig. 3. The assay conditions were the same as described before. The amount of enzyme protein used was 0.4 μg (Ammonium Sulfate Fraction III). The activity of the enzyme varied considerably depending on the type of buffer used. With Tris buffer, maximum activity was displayed at pH 7. Unfortunately, however, buffering action cannot be obtained with Tris below pH 7. In phosphate buffer the activity at pH 6 was considerably greater than that at pH 7. With phosphate buffer the optimum pH could not be determined, as its buffering capacity is small below pH 6. The rate was the same with these two buffers at pH 7. If, however, succinate buffer was used instead of phosphate, the activity was much less at pH 6. In the alkaline pH range, the rate decreased considerably with the increasing pH. As mentioned before, Tris (pH 7) was used in routine assays.

Time Course of Reaction—The extent of release of acid-soluble, ultraviolet-absorbing material from the various substrates

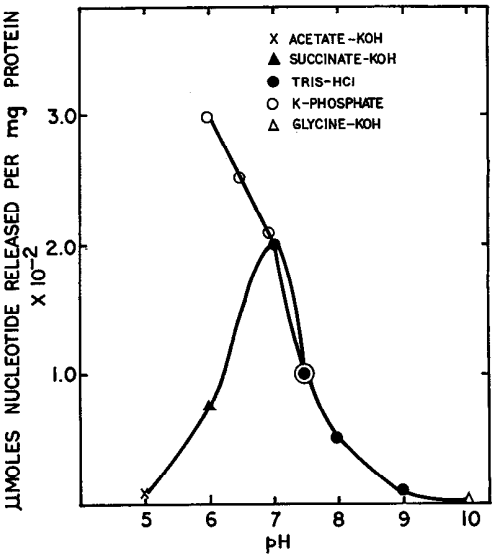

FIG. 3. Variation of the rate of hydrolysis of poly A with the pH of the incubation mixture.

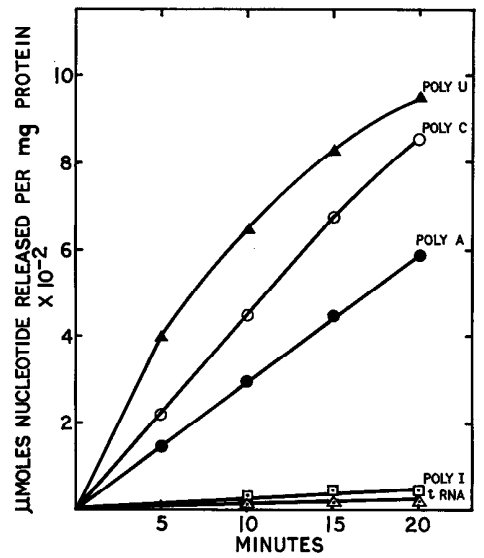

FIG. 4. Time course of hydrolytic breakdown of polyribonucleotides.

(poly A, poly U, poly C, poly I, and tRNA) at different periods of incubation is shown in Fig. 4. Each incubation was carried out in a total volume of 2 ml containing 200 μmoles of Tris (pH 7.0), 400 μg of the polyribonucleotide, and 1.2 μg of Ammonium Sulfate Fraction III. The incubation was carried out at 35° and a 0.25-ml aliquot was removed at requisite intervals. The subsequent steps of assay were the same as described before. With poly A or poly C as substrate, the reaction proceeded linearly with time, but with poly U as substrate the rate deviated somewhat from linearity. In the early stages the rate of the reaction with poly U was almost 3 times as fast as with poly A. The rate with poly C was about double that for poly A. Although the reaction rate was fastest with poly U, poly A was routinely used in the assay. Poly I, however, remained practically unhydrolyzed. K^+-activated phosphodiesterase, which is also specific for a single-stranded structure, is known not to hydrolyze poly I (13). It was assumed in that case that poly I had the ordered structure under the incubation conditions (20). It is not clear whether the same is true in the present case. tRNA also remained practically unhydrolyzed under these conditions (Fig. 4). In the presence of a large excess of the enzyme, tRNA was degraded to the extent of 40%. The secondary structure of tRNA might be responsible for this observation, as will be discussed later.

Stoichiometry of Hydrolysis of Homopolymers—Although the rates of hydrolysis of polyribonucleotides varied with the nature of the polyribonucleotide, the three homopolymers (poly A, poly C, and poly U) were converted entirely to products soluble in uranyl acid-trichloracetic acid in 20 to 30 min by the use of excess enzyme in the incubation mixture. The incubation was carried out at 37° in a total volume of 1.5 ml containing 150 μmoles of Tris, pH 7.0; polyribonucleotide, 0.9 μmole; and Ammonium Sulfate Fraction III (2, 1.5, and 1.0 μg for poly A, poly C, and poly U, respectively). Aliquots (0.25 ml) were removed at requisite intervals, the reaction was stopped, and the amount of ribonucleotide released was measured as described before.

Effect of Substrate Concentration on Activity of Enzyme—Under the identical incubation conditions mentioned above and in the presence of 0.16 μg of enzyme protein (Ammonium Sulfate Fraction III), the concentrations of the substrates, poly A and poly U, were varied. The results are shown in Fig. 5. With poly U, the rate of the reaction increased linearly with the increase in amount of the substrate up to about 50 μg, beyond which there was decrease in the rate with increase in the substrate concentration. The results with poly A were similar. In this case, however, the increase in the rate deviated from linearity beyond 4.5 μg. Beyond 50 μg there was marked decrease in the rate. Apparently higher substrate concentrations are inhibitory to the enzymatic hydrolysis of the polymers.

Effect of Secondary Structure of Homopolymers on Rate of Hydrolysis by Nuclease—From the previous experiments with poly A, poly U, and tRNA, it was apparent that this nuclease

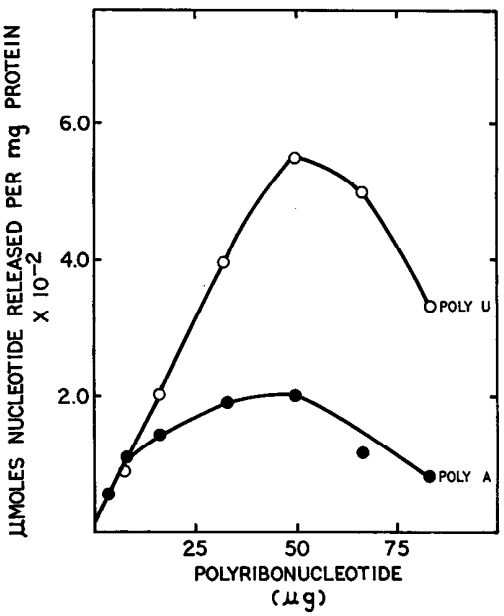

FIG. 5. Dependency of the rate of hydrolysis of polyribonucleotides on their concentrations.

attacked the single-stranded polymers such as poly A and poly U at much faster rates than it attacked the double-stranded nucleic acid, tRNA. In order to decide whether the secondary structure of nucleic acids is inhibitory to the nuclease, poly A and poly U mixed in various proportions in the presence of Mg^{++} were used as substrates for the enzyme. It is well known that this type of mixing in the presence of salts produces secondary interactions between the chains (21). Two sets of experiments were designed. In one set, increasing amounts of poly U were added while poly A was kept at a fixed concentration (13.5 μg), whereas in the other set an increasing amount of poly A was added while poly U was kept constant (13.5 μg). Other ingredients of the incubation mixture were the same except that NaCl (0.1 M) and $MgCl_2$ (0.001 M) were included. It was previously established that addition of $MgCl_2$ and NaCl at these concentrations did not affect the rate of the reaction as catalyzed by the partially purified enzyme. The results are presented in Fig. 6. It is evident that in both cases increasing the amount of one homopolymer added to the other decreased the rate of the reaction, but at higher concentrations there was tendency to regain the original activity. Thus, the rate became minimal at certain fixed concentrations of the two. In both cases this occurred when poly A and poly U were present in a ratio of 1:2. It is already known that, under the conditions mentioned above, poly A and poly U in these proportions produce maximum secondary interaction (21).

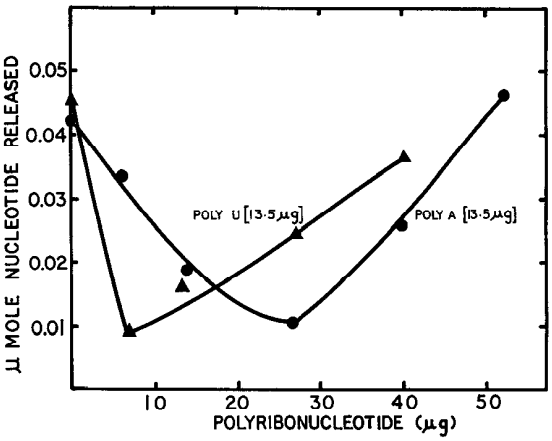

FIG. 6. Rate of hydrolysis of the mixtures of poly A and poly U present in various proportions.

Effect of Temperature on Stability of Enzyme—To study the effect of temperature on the stability of the enzyme, the Ammonium Sulfate Fraction III was diluted five times with 0.05 M Tris, pH 7.0, and the diluted solution was incubated at indicated temperatures (Fig. 7) for 15 min, after which the solutions were immediately cooled in ice. An aliquot of the incubated enzyme solution was used for assay under standard conditions. Fig. 7 shows the extent of inactivation at different temperatures. The enzyme withstood the incubation at 45°. At higher temperatures, however, it started to lose activity at increasing rates, and the inactivation was complete at 95°.

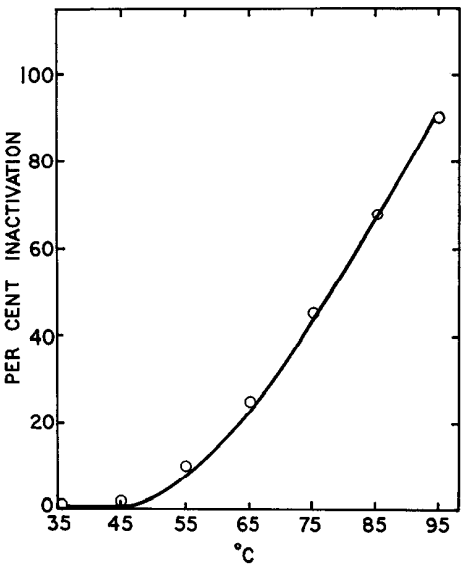

FIG. 7. Effect of temperature on the stability of the enzyme

Effect of Na^+, K^+, NH_4^+, Mg^{++}, and EDTA on Enzyme Activity—The nuclease activity, as measured in the extract of the cells of *S. typhimurium*, was found to be inhibited by Mg^{++} and considerably enhanced in the presence of EDTA. Similarly, the activity in the extracts was stimulated by the addition of Na^+, K^+, and NH_4^+. Therefore, the effects of the metal ions and EDTA on the activity of the purified RNase were studied. None of these had any effect on the purified nuclease. Subsequent studies have indicated that there is an inhibitor in the extracts which inhibits this nuclease activity, but only in the presence of Mg^{++}.[2] Evidence for the presence of K^+-activated phosphodiesterase type of activity (RNase II) in the extracts of *S. typhimurium* was also obtained. The presence of both K^+ and Mg^{++} is essential for the maximum activity of this enzyme.

Determination of Nature of Hydrolytic Breakdown of Homopolymers as Catalyzed by Nuclease—In order to determine whether the nuclease isolated from the extracts of *S. typhimurium* was an endonuclease or exonuclease, the following experiment was designed. The incubation conditions were the same as those already described except that less enzyme and a lower temperature of 25° were used, so that the reaction proceeded slowly. At different times, samples were removed, directly spotted on Whatman No. 3MM filter paper, and dried immediately so that further reaction was minimized. The solvent system used was isobutyric acid, NH_4OH, 0.1 M Na_2-EDTA, and H_2O in the proportions of 66:1:1.5:31.5, as described by Beers (22). The chromatogram was developed for 12 hours in the descending direction. Fig. 8 shows the nature of the products formed by the action of the enzyme. It is apparent that intermediate high molecular weight ultraviolet-absorbing materials were formed on short term incubations. With increasing time of incubation, however, increasing amounts of AMP were produced. On chromatography with a different solvent system (95% ethanol and 1 M ammonium acetate in the proportion of 4:6) modified from the method of Reddi (23), the oligonucleotides formed during the hydrolysis of poly A become nicely separated. In this case a mixture incubated for 30 min could be resolved into seven oligonucleotide bands. In all probability, the enzyme is of the endo type, because otherwise only AMP would have been expected as the product.

Identification of Final Product—In order to find out the nature of the final products formed by the degradation of poly A and poly U, a paper chromatographic method of separation of nucleotides was followed. For the complete degradation of the

[2] K. Chakraburtty and D. P. Burma, unpublished observations.

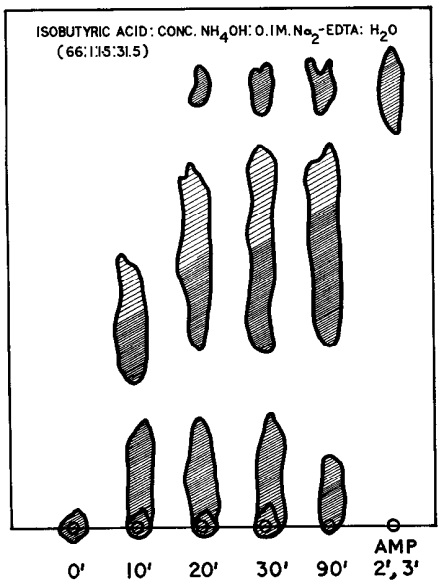

FIG. 8. Nature of the hydrolytic breakdown of poly A

homopolymers, excess of enzyme was used. A typical incubation mixture, containing 0.1 M Tris (pH 7.0), 50 μg of polyribonucleotide, and an excess of enzyme protein, was incubated for 1 hour at 35°. The reaction mixture was then directly spotted on Whatman No. 1 filter paper. Authentic samples of 2',3'-AMP and 5'-AMP were also run, along with the reaction mixture. The solvent system as described by Magasanik *et al.* (24) was used with slight modifications in composition (isobutyric acid and 0.5 M NH_4OH mixed in the proportion of 10:3). The chromatogram was developed in the descending manner. With AMP, the development was for 13 hours, while for UMP it required 24 hours for the separation of 2',3'- and 5'-nucleotides. It was evident from the chromatographic behavior of the products of degradation of poly A and poly U that the product in each case was 2',3'- and not 5'-nucleotide. This was further supported by the fact that the product was not hydrolyzed by snake venom 5'-nucleotidase.

The question arises, however, whether a mixture of 2'- and 3'-nucleotides is formed, or only one type of nucleotide is actually produced, or whether cyclic 2',3'-nucleotide is the product. To answer this question the solvent system (saturated ammonium sulfate-isopropanol-1 M sodium acetate (pH 6.0) mixed in the proportions of 80:2:18), as described by Lipsett (25) for the separation of 2'- and 3'-nucleotides was used. The chromatogram was allowed to run for 12 hours. Authentic samples of 2'- and 3'-AMP and 2',3'-cyclic-AMP were chromatographed. The major product of the hydrolysis had the same R_F as the cyclic nucleotide. This was confirmed by mixed chromatography as well. A small amount of 3'-nucleotide, however, was found to be present. The major product, suspected to be cyclic 2',3'-mononucleotide, was eluted from the paper and was again rechromatographed after the isolated material was hydrolyzed with 0.1 N HCl for 24 hours at 37°. This treatment led to the conversion of the material mostly to 3'-mononucleotide. A small amount of 2'-mononucleotide might have been formed as well. The same was found to be true for poly U. Thus, it appears that the major product of degradation of homopolymers is cyclic nucleotide, which is most probably slowly degraded to 3'-nucleotide. This was also confirmed by using cyclic nucleotides in the incubation mixture and adding excess of enzyme. On prolonged incubation, only small amounts of 3'-nucleotide were formed.

Hydrolysis of Cyclic Nucleotides—From the results already presented, it appears that cyclic nucleotides are very poorly hydrolyzed by the RNase from *S. typhimurium*. The cyclic nucleotides were tested as substrates for the RNase by using excess of enzyme and carrying out the incubation for 30 hours at 37°. Standard assay conditions were employed, except that 1 μmole of cyclic nucleotide was used as the substrate in place of poly A, and 10 μg of enzyme protein were added. Suitable controls without enzyme were also run to correct the final results for the spontaneous breakdown of the cyclic nucleotides. Aliquots from both the control and the incubation mixtures were spotted on Whatman No. 3MM filter paper. Isopropanol-ammonia-water in the ratio 7:1:2 (26) was used as the developing solvent. After chromatography the spots were located by ultraviolet light and eluted with water. Amounts were calculated from the absorption at 260 mμ. Under these conditions, 10% of both cyclic-AMP and cyclic-UMP were hydrolyzed, whereas cyclic-GMP and cyclic-CMP remained practically unhydrolyzed.

DISCUSSION

The enzyme which has been partially purified from the extracts of *S. typhimurium*, the properties of which have been described in this communication, behaves much like the RNase I of *E. coli*, particularly because it hydrolyzes preferentially single-stranded polyribonucleotides. This property of *E. coli* RNase I was demonstrated with naturally occurring polyribonucleotides as substrates (3). It appears, however, from the results of others and from those presented here that homopolymers such as poly A and poly U will also be rapidly broken down by the *E. coli* RNase I. As was mentioned in the introduction, the nuclease from *E. coli* has been purified about 730-fold (3), and the properties of the purified enzyme have been described. Although the *S. typhimurium* enzyme has been purified only about 150-fold, the preparation is practically free of other activities. No DNase activity was detectable, and no nucleotidases or nucleosidases were present, since, even after prolonged hydrolysis, nucleotides were the only products. It seems appropriate, therefore, to compare the properties of the enzymes from *E. coli* and *S. typhimurium*. The optimum pH of the *S. typhimurium* nuclease is in the neutral range (pH 7.0), provided the effect of phosphate on the hydrolytic breakdown is ignored, whereas the pH optimum of the *E. coli* enzyme lies in the alkaline range (pH 8.1). Phosphate enhances the activity of *S. typhimurium* nuclease, even in the acidic pH range. The activity of the *E. coli* nuclease was found to be enhanced by NaCl, KCl, and NaF, but inhibited by magnesium salts and by sodium dodecyl sulfate. The purified *S. typhimurium* nuclease, however, was not affected by these reagents. In crude extracts of *S. typhimurium*, the nuclease activity was stimulated by monovalent cations and inhibited by Mg^{++}, but K^+-activated phosphodiesterase type of activity was detectable in the extracts. Both *E. coli* and *S. typhimurium* enzymes behaved as endonucleases, and the primary product of hydrolytic breakdown was found to be 2',3'-cyclic phosphate, although traces of nucleoside 3'-phosphate were also formed. The *E. coli* nuclease hydrolyzes the cyclic adenylic and cytidylic acids about 5 times faster than cyclic guanylic and uridylic acids. In the initial stages of RNA digestion, the enzyme releases more

adenylic and uridylic acids than guanylic and cytidylic acids. The cyclic nucleotides of adenine and uracil were very poorly hydrolyzed, whereas neither cyclic guanylate nor cyclic cytidylate was hydrolyzed at all. The rates of hydrolysis of the homopolymers have been shown to be in the sequence poly U > poly C > poly A. Poly G was not tested because of its nonavailability.

The other important propety of *E. coli* RNase I is its strong affinity for ribosomes. Because of this, mixtaken conclusions as to the localization of the enzyme were made in the beginning. Spahr and Hollingworth's source of the purified enzyme was ribosomes which were disintegrated by treatment with urea (3). In the present work the affinity of the enzyme for the ribosomes was not tested, but from the source and mode of purification its association with and affinity for ribosomes seem to be unlikely. A direct test is necessary, however, before a final conclusion can be drawn. Thus, the enzymes may differ in some of their properties, although their modes of action appear to be practically the same.

The physiological function of this type of nuclease is practically unknown. It has already been noted that it does not seem justified to implicate RNase I in the degradation of messenger RNA. The only enzyme which seems suitable for this function is the K^+-activated phosphodiesterase. 5′-Nucleotides which are formed by the attack of this enzyme can be readily converted into nucleoside di- and triphosphates, and these may be reincorporated into RNA. In this way the cyclic process of the degradation of messenger RNA and its resynthesis could be continued. The 3′-nucleotides formed by the action of RNase I are unlikely to be involved in this process.

REFERENCES

1. Spahr, P. F., *J. Biol. Chem.*, **239,** 3716 (1964).
2. Elson, D., *Biochim. Biophys. Acta*, **27,** 216 (1958); **36,** 372 (1959).
3. Spahr, P. F., and Hollingworth, B. R., *J. Biol. Chem.*, **236,** 823 (1961).
4. Tal, M., and Elson, D., *Biochim. Biophys. Acta*, **76,** 40 (1963).
5. Artman, M., and Engelberg, H., *Biochim. Biophys. Acta*, **80,** 517 (1964).
6. Neu, H. C., and Heppel, L. A., *Proc. Nat. Acad. Sci. U. S. A.*, **51,** 1267 (1964).
7. Neu, H. C., and Heppel, L. A., *Biochem. Biophys. Res. Commun.*, **14,** 109 (1964).
8. Artman, M., and Engelberg, H., *Biochim. Biophys. Acta*, **95,** 687 (1965).
9. Gesteland, R. F., *J. Mol. Biol.*, **16,** 67 (1966).
10. Wade, H. E., *Biochem. J.*, **78,** 457 (1961).
11. Wade, H. E., and Lovett, S., *Biochem. J.*, **81,** 319 (1961).
12. Spahr, P. F., and Schlessinger, D., *J. Biol. Chem.*, **238,** PC2251 (1963).
13. Singer, M. F., and Tolbert, G., *Science*, **145,** 593 (1964).
14. Singer, M. F., and Tolbert, G., *Biochemistry*, **4,** 1319 (1965).
15. Khorana, H. G., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. VI*, Academic Press, New York, 1963, p. 649.
16. Monier, R., Stephenson, M. L., and Zamecnik, P. C., *Biochim. Biophys. Acta*, **43,** 1 (1960).
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
18. Andrews, P., *Biochem. J.*, **91,** 222 (1964).
19. Peterson, E. A., and Sober, H. A., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. V.*, Academic Press, New York, 1962, p. 3.
20. Rich, A., *Biochim. Biophys. Acta*, **29,** 502 (1958).
21. Felsenfeld, G., and Rich, A., *Biochim. Biophys. Acta.*, **26,** 457 (1957).
22. Beers, R. F., Jr., *J. Biol. Chem.*, **235,** 2393 (1960).
23. Reddi, K. K., *Nature*, **187,** 74 (1960).
24. Magasanik, B., Vischer, E., Doniger, R., Elson, D., and Chargaff, E., *J. Biol. Chem.*, **186,** 37 (1950).
25. Lipsett, M. N., *J. Biol. Chem.*, **240,** 3975 (1965).
26. Razzel, W. E., and Khorana, H. G., *J. Biol. Chem.*, **236,** 1144 (1961).