Acid-soluble Ribosomal Ribonuclease of *Escherichia coli**

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ABSTRACT: The ribonuclease of *E. coli* ribosomes which degrades ribonucleic acid (RNA) and polynucleotide homopolymers to oligonucleotides and nucleoside 2′,3′-monophosphates was purified with perchloric acid solution. After neutralization with KOH, the extracts exhibited high enzymatic activity. The relative specific activity was 48 for polycytidylic acid, 10 for polyadenylic acid, and 5 for s-RNA. Worthington BAPSF *Escherichia coli* alkaline phosphatase contains ribonuclease activity. Perchloric acid treatment of this fraction yielded an active enzyme with specific activity of 2000 for polycytidylic acid, 850 for RNA, and no activity with polyadenylic or polyuridylic acid. The enzymes showed no specific ion effects but were completely inhibited in high ionic strength media (over 0.15 M NaCl). Mercaptoethanol treatment inhibited the enzyme activity.

The acid-soluble ribonucleases were not adsorbed on diethylaminoethyl-cellulose but were bound on carboxymethyl-cellulose columns from which they were removed by a 0.1–0.3 M NaCl gradient in 0.05 M Tris, *p*H 7.2; the activities for polycytidylic and polyadenylic acids appeared in a single peak. The molecular weight of the acid-soluble ribonucleases was estimated by gel-filtration technique to be between 10,000 and 30,000. The *p*H optimum of the enzymes was at 8.0.

The ribonuclease which is associated with the ribosomes of *Escherichia coli* is not active in its natural combination with protein and nucleic acid (Elson, 1959). Disruption of the ribosome complex by high ionic strength media, urea, or EDTA yields an active ribonuclease (Elson, 1959; Spahr and Hollingworth, 1961). This enzyme activity was purified by Spahr and Hollingworth (1961), who found that it was located exclusively in the ribosome fraction and that it degraded RNA to nucleoside 2′,3′-cyclic phosphates and nucleoside 3′-monophosphates. Neu and Heppel (1964) have demonstrated that the conversion of *E. coli* cells to spheroplasts by lysozyme and EDTA released into the medium a ribonuclease which was similar to the ribosomal ribonuclease that was purified by Spahr and Hollingworth (1961). The enzyme purified from the spheroplast medium (Neu and Heppel, 1964) had a greater affinity for DEAE-cellulose columns than was found for the ribosomal enzyme.

We have found that ribosomal ribonuclease activity of *E. coli* is acid soluble and that it retains activity after acid treatment. This activity may be released from ribosomes or whole-cell extracts by treatment with cold dilute perchloric acid, and thus resembles the ribonuclease activity that was purified from the media of cultures of several bacilli by Rushizky *et al.* (1964). The acid-purified enzyme activity from *E. coli* degraded polycytidylic, polyuridylic, and polyadenylic acids to 2′,3′-cyclic mononucleotides and oligonucleotides. It was also found that the salt-fractionated *E. coli* alkaline phosphatase (Worthington BAPSF) contains the acid-soluble ribonuclease activity that degrades polycytidylic acid and RNA, but has no polyadenylic or polyuridylic nuclease activity.

Materials and Methods

Polyadenylic, polyuridylic, and polycytidylic acids were purchased from Miles Chemical Laboratory. Yeast s-RNA was purchased from General Biochemicals, Inc., and *E. coli* s-RNA and ribosomal RNA were prepared by a phenol extraction procedure (Hoagland *et al.*, 1958). Salt-fractionated *E. coli* alkaline phosphatase (BAPSF) was purchased from Worthington Biochemical Corp. DEAE-cellulose, Type 40, and CM-cellulose, Type 40, were obtained from Carl Schleicher & Schuell Co. Bio-gel 30 was purchased from Bio-Rad Laboratories.

E. coli cells were collected by centrifugation at the midlogarithmic growth phase of cultures in a glucose-salts medium. The cells were washed with 0.15 M sodium chloride and then stored at −10°. Five g of cells was disrupted by grinding in a mortar with 10 g of alumina at 4° (all subsequent operations were performed at 4°), and extracted with 17 ml of 0.01 M Tris buffer, *p*H 7.5, which contained 0.005 M $MgCl_2$. Alumina and debris were removed by centrifugation at 30,000 × *g* for 20 minutes. Ribosomes were collected from the 30,000 × *g* supernatant solution by centrifugation at 105,000 × *g* for 120 minutes. The ribosome pellet was suspended in

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3 ml of 0.01 M Tris buffer, *p*H 7.5. DNAase[1] was not employed in the preparation.

The acid-soluble ribonuclease was prepared from the ribosome fraction by adding 0.2 ml of 1 N $HClO_4$ to 3.0 ml of the suspension. The mixture was stirred with a glass rod to break up clumps of denatured protein and nucleic acid and it was then centrifuged at 10,000 × *g* for 10 minutes. The supernatant solution was adjusted to *p*H 7.0 with KOH and the insoluble $KClO_4$ was removed by centrifugation. The whole extract (30 000 × *g* supernatant) was similarly treated by adding 3 ml of 1 N $HClO_4$ to 15 ml of extract. The precipitate was thoroughly broken up with a stirring rod, the mixture was centrifuged and neutralized with KOH, and the insoluble perchlorate was removed by centrifugation. The total yield of enzyme activity was three to five times higher from the whole cell extract, and this appears to be a preferable preparative procedure. A small amount of acid-soluble nuclease activity was found in the 105,000 × *g* supernatant solution when polycytidylic acid was employed as a test substrate, but the bulk of ribonuclease activity was associated with the ribosomes, in agreement with previous work (Spahr and Hollingworth, 1961). The ribosome preparation was designated "Rib-Ac" and the whole extract preparation "30-Ac."

The salt-fractionated alkaline phosphatase preparation (BAPSF), obtained from Worthington Biochemical Corp., contained high ribonuclease activity. This activity was easily separated from the alkaline phosphatase activity by DEAE-cellulose column chromatography, which retained the phosphatase and did not absorb the nuclease, and by acid treatment which destroyed the phosphatase activity. The nuclease activity which was purified by passage through DEAE-cellulose (this fraction was designated BAPSF-DEAE) and that purified by acid treatment were the same when compared on the basis of substrate specificity, products of substrate degradation, inorganic salt effects, *p*H optima, and CM-cellulose chromatography. The nuclease purified by acid treatment had a higher specific activity and was more easily prepared, as follows: A vial of Worthington BAPSF fraction that contained 10 mg of protein was diluted to 5 ml with water and 1 ml of 1 N perchloric acid was added. The precipitate was removed by centrifugation, the supernatant was neutralized with KOH, and the insoluble perchlorate was removed by centrifugation. This fraction was designated "BAPSF-Ac."

Enzyme activity was assayed by measuring the formation of acid-soluble products from the degradation of polycytidylic acid, polyadenylic acid, and RNA. Polyuridylic acid was found to contain a high proportion of acid-soluble polymer and was therefore tested as a substrate by the detection of products on paper chromatography. In the standard assay for acid-soluble product formation the incubation mixture consisted of 0.2 mg substrate in 3 ml of 0.04 M Tris buffer,

[1] Abbreviation used in this work: DNAase, deoxyribonuclease.

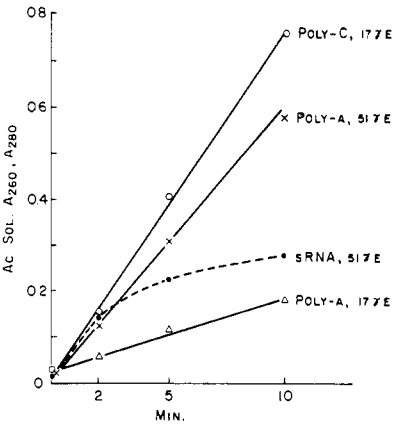

FIGURE 1: Degradation of polynucleotide by the acid-soluble ribonuclease of *E. coli* ribosomes (Rib-Ac fraction). The standard assay conditions are described in the methods section and the amount of protein in the enzyme fraction is indicated for each substrate.

*p*H 8.0, and an amount of enzyme which gave linear reaction rates over an initial 10-minute period at 37°. To terminate the incubation, 1 ml of 1 N $HClO_4$ was added and the mixture was centrifuged at 10,000 × *g* for 10 minutes at 4°. Absorbancy was measured at 260 and 280 mμ in the supernatant solution. A unit of enzyme activity was defined as equivalent to the formation of 0.1 A_{260} (or A_{280} for polycytidylic acid) per minute. Specific activity was calculated as units of activity per mg of protein which was estimated by the phenol-reagent method of Lowry *et al.* (1951). The amount of "protein" color in the enzyme preparations is very low and some heterocyclic compounds, as well as protein, may react in this determination. The value of specific activity calculations is limited to comparison of several fractions rather than an overall statement of enzyme purification.

The enzyme activities may also be assayed by the hyperchromic shift in ultraviolet absorbancy which is exhibited by the polymers upon enzymatic degradation. However, we discovered independently and alarmingly that the acid-soluble ribonucleases are adsorbed on glass, an observation also made by Neu and Heppel (1964), and that the precautions necessary for the employment of this assay restrict its routine utilization.

Paper chromatography in the solvent system 1-propanol–H_2O–concd NH_4OH, 60:10:30, was employed as described by Markham and Smith (1952) and Neu and Heppel (1964) for the separation and identification of nucleoside 2′,3′-cyclic monophosphates and nucleoside 3′-monophosphates. Nucleoside monophosphates were also analyzed by anion-exchange chromatography on Dowex 1 (formate) columns (Hurlbert, 1957). The separation of the oligonucleotide products of nuclease degradation of polycytidylic acid and RNA was achieved by NaCl-urea gradient elution from DEAE-cellulose columns as described by Tomlinson and Tener (1963).

Alkaline phosphatase activity was determined by the

TABLE 1: Specific Activity of Ribonuclease Preparation.

Fraction	Protein (μg/ml)	Substrate			
		s-RNA[a]	Poly-adenylic	Poly-cytidylic	Poly-uridylic[b]
Rib-Ac	340	5	10	48	+++
30-Ac	240	8	12	30	+++
BAPSF	2000	10	<2	23	0
-DEAE	154	250	0	800	0
-Ac	92	860	0	2000	0

[a] Linear reaction rates were not obtained with s-RNA so the 10-minute determination of acid-soluble absorbancy was arbitrarily employed (see Figures 1 and 2). [b] Activity was determined by paper chromatographic assay in the solvent system 1-propanol-H_2O-NH_4OH (60:30:30).

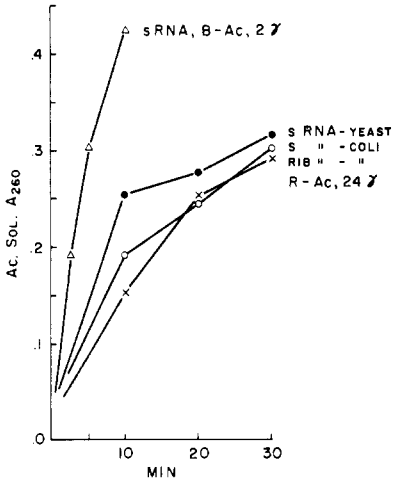

FIGURE 2: Degradation of RNA by acid-soluble nuclease. Standard assay conditions were employed to compare the rates of degradation of different RNA's by the acid-soluble ribosomal enzyme (R-Ac). A comparison of the rate of degradation of yeast s-RNA by the acid-purified Worthington BAPSF fraction (B-Ac) and the ribosomal enzyme (R-Ac) is shown for the amount of enzyme protein indicated on the graph. The substrate concentration, 200 μg in 3.0 ml of incubation mixture, was the same for each assay. △, 2 μg of B-Ac; ○, ×, ●, 24 μg of R-Ac.

method of Garen and Levinthal (1960) and phosphodiesterase activity by the method of Koerner and Sinsheimer (1957) with bisdinitrophenyl phosphate as a substrate. There was no alkaline phosphatase, phosphodiesterase, or deoxyribonuclease activity detectable in the acid-purified ribonuclease preparations described in this paper.

Results

The rate of formation of acid-soluble products from the degradation of polycytidylic acid, polyadenylic acid, and yeast s-RNA by the acid-soluble ribonuclease of *E. coli* ribosomes is shown in Figure 1. A comparison of the specific activity of the different preparations of ribonuclease in Table I shows that all the fractions contained small amounts of protein with high enzymatic activity, and that the Worthington BAPSF fraction contained relatively little polyadenylic nuclease activity. In the DEAE-nonadsorbable fraction derived from Worthington BAPSF and in the acid-purified Worthington BAPSF fraction there was no polyadenylic nuclease activity. Polyuridylic nuclease activity was also absent in these fractions when assayed for product formation by paper chromatography. It was found that the specific activity for degradation of polycytidylic acid and RNA could be increased 2- to 4-fold in each preparation by dialysis or precipitation of the enzyme activity with 0.7 saturated ammonium sulfate. However, the activity against polyadenylic acid was significantly lowered by this treatment in the Rib-Ac and 30-Ac fractions. This loss of polyadenylic nuclease activity could not be restored by adding several concentrations of Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, K^+, or Na^+ in the presence of sulfate, phosphate, or chloride anions. None of the enzyme activities was inhibited by EDTA.

The rate of degradation of s-RNA and ribosomal RNA by the acid-purified ribosomal enzyme is shown in Figure 2. The rates for yeast s-RNA and *E. coli* s-RNA were comparable and, as shown also in Figure 1, were apparently a composite of several reaction rates. Ribosomal RNA was degraded at about the same rate, but the reaction tended to be more linear in the early incubation period. The enzyme activity purified from the Worthington BAPSF fraction by acid treatment was highly active for the degradation of all RNA preparations tested, and, as shown in Figure 2, the rate of the reaction for yeast s-RNA was approximately linear in the 10-minute incubation period. In Figure 3 the relationship of enzyme activity to salt concentration of the medium is shown. In all the preparations high ionic strength of inorganic ions inhibited enzyme activity against polycytidylic acid, polyadenylic acid, and RNA.

TABLE II: Products of Ribonuclease Action.[a]

Enzyme Fraction	Substrate	Product[b] Nucleoside 3′-Monophosphate (%)	Nucleoside 2′,3′-Cyclic Phosphate (%)	Oligonucleotide[c] (%)
30-Ac	Polycytidylic	2–5	30	55
	Polyadenylic	2–5	25	63
	Polyuridylic	2–5	28	55
	s-RNA	0	0	58
BAPSF-Ac	Polycytidylic	<1	32	57
	Polyadenylic	0	0	0
	Polyuridylic	0	0	0
	s-RNA	0	0	100

[a] Incubation was for a period of 30 minutes at 37° of mixtures which contained 0.1 ml of substrate at a concentration of 2 mg/ml, 0.05 ml of 0.1 M Tris buffer, *p*H 8.0, and 0.1 ml of enzyme at a concentration sufficient to yield over 90% acid-soluble product in 10 minutes' incubation at 37°. For s-RNA the incubation was for 6 hours with 50 μg of the 30-Ac enzyme. [b] An aliquot (0.05 ml) of the incubation mixture was applied to Whatman No. 1 paper and the chromatogram was run for 16 hours in the solvent system 1-propanol-H_2O-NH_4OH (60:10:30). The products were located on paper in ultraviolet light, the spots were cut out and eluted with 0.1 N HCl, and the absorbancy of the eluate was measured at 260 and 280 mμ. The per cent recovery calculation was based on the absorbancy of the aliquot applied to the paper. The absence of mononucleotides in the digests of s-RNA was confirmed by chromatography on Dowex 1 (formate) columns. [c] The proportion of oligonucleotide was estimated from the difference between the total acid-soluble absorbancy and the absorbancy of the mononucleotide plus the cyclic mononucleotide fraction.

No specific anion or cation effects were observed. When Lineweaver-Burk reciprocal plots of velocity and substrate concentration were employed, the effect of salt concentration was found to be competitive and reversible. For polycytidylic acid degradation by the BAPSF-Ac fraction, the apparent K_s in 0.033 M Tris buffer, *p*H 8.0, was 1.5×10^{-5}. The addition of NaCl to give 0.046 M or $MgCl_2$ to give 0.023 M changed the apparent K_s to 2×10^{-2}.

Maximal activity of the acid-soluble ribonucleases was found in 0.04 M Tris buffer, *p*H 8.0. When 0.01 M buffer was employed, the addition of salt to give an ionic strength equivalent to 0.04 M Tris gave a comparable increase in activity and there were no specific cation or anion effects observed. The *p*H optimum for the degradation of polyadenylic acid occurred over a broader *p*H range (80% of maximum at 7.0 and 8.2) than that observed for polycytidylic acid or RNA (80% of maximum at 7.5 and 8.2).

In Table II the products of the degradation of the homopolymers and RNA by the acid-soluble ribonucleases are characterized. Under the conditions of these experiments where the ratio of enzyme to substrate was high (10–50 μg protein to 200 μg substrate), the principal product of the reaction from the homopolymers was nucleoside 2′,3′-cyclic monophosphates and oligonucleotides. The degradation of RNA led to the formation of oligonucleotides, and no nucleoside cyclic monophosphates or nucleoside 3′-monophosphates were found on prolonged incubation (6 hours; 37°). These results show that the mechanism of degradation of the homopolymer polynucleotide substrates by the acid-soluble ribonucleases is similar to that reported by Spahr and Hollingworth (1961) and

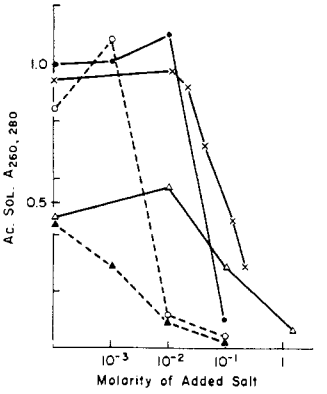

FIGURE 3: Effect of inorganic salts on acid-soluble nuclease activity under standard assay conditions. NaCl, ———; $MgCl_2$, – – – –; polycytidylic acid, ○, ●; s-RNA, △, ▲; polyadenylic acid, ×. The enzyme employed for polycytidylic and s-RNA was BAPSF-Ac, 2 μg protein, and for the degradation of polyadenylic acid the 30-Ac enzyme, 60 μg protein, was used; the incubation period was 20 minutes. The molarity is the final concentration of the added salt in 0.04 M Tris buffer, *p*H 8.0.

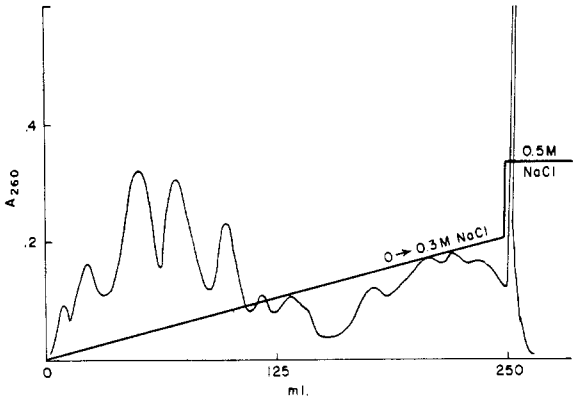

FIGURE 4: DEAE-cellulose column chromatography (Tomlinson and Tener, 1963) of a digest of 3 mg yeast s-RNA in 3.0 ml of 0.04 M Tris buffer and 180 μg protein of 30-Ac fraction. Incubation was for 2 hours at 37°. The mixture was added directly to a 12- × 1-cm column which had previously been washed with sodium chloride and water. The recovery of ultraviolet absorbancy was 78%. Yeast s-RNA in the absence of enzyme contained no ultraviolet absorbing components, aside from a small peak in the 0.5 M NaCl region, which appeared in the gradient fractions employed.

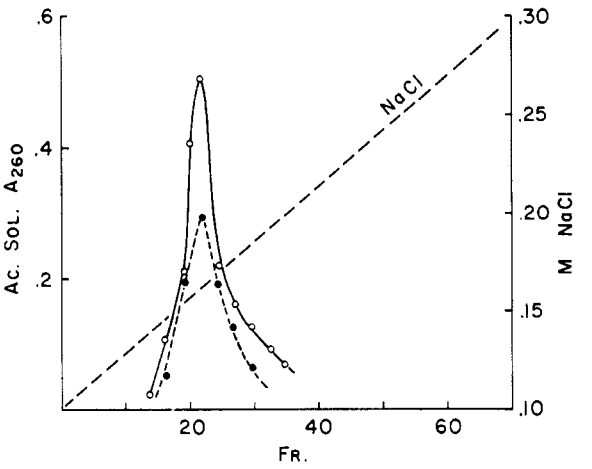

FIGURE 5: Chromatography of 30-Ac fraction on CM-cellulose. A column of CM-cellulose, Type 40, 30 × 1 cm, was equilibrated with 0.1 M sodium acetate buffer, *p*H 5.9. The 30-Ac fraction (15 ml) was adjusted to *p*H 4.5 with acetic acid and added to the column which was then washed with 30 ml of the acetate buffer. About 65% of the protein added to the column was recovered in the acetate buffer fraction. A linear gradient elution was then established with 100 ml of 0.1 M NaCl in 0.05 M Tris, *p*H 7.2, and 100 ml of 0.3 M NaCl in 0.05 M Tris, *p*H 7.2. The flow rate was about 1 ml/min and seventy fractions of 2.8 ml were collected. Polycytidylic nuclease activity was assayed with 0.1 ml of each fraction and polyadenylic nuclease with 0.2 ml.

Neu and Heppel (1964) for the ribonuclease activities which they purified from *E. coli*. When the ratio of the enzyme to the homopolymer substrate was low (~10 μg protein to 5000 μg substrate), the predominantly endonuclease activity of the enzyme was seen. Under these conditions over 90% of the acid-soluble products of the reaction of polycytidylic acid and RNA with BAPSF-Ac enzyme were recovered in the 3–12 nucleotide residue oligonucleotide region of the DEAE-cellulose chromatogram developed according to Tomlinson and Tener (1963). The amount of 2′,3′-cyclic monophosphate end groups in the oligonucleotide fractions was not determined. The action of the acid-soluble ribonucleases on RNA are exclusively of the endonuclease type under the conditions employed in the present studies, a finding illustrated in Figure 4 for the degradation of yeast s-RNA by the nuclease of the 30-Ac fraction.

The ribonuclease activities prepared from *E. coli* extracts and from the Worthington BAPSF fraction were not adsorbed on DEAE-cellulose, but they were strongly bound to CM-cellulose columns. Figure 5 illustrates the elution diagram of the nuclease activity which degraded polycytidylic and polyadenylic acids in the 30-Ac preparation adsorbed on CM-cellulose. Several conditions were explored in an attempt to separate the two activities, but in all cases so far studied polycytidylic and polyadenylic nuclease activity appeared in the same eluate fraction. It was also found that polycytidylic nuclease activity in the BAPSF-Ac fraction chromatographed in the same position as the activity from the 30-Ac fraction. In these studies it was observed that polycytidylic nuclease activity was not decreased during the operations, whereas about 20–40% of the polyadenylic activity was lost. The polycytidylic nuclease fraction retained activity for degrading RNA.

Gel-filtration technique was employed to estimate the molecular weight of the acid-soluble ribonucleases. Bio-gel 30, which has an exclusion limit of 30,000 mw, was suspended in distilled water and a column 28 cm × 13 mm was employed. A sample of DNA with a molecular weight greater than 1×10^6 was applied to the column in 1 ml of H_2O and the column was washed with water. DNA appeared in the effluent at 7.5–9.5 ml. A sample of cytidine 5-monophosphate was eluted in the fractions 18.5–27 ml. The enzyme activity of 1 ml of the Rib-Ac fraction, which degraded both polyadenylic and polycytidylic acids, appeared in a single peak in the 17–19 ml fractions. Since the enzyme activity was not dialyzable, it was estimated that the molecular weight of the enzyme was between 10,000 and 30,000.

The acid-soluble ribonucleases were inactivated by mercaptoethanol as shown in Table III. This suggests that these enzymes resemble pancreatic ribonuclease and depend on the integrity of disulfide linkages for activity (Anfinsen and Haber, 1961).

The enzyme activity which was prepared from the Worthington BAPSF preparation by acid treatment did not degrade polyadenylic or polyuridylic acid, but was

TABLE III: Mercaptoethanol Inhibition of Ribonuclease.[a]

Enzyme Fraction	Substrate	Mercaptoethanol (μmoles added)	Acid-soluble Product A_{280}	A_{260}
30-Ac	Poly-C	0	1.6	
	Poly-C	5	0.49	
	Poly-C	10	0.31	
	Poly-C	20	0.28	
30-Ac	Poly-A	0		1.1
	Poly-A	5		0.42
	Poly-A	10		0.34
	Poly-A	20		0.30

[a] The enzyme fraction, which contained 50 μg of protein, was added to 1.0 ml of 0.1 M Tris buffer, *p*H 8.0, and the indicated amount of mercaptoethanol. The mixture was incubated for 15 minutes. Two ml of H_2O and 200 mg of substrate were then added to each tube and the incubation was continued for 20 minutes.

highly active in the degradation of polycytidylic acid and RNA. The preference for cytidylic acid linkages in the degradation of s-RNA by this fraction was indicated by treatment of the digest with purified alkaline phosphatase (Worthington BAP-C) followed by alkaline hydrolysis. The only nucleoside which was then detected by paper chromatography was cytidine. The mononucleotides of a control and a nuclease-treated RNA digest were compared by chromatographic analysis after this treatment. From 7.5 mg of yeast s-RNA, 2.82 μmoles of cytidylic acid were recovered from the control sample and 1.98 μmoles of cytidylic acid were recovered from a replicate sample of nuclease-treated s-RNA. The difference in cytidylic acid residues, 1.04 μmoles, accounted for about 80% of the difference in nucleotide composition of the two samples. End-group analysis of endonuclease-treated RNA by these methods does not have the statistical validity of similar treatment of low molecular weight oligonucleotides, and the results must be viewed with this reservation. The specificity of the enzyme toward various trinucleotides has not been studied.

Discussion

The ribonuclease activity which is associated with *E. coli* ribosomes occurs in several different active forms, all of which are dissociated from the ribosome complex. The enzyme activity purified by Neu and Heppel (1964) from the medium of *E. coli* spheroplasts was retained on DEAE-cellulose columns and might consist of an association of the enzyme with some part of the ribosomal protein. The enzyme activity which was purified by Spahr and Hollingworth (1961) from *E. coli* ribosomes by ammonium sulfate fractionation was bound weakly to DEAE-cellulose. Both these enzymes differ from the nuclease activities we have prepared by acid treatment with respect to the inability of the acid-purified enzyme to adsorb on DEAE-cellulose and also with respect to the effect of added electrolytes. The enzyme purified by Spahr and Hollingworth (1961) and Neu and Heppel (1964) was found to be stimulated by concentrations of NaCl up to 0.3 M, whereas the nuclease activity we have purified by acid treatment is strongly inhibited at concentrations above 0.15 M NaCl. The latter effect is important practically in preventing the degradation of RNA in preparative procedures, and in analytical studies on the stepwise degradation of RNA employing the enzyme where its activity may be abruptly terminated by addition of suitable concentrations of electrolyte.

The nuclease activities described in this paper most closely resemble those reported by Rushizky *et al.* (1964) in acid solubility, affinity for CM-cellulose, and in apparent molecular weight. The ribonucleases purified by Rushizky *et al.* (1964) were prepared from the culture media of several bacilli, and in the light of the studies of Neu and Heppel (1964) these enzymes might also be considered to have been associated with the ribosomes of the cell at some stage of cell growth.

Whether the ribonuclease activities we have described in this paper represent several enzymes with different structural features and substrate specificities is not known. The preparation of an acid-soluble ribonuclease activity from the Worthington BAPSF fraction which degrades polycytidylic acid but not polyadenylic or polyuridylic acid might indicate that different enzymes with different substrate specificities occur. However, this apparent separation of activities could be owing to modification of the enzyme during preparative procedures in a way which results in loss of ability to degrade polyadenylic and polyuridylic acid. So far we have been unable to separate polycytidylic nuclease activity from polyadenylic nuclease activity on CM-cellulose columns. Since the acid-soluble nuclease activity is inhibited by mercaptoethanol, the loss of polyadenylic nuclease activity might be owing to rupture and rearrangement of disulfide linkages in preparative manipulations.

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Spectrophotometric Identification of Acyl Enzyme Intermediates*

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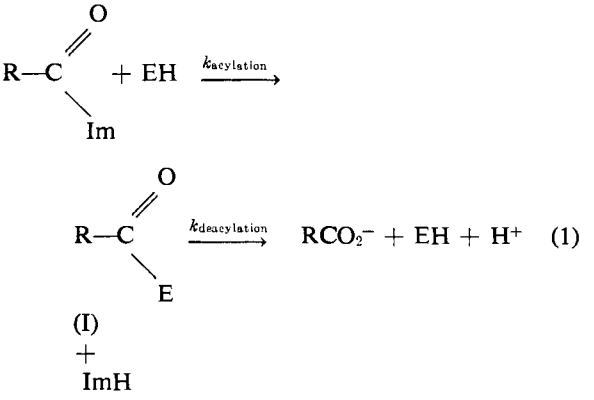
ABSTRACT: The ultraviolet spectra of a variety of β-arylacryloyl derivatives have been investigated in terms of the influence of the aryl substituent, the acyl substituent, and the solvent. The spectra of these derivatives have been compared with the corresponding spectra of the β-arylacryloyl derivatives of chymotrypsin and subtilisin, in an attempt to identify the acyl-acceptor group at the enzyme site. The "native" acyl enzyme spectrum in water differs in every case from that of a corresponding *O*-acylserine peptide in aqueous, hydrogen-bonding, or nonpolar solvents. The denatured acyl enzyme spectrum is in every case the same as that of a corresponding *O*-acylserine peptide. Of the potential amino acid acyl-acceptor side chains, only the imidazole group of histidine can form an acyl derivative which is compatible with the spectrophotometric characteristics of the corresponding native acyl enzyme. The acylimidazole spectrum in nonpolar solvents approaches that of the acyl enzyme for all β-arylacryloyl derivatives investigated. The slow rate of acyl enzyme hydrolysis at low *p*H (*p*H <4) is, however, inconsistent with the observed hydrolysis rates of acylimidazoles.

In a comprehensive set of papers, Bender and his collaborators (Bender, 1962a; Bender *et al.*, 1962a,b; Schonbaum *et al.*, 1961) demonstrated the utility of employing chromophoric acylating agents for the identification of intermediates in the chemical pathway of catalysis by proteolytic enzymes. Aside from the experimental convenience of following the rates of disappearance of substrate, appearance of enzyme-substrate intermediate, and appearance of final products individually by means of the characteristic ultraviolet absorption spectra of each of the components, the method presents a potential approach to the identification of the chemical nature of enzyme-substrate intermediates. An example reported by Bender *et al.* (1962a) serves as illustration: Table I lists the characteristic ultraviolet wavelength maxima and extinctions of a variety of derivatives of cinnamic acid. In the reaction of α-chymotrypsin with cinnamoylimidazole, an enzyme-substrate intermediate is readily detected. The kinetics of the reaction are in accord with the model of equation (1). By comparison of the data of Table I with the spectrum of the enzyme-substrate intermediate (compound I), the chemical nature of the acyl enzyme



(1)

linkage might possibly be inferred. Unfortunately, none of the model compounds of Table I corresponds very closely in its ultraviolet spectra to that of the acyl enzyme. The disparity between the spectrum of the acyl enzyme intermediate and that of the model compound, *N*-acetyl-*O*-cinnamoylserinamide (compound II), is particularly disappointing in light of the fact that an *O*-acetylserine peptide has been identified (Oosterbaan and Van Andrichem, 1958) among the proteolytic degradation products of *mono*acetyl chymotrypsin, an acyl enzyme isolated (Balls and Wood, 1956) under conditions similar to that employed in the spectral identification of cinnamoyl chymotrypsin. Wooten and Hess (1960) have found that even when non-ultraviolet absorbing acylating or phosphorylating agents are employed in the preparation of acyl or

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