HYDROLYSIS OF OLIGORIBONUCLEOTIDES BY AN ENZYME FRACTION FROM ESCHERICHIA COLI*

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This paper reports the isolation from Escherichia coli of an enzyme fraction that rapidly hydrolyzes adenine oligoribonucleotides, yielding 5'-AMP as the major product. Two ribonucleases from E. coli have been described previously. RNase I (Elson, 1959; Spahr and Hollingsworth, 1961) is an endonuclease that hydrolyzes polyribonucleotides and yields nucleoside 3'-monophosphates. RNase II (Spahr and Schlessinger, 1963; Spahr, 1964; Singer and Tolbert, 1964 and 1965; Nossal, Tolbert, and Singer, 1967) is an exonuclease that hydrolyzes polyribonucleotides from the 3'- termini and yields nucleoside 5'-monophosphates. Singer and Tolbert (1965) and Nossal et al. (1967) have reported that short adenine oligoribonucleotides are not hydrolyzed by RNase II.

Methods — E. coli Q13, a multiple mutant isolated in Dr. Walter Gilbert's laboratory and deficient in both RNase I and polynucleotide phosphorylase (Kivity-Vogel and Elson, 1967), was obtained through the courtesy of Dr. Gilbert. It was grown in enriched media (Canellakis, Gottesman, and Kammen, 1960), and the packed wet cells were ground with alumina (2.5 times the weight of wet cells). The alumina paste was extracted with a solvent (five times the weight of wet cells) of 10 mM Tris buffer, pH 7.8, containing 10 mM $MgCl_2$ and 20 mM NH_4Cl. The crude suspension was centrifuged for 15 minutes at 10,000 x g. The supernatant solution was centrifuged first for 30 minutes at 16,000 x g, and then for 4 hours at 28,000 rpm (30 rotor, Spinco Model L). To the final supernatant

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solution (called the supernatant fraction), 0.1 volume of 10% streptomycin sulfate was added, and after stirring for 30 minutes, the precipitate was removed by centrifugation. The protein fraction precipitating between 0.55 and 0.75 saturation with ammonium sulfate (pH 8.5) was then collected. The fraction was dissolved in 10 mM Tris buffer, pH 7.8, to a protein concentration of about 10 mg per ml and dialyzed for 24 hours against 10 mM Tris buffer, pH 7.8. Seven ml of the dialyzed ammonium sulfate fraction were applied to a DEAE-cellulose column (2 x 8 cm), and the column was eluted in a stepwise manner with 80 ml each of Tris buffer, pH 7.8, containing the following concentrations of KCl: 0.10 M, 0.15 M, 0.175 M, 0.20 M, and 0.30 M. Ten-ml fractions were collected. The $(pA)_2$-hydrolyzing activity was eluted in fractions 21-34. The active fractions were combined, and the 0.60 to 0.75 saturated ammonium sulfate fraction was collected as described earlier. The fraction was dissolved in 10 mM Tris buffer, pH 7.8, to a protein concentration of 2 mg per ml and dialyzed for 24 hours against 10 mM Tris buffer, pH 7.8. The dialyzed fraction was stored at -20°C.

The extent of hydrolysis of ^{14}C-$(pA)_2$ was used as a routine measure of enzyme activity. Reaction mixtures (0.05 ml) contained: ^{14}C-$(pA)_2$, 67 μM, 400 cpm/nmole; Tris buffer, pH 7.8, 100 mM; $MnCl_2$, 5 mM; and enzyme. After a 10-minute incubation period at 37°C, 10 μg of E. coli alkaline phosphatase (Worthington Biochemicals Corp.) were added, and the incubation was continued for another 10 minutes at 37°C. The reaction mixture was then diluted to 1 ml with water and applied to a Dowex-1-Cl column (1 x 2 cm). The column was eluted with 0.001 N HCl and the adenosine fraction collected. An aliquot of the fraction was counted with aqueous scintillation fluid in a Nuclear Chicago liquid scintillation spectrometer.

Oligonucleotides of the type $(pA)_n$ were made from ^{14}C-poly A by the action of a nuclease from Azotobacter agilis (Stevens and Hilmoe, 1960). Oligonucleotides of the type $(Ap)_n$ were prepared by partial alkaline hydrolysis of ^{14}C-poly A (Lane and Butler, 1959). Oligonucleotides of the type $(Ap)_nA$ were obtained by the action of E. coli alkaline phosphatase on either $(pA)_{n+1}$ or $(Ap)_{n+1}$. Oligonucleotides of the type $(Up)_n$ were prepared by controlled action of pancreatic ribonuclease on ^{3}H-poly U (Heppel, Ortiz, and Ochoa, 1957), and those of the type $(Up)_nU$ by the action of alkaline phosphatase on type $(Up)_{n+1}$. The oligonucleotides of different chain lengths were separated

and purified by paper chromatography on Whatman 3 MM paper. The best solvent for this purpose was 1-propanol:concentrated NH_4OH:H_2O (55/10/35, v/v/v).

For identification of the products of hydrolysis of the oligonucleotides, two solvent systems were used — the one described earlier, and isopropanol:concentrated NH_4OH:0.1 M boric acid (70/10/30, v/v/v) (as described in Schwarz BioResearch, Inc., Catalog, 1967).

$d(pA)_{3-6}$ was a gift from Dr. Ronald Rahn. Its hydrolysis was measured in a manner similar to that described above for ^{14}C-$(pA)_2$ except that inorganic phosphate was measured after the alkaline phosphatase treatment. The method of Chen, Toribara, and Warner (1956) as modified by Ames and Dubin (1960) was used for measuring inorganic phosphate.

RNase II was measured as described by Singer and Tolbert (1965).

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

Results and Discussion — It was initially observed that the supernatant fraction of E. coli B extracts hydrolyzes $(pA)_2$, yielding 5'-AMP. The same rate of hydrolysis was found with the supernatant fraction of E. coli Q13 extracts, and a 10-fold purification of the hydrolyzing activity was obtained by the procedure described under Methods. The final enzyme fraction contained 30% of the $(pA)_2$-hydrolyzing activity of the supernatant fraction.

The enzyme fraction rapidly hydrolyzes adenine oligoribonucleotides with a 5'-phosphomonoester end group or with no phosphomonoester end group. K_m and V_{max} values for compounds of these two types are shown in Table I. The compounds with a 5'-phosphomonoester end group have slightly higher K_m's and V_{max}'s than those with no phosphomonoester end group. With either type of compound, the K_m values decreased slightly with increasing chain length.

$(Ap)_4$ was used as a substrate to represent the type of compound with a 3'-phosphomonoester end group. Its rate of hydrolysis was only 10 to 20% of the rate of hydrolysis of $(pA)_4$. To test oligodeoxyribonucleotides as substrates, $d(pA)_{3-6}$ was used and found to be hydrolyzed at less than 10% of the rate of $(pA)_5$. $(Up)_2U$ and $(Up)_3U$ were hydrolyzed at rates similar to those of the corresponding adenine-containing compounds.

Reaction mixtures in which almost complete hydrolysis of ^{14}C-labeled

Table I

Kinetic Parameters for the Hydrolysis of Adenine Oligoribonucleotides by the Enzyme Fraction

Substrate	K_m ($M \times 10^{-4}$)	V_{max} [a] (μmoles)
$(pA)_2$	1.4	13.2[b]
$(pA)_3$	1.0	17.3
$(pA)_4$	0.3	9.6
$(pA)_5$	0.5	12.0
ApA	0.4	7.4[b]
$(Ap)_2A$	0.2	7.7
$(Ap)_3A$	0.1	6.5

[a] Adenosine formed per hr per mg of enzyme protein.

[b] The value calculated was twice this value. It was corrected to account for the fact that the hydrolysis of one phosphodiester bond results in the liberation of two molecules of adenosine. In the case of the other compounds, the reactions were run so that only 10 to 20% of the compound was hydrolyzed; so the hydrolysis of one phosphodiester bond probably results in the formation of only one molecule of adenosine. (If the enzyme were an endonuclease, some phosphodiester bonds of the tetramers and pentamers could be cleaved, yielding no adenosine.)

Reaction mixtures were similar to those with ^{14}C-$(pA)_2$ described under Methods. All compounds were ^{14}C-labeled. After a 10-minute period of incubation at 37°C, the reactions were stopped by heating for 3 minutes in a boiling water bath. The mixtures were then subjected to alkaline phosphatase treatment and ion-exchange chromatography as described under Methods. The amount of ^{14}C-adenosine formed was measured.

$(pA)_2$, $(pA)_3$, $(pA)_4$, and $(Ap)_3A$ had occurred were subjected to paper chromatography as described under Methods. 5'-AMP was the major product detected with all four substrates. No 3'-AMP was detected. With $(pA)_2$, $(pA)_3$, and $(pA)_4$, adenosine accounted for about 8% of the product, and with $(Ap)_3A$, for about 30% of the product. The adenosine formed from the first three compounds and the excess adenosine formed from the latter compound probably resulted from phosphatase contamination in the enzyme fraction.

The pH optimum for the hydrolysis of $(pA)_2$ was 8.0, with 70% of that rate being obtained at pH 7.0 or pH 8.9 (Tris buffers). The hydrolysis of $(pA)_2$ proceeds in the absence of added divalent cation, but the rate is stimulated 2-fold by Mg^{++}(10mM) and 3-fold by Mn^{++}(5mM).

Polyadenylic acid was also used as a substrate for the enzyme fraction. It was hydrolyzed maximally at a rate about 10% that of $(pA)_2$. Its rate of hydrolysis was increased 7-fold by the addition to the reaction mixtures of 0.1 M KCl. Since RNase II shows a strong KCl dependence (Spahr, 1964), it is possible that the hydrolysis of poly A was due to this enzyme still present in the enzyme fraction. (The enzyme fraction contains 3% of the RNase II activity of the supernatant fraction.) Neither 0.1 M KCl nor inorganic phosphate stimulated the hydrolysis of $(pA)_2$.

The enzyme activity described here differs from that of RNase II since purified RNase II does not attack short oligoribonucleotides such as $(pA)_2$ and $(pA)_3$ (Singer and Tolbert, 1965; Nossal et al., 1967). The enzyme fraction resembles venom phosphodiesterase (Khorana, 1961) and leukemic cell phosphodiesterase (Anderson and Heppel, 1960) in rapidly hydrolyzing oligoribonucleotides with a 5'-phosphomonoester end group. It differs from the two diesterases in that it rapidly hydrolyzes oligoribonucleotides with no phosphomonoester end group and has low, if any, activity on oligodeoxyribonucleotides. Further purification of the enzyme is being undertaken before studying its action on polynucleotides and its mode of hydrolysis of oligoribonucleotides.

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