The Deoxyribonucleases of *Escherichia coli*

III. STUDIES ON THE NATURE OF THE INHIBITION OF ENDONUCLEASE BY RIBONUCLEIC ACID*

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It was first observed by Kozloff (1) that the deoxyribonuclease activity of cell-free extracts of *Escherichia coli* could be increased by treatment with pancreatic ribonuclease. This stimulation was found to result from the destruction by the ribonuclease of an inhibitory ribonucleic acid. The phenomenon of cellular deoxyribonuclease inhibition is a widespread one, having been observed in yeast (2), mammalian tissues (3), and streptococci (4). The yeast and mammalian deoxyribonuclease inhibitors were identified as proteins; the streptococcal inhibitor was shown to have the properties of a ribonucleic acid.

Recent investigations of the nucleases of *Escherichia coli* (5, 6) have shown that the deoxyribonuclease activity in cell-free extracts of this organism is attributable to at least three different enzymes. Two of these have now been purified. It was, therefore, of interest to establish which of these enzymes is subject to inhibition by ribonucleic acid and what is the nature of the ribonucleic acid-enzyme interaction. The studies reported here show that the deoxyribonucleic acid-specific endonuclease described in the previous paper (6) is the only one of the known nucleases of *E. coli* which can be inhibited by ribonucleic acid. They further demonstrate that ribonucleic acids from a variety of sources, including *E. coli*, guinea pig liver, and tobacco mosaic virus, can serve as potent inhibitors of the purified endonuclease.

EXPERIMENTAL PROCEDURE

Materials and Methods

The *E. coli* endonuclease (carboxymethyl (CM) cellulose and Amberlite IRC-50 (XE-64) fractions) and DNA were prepared as described in the previous paper (6). Crystalline pancreatic RNase and DNase were purchased from the Worthington Biochemical Corporation. Crystalline horse heart cytochrome *c* was obtained from the Sigma Chemical Company. Crystalline bovine plasma albumin was obtained from the Armour Laboratories. Snake venom phosphodiesterase was prepared by the method of Sinsheimer and Koerner (7). *E. coli* phosphodiesterase was purified as described previously (5). Amino acid acceptor RNA and ribosomal RNA from *E. coli* (8, 9), amino acid acceptor RNA from guinea pig liver (10), and tobacco mosaic virus RNA (11) were gifts from Dr. Paul Berg. Polyadenylate and poly AGUC,[1] both synthesized by means of poly-

nucleotide phosphorylase from *Azotobacter vinelandii* (12), were kindly provided by Dr. Severo Ochoa. High molecular weight polyphosphate prepared by the method of Pfanstiel and Iler (13) was the gift of S. R. Kornberg.

Assay of Endonuclease Inhibitor—This assay measures the inhibition by RNA of the formation of acid-soluble products from P^{32}-labeled DNA by the *E. coli* endonuclease. The reaction mixture (0.3 ml) contained 20 μmoles of Tris buffer, pH 7.5; 2 μmoles of $MgCl_2$; 40 μg of cytochrome *c*; P^{32}-labeled *E. coli* DNA, 20 mμmoles of P (1 μc per μmole of P); 0.2 to 1.0 unit of RNA inhibitor; and 0.04 unit of Amberlite XE-64 fraction of endonuclease[2] (6). As a rule, three levels of inhibitor were run for each assay. After incubation for 30 minutes at 37°, acid-soluble P^{32} was measured as previously described (6). Alternatively, residual nuclease activity could be determined by observing the development of hyperchromicity as described before (6). One unit of inhibitor is defined as that amount which produces a decrease in rate such that $1/V - 1/V_0$ is equal to 1, where V is the velocity of reaction in the presence of inhibitor and V_0 is the velocity in its absence, when measured under the specified conditions of the assay.

RESULTS

Addition to the purified endonuclease of a crude extract of *E. coli* which had been heated at 100° for 20 minutes resulted in complete inhibition of nuclease activity. This inactivation was specifically attributable to the RNA in the boiled extract, since it could be alleviated by pretreatment of the extract with pancreatic RNase (Table I).

There are two major RNA components in *E. coli*, the lower molecular weight amino acid acceptor RNA, and a larger RNA component found in the ribosomes (8, 9). Both were found to be potent inhibitors of the endonuclease.

Kinetics of Inhibition by Amino Acid Acceptor RNA—A detailed examination of the kinetics of inhibition by amino acid acceptor RNA showed it to be a competitive inhibitor of the endonuclease. The dissociation constant of the enzyme inhibitor complex (K_i) calculated by the method of Lineweaver and Burk (14) (Fig. 1) was 9×10^{-9} M (expressed as moles of

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[1] The abbreviation used is: poly AGUC, copolymer composed of adenylic, guanylic, cytidylic, and uridylic acids.

[2] The CM-cellulose fraction, which has substantial RNase activity (6), could serve as well. The K_m of RNA for the contaminating ribonuclease is 3×10^{-3} M; the K_i of amino acid acceptor RNA for the endonuclease is 1×10^{-8} M. As a result, any RNA added to an RNase-contaminated endonuclease fraction is immediately bound by the endonuclease. In this form we have found the RNA to be relatively insensitive to the RNase.

<div style="text-align:center">TABLE I</div>

Demonstration of inhibitory RNA in boiled extract of E. coli

The incubation mixtures were set up as described under "Materials and Methods." Boiled extract was prepared by heating a crude extract at 100° for 20 minutes. After chilling at 0°, precipitated protein was removed by centrifugation for 10 minutes at 10,000 × *g*. The supernatant fluid was diluted 50-fold, and 0.02 ml, containing 0.11 optical density unit at 260 mμ, was added as indicated. RNase treatment was carried out in an incubation mixture (0.2 ml) containing 0.02 ml of boiled extract (5.5 optical density units at 260 mμ); 15 μmoles of Tris buffer, pH 7.5; and 50 μg of RNase. After incubation for 30 minutes at 37°, the solution was diluted 5-fold, and 0.02 ml was tested for inhibition in the standard assay. The CM-cellulose fraction of endonuclease was used in these experiments.

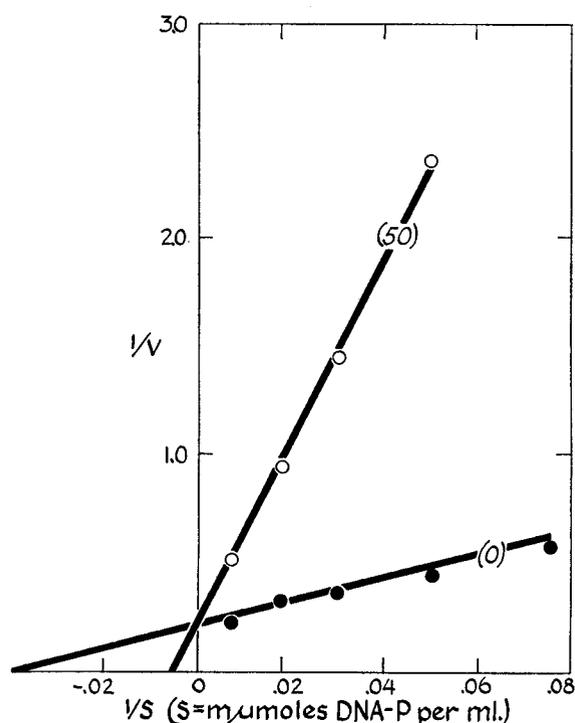| System	DNA-P made acid-soluble
	mμmoles
Endonuclease	3.18
Endonuclease + boiled extract	<0.10
Endonuclease + RNase-treated boiled extract	3.21

FIG. 1. Competitive inhibition of *E. coli* endonuclease by amino acid acceptor RNA from *E. coli*. The numbers in parentheses give the concentration of amino acid acceptor RNA in micromicromoles of P per ml. 1/*V* is the reciprocal of the millimicromoles of DNA-P made acid-soluble in 30 minutes.

RNA nucleotide). This is to be compared with a value of 2.5 × 10⁻⁵ M, which is the K_m observed for DNA (expressed as moles of DNA nucleotide). A plot of the reciprocal of the reaction velocity against inhibitor concentration according to the method of Dixon (15) gave a value of 1.0 × 10⁻⁸ M for the K_i, which is in good agreement with that calculated from the Lineweaver-Burk expression (Fig. 2). Moreover, this type of plot provided a

means of assaying quantitatively the inhibitor activity. When $1/V - 1/V_0$ is plotted against concentration of Inhibitor I, a linear relationship is obtained (Fig. 3).

Magnesium ion is required for inhibition of the endonuclease by amino acid acceptor RNA. Thus, when the magnesium ion concentration normally present in the incubation mixture is reduced by a factor of 100 and cytochrome *c* is omitted, inhibition is no longer observed. It can be partially restored by the addition of cytochrome *c* (Table II).

Susceptibility of Inhibitor to RNase and Snake Venom Diesterase—Pretreatment of purified amino acid acceptor RNA with RNase results in a loss of its activity as an inhibitor of the endonuclease (Fig. 4). At a concentration of RNase (3.7 × 10⁻⁴ μg per ml) at which 50% of the RNA was converted to acid-soluble products, essentially all inhibitor activity was lost.

The inhibitor is also destroyed by the action of snake venom phosphodiesterase, another enzyme which attacks RNA. In this case, when 23% of the RNA had been converted to acid-soluble fragments by the action of this enzyme, a 34% decrease in inhibitor activity was observed. When 40% of the RNA had become acid-soluble, only 25% of the inhibitor activity remained. It is noteworthy that the endonuclease inhibitor activity of this RNA is considerably less sensitive to hydrolysis by these two enzymes than is its amino acid-binding activity. For example, venom phosphodiesterase digestion of the RNA results in essentially complete loss of amino acid acceptor activity when as few as 5% of the nucleotides have been cleaved off (16). Since this enzyme carries out an exonucleolytic attack beginning at the 3'-hydroxyl end of the RNA chain (17), the very rapid inactivation of acceptor activity is consistent with the known requirement for an adenosine residue at this end of the chain (18). The
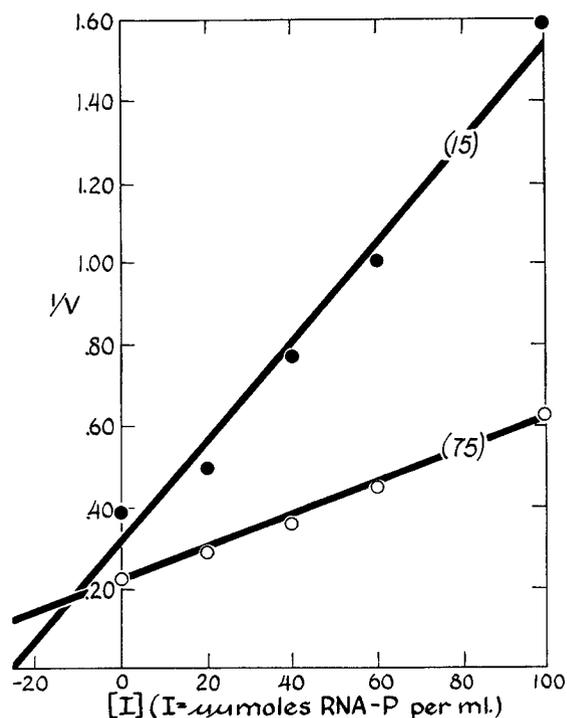

FIG. 2. Determination of K_i for amino acid acceptor RNA from *E. coli*. The numbers in parentheses are the concentrations of DNA in millimicromoles of P per ml. 1/*V* is the reciprocal of the millimicromoles of DNA-P made acid-soluble in 30 minutes.

presence of a specific residue at this end of the RNA molecule is clearly not essential for its activity as an inhibitor of the *E. coli* endonuclease.

Inhibitor Activity of Other RNA's—Examination of a variety of RNA preparations showed the amino acid acceptor RNA from *E. coli* to be the most active of those tested; however, all the RNA's examined were to some extent active (Table III). Amino acid acceptor RNA from guinea pig liver showed approximately one-third as much activity as the corresponding RNA from *E. coli*. High molecular weight RNA from *E. coli* ribosomes or
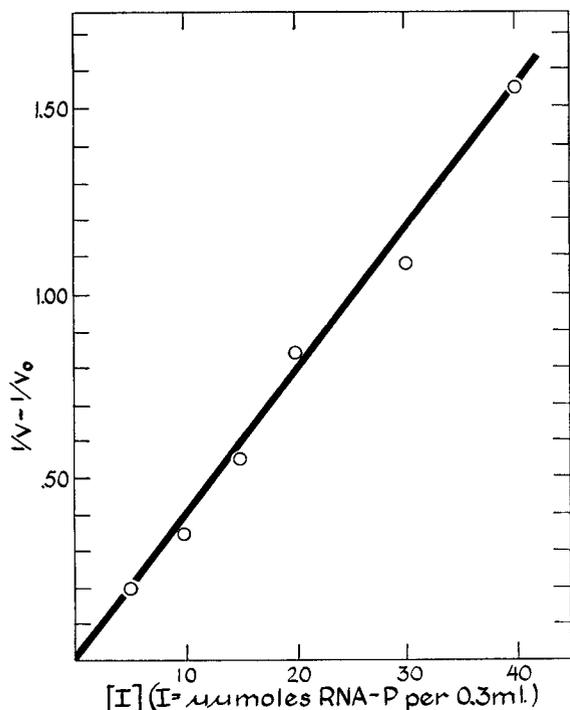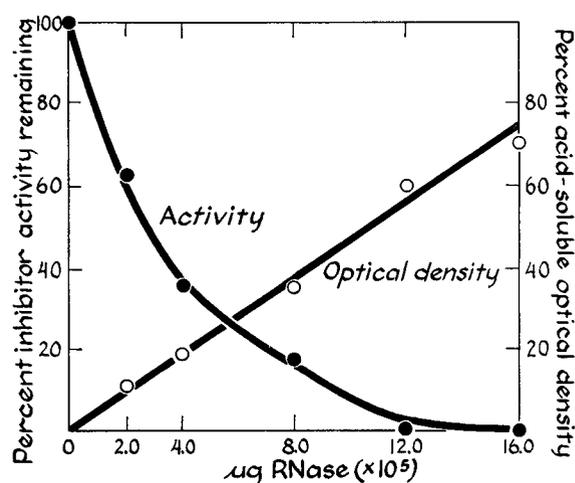
FIG. 4. Destruction of inhibitor activity of amino acid acceptor RNA from *E. coli* by pancreatic RNase. The incubation mixtures (0.3 ml) contained 10 μmoles of Tris buffer, pH 7.5; 0.5 mg of crystalline bovine plasma albumin; 60 mμmoles of RNA-P, and the indicated amounts of RNase (diluted in albumin, 1 mg per ml). After 30 minutes at 37°, 0.02-ml aliquots were removed for assay of inhibitor activity. To 0.2 ml of the remainder were added 0.01 ml of a solution of albumin (50 mg per ml), 0.29 ml of water, and 0.5 ml of 1 N perchloric acid. The tubes were kept at 0° for 5 minutes, then centrifuged at 10,000 × *g* for 5 minutes. The optical density of the supernatant fluids at 260 mμ was determined. In calculating the percentage of the total optical density made acid-soluble, no correction was made for the hyperchromic effect resulting from the hydrolysis of the RNA.

FIG. 3. Assay of inhibitor activity of amino acid acceptor RNA from *E. coli*. $1/V$ is the reciprocal of the reaction velocity (millimicromoles of DNA-P made acid-soluble in 30 minutes) in the presence of inhibitor; $1/V_0$ is the reciprocal of the velocity in the absence of inhibitor.

TABLE II

Effect of magnesium ion concentration on inhibition of endonuclease by amino acid acceptor RNA

The incubation mixtures (0.30 ml) contained 20 μmoles of Tris buffer, pH 7.5; MgCl$_2$ as indicated; amino acid acceptor RNA, 3 mμmoles of P where indicated; P^{32}-labeled *E. coli* DNA; 20 mμmoles of P; and 0.04 unit of endonuclease (CM-cellulose fraction). The assay was then carried out as described under "Materials and Methods."

Magnesium concentration	Acid-soluble nucleotide formed	
	−Inhibitor	+ Inhibitor
moles/liter	*mμmoles*	
7 × 10^{-3}	2.0	<0.02
7 × 10^{-4}	0.9	<0.02
7 × 10^{-5}	0.7	0.7
7 × 10^{-5}*	1.6	1.0

* Cytochrome *c* (40 μg) was added to the incubation mixture.

TABLE III

Relative inhibitor activity of various RNA preparations

Inhibitor	Approximate molecular weight	Inhibitor units per mμmole of RNA nucleotide	Inhibitor units per mμmole of RNA
Amino acid acceptor RNA (*E. coli*)	3 × 10^4 (8)	39	3900
Ribosomal RNA (*E. coli*)	1 × 10^6 (9)	0.4	1200
Tobacco mosaic virus RNA	2 × 10^6 (11)	0.2	1200
Amino acid acceptor RNA (guinea pig liver)	3 × 10^4 (10)	14	1400
Poly AGUC	1 × 10^5	0.1	30
Polyadenylate	1 × 10^5	<0.002	<0.5
Polyphosphate	>1 × 10^4 (13)	<0.002	<0.5

tobacco mosaic virus was also approximately one-third as active as the *E. coli* acceptor RNA when compared per mole of RNA rather than per mole of nucleotide. In contrast, the RNA-like polymers synthesized by means of polynucleotide phosphorylase were relatively inert. Poly AGUC showed only slight inhibitor activity, and polyadenylate, even at very high concentrations, was completely inactive. Examination of both of these polymers in the ultracentrifuge[3] showed them to be polydisperse ma-

[3] We are grateful to Dr. Ross Inman for performing these studies.

terials with approximate weight average molecular weights of about 1×10^5. Polyphosphate, another high molecular weight polyanion, was also found to be inert as an inhibitor of the endonuclease. On the contrary, this compound caused a 3-fold stimulation in endonuclease rate when added in concentrations as high as 20 μmoles of P per ml.

Specificity of RNA for Endonuclease—Other enzymes which attack DNA or polydeoxynucleotides are unaffected by amino acid acceptor RNA from *E. coli*. Incubation of pancreatic DNase (1 unit)[4] or *E. coli* phosphodiesterase (1 unit) each with 10 mμmoles of amino acid acceptor RNA-P did not diminish their nuclease activity. Snake venom phosphodiesterase was similarly unaffected by the presence of RNA when assayed for its activity on a mixture of deoxyoligonucleotides.

Inhibition of the *E. coli* endonuclease, which is a basic protein (6), by amino acid acceptor RNA is not affected by the presence in the incubation mixture of a very large excess of another basic protein, cytochrome *c*, which is routinely added to stabilize the endonuclease. Thus, the same inhibition in the activity of 0.01 μg of enzyme protein (approximately 60%) by amino acid acceptor RNA was observed in the presence or absence of 40 μg of cytochrome *c*.

DISCUSSION

Of the deoxyribonucleases now known to be present in *E. coli*, the endonuclease described here and in the previous paper (6) is the only one which is sensitive to inhibition by RNA. Although many RNA's are capable of inhibiting the purified *E. coli* endonuclease, the inhibition exhibits certain specific features. Thus, the phenomenon appears to be confined to an interaction between the *E. coli* endonuclease and naturally occurring RNA. On the one hand, other nucleases which attack DNA, such as pancreatic DNase and the *E. coli* and snake venom phosphodiesterases, are unaffected by RNA. Moreover, proteins, such as cytochrome *c*, which have dimensions and ionic properties similar to the *E. coli* endonuclease do not interfere with the inhibition of the endonuclease by RNA, even when they are present in very great excess. On the other hand, the enzymatically synthesized polymers which resemble RNA in a number of respects (12) appear to lack features characteristic of the naturally occurring RNA's which permit the natural RNA's to act as inhibitors.

It should be pointed out that, although the amino acid acceptor and ribosomal RNA's from *E. coli* are both potent inhibitors of the purified *E. coli* endonuclease, the possibility cannot be excluded that there exist within the cell other, more potent, RNA's whose specific function is, indeed, the immobilization of this nuclease.

In this regard, the relevance of the RNA inhibition of the purified endonuclease to the deoxyribonuclease activity of extracts of phage-infected bacteria deserves some comment. It was first demonstrated by Pardee and Kunkee (19) that extracts of *E. coli* infected with bacteriophage T2 exhibit a 2- to 3-fold greater deoxyribonuclease activity than uninfected cell extracts. This increase in total deoxyribonuclease activity was attributed by Kozloff (1) to a partial destruction of an inhibitory RNA,

which he discovered as a result of these studies. The experiments reported here would suggest that the increase in deoxyribonuclease activity following phage infection is not entirely attributable to the destruction of a specific RNA. Thus, it is conceivable that if the RNA which normally binds the endonuclease within the cell were in some way destroyed as a result of the infection, the amino acid acceptor or ribosomal RNA's,[5] both potent inhibitors of the endonuclease, could immediately bind the endonuclease, rendering it inactive.

Recent studies of the enzymatic activities of phage-infected cells (21) have demonstrated the induction, as a result of the infective process, of new enzymes specific to the infected cell. In addition, the increased activity of enzymes already present at the time of infection has been observed (21, 22). It is, therefore, not inconceivable that the increase in over-all deoxyribonuclease activity found in phage-infected cells may in fact be due to an increased synthesis of existing nucleases, or perhaps to synthesis of deoxyribonucleases unique to the phage-infected bacterium.

SUMMARY

The *Escherichia coli* endonuclease is found in extracts bound to an inhibitory ribonucleic acid.

1. When purified, this enzyme is free of inhibitor and can be inhibited by a variety of ribonucleic acids from *Escherichia coli*, guinea pig liver, and tobacco mosaic virus. Of those tested, the amino acid acceptor ribonucleic acid from *Escherichia coli* is the most active. The kinetics of the enzyme inhibited by the amino acid acceptor ribonucleic acid obey the equations for a competitive inhibition, and the calculated K_i is of the order of 1×10^{-8} M (ribonucleic acid nucleotide).

2. The ribonucleic acid-like polynucleotides synthesized by polynucleotide phosphorylase are essentially inactive as inhibitors, as is high molecular weight polyphosphate.

3. The activity of other deoxyribonucleic acid-cleaving enzymes, including pancreatic deoxyribonuclease and the *E. coli* and snake venom phosphodiesterases, is not affected by ribonucleic acid.

4. Other basic proteins such as cytochrome *c*, when present in great excess, do not prevent the inhibition of the endonuclease (also a basic protein) by amino acid acceptor ribonucleic acid.

The relevance of these observations to the changes in over-all deoxyribonuclease activity after phage infection of *Escherichia coli* is discussed.

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[4] A unit of pancreatic DNase activity is defined as the amount which will produce 0.1 μmole of acid-soluble DNA nucleotide in 30 minutes under the conditions used for assay of the *E. coli* endonuclease.

[5] The level of RNA in *E. coli* following infection with the T-even bacteriophages remains relatively unchanged (20).

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