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POLYGUANYLIC ACID-INHIBITED RIBONUCLEASE OF *KLEBSIELLA*

II. STUDIES WITH SYNTHETIC POLYRIBONUCLEOTIDES

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SUMMARY

Purified *Klebsiella* ribonuclease (ribonucleate nucleotido-2'-transferase (cyclizing), EC 2.7.7.17) is able to hydrolyze poly(A), poly(U) and poly(C) more readily than yeast RNA, but cannot attack poly(G), poly(I), poly(X), poly(hU), poly(A)·poly(U) and poly(I)·poly(C). Hydrolysis of yeast RNA, poly(U), and poly(A) by this nuclease is inhibited markedly by poly(G) and to a much lesser extent by poly(I). Another compound, poly(X), is more potent than poly(I) as an inhibitor of RNA hydrolysis but less potent in inhibiting poly(U) hydrolysis. Relatively high concentrations of poly(hU) will inhibit poly(U) hydrolysis but have no effect when yeast RNA is the substrate. A number of mononucleotides were tested and have no effect on RNA hydrolysis by *Klebsiella* nuclease. Several other nucleases were found to be inhibited to a greater or lesser extent by poly(G) and poly(I). The data suggest that the nuclease prefers molecules of unordered secondary structure as substrates, and that ordered molecules are not attacked and may actually be inhibitors.

INTRODUCTION

In the preceding paper, the purification and general properties of an endonuclease (ribonucleate nucleotido-2'-transferase (cyclizing), EC 2.7.7.17) from *Klebsiella* sp. were described. Synthetic ribonucleic acid polymers were used initially to attempt to define the residue specificity of the enzyme. Significantly, while the nuclease does not manifest specificity in the hydrolysis of native RNA, it has a marked specificity against synthetic polymers. It, in addition, is inhibited by several of the synthetic

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Abbreviations: poly(A), polyadenylic acid; poly(C), polycytidylic acid; poly(G), polyguanylic acid; poly(U), polyuridylic acid; poly(I), polyinosinic acid; poly(A)·poly(U), polyadenylic·polyuridylic acid (double-stranded); poly(I)·poly(C), polyinosinic·polycytidylic acid (double-stranded); poly(A)·poly(U)·poly(U), polyadenylic·polyuridylic·polyuridylic acid, (triple-stranded); poly(X), polyxanthylic acid; poly(hU), polydihydrouridylic acid; mM P, mmoles per l of polynucleotide phosphorus; μM P, μmoles per l of polynucleotide phosphorus.

polymers tested: poly(G), poly(I), poly(X) and poly(hU). The results of these experiments with synthetic nucleic acid polymers are presented below.

MATERIALS AND METHODS

Chemicals

Synthetic homopolymers were bought from the Sigma Chemical Co. (St. Louis, Mo.) or Miles Laboratories (Elkhart, Ind.). Ribonucleoside and deoxyribonucleoside phosphate compounds were obtained from the Sigma Chemical Co. Poly(A)·poly(U), poly(I)·poly(C) and GCG tricodon were products of the Miles Laboratories. Bovine pancreatic ribonuclease A (crystalline) was purchased from the Worthington Biochemical Corp. (Freehold, N.J.).

Ribonuclease assay

Ribonuclease assay was described in the preceding paper[1]. Assays of activity against synthetic polymers were performed in the same way but at 25 °C. When poly(U) was the substrate, incubation time was 7.5 min and 20 mM lanthanum nitrate in 12% $HClO_4$ was used as the precipitant in place of 12% $HClO_4$ alone. When activity against poly(C) was assayed, absorbances were measured at 280 nm rather than 260 nm.

RESULTS

Activity against synthetic polymers

The purified nuclease was tested for its activity against a variety of synthetic polymers, both single- and double-stranded, and the results are shown in Table I. Activities against poly(U), poly(A), and poly(C) are 7, 3 and 2 times control (yeast RNA), respectively. No activity was found against poly(G), poly(I), poly(X) and poly(hU); or against the double-stranded compounds, poly(A)·poly(U) and poly(I)·poly(C) and triple-stranded poly(A)·poly(U)·poly(U).

TABLE I

ACTIVITY OF *Klebsiella* ENDONUCLEASE AGAINST SYNTHETIC RIBONUCLEIC ACID

Assays were performed at 25 °C with 0.6 unit of enzyme per ml. Concentrations of synthetic polymers were 0.5 mM P.

Substrate	*Relative activity (%)*
Yeast RNA (control)	100
Poly(A)	335
Poly(C)	216
Poly(G)	0
Poly(U)	716
Poly(I)	0
Poly(A)·poly(U)	0
Poly(I)·poly(C)	0
Poly(A)·poly(U)·poly(U)	0
Poly(X)	0
Poly(hU)	0

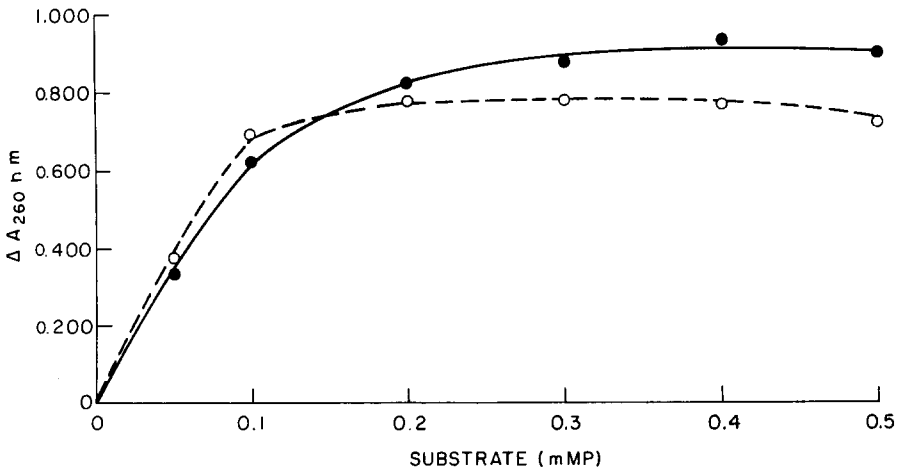


Fig. 1. *Klebsiella* nuclease·poly(U) and poly(A) substrate saturation curves. Assays performed as indicated in text with 0.6 unit of nuclease per reaction using poly(U) (●—●) or poly(A) (○--○) as substrates in place of yeast RNA. Substrate concentration was varied as indicated.

In Fig. 1 are shown the saturation curves for *Klebsiella* nuclease when poly(U) and poly(A) are substrates. Saturation occurs in both cases at the relatively low substrate level of about 0.2 mmoles per l of polynucleotide phosphorus (mM P).

Both poly(A) and poly(U) manifest substrate inhibition at relatively high concentrations (Table II). Poly(A) substrate inhibition is more profound, its hydrolysis being inhibited virtually completely at a concentration of 4 mM P, while poly(U) hydrolysis at the same concentration is inhibited only 57%, and only 75% at the concentration of 8 mM P. When equivalent concentrations of yeast RNA were used, substrate inhibition was either slight or did not occur.

Inhibition by poly(G) and poly(I) of substrate hydrolysis by Klebsiella nuclease

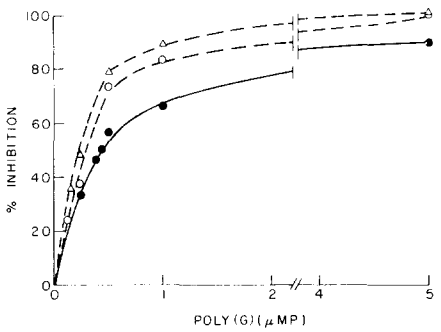
50% inhibition of RNA, poly(U) and Poly(A) hydrolysis occurs at poly(G) concentrations of 0.5, 0.375 and 0.25 μmoles per l of polynucleotide phosphorus (μM P) respectively (Fig. 2). The hydrolysis of the synthetic polynucleotides is thus some-, what more sensitive to poly(G) inhibition than is the native nucleic acid.

TABLE II

SUBSTRATE INHIBITION OF *Klebsiella* NUCLEASE

Assays performed as indicated in the section on Materials and Methods, using 0.6 unit of nuclease per ml of reaction volume.

Substrate	*(mM P)*	*Inhibition of nuclease activity (%)*
Poly(A)	0.4	—
	4.0	95
	8.0	99
Poly(U)	0.4	—
	4.0	57
	8.0	75



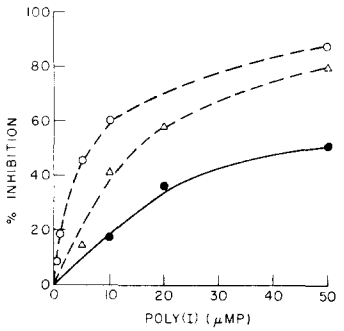


Fig. 2. Poly(G) inhibition of yeast RNA, poly(U) and Poly(A) hydrolysis by *Klebsiella* nuclease. Assays performed as indicated in text, using 0.6 unit of enzyme per reaction. Concentration of yeast RNA was 250 μg per reaction. Poly(A) and poly(U) concentrations were 0.5 mM P. Poly(G) concentration was varied as indicated. ●—●, yeast RNA. ○---○, poly(U). △---△, poly(A).

Fig. 3. Poly(I) inhibition of yeast RNA, poly(U) and poly(A) hydrolysis by *Klebsiella* nuclease. Assays performed as in Fig. 2. Poly(I) concentration was varied as indicated. ●—●, yeast RNA; ○---○, poly(U); △---△, poly(A).

Poly(I) is much less potent than poly(G), 50% inhibition of RNA, poly(U) and poly(A) hydrolysis occurring at poly(I) concentrations of 50, 7.5 and 15 μM P, respectively (Fig. 3). In contrast to poly(G), which inhibits hydrolysis of these substrates to about the same extent, poly(I) is much more potent in its effects upon hydrolysis of poly(U) and poly(A) than it is upon RNA hydrolysis.

When double reciprocal plots of yeast RNA concentration against reaction velocity are made in the presence and absence of inhibitory polymers, the inhibition is clearly competitive in the case of poly(G) (Fig. 4) and probably competitive in the

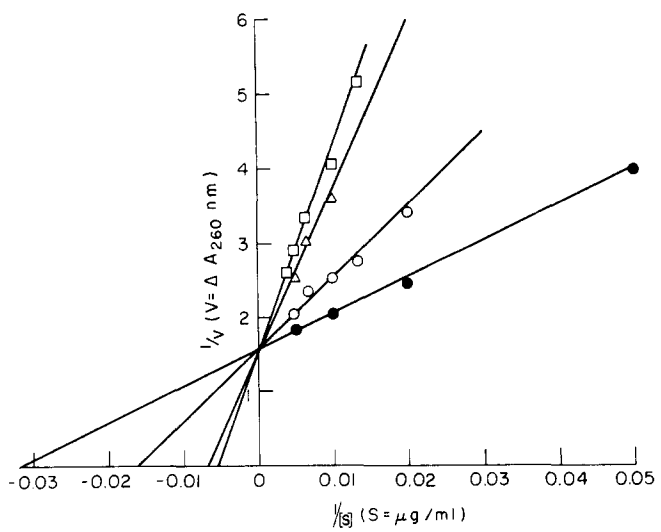


Fig. 4. Poly(G) and poly(X) inhibition of yeast RNA hydrolysis: Lineweaver–Burk plots. Assays performed with 0.6 unit of nuclease per reaction. ●—●, control; ○—○, poly(G), 0.25 μM P; △—△, poly(G), 0.5 μM P; □—□, poly(X), 2.5 μM P.

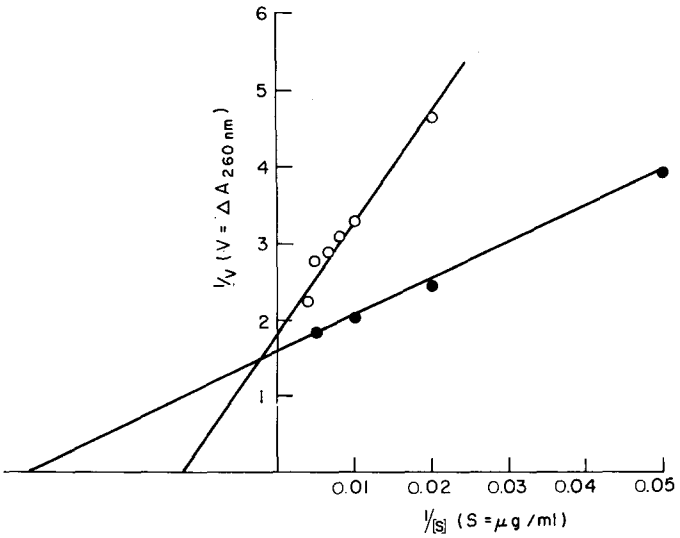


Fig. 5. Poly(I) inhibition of yeast RNA hydrolysis: Lineweaver–Burk plots. 0.6 unit of enzyme per reaction. ●—●, control; ○—○, poly(I), 25 μM P.

case of poly(I) (Fig. 5). In Fig. 6 is shown the double reciprocal plot of poly(U) concentration against initial reaction velocity with and without inhibitory polymers. Both poly(G) and poly(I) are clearly competitive inhibitors of poly(U) hydrolysis.

Poly(C) effect on inhibition poly(G)

Since poly(C), which is attacked by *Klebsiella* nuclease, is known to form complexes with poly(G), the effect of this compound on poly(G) inhibition of RNA hydrolysis was tested. When equal concentrations of poly(C) and poly(G) were added to a reaction, the inhibition of RNA hydrolysis by poly(G) was unaffected. This suggests that poly(G) can bind to the nuclease even though it is complexed with poly(C).

Inhibition by poly(X) and poly(hU) of substrate hydrolysis by Klebsiella nuclease

In contrast to poly(G) and poly(I), poly(X) inhibits hydrolysis of yeast RNA

TABLE III

INHIBITION OF *Klebsiella* NUCLEASE BY POLY(U) AND POLY(hU)

Assays were performed as indicated in previous tables and in the text.

Substrate	*Polynucleotide concn (μM P) producing 50% inhibition*	
	Poly(X)	*Poly(hU)*
RNA	5	Not inhibitory
Poly(U)	25	125

more potently than it does poly(U) hydrolysis (Table III). 50% inhibition of yeast RNA hydrolysis by *Klebsiella* nuclease occurs at a poly(X) concentration of 5 μM P, while poly(U) hydrolysis is not inhibited to this extent until poly(X) concentration has reached 25 μM P. Poly(hU) at a relatively high level does not affect yeast RNA hydrolysis, and it is the least potent of the synthetic polynucleotide compounds in its effect upon poly(U) hydrolysis (Table III). Poly(X) inhibition of RNA hydrolysis (Fig. 4) and poly(U) hydrolysis (Fig. 6) is competitive as is poly(hU) inhibition of poly(U) hydrolysis (Fig. 6).

Effects of nucleotides on endonuclease activity

The 2':3'-, 3'- and 5'-monophosphates of adenosine, cytidine, uridine and guanosine, and the 5'-monophosphates of deoxyadenosine, deoxycytidine, deoxy-

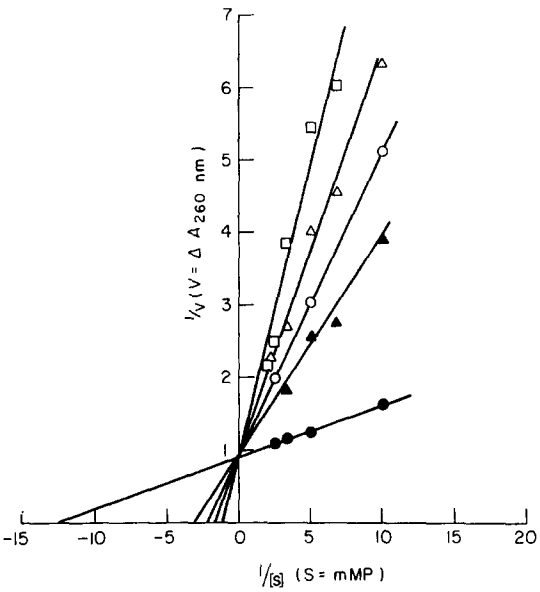


Fig. 6. Inhibition of poly(U) hydrolysis by poly(G), poly(I), poly(X) and poly(hU): Lineweaver-Burk plots. ●—●, control; ○—○, poly(G), 0.25 μM P; ▲—▲, poly(I), 5 μM P; △—△, poly(hU), 75 μM P; □—□, poly(X), 12.5 μM P.

guanosine and thymidine, at a concentration of 0.1 mM do not inhibit yeast RNA hydrolysis by *Klebsiella* nuclease. ATP, GTP ,GDP and the tricodon GCG also have no effect on *Klebsiella* nuclease activity.

Effects of poly(G) and poly(I) on other ribonucleases

Several other ribonucleases were found to be inhibitable by poly(G) and poly(I) (Table IV). Two bacterial ribonucleases, *Enterobacter* nuclease[2] and *Citrobacter* nuclease (W. E. Mitch, Jr and C. C. Levy, in preparation) showed the same order of sensitivity to poly(G) inhibition as did *Klebsiella* nuclease, but bovine pancreatic ribonuclease A was inhibitable only at poly(G) concentrations 200 times greater. On the other hand, *Enterobacter* nuclease was inhibited by poly(I) to the same extent

TABLE IV

EFFECT OF POLY(G) AND POLY(I) ON ACTIVITIES OF OTHER RIBONUCLEASES

Assays were performed as indicated in the section on Materials and Methods, using yeast RNA as substrate. For bovine pancreatic ribonuclease A and *Enterobacter* enzyme 1.0 unit of enzyme per reaction was used. For *Citrobacter* nuclease, 0.6 unit was used.

Polynucleotide		*Inhibition of RNA hydrolysis (%)*		
		Enterobacter nuclease	*Citrobacter nuclease*	*Bovine pancreatic ribonuclease A*
Poly(G)	0.5 μM P	60	72	—
	50.0 μM P	—	93	14
	125.0 μM P	—	—	59
Poly(I)	50.0 μM P	47	21	15
	125.0 μM P	—	47	44

as *Klebsiella* nuclease, but bovine pancreatic ribonuclease A and *Citrobacter* nuclease were inhibited significantly only at much higher concentrations of poly(I).

DISCUSSION

Although *Klebsiella* endonuclease hydrolyzes native yeast RNA completely, showing no base specificity, its activity against synthetic ribonucleic acid polymers is variable. Poly(U), the synthetic substrate most rapidly utilized, is degraded about 7 times as fast as native RNA. Poly(A) is hydrolyzed at 3 times and poly(C) at 2 times the rate for RNA. However, poly(G), poly(I) and poly(X), homopolymers believed to have helical configurations in solution[3–5], do not serve as substrates, nor does poly(hU), the secondary structure of which is not yet known. Double-stranded polymers such as poly(A)·poly(U), poly(I)·poly(C) and DNA[1] also are not hydrolyzed. These data suggest that secondary and tertiary molecular structures of the substrate are of great importance in determining the activity of the enzyme. Similar observations have been made by Singer and Tolbert[6] and Chakrabortty and Burma[7] using other bacterial endonucleases. The fact that native RNA can be completely broken down, whereas poly(G) is not used is further evidence that non-utilization of poly(G) is due to molecular configuration rather than to its nucleotide composition.

The further finding that poly(G), poly(I), poly(X) and poly(hU) are competitive inhibitors of nuclease activity was unexpected. Since guanosine mononucleotides and the trinucleotide diphosphate codon GCG do not inhibit nuclease activity, it is likely that the inhibition is related to the ordered secondary structure of the inhibitory molecules. The fact that inhibition of yeast RNA hydrolysis occurs at substrate to inhibitor ratios of greater than 1000:1 for poly(G) and 20:1 for poly(I) suggests that the inhibition is due to an effect upon the enzyme rather than to a structural alteration of the substrate secondary to formation of complexes with the inhibitor. Inhibition of nuclease activity by polyarabinouridylic acid has been reported, but at substrate to inhibitor ratios of a much smaller magnitude[8,9]. An attempt to prevent poly(G) inhibition by adding an equimolar amount of poly(C) to cause poly(C)·poly(G) complexing was unsuccessful, suggesting that the poly(C)·poly(G) complex is also able to bind to the nuclease.

In general, the hydrolysis of synthetic polyribonucleotides is inhibited more strongly than is hydrolysis of yeast RNA. The reverse is true with respect to poly(X) inhibition, when comparing poly(U) and RNA hydrolysis. The fact that poly(X) will complex with poly(U) may be of significance in explaining this inconsistency. Possibly poly(U)·poly(X) (the multistranded helical compound) is unable to bind to the enzyme as effectively as does poly(X) alone. The relatively weak inhibition of poly(U) hydrolysis by poly(hU) may be related to its primary structure similarity to poly(U) rather than to the presence of a helical secondary structure, since this polymer does not affect yeast RNA hydrolysis.

The fact that two other bacterial endonucleases isolated previously in this laboratory are also inhibitable by poly(G) and poly(I) suggests that this property may be common to enzymes of this type. Regulation of ribonuclease activity intracellularly in cells lacking lysosomes or other means of segregation of degradative enzymes may be in part a result of binding and inhibition of the enzyme by helical ribonucleotide sequences.

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