Biochem. J. (1981) **193**, 311–324
Printed in Great Britain

Poly(adenylic acid) in small amounts, free or covalently linked to substrate, protects RNA from hydrolysis by ribonuclease

Timothy P. KARPETSKY, Kren K. SHRIVER and Carl C. LEVY
Laboratory of Molecular Biology, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Baltimore, MD 21201, U.S.A.

(*Received 15 April 1980/Accepted 27 August 1980*)

Short lengths (18 residues) of poly(A), covalently linked to the 3′-termini of *Escherichia coli* 5S rRNA, induce powerful inhibitions (38–87%) of the activities of RNAases (ribonucleases) from *Citrobacter* sp., *Enterobacter* sp., bovine pancreas, human spleen and human plasma. As the polypurine chain length is extended, enzyme activity declines. Furthermore, poly(A) sequences, present only on a small subpopulation of RNA, and accounting for less than 1% of total RNA, serve to protect all RNA, polyadenylated or not, from enzyme-catalysed degradation. The quantity of 3′-terminal adenylic acid residues, relative to the amount of substrate, determines enzyme activity. The exact distribution of a fixed amount of poly(A) residues on the 3′-termini of substrate molecules is unimportant in this respect. Comparison of the efficacies of inhibition of RNAase activity, by using linked poly(A) and similar quantities of free poly(A), revealed that although the free polypurine inhibits RNAase activity, covalent linkage of poly(A) to RNA is more advantageous to the stability of an RNA substrate. However, the ratio of inhibited activities obtained by using linked or free poly(A) may change considerably with alterations in either substrate concentration or polyadenylic acid segment length.

Poly(A) is normally found *in vivo* linked at the 3′-terminus to some viral RNA species, to mRNA and, in the eukaryotic cell, to heterogeneous nuclear RNA. Since these RNA molecules present inherent difficulties in the study of poly(A) function, both with respect to their non-homogeneity (Levy *et al.*, 1973; Hieter *et al.*, 1976) and to the limited quantities of material available, our approach to the study of polyadenylated RNA was to synthesize model compounds in which an RNA was linked at its 3′-terminus to poly(A). The RNA chosen was a well-characterized one (*Escherichia coli* 5S rRNA) that was available as a homogeneous preparation in relatively large quantities. Poly(A) segments of varying length were added to the 3′-terminus of the RNA enzymically, and the polyadenylated substrate molecule was then examined under a variety of experimental conditions by using a ribonuclease from human spleen (Hieter *et al.*, 1976). The results of these studies suggested a mechanism whereby poly(A), acting perhaps in concert with other factors, maintains the stability of an RNA to which it is linked by virtue of its ability to inhibit RNAase activity (Levy *et al.*, 1975). That the effect of poly(A) was specific was indicated by the finding that substitution of tracts of poly(C) for the polypurine did not prevent the subsequent enzymic degradation of the RNA to which the tracts were linked (Hieter *et al.*, 1976). Further studies indicated that covalent linkage of poly(A) to RNA yields a molecule having a structure distinct from that expected based on the simple sum of its component parts (Karpetsky *et al.*, 1980*a*). With some ribonucleases, under certain reaction conditions, profound differences in enzyme activity were found on comparing free homopolymeric poly(A) with tracts of the polypurine covalently linked to substrate.

As might be anticipated, these findings raised more questions than could be answered in our initial studies. It would be of interest to know, for example, just how general the protective effect of an inhibitor linked to substrate is. Does the efficacy of protection vary widely if enzymes from different sources are used? Further, how efficiently does a small quantity

Abbreviations used: RNAase, ribonucleases; 5S [^{3}H]rRNA·$(A)_n$, ^{3}H-labelled *Escherichia coli* 5S rRNA having 3′-terminal tracts of poly(A) containing, on the average, *n* adenylic acid residues; SDS, sodium dodecyl sulphate.

0306-3275/81/010311-14$01.50/1

of polyadenylated molecules protect a much larger population of non-polyadenylated RNA? What advantage is there in linking poly(A) tracts to RNA? Could a similar inhibition of RNAase activity be attained by the same amount of free homopolymeric poly(A)? And, finally, at the molecular level, what types of mechanisms control the inhibition of RNAases mediated by poly(A) and, therefore, the ambient concentrations of substrate? These questions are addressed in this and the following paper (Karpetsky *et al.*, 1980*b*), wherein the mechanisms of poly(A) inhibition of RNAase activity are considered along with ways to control this inhibition without changing the concentrations of enzyme, RNA, or poly(A).

Experimental

Materials and general methods

Polynucleotide phosphorylase from *Micrococcus luteus* was obtained from two sources, P-L Biochemicals (Milwaukee, WI, U.S.A.) and Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied adenosine 5′-diphosphate, sodium salt (grade 1 from equine muscle) and ribonuclease A (type X-A, from bovine pancreas). Miles Laboratories (Elkhart, IN, U.S.A.) prepared polyuridylic acid, crystallized bovine serum albumin (RNAase-free as determined by using the standard assay system for RNAase A), *Escherichia coli* K12 5S rRNA, *E. coli* RNAase$_I$, 16S and 23S rRNA mixture, *E. coli* K12 tRNA (4S), Qβ-viral RNA, MS2-viral RNA, *E. coli* K12 frozen cell paste (mid-exponential-phase harvest), and poly(A) segments of defined length. These were characterized by sedimentation velocity (s_{20}), with 0.02M-potassium phosphate buffer, pH 7.0, containing 1.0M-NaCl. The average number of adenylic acid residues per chain, the 50% range of sedimentation values, and the corresponding sedimentation coefficients were: $\overline{16}$ (12–23), 1.5; $\overline{33}$ (24–43), 2.1; $\overline{44}$ (35–51), 2.4; $\overline{54}$ (42–66), 2.6; $\overline{90}$ (71–109), 3.3; $\overline{96}$ (69–122), 3.42; and $\overline{410}$ (320–490), 6.7. Poly(C) and oligoriboadenylic acid pentamer (A_5) were obtained from P-L Biochemicals. New England Nuclear Corp. (Boston, MA, U.S.A.) supplied [5,6-^{3}H]uridine and Protosol (0.5M solution). Baker's yeast was purchased from Anheuser-Busch (Baltimore, MD, U.S.A.). Bio-Rad Laboratories (Richmond, CA, U.S.A.) supplied acrylamide, bisacrylamide *NNN′N′*-tetramethylethylenediamine and ammonium persulphate.

The following enzymes were obtained as homogeneous preparations by methods described previously: human plasma ribonuclease (Schmukler *et al.*, 1975) (specific activity 21000 units/mg), human spleen ribonuclease (Neuwelt *et al.*, 1976) (specific activity 88000 units/mg), *Citrobacter* sp. ribonuclease (Levy *et al.*, 1973) (specific activity 50000 units/mg) and *Enterobacter* sp. ribonuclease (Levy & Goldman, 1970) (specific activity 60000 units/mg). All of these enzymes are pyrimidine-specific and none, under the assay conditions employed, hydrolyse poly(A).

Assays of enzyme activity, performed for an analysis of the kinetics of RNAase inhibition, were done in triplicate, and the average value was used in the determination of constants.

Measurements of enzyme activity

Ribonuclease A. The standard assay system (1.0ml) contained 16.8nmol (as phosphate residues) of *E. coli* 5S [^{3}H]rRNA (specific activity 190c.p.m./nmol), 5μmol of Tris/HCl buffer, pH 7.6, and 1.03ng of enzyme. After incubation of the reaction mixture for 7.5min at 37°C, the reaction was terminated by the addition of 1.0ml of 12% (v/v) $HClO_4$ containing 20mM-$La(NO_3)_3$. After cooling in an ice bath for 20min, the mixture was centrifuged at 39900***g*** for 15min and a portion (1.8ml) of the radioactive acid-soluble nucleotides released during the reaction was mixed with 15ml of Aquasol. The ^{3}H content was then measured in a Searle Isocap/300 6872 liquid-scintillation spectrometer. Although it has been noted that poly(A) can be hydrolysed by RNAase A (Iqbal, 1975), the reaction conditions described were such that on use of the polypurine as substrate, no evidence for hydrolysis could be seen.

Human plasma ribonuclease. Enzyme activity was measured by using the standard assay system described above, except that the reaction mixtures were incubated for 5min and contained 24.7 units of enzyme. An enzyme unit is defined as that amount of enzyme activity required to increase the A_{260} by 0.1 unit under the assay conditions described previously, with 1.5μmol of poly(C) as substrate (Schmukler *et al.*, 1975).

Human spleen ribonuclease. Assay conditions were similar to those described in the standard assay system, except that the reaction mixtures, containing 10μmol of sodium phosphate buffer, pH 6.2, and 6.6 enzyme units, were incubated for 15min. An enzyme unit is defined as that amount of enzyme activity required to increase the A_{260} by 0.1 unit under the assay conditions described previously, using 0.6μmol of poly(U) as substrate (Levy *et al.*, 1974).

Citrobacter ribonuclease. The reaction mixture contained 16.8nmol (as phosphate residues) of *E. coli* 5S [^{3}H]rRNA (specific radioactivity 190c.p.m./nmol), 10μmol of Tris/HCl buffer, pH 7.6, and 6.4 units of enzyme. The amount of enzyme activity required to increase the A_{260} by 0.1 unit under conditions described previously, using 0.6μmol of poly(U), is defined as one enzyme unit

(Levy *et al.*, 1973). After incubation for 5 min at 37°C, the procedures described in the standard assay system were utilized.

Enterobacter ribonuclease. The standard assay system described above for RNAase A was employed, except that the reaction mixtures contained 20.1 units of enzyme (Levy & Goldman, 1970) and were incubated for 15 min. One enzyme unit is defined as that amount of enzyme activity required to increase the A_{260} by 0.1 unit under assay conditions described previously, with 0.5 μmol of poly(C) as substrate (Levy *et al.*, 1973).

Ribonucleic acid from yeast

RNA was prepared as described by Crestfield *et al.* (1955).

Preparation of E. coli 5 S [^{3}H]rRNA

Frozen cell paste (0.5 g) of *E. coli* K12 from mid-exponential phase was grown in 100 ml of the medium described by Garen & Levinthal (1960). After 18 h at 37°C, the culture was transferred to 500 ml of fresh media, grown for an additional 16 h and then transferred to 2.5 litres of media. After 5 h of growth the culture was inoculated with 105 mCi of [5,6-^{3}H]uridine. Incubation continued for 4 more h, at the end of which the cells (5.6 g wet wt.) were collected by centrifugation, washed, first by resuspension in growth media, and then twice more in 0.1 M-Tris/HCl, pH 7.6. The cell mass, suspended in 7 ml of this buffer, was treated with water-saturated redistilled phenol to extract RNA in accord with the procedure of Hindley (1967). 5 S [^{3}H]rRNA was separated from other RNA species by the SDS gel-filtration method (with Sephadex G-75) described by Robins & Raacke (1968). Both the identification of fractions from the Sephadex column containing 5 S rRNA, as well as the degree of purity of the RNA sample, were established by using the tandem polyacrylamide gels described by Yu (1973). 5 S [^{3}H]rRNA in this system migrated with an R_F identical with that of a 5 S RNA standard (0.76) and was well separated from *E. coli* 4 S, 16 S and 23 S rRNA standards which had R_F values of 0.86, 0.21, and 0.04 respectively. The radiochemical purity of the 5 S [^{3}H]rRNA was established by slicing the gel into 1.0 mm segments (model SL-280 gel slicer; Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) and digesting each segment overnight at 37°C with 0.5 ml of 90% (v/v) aqueous Protosol. The ^{3}H content of each segment was then determined by liquid-scintillation counting. No detectable 4 S, 16 S or 23 S rRNA contaminated the purified 5 S [^{3}H]rRNA sample (limit of detection 0.3%). The specific radioactivity of *E. coli* 5 S [^{3}H]rRNA was 15000 c.p.m./nmol of phosphate residue.

Polynucleotide-phosphorylase-catalysed addition of adenylic acid residues to the 3'-termini of 5 S rRNA

Polynucleotide phosphorylase can catalyse the addition of nucleotide residues to a polynucleotide (primer-dependent reaction) and can mediate the synthesis of new polymer strands from nucleotides (primer-independent reaction) (Singer & Guss, 1962; Chou & Singer, 1971). In order to determine the ratio of primed to unprimed synthesis, two reaction mixtures containing polynucleotide phosphorylase were utilized that differed only in that one contained a primer (5 S [^{3}H]rRNA) and the other did not (Hieter *et al.*, 1976). The release of P_i served as a measure of the extent of either primed or unprimed polymerization (Chen *et al.*, 1956). Previously, adenylic acid residues could be coupled to the 3'-termini of 5 S [^{3}H]rRNA by using primer-dependent polynucleotide phosphorylase with little contamination by free poly(A) (Hieter *et al.*, 1976). Recently, however, the use of commercial preparations of this enzyme yielded a product that contained significant quantities of homopolymer and that was therefore not suitable for our purposes. This problem, caused by rapid unprimed polymerization of monomer, was overcome by using non-primer-dependent enzyme and increasing the ADP/Mg^{2+} ratio from 4 (Singer & Guss, 1962) to 2000 (Chou & Singer, 1971), and by lowering the concentration of enzyme from 2.2 units to 0.62 units/ml. These changes brought about an increase in the ratio of primer-dependent to primer-independent activity from 5 to 66, giving rise to a product that had virtually no detectable free homopolymer. For polymerization involving large quantities of RNA, the reaction mixture (42 ml) contained 62.2 μmol of *E. coli* 5 S [^{3}H]rRNA (specific radioactivity: 1.90×10^2 c.p.m./nmol), 4.2 mg of bovine serum albumin, 840 μmol of ADP, 420 nmol of $MgCl_2$, 21 mmol of Tris/HCl buffer, pH 9.0, and 10.5 units of polynucleotide phosphorylase (P-L Biochemicals, Type 15, lot X-3453-A). When this reaction mixture was incubated at 37°C the release of P_i (Chen *et al.*, 1956) was found to be linear for at least 150 min. At 3, 12, 24, 36, 48, 60, 70 and 120 min intervals during the course of the reaction, portions were removed and frozen to stop the reaction. To obtain a sample of *E. coli* 5 S [^{3}H]rRNA at zero time, the RNA was added to a reaction mixture that did not contain polynucleotide phosphorylase.

These portions were placed individually on Sephadex G-50 columns that had been equilibrated previously with 1.0 mM-Tris/HCl buffer, pH 7.6 (the bed volume was 20 times the sample size). Elution was performed with equilibrating buffer and fractions 5.0 ml in volume were collected at a flow rate of 12 ml/h. Radioactivity was found in the void-volume fractions and those fractions with the highest

radioactivities were combined for subsequent use. The following lengths of poly(A) sequences were coupled to 5S [^{3}H]rRNA: $\overline{5}$, $\overline{18}$, $\overline{31}$, $\overline{41}$, $\overline{59}$, $\overline{73}$, $\overline{100}$ and $\overline{151}$. Each of these 5S [^{3}H]rRNA·$(A)_n$ migrated as a single band on gel electrophoresis and, from quantification of band intensities, the 50% range of lengths was no greater than the average length ±27% of the average length.

Quantification procedures

Since the specific radioactivity of the 5S [^{3}H]-rRNA portion of all polymers is identical, all reaction mixtures containing equal quantities of polymer chains of 5S [^{3}H]rRNA or 5S [^{3}H]rRNA·$(A)_n$ should, and did, have equal ^{3}H contents. The average lengths of poly(A) segments coupled to 5S [^{3}H]-rRNA were determined by the amount of P_i released during synthesis and by comparisons of the absorbances obtained at high temperatures. The poly(A) segment lengths obtained by these two methods were in excellent agreement (correlation coefficient = 0.98). The average length of polymer chains of free poly(A) was measured by ultracentrifugation. To verify that solutions of 5S [^{3}H]-rRNA·$(A)_n$ had the same quantity of adenylic acid residues as solutions containing poly(A), A_∞ values for the polymers were compared. At high temperatures (>95°C), it is assumed that 5S [^{3}H]rRNA·$(A)_n$, 5S [^{3}H]rRNA and poly(A) are unstacked, and that A_∞ values represent the absorbances of these totally denatured polymers. Thus subtracting A_∞ for 5S [^{3}H]rRNA from the A_∞ values for 5S [^{3}H]rRNA·$(A)_n$ results in derived A_∞ values for poly(A). Plots of these derived A_∞ values against the experimentally determined A_∞ values for exogenous poly(A) should be linear with a slope of 1.00 and an intercept of 0.00, if the quantities of free poly(A) and poly(A) linked to 5S [^{3}H]rRNA·$(A)_n$ are identical [small deviations from these ideal values for slope and intercept are expected, because the chain lengths of poly(A) are not precisely identical in the two solutions; for example, 5S [^{3}H]rRNA·$(A)_{\overline{31}}$ was compared with $(A)_{\overline{33}}$]. In fact, a slope of 1.05, an intercept of 0.001 and a correlation coefficient of 0.996 were determined by application of a linear-regression program to the experimental data. Thus the quantities of 5S [^{3}H]rRNA and adenylic acid residues present in solutions of 5S [^{3}H]rRNA·$(A)_n$, and in solutions of 5S [^{3}H]rRNA + poly(A), were, for all intents and purposes, the same.

Results

Covalent addition of poly(A) to the 3'-terminus of 5S [^{3}H]rRNA protects the non-polyadenylated portion of the molecule from RNAase-mediated hydrolysis

RNAases from human plasma and from *Citrobacter* sp. were studied by using the linked substrate inhibitor 5S [^{3}H]rRNA·$(A)_n$. As shown in Fig. 1, covalently linked poly(A) is an efficient inhibitor of the human and bacterial RNAases, which differ considerably in specificity, cleaving at cytidylate and uridylate residues respectively. Use

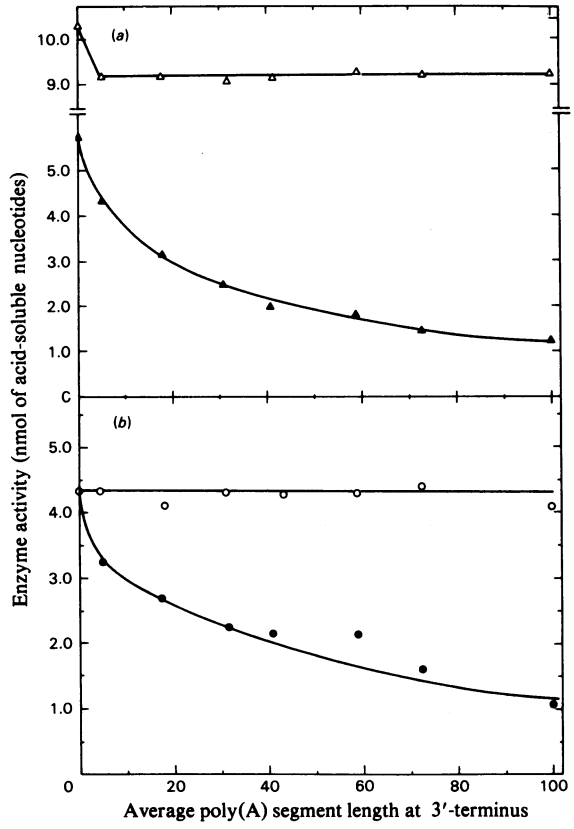


Fig. 1. *Inhibition of human plasma RNAase or Citrobacter RNAase by 5S [^{3}H]rRNA containing 3'-terminal poly(A) segments of varying length*

(*a*) The standard assay reaction mixture for human plasma RNAase was prepared except that 24.7 units of enzyme were used and the incubation time was increased to 7.5 min. Individual reaction mixtures contained 0.14 nmol (3200 c.p.m.) of polymer chains of either 5S [^{3}H]rRNA, or one of the seven 5S [^{3}H]rRNA·$(A)_n$ species (▲). When the effects of spermidine were studied, enzyme activity was measured as described above, except that 20 nmol of spermidine were added to reaction mixtures (△). (*b*) Enzyme activity was measured by using the standard assay system for *Citrobacter* RNAase, except that each reaction mixture contained 4.3 units of enzyme and 0.14 nmol of polymer chains of different 5S [^{3}H]rRNA·$(A)_n$ substrate molecules (●). When the effects of spermidine were examined, 200 nmol of the polyamine were added to the reaction mixtures (○).

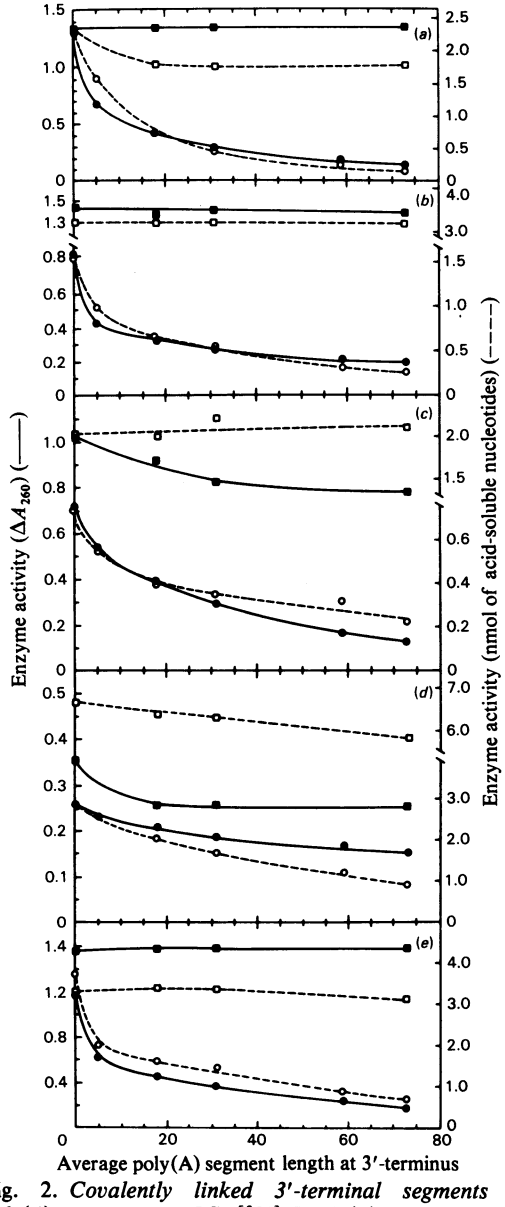


Fig. 2. *Covalently linked 3'-terminal segments of poly(A) present in 5S [^{3}H]rRNA·$(A)_n$ protect non-polyadenylated substrates from RNAase digestion*

Assay conditions for each enzyme were as described in the Experimental section, except that the reaction mixtures contained 0.14nmol of polymer chains (16.8–27.0nmol of phosphate residues) of 5S [^{3}H]rRNA·$(A)_n$ substrate molecules of various poly(A) lengths and a second, non-polyadenylated substrate. The amount of this second substrate as well as the identity of the enzyme in the individual reaction mixtures were as follows: (*a*) ribonuclease A, 375nmol of poly(C); (*b*) human plasma ribonuclease, 275nmol of poly(C); (*c*); human spleen ribonuclease, 500nmol of yeast ribonucleic acid; (*d*) *Citrobacter* ribonuclease, 152nmol of *E. coli* 5S rRNA; (*e*) *Enterobacter* ribonuclease, 375nmol of poly(C). Quantification of the extent of hydrolysis of each type of substrate molecule was made possible by using a large excess (9–30-fold) of non-polyadenylated substrate, relative to the concentration of 5S [^{3}H]rRNA·$(A)_n$. The amount of degradation of the predominant RNA species was determined spectrophotometrically, with little interference from the polyadenylated substrates, whereas the quantity of 5S [^{3}H]rRNA·$(A)_n$ hydrolysed was measured by using standard radioactivity quantification procedures. Thus the assay procedure for each enzyme was as described in the Experimental section, except that, after centrifugation, the reaction mixture (2.0ml) was divided into two portions. The ^{3}H content of one (1.5ml) was measured (○), and the A_{260} was determined with the remainder (0.5ml) of this solution (●). When the effects of spermidine were examined, 200nmol of the polyamine were added to reaction mixtures prepared as described above. Both incubation conditions and the measurement of enzyme activity [i.e. ^{3}H content (□) and A_{260} (■)] were also as described above.

of 5S [^{3}H]rRNA·$(A)_5$, in which the polyadenylic acid portion accounts for only 4% of the total polynucleotide, resulted in a 25% inhibition of RNAase activity with either enzyme (Fig. 1). Increasing the length of the poly(A) segment to 41 caused more than 50% inhibition of activity in either enzyme. Because of the reciprocal relationship between the average poly(A) segment length and enzyme activity, as the poly(A) segment is lengthened, changes in enzyme activity will diminish. Thus a significant alteration in poly(A) length may result in a relatively small change in enzyme inhibition. If the poly(A) segment length is increased from 41 to 100 nucleotides, for example, the inhibition of human plasma RNAase activity is increased only from 64 to 76%. Conversely, if the length of the poly(A) tract is initially relatively long, its substantial shortening can result in only a minor restoration of activity.

The inhibition of RNAase activity is readily reversed by low concentrations of spermidine (Fig. 1). At a concentration of 200μM, spermidine restores 100% of *Citrobacter* sp. RNAase activity, even in the presence of very long poly(A) segments. A lower concentration of the polyamine (20μM) not only overcomes the inhibition of human plasma RNAase, but also induces a considerable stimulation (80%) of substrate hydrolysis.

Poly(A) protects not only the RNA to which it is covalently linked, but unpolyadenylated RNA as well, from RNAase-mediated hydrolysis

Although intermolecular stabilization of non-polyadenylated RNA by substrate molecules containing covalently linked poly(A) has been demonstrated in a general sense with one RNAase previously (Hieter *et al.*, 1976), little information

is available on the sensitivity of this effect. To gain this type of information, RNA mixtures consisting of low concentrations of polyadenylated substrates and large excesses of non-polyadenylated RNA species [yeast RNA, poly(C), or 5S rRNA] were incubated with each of five different RNAases (Fig. 2). The amount of covalently linked $(A)_{\overline{5}}$ ranged from 0.14% to at most 0.46% of the total RNA present. Yet, irrespective of the source of the enzyme, or of the identity of non-polyadenylated polynucleotide, the presence of the poly(A) segment on one substrate is sufficient to protect all the substrate molecules from hydrolysis. This intermolecular stabilization serves to protect phage and viral RNA species as well (Table 1). Use of 5S [^{3}H]rRNA·$(A)_{\overline{59}}$, for example, although it comprises only 6% of the total RNA, can cause at least 44% inhibition of the hydrolysis of Qβ- or MS2-viral RNA.

The size of the poly(A) tract involved in this stabilization of substrate is also of interest. With the exception of the *Citrobacter* RNAase, the use of terminal polyadenylic acid sequences as short as $\overline{18}$ residues caused more than 43% decrease in hydrolysis of both the non-polyadenylated and poly(A)-containing substrates (Fig. 2). The most significant changes in enzyme activity occur as the average poly(A) segment length is increased from 0 to $\overline{31}$. Only minor changes in inhibition occur on lengthening the polypurine tract past $\overline{31}$ residues. Conversely, when the average number of adenylic acid residues is decreased from $\overline{73}$ to $\overline{31}$, the amount of inhibition decreases on average only 14%.

The inhibition of hydrolysis of both polyadenylated and non-polyadenylated substrates by the poly(A) tracts occurs to approximately the same extent. Although spermidine reverses the poly(A) inhibition of RNAase activity, in a number of cases the polyamine may also stimulate enzyme activity, so that large differences in the rates of hydrolysis of different substrates become apparent [Figs. 1(*a*), and 2(*c*)–(*e*)].

Similar inhibitions of RNAase activity are achieved, irrespective of the manner in which a fixed quantity of adenylic acid residues are distributed on the 3′-termini of a fixed substrate population

Once it was established that poly(A) segments protect all RNA molecules in solution from enzyme hydrolysis, we decided to examine whether it was the distribution of those segments on the 3′-termini of RNA, or the ratio between the amount of poly(A) and the amount of substrate, that played a greater role in determining the efficacy of RNAase inhibition. To examine the problem, a fixed amount of poly(A) was distributed among a constant number of substrate molecules in several ways. Solutions for example containing the same number of molecules of 5S [^{3}H]rRNA and 5S [^{3}H]rRNA·$(A)_{\overline{73}}$, or 5S [^{3}H]rRNA·$(A)_{\overline{18}}$ and 5S [^{3}H]rRNA·$(A)_{\overline{59}}$, or 5S [^{3}H]rRNA·$(A)_{\overline{31}}$ and 5S [^{3}H]rRNA·$(A)_{\overline{41}}$, have identical total concentrations of 5S [^{3}H]rRNA

Table 1. *Poly(A) segments at the 3′-terminus of 5S [^{3}H]rRNA protect unpolyadenylated Qβ- or MS2-viral RNA species from RNAase-mediated degradation*

Enzyme activity for each ribonuclease was measured by using the procedures detailed in the Experimental section, except that individual reaction mixtures contained 3.0 A_{260} units of either Qβ- or MS2-viral RNA in addition to 0.14 nmol of polymer chains of 5S [^{3}H]rRNA·$(A)_n$. Also, in cases involving ribonuclease A, 4.56 ng of the enzyme were utilized. The incubation time for reaction mixtures containing human plasma ribonuclease was 15 min. In all cases, after centrifugation, the reaction mixture (2 ml) was divided into two portions. The ^{3}H content of one (1.5 ml) was measured, and the A_{260} of the other (0.5 ml) was determined. Listed below are the ^{3}H contents (expressed as nmol of acid-soluble nucleotides) and the absorbances (ΔA_{260}) found for the different reaction conditions indicated.

		Enzyme activity			
		Qβ RNA		MS2 RNA	
Enzyme	Average poly(A) segment length at 3′-termini of *E. coli* 5S [^{3}H]rRNA	ΔA_{260}	Acid-soluble nucleotides (nmol)	ΔA_{260}	Acid-soluble nucleotides (nmol)
Ribonuclease A	0	0.157	0.53	0.065	0.21
	$\overline{5}$	0.100	0.32	0.047	0.16
	$\overline{59}$	0.040	0	0.026	0
Human plasma ribonuclease	0	0.404	2.0	0.202	1.25
	$\overline{5}$	0.313	1.7	0.143	0.61
	$\overline{59}$	0.137	0.61	0.097	0.33
Human spleen ribonuclease	0	0.340	1.7	0.260	1.6
	$\overline{5}$	0.260	1.5	0.210	1.4
	$\overline{59}$	0.190	1.2	0.130	0.84

(as 5S [^{3}H]rRNA·$(A)_n$ or free 5S [^{3}H]rRNA), and virtually identical total concentrations of 3′-terminal adenylate tracts. However, the distribution of these poly(A) segments among the RNA molecules is distinct in every case. The addition of RNAase A to these different mixtures resulted in enzyme activities that were essentially the same (Table 2). This would suggest that similar inhibitions of enzyme activity are obtained if the ratio of the total poly(A) concentration to the total substrate concentration is maintained constant, irrespective of the manner in which poly(A) segments are distributed among substrate molecules.

Consistent with these findings is the observation that changes in the ratio are reflected in alterations in enzyme inhibition. If the total poly(A) concentration is maintained at 14.02 μM-phosphate residues, for example, and the final substrate concentration is increased from 16.8 μM (Table 2, line 14) to 50.4 μM (Table 2, lines 17 and 18), and then to 84.0 μM (Table 2, lines 21 and 22), enzyme activities rise from an initial value of 1.0 nmol of acid-soluble nucleotides to a final one of 5.0 nmol.

Kinetics of poly(A)-induced inhibition of RNAase activity

The efficiencies of free and linked poly(A) with respect to enzyme inhibition were compared by using RNAase from human spleen and bovine pancreas. In the presence of either enzyme (Figs. 3 and 5), the hydrolysis of the non-polyadenylated portion of 5S [^{3}H]rRNA·$(A)_n$ proceeds in a manner similar to that of 5S [^{3}H]rRNA. If K_m or $V_{max.}$ differed significantly for the two substrates, then plots of $1/v$ against 1/[total 5S [^{3}H]rRNA], for individual fixed concentrations of 5S [^{3}H]rRNA·$(A)_n$, should deviate from linearity. This would occur because reaction mixtures vary in initial substrate composition from exclusively 5S [^{3}H]rRNA·$(A)_n$ to mixtures of 5S [^{3}H]rRNA·$(A)_n$ and 5S [^{3}H]rRNA. No such deviations were noted. Instead, reciprocal plots are linear over the range of concentrations examined.

Table 2. *Amount of inhibition of RNAase-A-mediated hydrolysis of substrate is not dependent on the distribution of 3′-terminal adenylic acid residues present in the reaction mixture*

Enzyme activity was measured by using the standard assay system for ribonuclease A, except that 20 ng of enzyme and the various amounts of 5S [^{3}H]rRNA·$(A)_n$ indicated below were added to the individual reaction mixtures.

Amount (nmol) of 5S [^{3}H]rRNA·$(A)_n$ polymer chains present								Total 5S [^{3}H]-rRNA concentration (free and as 5S [^{3}H]rRNA·$(A)_n$) (nmol of nucleotide residues*)	Total 3′-terminal poly(A) concentration (nmol of adenylic acid residues ±6%*)	Enzyme activity (nmol of acid-soluble nucleotides)
n ... 0	$\bar{5}$	$\overline{18}$	$\overline{31}$	$\overline{41}$	$\overline{59}$	$\overline{73}$	$\overline{100}$			
0.14								16.8	0	8.0
0.28								33.6	0	10.9
0.42								50.4	0	18.7
0.56								67.2	0	24.4
0.70								84.0	0	28.5
						0.14		16.8	10.2	2.2
0.14						0.14		33.6	10.2	2.8
		0.14			0.14			33.6	10.2	2.6
			0.14	0.14				33.6	10.2	2.6
0.28						0.14		50.4	10.2	3.3
	0.28				0.14			50.4	10.2	3.1
0.42						0.14		67.2	10.2	3.9
	0.14	0.28	0.14					67.2	10.2	4.3
							0.14	16.8	14.0	1.0
0.14							0.14	33.6	14.0	1.9
				0.14	0.14			33.6	14.0	2.5
0.28							0.14	50.4	14.0	2.9
			0.28	0.14				50.4	14.0	3.1
0.42							0.14	67.2	14.0	3.3
	0.28		0.14		0.14			67.2	14.0	4.2
0.56							0.14	84.0	14.0	5.0
	0.28	0.14	0.14	0.14				84.0	14.0	4.8

* Values in these columns are obtained by multiplying the amount (nmol) of 5S [^{3}H]rRNA·$(A)_n$ polymer chains by either 120 (the number of nucleotides in 5S rRNA) or *n* [for poly(A)].

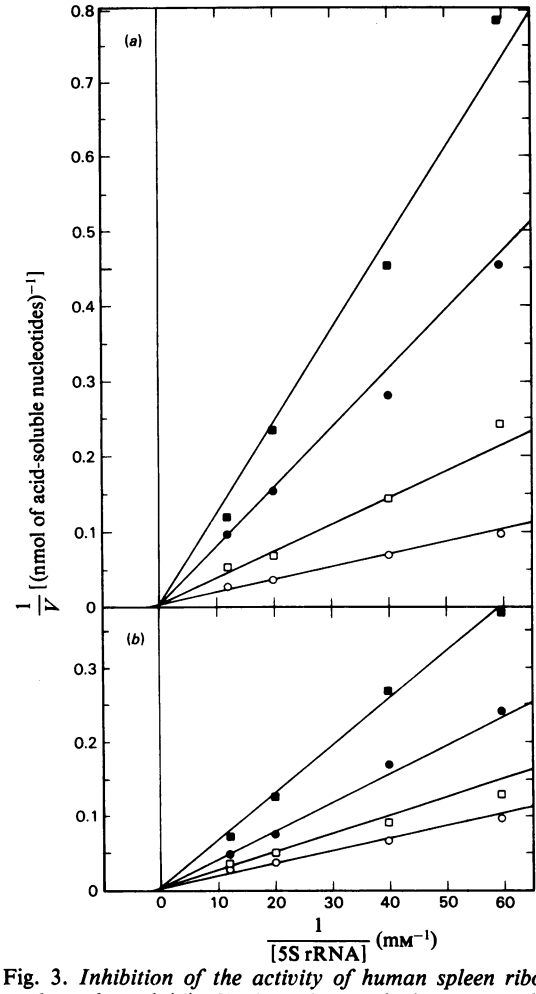


Fig. 3. *Inhibition of the activity of human spleen ribonuclease by poly(A); K_i depends on whether or not the polyadenylic acid segments are covalently linked to the 3'-termini of substrate (E. coli 5S [^{3}H]rRNA)*

Enzyme activity, which was linear both with respect to enzyme concentration and to time of incubation, was measured by using the standard assay system for human spleen ribonuclease with the following exceptions. The incubation time was decreased to 5min and, for the experiment in (*a*), all reaction mixtures contained 0.14nmol of polymer chains of 5S [^{3}H]rRNA·$(A)_n$, the non-polyadenylated portion of which corresponded to 16.8nmol of nucleotide residues of 5S [^{3}H]rRNA. Quantities of 5S [^{3}H]-rRNA were added to achieve the total concentrations of substrate indicated. The average poly(A) segment lengths at the 3' termini of 5S [^{3}H]rRNA·$(A)_n$ were: none (○); $\overline{18}$ (□); $\overline{59}$ (●); $\overline{100}$ (■). The experiment in (*b*) was performed in a similar manner, except that the poly(A) segments were not covalently linked to 5S [^{3}H]rRNA. The same quantity (0.14nmol) of polymer chains of poly(A) was added to the reaction mixtures. The average numbers of adenylic acid residues per polymer chain of poly(A), however, were as follows: none (○); $\overline{16}$ (□); $\overline{54}$ (●); $\overline{96}$ (■). Solid lines represent the computer-generated 'best fit' of all data in either (*a*) or (*b*) to eqn. (1). The MLAB program (Knott & Reece, 1972; Knott & Shrager, 1972) was used in these determinations. The values of the kinetic constants utilized to generate these drawings are listed in Table 3. See the text for additional details.

Human spleen ribonuclease

The use of a fixed concentration of polymer chains (0.14μM) of 5S [^{3}H]rRNA·$(A)_n$, but varying n (0, $\overline{18}$, $\overline{59}$ and $\overline{100}$ residues) gives rise to an increased inhibition of enzyme activity as n is made larger. Reciprocal plots of the data obtained with different concentrations of 5S [^{3}H]rRNA are characteristic of competitive inhibition (Fig. 3*a*). The values of kinetic constants (Table 3) were determined by fitting eqn. (1):

$$\frac{1}{V} = \frac{1}{V_{max.}} + \frac{K_s \cdot (1 + [I]/K_i)}{[S]\,V_{max.}} \qquad (1)$$

which describes competitive inhibition (Webb, 1963), to these data, with the MLAB Program (Knott & Reece, 1972; Knott & Shrager, 1972), where [S] and [I] represent the total concentrations of 5S [^{3}H]rRNA {whether free or as 5S [^{3}H]rRNA·$(A)_n$} and 3'-terminal adenylic acid residues respectively). K_s, K_i and $V_{max.}$ have their usual meaning (Scheme 1). The solid lines in Fig. 3(*a*), which fit the experimental data well, were drawn by using these constants and eqn. (1). Attempts to fit eqn. (2):

$$\frac{1}{V} = \frac{[I] + \alpha K_i + \alpha \cdot K_s([I] + K_i)/[S]}{V_{max.}(\alpha K_i + \beta[I])} \qquad (2)$$

which is descriptive of a more general type of inhibition [embracing competitive, non-competitive, mixed inhibitions etc. as special cases (Webb, 1963)] resulted in values for α in excess of 10^4 (see Scheme 1). Thus ternary complexes (such as EIS), if formed at all, do so in negligible amounts, and the inhibition of human spleen RNAase by coupled poly(A) segments may be regarded as competitive.

The effects of different chain lengths of free poly(A) on enzyme activity are plotted in Fig. 3(*b*). Application of eqns. (1) and (2) to these data suggests that the inhibition of the spleen enzyme by free poly(A) is also competitive. The kinetic constants obtained by computer 'best fit' (Table 3), however, indicates that the K_i for poly(A) linked to 5S [^{3}H]rRNA, is less than one-half the K_i determined by using free poly(A). For the same number of adenylic acid residues, then, covalent attachment to 5S [^{3}H]rRNA gives rise to a more efficient inhibitor of RNAase activity than does the free

Table 3. *Kinetic constants for the inhibition of RNAase activity induced by homopolymeric poly(A) or poly(A) linked to 5S rRNA (see Scheme 1)*

The MLAB program (Knott & Reece, 1972; Knott & Shrager, 1972) was used to fit eqns. (1) and (2) to data obtained by using RNAases from human spleen (Fig. 3) and bovine pancreas (Fig. 5). All values result from 'best fits', i.e., the non-linear least-squares error is minimized by using these values. That these values represent unique solutions is implied by the fact that the same final values were obtained in successive computer runs in which different arbitrary numbers were initially assigned to each constant. 5S rRNA + poly(A) denotes that poly(A) is not covalently linked to 5S [^{3}H]rRNA, whereas, 5S rRNA·$(A)_n$ indicates that poly(A) is coupled to substrate. See the text for further details.

	Human spleen RNAase		RNAase A	
Constant	5S rRNA + poly(A)	5S rRNA·$(A)_n$	5S rRNA + poly(A)	5S rRNA·$(A)_n$
K_s (μM)	624	624	126	126
K_i (μM)	4.7	2.3	2.7	4.0
αK_s (μM)			294	58.6
αK_i (μM)			6.3	1.9
$V_{max.}$ (nmol of acid-soluble nucleotides)	368	368	84.2	84.2
$\beta V_{max.}$ (nmol of acid-soluble nucleotides)			73.7	4.53

homopolymer. This can best be illustrated when the predicted ratios of the enzyme activities obtained with 5S [^{3}H]rRNA·$(A)_n$ or 5S [^{3}H]rRNA + poly(A) are plotted as a function of poly(A) concentration (Fig. 4). The constants listed in Table 3 for the human spleen RNAase were used, as was eqn. (3):

$$\frac{V_{[5\text{S rRNA}\cdot(A)_n]}}{V_{[5\text{S rRNA + poly(A)}]}} = \frac{[S] + K_s(1 + [I]_{ncl}/K_{i_{ncl}})}{[S] + K_s(1 + [I]_{cl}/K_{i_{cl}})} \quad (3)$$

where [S] represents the total concentration of 5S [^{3}H]rRNA, and $[I]_{ncl}$, $K_{i_{ncl}}$ and $[I]_{cl}$, $K_{i_{cl}}$ represent the concentration of poly(A) (and the corresponding K_i) not covalently linked or covalently linked to 5S [^{3}H]rRNA respectively. The data illustrated in Fig. 4 indicate that the ratio of activities depends strongly on the total number of adenylic acid residues, first decreasing sharply for low quantities of poly(A), then asymptotically approaching 0.48 as a limit for very long poly(A) chains, regardless of substrate concentration. For short poly(A) tracts, an increase in substrate concentration ($>10^3$ fold) raises the ratio of activities by a small factor.

Bovine pancreatic ribonuclease

When free poly(A) is used as the inhibitor, graphical representation of the data superficially suggests competitive inhibition (Fig. 5*a*). Attempts to fit eqn. (1) to the data of Fig. 5(*a*) were unsatisfactory because the computer-determined 'best fit' resulted in an unacceptably large error expressed as the sum of the squares. This error was greatly decreased when the inhibition was assumed to occur by a partially competitive mechanism (eqn. 2, $\beta = 1.0$; see Scheme 1). However, the minimum least-squares value was obtained with $\beta = 0.875$ in eqn. (2). The values of kinetic constants computed in this manner are listed in Table 3 and shown as solid lines, which fit the experimental results well (Fig. 5*a*).

When the polyadenylated substrate is used as the inhibitor, reciprocal plots of the data indicate the impossibility of a common intersection point on the ordinate (Fig. 5*b*). Lines drawn through the data points intersect near the abscissa, in the lower left quadrant. Use of non-competitive or partially non-competitive (eqn. 2, $\alpha = 1.0, \beta = 0$) (eqn. 2, $\alpha = 1.0$, $0 < \beta < 1.0$)-inhibition models gave poor 'fits' of predicted enzyme-activity values to experimental data. A considerable decrease in error was attained by direct application of eqn. (2) to the experimental data. As a result of this treatment, the solid lines in Fig. 5(*b*) were generated by using eqn. (2) and the appropriate constants (Table 3).

In sum, then, differences in the results obtained from the use of free or linked poly(A) can be attributed primarily to the disparity in $\beta V_{max.}$ values. With the bound inhibitor, $\beta V_{max.}$ is only 5.4% of the value obtained with free poly(A) (Table 3).

The superiority of bound poly(A) as an inhibitor of RNAase A activity may be seen in Fig. 6. This Figure was derived by using the values for kinetic constants listed in Table 3, and eqn. (4):

$$\frac{V_{[5\text{S rRNA}\cdot(A)_n]}}{V_{[5\text{S rRNA + poly(A)}]}} = \frac{V_{max.}[S](\alpha_{cl}\cdot K_{i_{cl}} + \beta_{cl}\cdot[I]_{cl})/[[S][I]_{cl} + \alpha_{cl}(K_{i_{cl}}\cdot[S] + K_s\cdot[I]_{cl} + K_s\cdot K_{i_{cl}})]}{V_{max.}[S](\alpha_{ncl}\cdot K_{i_{ncl}} + \beta_{ncl}\cdot[I]_{ncl})/[[S][I]_{ncl} + \alpha_{ncl}(K_{i_{ncl}}\cdot[S] + K_s\cdot[I]_{ncl} + K_s\cdot K_{i_{ncl}})]} \quad (4)$$

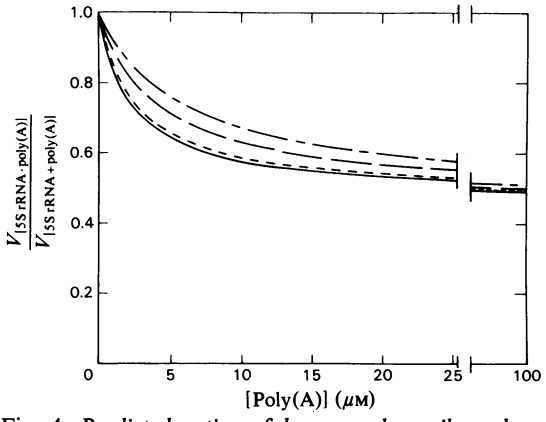


Fig. 4. *Predicted ratios of human spleen ribonuclease activities obtained by using poly(A) linked to substrate ($V_{[5S\ rRNA\cdot(A)_n]}$), or homopolymeric poly(A) not attached to substrate ($V_{[5S\ rRNA+poly(A)]}$), as a function of poly(A) concentration*

The lines were generated by computer with the appropriate constants listed in Table 3 and eqn. 3, where the concentrations of free poly(A) ($[I]_{ncl}$) and covalently linked poly(A) ($[I]_{cl}$) are assumed to be the same and equal to the values indicated on the abscissa. Each line represents a different total concentration of 5S rRNA substrate: ——, 1.0μM; – – – –, 100μM; ——, 500μM; ——, 1000μM. See the text for further information. The values shown on the abscissa represent the concentrations of adenylic acid residues, whether free or linked to substrate, and may be transformed into a scale of average numbers (n) of adenylic acid residues per poly(A) polymer chain by dividing the concentrations of residues by the concentrations of polymer chains of poly(A). Thus, if the polymer chain concentration is 0.14μM, the major divisions of the abscissa (n) become 0, 36, 71, 107, 143, 179 and 714 adenylic acid residues/poly(A) chain. This type of scale is useful in envisioning the effect of chain length on the ratio of enzyme activities. See Table 4 for a comparison of experimentally determined and predicted ratios.

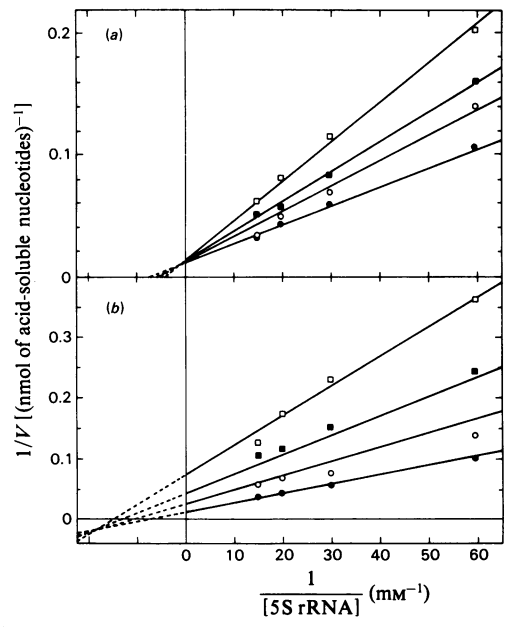


Fig. 5. *Inhibition of the activity of ribonuclease A by poly(A); the apparent mechanism of inhibition depends on whether or not the polyadenylic acid segments are linked to the 3'-termini of substrate (E. coli [³H]rRNA)*

Enzyme activity was linear with respect to both time and enzyme concentration, and was determined by using the standard assay system for ribonuclease A, with the following exceptions. Individual reaction mixtures contained 12μmol of Tris/HCl buffer, pH 7.6, and 2.0ng of enzyme and were incubated for 5min at 37°C. (*a*) 5S [³H]rRNA was added to give the concentrations indicated. Poly(A) (0.14nmol of polymer chains), having the indicated average number of adenylic acid residues per polymer chain, was also present: none (●); 16 (○); 28 (■) 96 (□). (*b*) Each reaction mixture contained 0.14nmol of polymer chains of 5S [³H]-rRNA·$(A)_n$ (corresponding to 16.8nmol of nucleotide residues of 5S [³H]rRNA), in addition to sufficient *E. coli* 5S [³H]rRNA to achieve the total substrate concentrations indicated. The average lengths of the 3'-terminal poly(A) segments were: none (●); 18 (○); 41 (■); 100 (□). Solid lines represent the computer-generated 'best fit' of the data in (*a*) or (*b*) to eqn. (2) [the MLAB program (Knott & Reece, 1972; Knott & Shrager, 1972) was used], and were drawn by using the appropriate constants listed in Table 3. See the text for further information.

where the subscripts $_{cl}$ and $_{ncl}$ refer to segments of poly(A) covalently linked, or not, to 5S [³H]rRNA respectively. [S] and [I] represent the total concentrations of 5S [³H]rRNA and poly(A) respectively, and $V_{max.}$, α, K_I, β and K_S have their usual meaning (Scheme 1).

Fig. 6 demonstrates that, with respect to the inhibition of RNAase A activity, an increase in the concentration of 5S [³H]rRNA causes a decline in the ratio of activities obtained with linked or free poly(A). Thus, on increasing substrate amount, there is a concomitant increase in the inhibitory advantage that covalent linkage confers on poly(A). Under similar conditions exactly the opposite result was obtained with the spleen enzyme, in that the inhibitory prowess of free poly(A), relative to the linked polypurine, rose as the quantity of substrate increased. The values of individual ratios, predicted by using eqns. (3) or (4) correspond well to the experimentally determined ratios for either the spleen enzyme or for RNAase A (Table 4). These

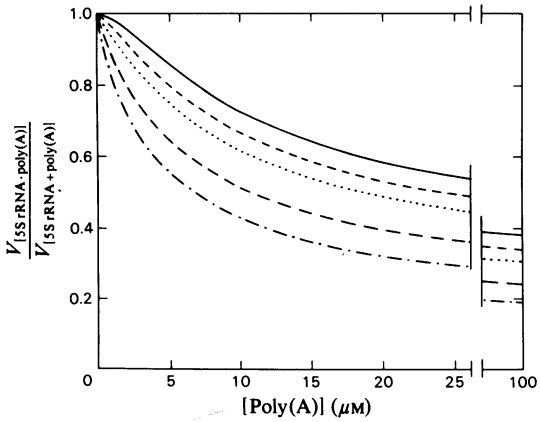


Fig. 6. *Predicted ratios of ribonuclease A activities obtained using poly(A) linked to substrate* ($V_{[5S\ rRNA\cdot(A)_n]}$), *or free poly(A)* ($V_{[5S\ rRNA+poly(A)]}$), *as a function of poly(A) concentration*

The lines, representing different, total concentrations (as phosphate residues) of 5S rRNA substrate (——, 1.0μM; – – –, 16.8μM; ····, 33μM; — —, 62μM; —·—·, 100μM), were generated by computer with eqn. (4) and the constants given in Table 3. See the legend to Fig. 4 for further details and for information regarding the transformation of poly(A) concentrations into a scale representing the average numbers of adenylic acid residues per poly(A) chain. A comparison of experimentally determined and predicted ratios is made in Table 4.

findings suggest that the advantage, in terms of inhibition, of one type of poly(A) over another is not fixed, and, in fact, may vary considerably with changes in substrate concentration or poly(A) segment length.

Discussion

By using substrate linked to inhibitor in the form of 5S [^{3}H]rRNA·$(A)_n$, it was possible to show that, as the average number of adenylic acid residues per polymer molecule becomes larger, increases in the inhibition of *Citrobacter* sp. and human plasma RNAase activities are realized. The inverse relationship between activity and poly(A) segment length is similar for these enzymes and for the human spleen RNAase examined previously (Hieter *et al.*, 1976) and suggests a general phenomenon. An interesting feature of this relationship, not noted before, is that a change in the number (n) of 3′-terminal adenylic acid residues will induce quite different changes in RNAase activity, depending on the value of n. In all cases, for example, the greatest decreases in enzyme activity are found as n is increased from 0 to approximately $\overline{40}$ residues per molecule. For $n > \overline{60}$, although substantial lengthening of the poly(A) segment may occur, there is little additional change in the rate of RNAase-catalysed hydrolysis of substrate. Similar results were obtained with five RNAases if 5S [^{3}H]rRNA·$(A)_n$ were used in conjunction with a non-polyadenylated substrate.

An inverse relationship between enzyme activity and poly(A) segment length could be beneficial to the stability of RNA. Because the average poly(A) tract may vary in length with time (Sheiness & Darnell, 1973; Slater *et al.*, 1973; Wilt, 1973, 1977; Diez & Brawerman, 1974; Jeffrey & Brawerman, 1974; Lodish *et al.*, 1974; Dolecki *et al.*, 1977), large changes in the rate of enzymic degradation of RNA would occur only if the average number of adenylic acid residues per molecule were low. For cases where n is large, alterations of n will affect RNAase activity in a relatively minor way, and the rate of hydrolysis of substrate will remain low and effectively constant.

In this connection, Deshpande *et al.* (1979) have constrasted the protein-synthesizing capacity of polyadenylate-'rich' and polyadenylate-'poor' mRNA species in *Xenopus* oocytes. Since about the same amount of protein was found when either mRNA was used, those authors consider that the size of the poly(A) tract on the mRNA does not influence stabilization of the messenger. However, the polyadenylate-'poor' mRNA had some 40 adenylic acid residues, and in view of what has been discussed above, those 40 residues may offer much the same protection against RNAase activity as the 170 adenylic acid residues on the polyadenylate-'rich' mRNA. Certainly 5S rRNA and mRNA are not equivalent entities, and other factors known and unknown may well influence mRNA stability in the *Xenopus* system. However, the contrast between 40 and 170 adenylic acid residues, with respect to the inhibition of RNAase hydrolysis, is a limited one, because both these segment lengths favour significant inhibition of the activities of the bacterial, mammalian, and human RNAases tested.

The variation in naturally occurring poly(A) segment length, as well as the absence of terminal poly(A) tracts from a variety of mRNA species (Adesnik & Darnell, 1972; Milcarek & Penman, 1974; Nemer *et al.*, 1974; Fromson & Verma, 1976; Lewis *et al.*, 1976), raised another question that could be explored with the polyadenylated model compounds: namely, does the presence of poly(A) on some RNA strands help to stabilize other non-polyadenylated RNA molecules? In the present studies, polyadenylated RNA as well as a variety of non-polyadenylated substrates were incubated with different RNAases. In each case the poly(A) segment inhibited the activity of the enzyme under study, thus protecting from hydrolysis about the same proportion of non-polyadenylated as well as

Table 4. *Ratio of enzyme activities obtained with 5 S [^{3}H]rRNA·$(A)_n$ to activities obtained with 5 S [^{3}H]rRNA + poly(A)*
The average number of adenylic acid residues per polymer chain of 5 S [^{3}H]rRNA·$(A)_n$, or per strand of homopolymeric poly(A) are represented by n or m, respectively. Ratios of activities were calculated by using the experimental data shown in Fig. 3 (human spleen RNAase) or Fig. 5 (RNAase A). Values in parentheses represent the predicted ratio of enzyme activities computed by using eqn. (3) (human spleen RNAase) or eqn. (4) (RNAase A), the concentrations of substrate listed and the appropriate kinetic constants (Table 3). The mean errors of the predicted values, relative to experimentally determined ratios, were as follows: human spleen RNAase, 9.6%; bovine pancreatic RNAase, 8.8%. See the text for further details.

Enzyme	Total 5 S [^{3}H]rRNA (μM of phosphate residues)	n/m	Enzyme activity with 5 S[^{3}H]rRNA·$(A)_n$ / Enzyme activity with 5 S[^{3}H]rRNA + $(A)_m$
Human spleen ribonuclease	16.8	$\overline{18}/\overline{16}$	0.52 (0.69)
	25.2		0.63 (0.69)
	50.4		0.70 (0.70)
	84.0		0.70 (0.71)
	16.8	$\overline{59}/\overline{54}$	0.53 (0.49)
	25.2		0.60 (0.49)
	50.4		0.49 (0.50)
	84.0		0.51 (0.50)
	16.8	$\overline{100}/\overline{96}$	0.48 (0.52)
	25.2		0.59 (0.53)
	50.4		0.54 (0.53)
	84.0		0.62 (0.53)
RNAase A	16.8	$\overline{18}/\overline{16}$	1.0 (0.82)
	33.6		0.91 (0.77)
	50.4		0.73 (0.74)
	67.2		0.60 (0.71)
	16.8	$\overline{41}/\overline{28}$	0.67 (0.69)
	33.6		0.55 (0.63)
	50.4		0.50 (0.58)
	67.2		0.50 (0.55)
	16.8	$\overline{100}/\overline{96}$	0.56 (0.57)
	33.6		0.50 (0.50)
	50.4		0.47 (0.46)
	67.2		0.50 (0.42)

polyadenylated substrates. It should be noted that the polyadenylated segments could constitute as little as 0.2% of all the RNA present and yet, depending on the length of the poly(A) segment, render very efficient protection. The mechanism, therefore, of poly(A) stabilization of RNA, by virtue of the inhibition of RNAase activity, offers a function for poly(A) that does not require that every molecule be polyadenylated.

Although the RNAase-catalysed hydrolysis of each substrate tested was inhibited by the presence of poly(A) segments, the addition of low concentrations of spermidine reversed inhibition by significant amounts in every case. However, the hydrolysis of polyadenylated and non-polyadenylated substrates may be affected to different extents by the presence of the polyamine. For example, the degradation of one polymer may be stimulated relative to a second substrate. Also, for one polynucleotide, the polyamine may abolish any sensitivity of enzyme activity to poly(A) segment length, whereas for another substrate, activity will decline in the presence of polyamine as longer poly(A) tracts are used. These differences in polyamine effects prompted an investigation, reported in the following paper (Karpetsky *et al.*, 1980*b*), of variation in inhibition reversal with changes in enzyme identity for a number of polyamine analogues.

Apparently, the distribution of poly(A) segments on the 3′-termini of RNA plays little or no role in determining the amount of inhibition of RNAase activity attained. If the total concentration of poly(A) relative to that of substrate was the same, irrespective of the distribution of the poly(A) segments on substrate molecules, similar inhibitions of enzyme activity could be achieved. When the ratio of poly(A) to substrate concentrations was varied, large fluctuations in the inhibition of RNAase activity were observed. The total quantity of poly(A) present then, relative to that of available substrate, far outweighs in importance the exact distribution of these homopolymeric segments on the termini of RNA.

These results are of particular interest, because, previously, from the results of T_m ('melting' temperature) measurements, differences were found in

the structures of 5S [^{3}H]rRNA·$(A)_n$ as n was increased (Karpetsky *et al.*, 1980*a*). The implication of the present data is that, for the activity of RNAase A, the differences among these substrate molecules are not particularly important. However, the covalent linkage of poly(A) to 5S [^{3}H]rRNA does have an important effect on the inhibitory prowess of the polypurine.

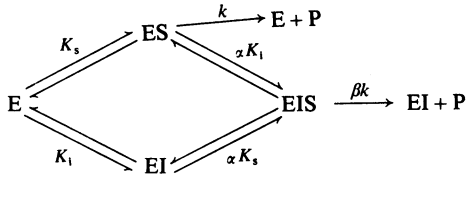
For RNAases from human spleen or bovine pancreas, poly(A) linked to RNA was a superior inhibitor compared with the same quantity of free homopolymer. Although, for example, poly(A), whether bound to 5S [^{3}H]rRNA or not, is a competitive inhibitor of the spleen enzyme, covalent linkage of polypurine segments to the substrate resulted in a 51% decrease in K_i.

With respect to RNAase A, a mechanism (Scheme 1) more general than that of competitive inhibition was found to apply to inhibitions of its activity caused by either form of poly(A). This mechanism assumes the formation of an enzyme–substrate–inhibitor complex, which, although unusual for an enzyme as small as RNAase A, is not unknown. Analogous complexes, for example, involving multiple substrate-binding sites on bovine pancreatic RNAase, and nucleotides or proteins, have been discussed by others (Walker *et al.*, 1975, 1976). Recently, a human placental protein, of mol.wt. about 50000, was found to be a non-competitive inhibitor of the pancreatic enzyme. In those studies, complex-formation between the enzyme, yeast RNA, and the large protein inhibitor was inferred (Blackburn *et al.*, 1977). These precedents suggest the availability of multiple binding sites on the RNAase A molecule, both for nucleotides and for the formation of complexes between the enzyme and two large macromolecules. Our results suggest that use of poly(A) linked to substrate favours an enzyme–inhibitor–substrate complex that turns over to product much slower than if similar quantities of homopolymeric poly(A) are employed.

Because the reason for the superiority of linked polyadenylic acid differed as the identity of the enzyme was changed, the response of the ratio of activities obtained by using linked or free poly(A) was distinct for each enzyme. With increases in substrate concentration, this ratio may rise (human spleen RNAase) or fall (RNAase A), although superior inhibitions will always be obtained with 3′-terminal segments of poly(A). For each RNAase, the degradation of substrate, linked to poly(A) or not, was governed by virtually identical kinetic constants. For both enzymes, then, it is possible to relate the differences in inhibition, found using covalently linked or free poly(A), directly to differences between these two types of poly(A) segments. On this basis, it may be that poly(A) undergoes a structural change on addition to 5S [^{3}H]-rRNA (Karpetsky *et al.*, 1980*a*). Under the experimental conditions employed, poly(A) assumes a single-stranded random-coil structure containing regions of stacked bases (Stannard & Felsenfeld, 1975). The potential exists, therefore, for favouring one set or range of conformations over others as a consequence of linkage. Furthermore, although interactions between free poly(A) and regions of 5S [^{3}H]rRNA may be slight, the proximity of the two polynucleotides, as a result of covalent linkage, could overcome an entropic barrier to interaction so that modifications in the three-dimensional structure of poly(A) or 5S [^{3}H]rRNA may occur. In this context, it is noteworthy that residues 74–89 of 5S [^{3}H]rRNA contain 38% uridylic acid (Barrell & Clark, 1974), and may constitute such a region for interaction with poly(A).

In any case, the results of the present and previous investigations (Hieter *et al.*, 1976; Karpetsky *et al.*, 1980*a*) indicate clearly the complicated nature of the consequences of interaction of poly(A) and ribonuclease. Although inhibition of enzyme activity by poly(A) emerges as a general effect, no less important are the observations that covalent linkage of poly(A) to substrate produces a molecule differing from RNA plus free polypurine homopolymer in both structure and effect on enzyme activity. It is also significant that, for each RNAase, different factors may be critical for inhibition or inhibition reversal and that hard-and-fast rules regarding the mechanisms of these effects or the conditions under which they may be achieved are subject to many exceptions.

In the system we have constructed, the use of RNA models containing linked poly(A) tracts of various lengths has enabled us to explore several of the problems relating to the function of poly(A) that could be undertaken only with difficulty with naturally occurring polyadenylated cellular RNA. We recognize that the types of RNA are probably structurally distinct, or differ in various other ways. We recognize too that there are doubtless other factors, inherent to the make-up of cells, which must be considered in any assessment of what the function of poly(A) may be. Within these con-



Scheme 1. *General mechanism of enzyme inhibition*

straints, however, much that has been learned about the possible functions of poly(A) would appear to be applicable, so much so that the statement made several years ago, that poly(A) is 'a sequence looking for a function' (Lewin, 1975), does not appear to have validity.

We express our gratitude to Mr. Philip Hieter for assistance in preparing large quantities of 5S [^{3}H]rRNA·$(A)_n$.

References

Adesnik, M. & Darnell, J. E. (1972) *J. Mol. Biol.* **67**, 387–406

Barrell, B. G. & Clark, B. F. C. (1974) *Handbook of Nucleic Acid Sequences*, p. 68, Joynson-Bruvvers, Oxford

Blackburn, P., Wilson, G. & Moore, S. (1977) *J. Biol. Chem.* **252**, 5904–5910

Chen, P. S., Jr., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756

Chou, J. Y. & Singer, M. F. (1971) *J. Biol. Chem.* **246**, 7497–7504

Crestfield, A. M., Smith, K. C. & Allen, F. W. (1955) *J. Biol. Chem.* **216**, 185–193

Deshpande, A. K., Chatterjee, B. & Roy, A. K. (1979) *J. Biol. Chem.* **254**, 8937–8942

Diez, J. & Brawerman, G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4091–4095

Dolecki, G. J., Duncan, R. F. & Humphreys, T. (1977) *Cell* **11**, 339–344

Fromson, D. & Verma, D. P. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 148–151

Garen, A. & Levinthal, C. (1960) *Biochim. Biophys. Acta* **38**, 470–483

Hieter, P. A., Le Gendre, S. M. & Levy, C. C. (1976) *J. Biol. Chem.* **251**, 3287–3293

Hindley, J. (1967) *J. Mol. Biol.* **30**, 125–136

Iqbal, Z. M. (1975) *Mol. Cell. Biochem.* **9**, 17–20

Jeffrey, W. R. & Brawerman, G. (1974) *Biochemistry* **13**, 4633–4637

Karpetsky, T. P., Shriver, K. & Levy, C. C. (1980*a*) *J. Biol. Chem.* **255**, 2713–2721

Karpetsky, T. P., Shriver, K. & Levy, C. C. (1980*b*) *Biochem. J.* **193**, 325–337

Knott, G. D. & Reece, D. K. (1972) *Proc. ONLINE '72 Int. Conf.* **1**, 497–526

Knott, G. D. & Shrager, R. I. (1972) *SIGGRAPH Notices* **6**, 138–151

Levy, C. C. & Goldman, P. (1970) *J. Biol. Chem.* **245**, 3257–3262

Levy, C. C., Mitch, W. E. & Schmukler, M. (1973) *J. Biol. Chem.* **248**, 5712–5719

Levy, C. C., Hieter, P. A. & Le Gendre, S. M. (1974) *J. Biol. Chem.* **249**, 6762–6769

Levy, C. C., Schmukler, M., Frank, J. J., Karpetsky, T. P., Jewett, P. B., Hieter, P. A., Le Gendre, S. M. & Dorr, R. G. (1975) *Nature (London)* **256**, 340–342

Lewin, B. (1975) *Cell* **4**, 11–20

Lewis, F. S., Rutman, R. J. & Avadhani, N. G. (1976) *Biochemistry* **15**, 3367–3372

Lodish, H. F., Jacobson, A., Firtel, R., Alton, T. & Tuchman, J. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 5103–5108

Milcarek, C. & Penman, S. (1974) *J. Mol. Biol.* **89**, 327–338

Nemer, M., Graham, M. & Dubroff, L. M. (1974) *J. Mol. Biol.* **89**, 435–454

Neuwelt, E. A., Frank, J. J. & Levy, C. C. (1976) *J. Biol. Chem.* **251**, 5752–5758

Robins, H. I. & Raacke, I. D. (1968) *Biochem. Biophys. Res. Commun.* **33**, 240–246

Schmukler, M., Jewett, P. B. & Levy, C. C. (1975) *J. Biol. Chem.* **250**, 2206–2212

Sheiness, D. & Darnell, J. E. (1973) *Nature (London) New Biol.* **241**, 265–268

Singer, M. F. & Guss, J. K. (1962) *J. Biol. Chem.* **237**, 182–189

Slater, I., Gillespie, D. & Slater, D. W. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 406–411

Stannard, B. S. & Felsenfeld, G. (1975) *Biopolymers* **14**, 299–307

Walker, E. J., Ralston, G. B. & Darvey, I. G. (1975) *Biochem. J.* **147**, 425–433

Walker, E. J., Ralston, G. B. & Darvey, I. G. (1976) *Biochem. J.* **153**, 329–337

Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors*, vol. 1, pp. 55–65, Academic Press, New York

Wilt, F. H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2345–2349

Wilt, F. H. (1977) *Cell* **11**, 673–681

Yu, R. S. T. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 125–130