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INHIBITION AND SUBSTRATE COMPETITION KINETICS IN ANALYSIS OF PORCINE THYROID ALKALINE RIBONUCLEASE'S SPECIFICITY TOWARD SYNTHETIC RNA'S AND tRNA

BARBARA E. CRUTE[†], JAMES A. MARKSTEIN[‡] and FREDRICK J. KULL[¶,*]

Department of Biological Sciences, State University of New York at Binghamton, New York 13902-6000, USA

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Inhibition and substrate competition kinetics demonstrated that tRNA is a highly preferred substrate of thyroid alkaline RNase. The pyrimidine-specific RNase cleaved poly(C) 2.8×10^5 faster than poly(U). $k_{cat}:K_M$ ratios for tRNA and poly(C) based on molecular weights failed to predict preference when both were present. Competition experiments between poly(C) and tRNA revealed tRNA was a tight-binding competing substrate and the cytidylate residues in the 3′-CCA terminus of tRNA were preferred about 280 : 1 over those in poly(C). Poly(U) was competitive with tRNA. When poly(C) was the substrate, inhibition type by poly(G) depended on poly(G) concentration. Neither tRNA lacking its 3′ terminal cytidylyl(3′-5′)adenosine and terminating in a 2′:3′ cCMP residue, tRNA lacking its 3′ terminal 5′AMP residue, guanosine, nor guanylyl(3′-5′)guanylyl(3′-5′)guanosine were inhibitors. Product inhibition by adenosine and 2′:3′ cCMP showed the kinetic mechanism for cleavage of tRNA was ordered uni bi.

Keywords: Porcine thyroid RNase; Kinetic mechanism; tRNA specificity; Tight-binding, competitive, substrate inhibition; Competition kinetics

* Corresponding author. Tel.: (603) 646-1552. Fax: (603) 646-3946.
Current addresses: [†]Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire, 03756, USA; [‡]Department of Biological Sciences, State University of New York: College at Oneonta, Oneonta, New York, 13820, USA; [¶]Department of Chemistry, Dartmouth College, Hanover, New Hampshire, 03755, USA

ABBREVIATIONS

A – adenosine; CA – cytidylyl(3′-5′)adenosine; GGG – guanylyl(3′-5′) guanylyl(3′-5′)guanosine; tRNACp> – tRNA lacking its 3′ terminal cytidylyl(3′-5′)adenosine and terminating in a 2′:3′ cCMP residue; tRNACC – tRNA lacking its 3′ terminal 5′AMP residue.

INTRODUCTION

Alkaline RNase is a widely distributed cytosolic, intracellular RNase mostly present in an inactive (latent) state through being complexed to a proteinaceous inhibitor.[1–3] Although mammalian alkaline RNase has been purified to homogeneity by three different procedures,[4–6] its amino acid sequence has not been determined. Therefore, this enzyme is not presently considered a member of the RNase superfamily[7] epitomized by pancreatic RNase A, the archetype of the superfamily. Among the many ways alkaline RNase differs from pancreatic RNase A, perhaps the most distinctive is in its natural association with RNase inhibitor. The RNA target(s) of alkaline RNase, its role in RNA metabolism, and its regulation remain unresolved. However, because the enzyme : inhibitor complex bears similarity to enzyme complexes of regulatory and catalytic subunits, it seems reasonable the enzyme plays some key cellular role.

The thrust of the preliminary research which led to the results described in this paper was to compare previous studies done with alkaline RNase purified from the latent complex using non-denaturing procedures[4] with alkaline RNase purified using phenol : SDS treatment.[6] The latter preparations of alkaline RNase are homogeneous as judged using protein-specific stains on SDS-PAGE as a criterion, but contain significant amounts of closely associated RNA prior to electrophoresis. This RNA might contribute to alkaline RNase's remarkable stability when exposed to proteinase K and many common protein denaturants, including : SDS : phenol, SDS : chloroform : isoamyl alcohol, SDS : phenol : chloroform : isoamyl alcohol, ethanol, 7 M urea, 3 M guanidinium : Cl, heat (100°C), heat (80°C) in the presence of 100 mM NaCl.[6]

Previous studies showed alkaline RNase is a single-strand specific endoRNase that cleaves native (rRNA, tRNA) and synthetic RNA's [poly(U), poly(C)] to yield 3′ terminal pyrimidine residues. The sole mononucleotide product arising from incubation with poly(C) is 2′:3′ cCMP and the enzyme is unable to catalyze its hydrolysis to a monoester.[4–5] The only

detectable products present after incubation with tRNA are cytidylyl (3′-5′)adenosine (CA), 2′:3′ cCMP, adenosine (A) and a high M_r species lacking CA and terminating in a 2′:3′ cCMP residue (tRNACp>). The high M_r product can be converted back to functional tRNA using liver extracts or sequential treatments using an array of purified mammalian enzymes: 2′:3′ cyclicnucleotide 3′ phosphodiesterase, alkaline phosphatase, and tRNA nucleotidyl transferase.[8]

A preliminary survey concerning the substrate specificity of alkaline RNase purified using phenol:SDS confirmed earlier work[4-5] with one exception; cleavage of poly(U) was negligible. Therefore, this apparent discrepancy and the enzyme's limited cleavage of tRNA were examined in detail.

This paper reports that the specificity of alkaline RNase prepared using phenol:SDS is indeed identical to that of the alkaline RNases previously purified using non-denaturing techniques.[4] Direct competition experiments led to the generalization that comparison of $k_{cat}:K_M$ ratios does not necessarily predict substrate preference. A kinetic analysis of the enzyme's action toward poly(U), poly(C), and tRNA revealed it was highly specific for cytidylate residues, especially those in tRNA. Substrate competition kinetics showed that tRNA acted as a tight-binding substrate inhibitor of poly(C) cleavage. Competition experiments also suggested that two forms of enzyme might exist; one that cleaved both poly(C) and tRNA and another which could only cleave tRNA. Poly(G) competitively inhibited cleavage of tRNA but was a mixed inhibitor when poly(C) was substrate; K_{ii}'s depended on poly(G) concentration. Neither tRNACp>, tRNA lacking its 3′ terminal 5′AMP residue (tRNACC), guanosine, nor guanylyl (3′-5′)guanylyl(3′-5′)guanosine (GGG) inhibited the enzyme. Product inhibition studies using A and 2′:3′ cCMP showed the kinetic mechanism for cleavage of tRNA was ordered uni bi.

MATERIALS AND METHODS

Materials

Porcine thyroids were obtained and maintained as previously described.[4] Benzoyl-DEAE-cellulose (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA.), DEAE-cellulose [(DE-32) Whatman Inc., Clifton, NJ, USA.], DEAE-Trisacryl M (LKB, Gaithersburg, MD, USA.), and ion exchange resins (Bio-Rad, Rockville Centre, NY, USA.) were pre-cycled

and pre-equilibrated as recommended by the manufacturers. Sephadexes were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). SDS (analytical grade) was from Serva Fine Biochemicals, Inc. (Westbury, NY, USA.). Plates for thin-layer chromatography were obtained from Analtech, Inc. (Newark, DL, USA.). Synthetic polynucleotides, nucleotides, and nucleosides were purchased from PL Biochemicals, Inc. [Pharmacia Fine Chemicals (Uppsala, Sweden)] and/or Sigma Chemical Company (St. Louis, MO, USA.), or prepared as described below. Calf intestine alkaline phosphatase was also obtained from Sigma.

The [^{14}C]amino acid hydrolysate, used to prepare substrate, and individual [^{14}C]-labeled amino acids used to test the purity of liver tRNA, were from Amersham-Searle (Arlington Heights, IL, USA.). The protein reagent for Bradford protein assays was purchased from Bio-Rad (Rockville Centre, NY, USA.). Except for brewer's yeast tRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA.), native nucleic acids were prepared as previously[5] or as described below. All other chemicals were of the highest grade commercially available.

Isolation of Alkaline RNase

Alkaline RNase was isolated from porcine thyroids using phenol extraction in the presence of 2% SDS.[6] After chromatography on benzoyl-DEAE-cellulose and Sephadex G-100 this procedure yields a single protein-staining band after SDS-PAGE, however, as isolated, the protein is associated with RNA. Electrophoresis under both denaturing and native conditions, and chromatographic behavior[6] suggest the protein is identical to the major alkaline RNase purified from the alkaline RNase : RNase inhibitor complex using non-denaturing techniques.[4] The protein and RNA composing the alkaline RNase complex could be separated by column chromatography (1×15 cm) using DEAE-Trisacryl (NaCl gradient, 0–0.5 M; 1.0 mM $MgCl_2$; 10.0 mM Tris-HCl, pH 8.0).[8]

Purification and Analysis of Porcine Liver tRNA

Porcine liver obtained from a local *abattoir* was used to prepare tRNA[9] and its quality was assessed by measuring its acceptance of three different amino acids in a cell-free aminoacylation system (porcine liver) as described by the same workers except amino acid concentrations were 250 μM. Total acceptances (pmol, A_{260} unit^{-1}) were: valine, 99.6; glutamate, 183.1; leucine, 163.9. These acceptances equaled or exceeded those previously

reported indicating that the tRNA preparation was greater than 72% pure.[9]

Preparation of Substrate

Brewer's yeast tRNA, aminoacylated with a mixture of [^{14}C]-amino acids, was prepared as previously[10] except mixtures were adjusted to 0.2 M sodium acetate, pH 5.5, prior to extraction using 85% aqueous phenol titrated with NaOH to pH 5.5.

Assays

Previous work demonstrated that alkaline RNase does not discriminate between aminoacylated and free tRNA.[4–6,10,11] Therefore, RNase activity was measured using the above aminoacylated tRNA substrate.[10] Typical assays (180 µl) at room temperature (facility controlled at 23°C) were 50 mM Tris-HCl, pH 8.1 and contained 200 µg substrate ml^{-1}. Portions (50–75 µl) were removed at appropriate times, and assayed[12] using a Beckman Liquid Scintillation 7500 Spectrophotometer.

Cleavage of poly(C) and poly(U) [average M_r's: 1.77×10^5 and 6.2×10^4, respectively (communicated by Pharmacia) was measured spectrophotometrically[13] {using molar extinction coefficients of 1.13×10^4 [280 nm (A_{280})] and 1.00×10^4 (A_{262}), respectively}, except the buffer was 50 mM Tris-HCl, pH 8.1, and reactions were stopped by addition of an equal volume of 2 N perchloric acid containing 20 mM lanthanum acetate.

For both the above assays, one unit of RNase activity is defined as the amount able to cleave 1 µmol min^{-1} of substrate to acid soluble products.

All absorbances were obtained using a Beckman 25B spectrophotometer and refer to a 1.0 cm path length. Absorbance at 260 nm (A_{260}) and A_{260}:A_{280} ratios were used to locate and estimate approximate concentrations of nucleic acids and their cleavage products eluting from columns. Absorbance at 280 nm was used as a rough measure of protein concentration. For more specific measurements, the microassay method of Bradford[14] was used with BSA as standard.

General Specificity using Gel Filtration Chromatography

To test the general specificity of alkaline RNase different native and synthetic substrates (5.0 A_{260} units each) were incubated, chromatographed, and analyzed as previously described.[4]

Preparation of CA

CA was prepared following the protocol of Heppel *et al.*[15] except the incubation mixture was strip-chromatographed on Whatman 3MM paper[16] using reactants as reference standards. Material in bands migrating differently than standards was located by ultraviolet light (Ultra-Violet Products, Inc., San Gabriel, CA), cut from the dried paper, eluted with water, filtered, condensed by rotary evaporation, and analyzed spectrophotometrically relative to known spectra for ACp[17] and by thin-layer chromatography relative to standards (120 min; 2-propanol : water : concentrated aqueous NH_3; 7 : 2 : 1) before and after base hydrolysis (0.4 N KOH, 90°C, 45 min).

Competition Kinetics

Competition experiments between poly(C) and tRNA are described in RESULTS. Competition kinetics between poly(U) and tRNA were done as follows. Assays[5] contained 0.8 units of alkaline RNase and variable concentrations of tRNA each in the absence or presence of either 25, 50, or 75 μM poly(U) (based on molarity of uridylate residues). Double reciprocal plots of averaged duplicate trials ("best fit") showed competitive inhibition.

The effects of poly(G) on the cleavage of tRNA by alkaline RNase were as described for the cleavage of poly(C) (Figure 4) except poly(G) was at either 11.8 or 23.6 μM (based on molarity of guanylate residues); the assay measured loss of tRNA.

Preparation of GGG

Poly(G) (10 mg) was incubated with 100 units RNase TI (Sigma Chemical Company, St. Louis, MO, USA; Grade IV)[18] for 1 h to prepare GGG. Following incubation the mixture was applied directly to a DEAE-cellulose column and GGGp was located, isolated, desalted, and condensed as previously described.[19] The 3′ terminal phosphate of the GGGp was removed by incubation with 1.0 unit of alkaline phosphatase (1 h, 37°C) in a volume of 1.0 ml (0.05 M Tris-HCl, pH 9.0). The resulting GGG was isolated from the mixture using a DEAE-cellulose column.[19]

Preparation of tRNACp >

The high M_r product from a 15 min incubation of tRNA with alkaline RNase (putative tRNACp>) was prepared as follows.[8] Transfer RNA

(640 nmol) was incubated with 300 units of alkaline RNase in 50 mM Tris-HCl, pH 8.0 at 23°C in a volume of 4.96 ml. After 15 min the reaction was stopped by addition of 5.0 M HCl to pH 5.0. The mixture (5.0 ml) was then added to a Sephadex G-25 column (2.5 × 45.5 cm) and eluted with water. Excluded fractions were combined and immediately added to an equal volume of 85% aqueous phenol, pH 7.0, stirred for 20 min and centrifuged (10 min, 27,000 ×g, 23°C). After the aqueous phase was extracted a second time with phenol, high M_r products were precipitated from the aqueous phase, previously adjusted to 0.2 M NaCl, by addition of 3 vol 95% ethanol. After overnight storage at −20°C and centrifugation as before, the precipitate was dissolved in 21 ml 10 mM Tris-HCl, pH 8.0, 1.0 mM $MgCl_2$, added to a DEAE-Trisacryl column (1.0 × 15 cm) pre-equilibrated in the same buffer, and washed with 40 ml of the same buffer, all at 23°C. The column was then eluted with a 0 to 1.0 M NaCl gradient in the same buffer. Fractions from the symmetrical A_{260} peak having A_{260} greater than 0.6 were combined and the RNA condensed by ethanol and centrifugation as before. The pellet was dissolved in the same buffer, and following overnight dialysis against the same buffer, was stored at −20°C. The product which was inactive as substrate for aminoacylation was convertible to active tRNA when treated sequentially with 2′:3′ cyclicnucleotide 3′ phosphodiesterase, alkaline phosphatase, and tRNA nucleotidyl transferase as described by Markstein.[8]

Preparation of tRNACC

The methods of Neu and Heppel[20] as modified by Deutscher[21] were used to prepare tRNA lacking its 3′ terminal 5′AMP residue (tRNACC). This material was generously donated by Mr. Joseph Del Pizzo.

RESULTS

Specificity of Alkaline RNase for Poly(C) and Poly(U)

Preliminary studies on the specificity of alkaline RNase,[6] including those which showed limited degradation of porcine liver tRNA [Figure 1(a)], confirmed previous results[4,5] with one exception. Although alkaline RNase catalyzed extensive degradation of poly(C), cleavage of poly(U) was questionable [Figures 1(b) and (c)], this apparent discrepancy was traced to differences in the amount of enzyme used here and previously. Figures 1(d) and (e) show results obtained by incubating poly(C) and poly(U) with

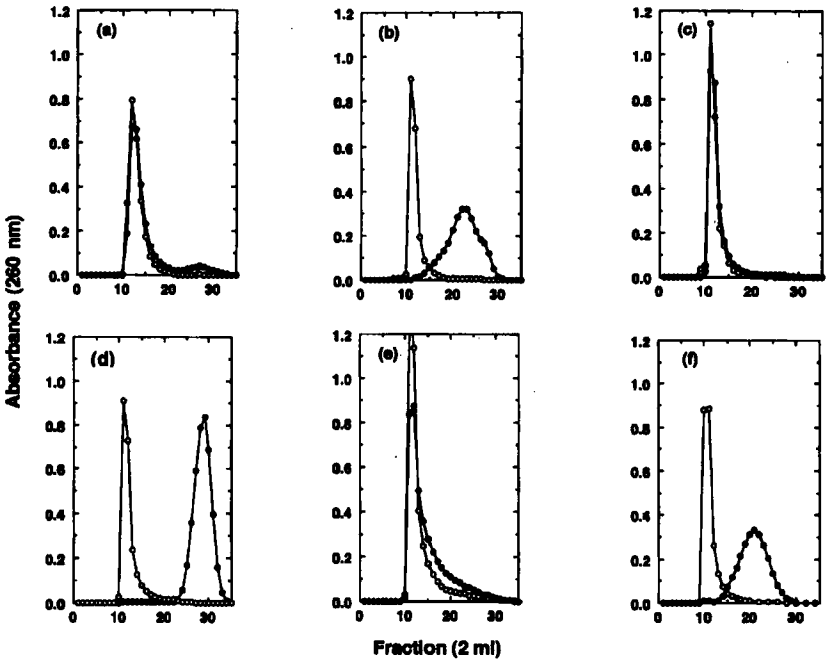


FIGURE 1 Activity of alkaline RNase toward natural and synthetic RNA's. Potential substrates were incubated for 2 h (23°C) alone (open circles) or in the presence of various units of alkaline RNase (solid circles) and added to either a G-50 Sephadex (a–c; 1.0 unit of alkaline RNase) or a G-75 Sephadex (d–f) column as described by Button *et al.*[4] (a) porcine thyroid tRNA; (b) poly(C); (c) poly(U); (d) poly(C), 10 units; (e) poly(U), 10 units; (f) poly(U), 50 units.

10 units and Figure 1(f) incubation of poly(U) with 50 units of alkaline RNase. Only at the latter level of enzyme did the cleavage of poly(U) approximate that obtained with poly(C) using 1 unit of enzyme. Even then, cleavage products of poly(U) eluted six fractions before those from poly(C) [Figures 1(d) and (f)] and mononucleotide controls (not shown) indicating that the majority of products arising from cleavage of poly(U) were larger than mononucleotides.

Rates of cleavage of poly(C) and poly(U) were also compared. Various amounts of alkaline RNase (3.75, 12.5, 25, 50 units) were incubated with limiting poly(U) (4.95 μM), each for 5, 30, 60, and 120 min. No activity was observed after 5 min at any level of alkaline RNase and little at the two highest amounts after 30 min. Rates per unit of alkaline RNase (μmol of UMP residues solubilized $min^{-1} ml^{-1}$ incubation) calculated from the 60 and 120 min incubations for all four concentrations of alkaline RNase were similar ($4.5–10.0 \times 10^{-8}$, range; 7.8×10^{-8}, average of all eight trials). The same calculation done using poly(C) yielded an average rate per unit

of 2.20×10^{-3} μmol min^{-1} ml^{-1}. Therefore, at equivalent concentrations of polymers, alkaline RNase cleaved poly(C) at a rate 2.8×10^4 times greater than poly(U).

Kinetic Parameters for tRNA, Poly(C), and Poly(U)

Figure 1 shows the results of two hour incubations of alkaline RNase with two synthetic RNA's, poly(C) and poly(U), and tRNA at concentrations of 0.76, 2.48, and 8.00 μM, respectively. Over this time interval, based on absorbance, it appeared the enzyme cleaved much more poly(C) than tRNA and poly(U) and more tRNA than poly(U).

The $k_{cat}:K_M$ ratio is widely used as the parameter of choice in deciding for which of several substrates a multi-substrate enzymes is more specific (or for which it would show preference).[22] However, there are caveats that should be considered. In order to determine the K_M used in the calculation, one must know the molar concentration of the substrate. While the molarity of the substrate can be determined from the mass used and the M_r of various macromolecules, a problem immediately arises when working with a nucleotide-specific nuclease such as alkaline RNase which could potentially bind and cleave at multiple sites. That is, should one calculate K_M's based on the M_r of the molecule or on the molarity of the residues comprising the macromolecule? Whereas which to choose is purely academic when comparing two rather similar macromolecules such as poly(U) and poly(C) both of which have more-or-less equal binding and cleavage potential, it could be of significant importance when comparing an enzyme's relative activity toward a synthetic and a natural RNA which has many fewer such sites as well as a variety of double-stranded regions. Also, the relative affinities of the two substrates for the enzyme could have a profound effect on preference if their magnitudes differed greatly.

To examine the significance of these points, k_{cat}'s were measured for the three substrates: poly(U), poly(C), and $k_{cat}:K_M$ ratios were obtained using K_M's calculated on a molar basis (mol polymer, L^{-1}) (Table I). It will be shown below that these ratios failed to predict alkaline RNase's empirical preference.

Relationships Between Poly(C) and tRNA Substrates and Validity of the $k_{cat}:K_M$ Ratio

From Table I it appeared that the preliminary order of specificity determined from the extensive incubations shown in Figure 1: poly(C) > tRNA

TABLE I Kinetic parameters for the action of alkaline RNase on poly(U), poly(C), and tRNA

Substrate	k_{cat}	K_M	$k_{cat} : K_M$
Poly(U)	7.8×10^{-1}	1.80×10^{-7}	4.33×10^{6}
Poly(C)	2.18×10^{4}	3.71×10^{-7}	5.88×10^{10}
tRNA	4.79×10^{2}	5.97×10^{-6}	8.02×10^{7}

K_M's (M) were based on three [poly(U)] or four [poly(C) and tRNA] different trials. Standard deviations were: 1.63×10^{-8}, 7.51×10^{-8}, and 1.84×10^{-7} M, respectively. Each K_M value was determined from least squares analysis of data obtained using Lineweaver-Burk plots. Turnover numbers (k_{cat}, min^{-1}) for poly(U), poly(C) and tRNA (mol substrate degraded min^{-1} mol^{-1} enzyme) were calculated based on the assumption of pure enzyme of 51,000 M_r.[4-6,23] Units of $k_{cat} : K_M$ are $min^{-1} M^{-1}$.

poly(U) was substantiated by rate measurements. In order to further compare alkaline RNase's relative "preference" for poly(C) and tRNA, specificity indices were calculated for each substrate in three ways: mol of polymer; mol of residues mol^{-1} of polymer; mol of assumed cytidylate residues (those in non-hydrogen bonded regions) mol^{-1} of polymer (Table II).

The ratio of specificity indices for the two substrates [poly(C) : tRNA; specificity factors] obtained for each way $k_{cat} : K_M$'s were calculated suggested alkaline RNase would prefer poly(C) over tRNA if both were present in the same incubation.[22] The specificity factors further predicted that poly(C) should be a very effective competing substrate when tRNA degradation was observed, whereas tRNA should not significantly inhibit the degradation of poly(C) except at very high concentrations. However, when competition experiments were carried out, opposite results were obtained. [In the following description of these experiments, apparent inhibitor constants (K_i's) are designated by subscripts referring to whether they are for steady-state forms affecting slopes (s) or intercepts (i) of double reciprocal plots. These apparent inhibitor constants actually represent K_M's in cases where complexes are catalytically productive.]

When alkaline RNase was incubated with tRNA (1–5 μM) in the presence of poly(C) at concentrations of 0.034 μM and 0.068 μM, little inhibition of cleavage of tRNA was observed; not the 80–90% predicted from Table II. [e.g.: specificity factor × concentration of poly(C) equals the effective molarity of poly(C) ($733 \times 0.068\,\mu M = 49.8\,\mu M$); the effective molarity of poly(C) divided by the concentration of tRNA equals the effective molar excess of poly(C) to tRNA (49.8 μM poly(C)/5 μM tRNA = 9.97); the predicted relative activity toward tRNA would be 1/9.97, ≅0.1, ≅90% inhibition] Furthermore, when alkaline RNase was incubated with 1.04 μM poly(C) in the presence of tRNA at concentrations of 0.91 μM and 1.83 μM, significant inhibition of the cleavage of poly(C)

TABLE II Kinetic parameters for alkaline RNase's action on poly(C) and tRNA

Parameter	*Substrate*		*Specificity Factor*
	Poly(C)	*tRNA*	
k_{cat}	2.18×10^4	4.79×10^2	
K_M^1	3.71×10^{-7}	5.97×10^{-6}	
K_M^2	2.15×10^{-4}	4.18×10^{-4}	
K_M^3	2.15×10^{-4}	2.99×10^{-5}	
$k_{cat} : K_M^1$	5.88×10^{10}	8.02×10^7	733
$k_{cat} : K_M^2$	1.01×10^8	1.15×10^6	87.3
$k_{cat} : K_M^3$	1.01×10^8	1.60×10^7	6.31

M_r's used: tRNA, 2.5×10^4; poly (C), 1.77×10^5; cytidylate residue mass, 305. The average number of residues per tRNA was taken as 70 and of cytidylate residues in single-stranded regions of the average tRNA molecule as 5. Poly(C) would have 579 residues mol^{-1}. Values of k_{cat} are expressed as mol substrate converted min^{-1} mol^{-1} enzyme (min^{-1}). K_M^1's (M) were calculated on the basis of M_r, K_M^2's on moles of residues mol^{-1}, and K_M^3's on the moles of cytidylate residues mol^{-1}. Specificity factors were obtained by dividing k_{cat}:K_M ratios (min^{-1} M^{-1}) of poly(C) by that for tRNA.

occurred; the opposite experimental result than predicted from the theoretical calculations in Table II. Because in the latter experiment inhibition was linear with respect to tRNA concentration (19% at 0.91 μM; 38% at 1.83 μM), the concentration of tRNA that would have given 50% inhibition (the point where alkaline RNase showed equal preference toward each competing substrate) was estimated by extrapolation to be 2.4 μM, not 1341.4 μM as would be predicted from Table II ($733 \times 1.83\,\mu M = 1341.4\,\mu M$). Thus, based on substrate molarities an empirical specificity factor of tRNA : poly(C) of 559 existed (1341.4 μM/2.4 μM); a very different value from the theoretical value of 1.36×10^{-3}(1/733).

From these preliminary competition experiments it was clear that if only $k_{cat} : K_M$ ratios calculated on a molar substrate basis were used as measures of substrate preference, the wrong conclusion regarding empirical substrate preference would have been reached.

To design further experiments comparing the enzyme's preference and specificity it was necessary to more accurately quantify effective concentrations. This was done as follows. Because k_{cat}'s are empirically determined and alkaline RNase's K_M for tRNA is known with some certainty[4,5,10,23] the $k_{cat} : K_M$ ratio for tRNA could be assumed to be correct. Therefore, the same $k_{cat} : K_M$ ratio could be assumed for poly(C) to calculate an effective K_M for poly(C). This worked out to be 272 μM. The average poly(C) molecule ($M_r = 1.77 \times 10^5$) contains 579 residues. Therefore, if the K_M for poly(C) is calculated using cytidylate residue molarity instead of molecular molarity, K_M is 215 μM (K_M^2, Table II). This value is in reasonable agreement with that just estimated by equating the $k_{cat} : K_M$ for poly(C) with that for tRNA. Use of K_M's for poly(C) and other synthetic RNA's,

calculated on the basis of residue molarity proved adequate in designing more quantitative experiments and were used in all the following experiments which used synthetic RNA's.

In order to better understand the discrepancies between theoretical calculations and experimental results, the effects of the presence of poly(C) on cleavage of tRNA were determined more quantitatively [Figure 2(a)]. Poly(C) proved to be a weak, competing substrate with a K_{is} (K_M) of 289 μM. However, the converse of this experiment [i.e., poly(C) varied; effects of competing tRNA] showed unexpected inhibition patterns [Figure 2(b)] in that they resembled mixed rather than competitive inhibition. This result suggested the presence of an additional productive steady-state enzyme-substrate form. The average K_{ii} for 13 such determinations was 5.71 μM, close to the K_M for tRNA (5.97 μM), whereas the average K_{is} was 0.93 μM, about one-sixth of the empirically determined K_M.

However, K_{is} and K_{ii} values were not constant over the range of tRNA concentrations. Instead, the values of K_{ii}'s increased linearly with increasing tRNA concentration whereas K_{is} values decreased hyperbolically. This result clearly indicated that tRNA was not behaving like a classical competing substrate in competition with poly(C) for the enzyme.

The above preliminary conclusion was verified by replotting the data from one such experiment (Figure 3). This type of plot, which is analogous to a Dixon plot of reciprocal velocity versus inhibitor concentration, showed that the competition was exponential rather than linear. Therefore, these data could not be analyzed using conventional Michaelis-Menten assumptions and treatments. The parabolic curves had two ramifications: complex interactions were taking place among alkaline RNase, tRNA, and poly(C) and the affinity between alkaline RNase and tRNA was much greater than between alkaline RNase and poly(C). That is, it appeared that tRNA was acting as a tight-binding competing substrate. Therefore, the data were re-analyzed analogously to tight-binding inhibition kinetics[25] (Inset to Figure 3). This analysis revealed the following: (1) tRNA's inhibition of cleavage of poly(C) was competitive, (2) the K_M for poly(C) (225 μM) agreed with those previously estimated, and (3) the K_M for tRNA (0.67 μM) was an order of magnitude lower than in the absence of poly(C) (5.97 μM) but close to the average K_{is} value obtained using classical inhibition kinetics (0.93 μM).

Effects of Poly(U), Poly(A), Poly(G), GGG, and Guanosine

Poly(U) acted as a classical competitive inhibitor of tRNA cleavage. Based on residue molarity, a K_M of 36.4 μM was measured. Cleavage of poly(U)

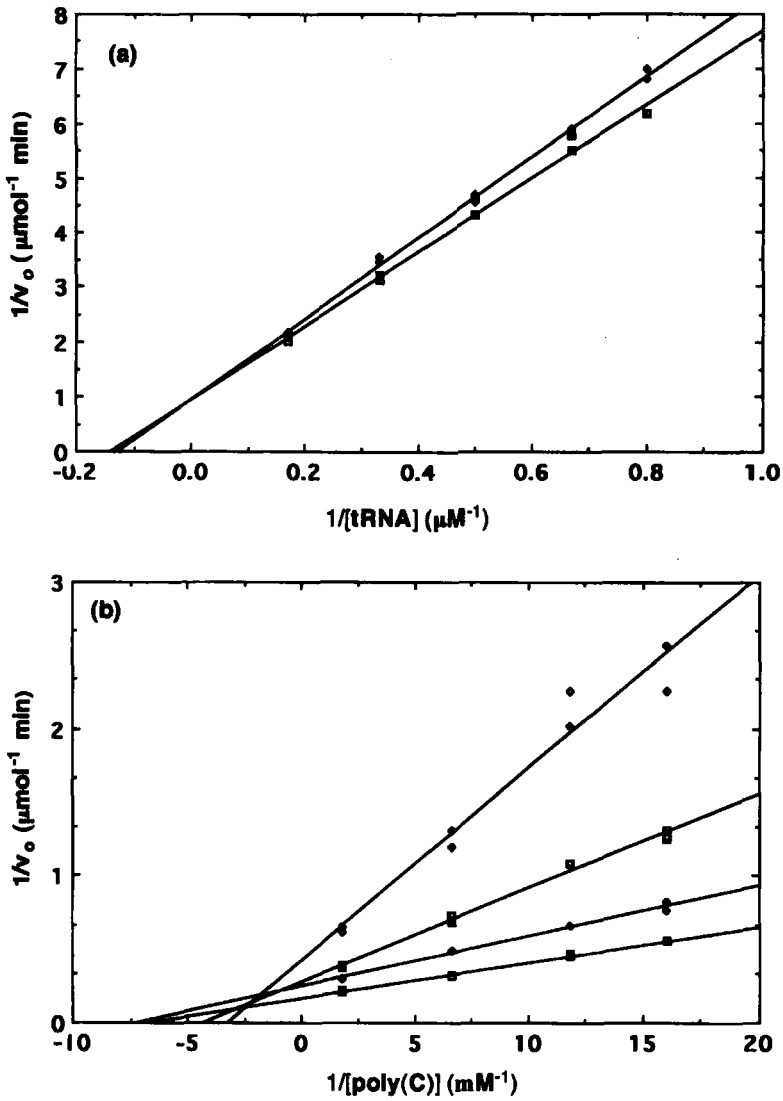


FIGURE 2 Lineweaver-Burk plots for determination of K_M's of alkaline RNase for tRNA and poly(C) and effects of substrates competing for alkaline RNase. (a) tRNA as substrate; poly(C) competing. Duplicate incubations contained 1.6 units of alkaline RNase and varying concentrations of tRNA either in the absence (squares) or presence (diamonds) of poly(C) at 40 μM. Initial velocities are expressed as μmol aminoacyl-tRNA destroyed $min^{-1} ml^{-1}$ incubation mixture and substrate is μM. (b) poly(C) as substrate; tRNA competing. Assay conditions were the same as in (a) except poly(C) was used as substrate (Methods) at the concentrations indicated (from bottom to top) in the absence (squares) or presence of tRNA [1.03 (diamonds), 2.11 (squares), 4.24 μM (diamonds)] and they contained 4.0 units of enzyme. Initial velocities are expressed as μmol of cytidylate residues solubilized $min^{-1} ml^{-1}$ incubation mixture and substrate is mM.

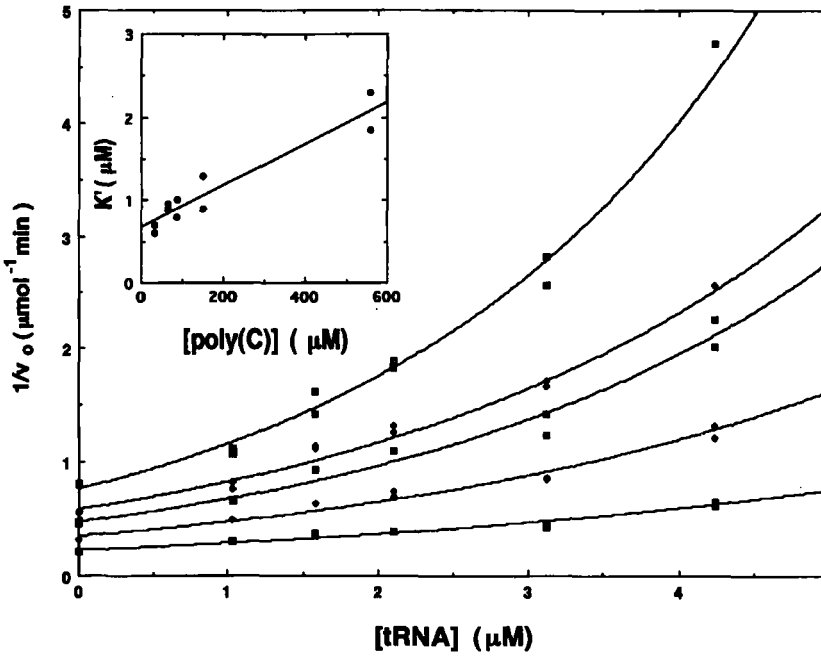


FIGURE 3 Competing inhibition by tRNA of the cleavage of poly(C) catalyzed by alkaline RNase. Reciprocal initial velocities are plotted as a function of tRNA concentrations. Incubations done as described in Figure 2(b) contained the amounts of poly(C) and tRNA indicated. Nonenzymatic controls for tRNA and poly(C) at each concentration of each substrate and enzymatic controls for tRNA alone at each concentration were taken into account in determining initial velocities. In the case of the latter, absorbance changes were negligible, varying linearly from 0.008 at the lowest tRNA concentration to 0.031 at the highest. In the Inset the same data were treated according to Dixon.[24] Two or more K′ values were estimated from plots of velocities versus tRNA concentrations for each concentration of poly(C) and as shown varied with concentration, a criterion of competitive, tight-binding inhibition. The ordinate intercept yields a value for alkaline RNase's K_M for tRNA and the abscissa intercept is the $-K_M$ for poly(C).

was not observed when tRNA was present in incubations at even very low concentrations.

Neither poly(A) nor poly(G) are cleaved by alkaline RNase. However, while poly(A) does not inhibit alkaline RNase[4] poly(G) inhibits both cleavage of tRNA and poly(C). Poly(G) behaved as a classical competing inhibitor of the cleavage of tRNA (K_{is} of 20 µM based on the molarity of guanylate residues). However, when competing with poly(C) the results shown in Figure 4 were obtained.

These data indicated that the mode of inhibition of the cleavage of poly(C) by poly(G) was dependent upon the concentration of poly(G) in

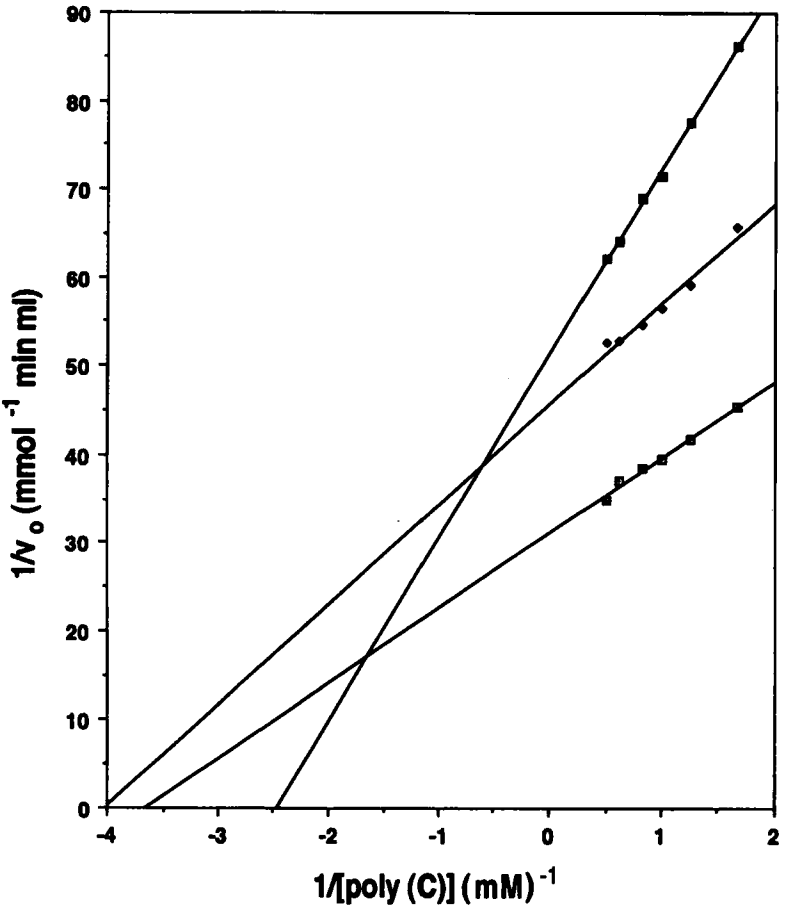


FIGURE 4 Inhibition by poly(G) of the cleavage of poly(C) by alkaline RNase. Assays in 0.3 ml contained the concentrations of poly(C) indicated and 4 units of alkaline RNase. Velocities are expressed as μmol product formed min^{-1} ml^{-1} assay. Assays were done in the absence (open squares; bottom line) and presence of either 23.6 μM (closed diamonds) or 47.1 μM poly(G) (closed squares) (based on guanylate residue weight). The lines shown are computer-generated "best fit" from averaged duplicate trials.

the assay. At low concentrations the inhibition was essentially simple, linear, mixed (K_{is}, 59.4 μM; K_{ii} 53.8 μM). At higher concentrations it was mixed with the intersection point shifted from near the abscissa to close to the ordinate (K_{is}, 30.3 μM; K_{ii}, 76.8 μM). The K_M for poly(C) obtained in these experiments was 255 μM. Neither GGG at 100 μM nor guanosine at concentrations up to 833 μM (higher concentrations could not be tested because of guanosine's insolubility) had any effect.

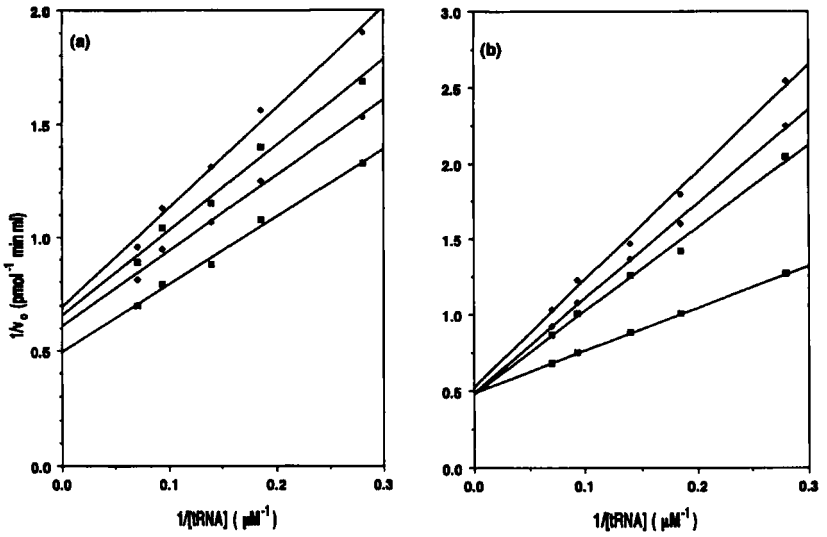


FIGURE 5 Product inhibition of the cleavage of tRNA by alkaline RNase. Initial velocities are expressed as pmol tRNA cleaved min^{-1} ml^{-1} (a) Inhibition by A: (from bottom to top) open squares, no added A [K_M (tRNA), 6.2 μM]; closed diamonds, 5 mM A; closed square, 10 mM A; open diamonds, 20 mM A. (b) Inhibition by 2′:3′ cCMP: (from bottom to top) open squares, no added 2′:3′ cCMP [K_M (tRNA), 6.0 μM]; closed squares, 65.7 μM 2′:3′ cCMP; open diamonds, 87.3 mM 2′:3′ cCMP; closed diamonds, 110.5 mM 2′:3′ cCMP. For both products, duplicate incubations, in 75 μl, contained 1.25 units of alkaline RNase and were for 7 min. The lines shown are computer-generated "best fit" from averaged duplicate trials.

Inhibition by Products and Kinetic Mechanism

Figure 5 shows the kinetics of incubating alkaline RNase with substrate tRNA in the presence of mM concentrations of two of its major products: A and 2′:3′ cCMP. Adenosine behaved as a mixed inhibitor (K_{is}, 52.8 mM; K_{ii}, 33.7 mM) whereas 2′:3′ cCMP was competitive (K_{is}, 70.5 mM).

Similar product inhibition studies with CA and tRNACp >, the enzyme's other major products, could not be done because sufficient amounts of these were unavailable to test at concentrations comparable to commercially available products. Nevertheless, it was important to determine if tRNACp > could effectively bind to the enzyme in the presence of equal molar amounts of tRNA. That is, to test the question as to whether it could inhibit the forward reaction through competition with tRNA. Results from experiments performed under these conditions showed no inhibition of the cleavage of tRNA by tRNACp > in concentrations (5.61 μM) approximately equal to the enzyme's K_M for intact tRNA

(5.97 μM). At concentrations up to 7.5 μM, tRNACC also failed to inhibit cleavage of tRNA. Therefore, these experiments provided good evidence that the binding of tRNA to the enzyme's catalytic site involved the intact 3′-CCA terminus of tRNA and localized the portion of intact tRNA catalytically bound by the enzyme as the 3′ terminal 5′AMP residue of tRNA.

Finally, incubation of 144 μmol tRNACC with 100 units of alkaline RNase for 15 min followed by chromatography on G-25 (see Methods) did not produce detectable cytidine. That is, tRNACC was not a substrate for the enzyme.

DISCUSSION

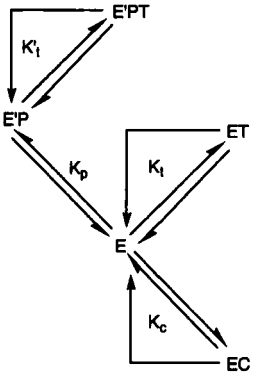
Both poly(U) and poly(C) are mostly present as single-stranded forms under the conditions of the assays employed here.[26,27] Therefore, initial trials with poly(U) and poly(C) were surprising when compared to previous results[4,5] in that little or no cleavage of poly(U) by alkaline RNase was observed. Subsequent experiments revealed that activity toward poly(U) was undetectable unless incubation times were lengthy and the amount of alkaline RNase was greatly increased. Ultimately alkaline RNase was shown to cleave poly(C) 28,000 times faster than poly(U). This degree of specificity for cytidylate over uridylate residues is in contrast to the pancreatic- and nonpancreatic-type human RNases compared by Sorrentino and Libonati.[28] An alkaline RNase from chicken liver has been isolated that is also rather specific for cytidylate residues.[29] However, that enzyme also catalyzes significant cleavage of poly(U) and poly(A,U). Among other criteria by which the chicken liver enzyme and thyroid latent alkaline RNase differ are pH optima and M_r.

Even though poly(C) was the only substrate tested that seemed to serve as a good substrate for thyroid alkaline RNase, further investigation of the enzyme's apparently limited activity toward tRNA was done for the following reasons. First, the enzyme assay used was the same as was used during the original purifications of alkaline RNase from homogenates containing other cellular nucleases,[4,5] and for many other studies.[4–6,8,10,23] Because this assay utilizes a tRNA substrate,[10] it seemed possible that it might have fortuitously selected an enzyme specific for tRNA out of the *milieu* of cellular nucleases. Second, the finding that alkaline RNase was associated with RNA[6] suggested a possible similarity to RNase P, a pre-tRNA processing ribozyme from *Escherichia coli*[30] Third, alkaline RNase was quite specific for cytidylate residues in single-stranded RNA's, a structural feature found in all functional tRNA's penultimate to their 3′ termini.

Although specificity factors calculated on the basis of M_r indicated a 733 greater specificity for poly(C) than tRNA, preliminary competition experiments, also using molarities calculated using M_r's, showed that the enzyme actually preferred tRNA over poly(C) by a factor of 559:1. In order to equate $k_{cat}:K_M$ ratios for these two substrates, the K_M for poly(C) was recalculated based on the molarity of cytidylate residues.

The competition between tRNA and poly(C) could not be adequately analysed by Lineweaver-Burk analysis. Poly(C) behaved as a classic competing substrate when cleavage of tRNA was measured while tRNA inhibition of poly(C) cleavage showed apparent mixed inhibition. This observation could be interpreted to mean that incubations contained two forms of enzyme capable of cleaving tRNA, but only one which cleaved poly(C). Further analyses revealed tRNA's inhibition was not linear because it behaved as a tight-binding competitive substrate when cleavage of poly(C) was measured. This analysis yielded a K_M value for poly(C) very close to the estimated value, however, the K_M value for tRNA (0.67 μM) was about an order of magnitude lower than that determined here and by others[4,5,10,23] in the absence of poly(C). This lower value was close to the average K_{is} (K_M) obtained using conventional double reciprocal plots of data from the competition experiments (0.93 μM).

Because a kinetic treatment of competing substrates yielding results such as were observed has apparently not found its way into the literature, various possibilities of alkaline RNase's combinations with poly(C) and tRNA were considered. One such working mechanism which is consistent with the available evidence is shown in Scheme 1. In this mechanism two catalytically active forms of alkaline RNase exist (E and E′P) having different kinetic parameters. The ten-fold lower value for the K_M (tRNA) obtained from the tight-binding substrate analysis of the competing substrates represents the K_M for the sole form of alkaline RNase capable of cleaving poly(C), E. On the other hand, the K_M obtained for tRNA in the absence of poly(C) is the sum of E and E′P's individual K_M's (K_t and K_t'). This mechanism also predicts that alkaline RNase would either have to be capable of simultaneously binding its RNA component, P, and cleaving tRNA or that P is displaced by tRNA prior to a new cleavage round. In addition to the kinetic evidence supporting the latter hypothesis is the presence of RNA in highly purified preparations of alkaline RNase that approaches the size of tRNA. The presence of this RNA could account for a number of alkaline RNase's more unusual properties (i.e. general behavior as a nucleic acid.[6] (In this regard see references 31 and 32).



SCHEME 1 In this mechanism C(c) stands for poly(C), T(t) for tRNA, P(p) for either a product of tRNA degradation or the RNA present in the alkaline RNase preparation[6] and K_c and K_t are the respective K_M's for poly(C) and tRNA. K_t' is the K_M for a putative second active form of alkaline RNase, E'P, and K_p is the dissociation constant of E'P.

Poly(U) was a poor substrate of alkaline RNase even though it had a somewhat lower K_M than poly(C). Poly(U)'s K_M calculated on a residue basis is 3.64×10^{-5} M and its $k_{cat}:K_M$ ratio is 2.14×10^4. If the $k_{cat}:K_M$ ratio for poly(C) is 5.88×10^{10}, the specificity factor of poly(C):poly(U) is 2.74×10^6. However, if the $k_{cat}:K_M$ ratio for poly(C) is taken to be 1.01×10^8, i.e. both based on residue molarity, the specificity factor is 4.72×10^3. Competition experiments between poly(U) and poly(C) were not done. Compared to the $k_{cat}:K_M$ ratio for tRNA the specificity factor of tRNA:poly(U) is 3.8×10^3.

From these experiments it can be concluded that alkaline RNase exhibits a high degree of specificity and preference for tRNA over poly(C). In order for such a preference to be reflected in the $k_{cat}:K_M$ ratios for the two substrates, that for poly(C) would have to be 1.43×10^5. Assuming a k_{cat} for poly(C) of 2.18×10^4 mol min^{-1}, K_M would have to be 152 mM. To the extent that K_M can be considered a measure of affinity, it would seem that alkaline RNase's preference for tRNA rests more in its relative affinity than in k_{cat}.

It should be reiterated that a certain degree of caution must be exercised in ascribing preference for one substrate over another for multi-substrate enzymes based solely on $k_{cat}:K_M$ ratios. One must do direct competition experiments to verify that the specificity factor, the theoretical ratio of $k_{cat}:K_M$ ratios for two substrates, is borne out.

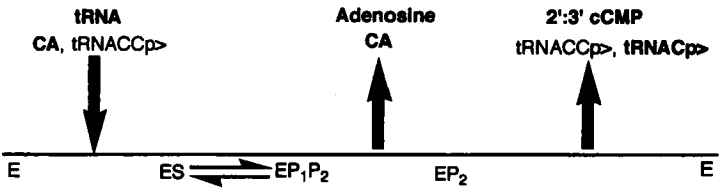
A less general but more relevant point to the work described here is that factors other than non-specific recognition of cytidylate residues were

involved in alkaline RNase's catalytic activity toward tRNA. If alkaline RNase lacked specificity toward cytidylate residues present in a macromolecular substrate, poly(C) would be preferred in competition experiments. Such was not the case when both poly(C) and tRNA were available. The preferred substrate was tRNA. Incubation of tRNA with alkaline RNase yields only 2′:3′ cCMP, A, CA, and a high M_r product, probably tRNACp>, because it can be repaired to functional tRNA by enzymatic treatments.[8] Thus, the target for the preference toward tRNA shown here appears to be localized in the invariant adjacent cytidylate residues located in tRNA's 3′ terminus. To emphasize the degree of the enzyme's specificity for tRNA, either of the 2 cytidylate residues in tRNA's-CCA terminus was preferred 280:1 over the 579 in the average poly(C) molecule. Perhaps coincidentally, the preference factor calculated from competition experiments using molar concentrations of tRNA and poly(C) was 559.

The observation that alkaline RNase neither acted on, nor was inhibited by tRNACC, suggested that the terminal 5′AMP of tRNA's 3′ terminus was involved in the enzyme's recognition and binding of tRNA. Taken together, these results show that tRNA, CA, and perhaps tRNACCp> (which was not tested) are substrates of alkaline RNase whereas tRNACC is not.

Of other potential substrates tested, the enzyme cleaved neither poly(A) nor poly(G). While poly(A) was not an inhibitor, poly(G) did inhibit and therefore was bound by alkaline RNase. It is perhaps worth noting that the mode of poly(G) inhibition of the cleavage of poly(C) changed as the concentration of poly(G) increased. This result was interpreted to mean that the higher concentrations favored the formation of poly(C)·poly(G) complexes[27] to which alkaline RNase could bind but because of its single-strand specificity might not cleave. Neither tRNACC, tRNACp>, GGG, nor guanosine were effective inhibitors of the cleavage of tRNA by alkaline RNase which tends to rule out the attractive possibility that the adjacent invariant guanosine residues in the D loop of tRNA are important in binding to the enzyme.

Both 2′:3′ cCMP and A were inhibitors. The high K_i values measured for these products (actually K_M's) are in accordance with those predicted by the Haldane equation. The concentrations of products of most hydrolytic and cleavage enzymes (i.e. reactions having large K_{eq}'s) that are necessary to effect inhibition of the initial rates of their forward reactions are much larger than the K_M's of the substrates for the forward reaction. The product inhibition studies revealed the kinetic mechanism of alkaline



SCHEME 2 Proposed kinetic mechanism of alkaline RNase. Because A and 2′:3′ cCMP are produced in equal amounts throughout time courses of cleavage,[8] it seems likely that if A is produced by the initial cleavage rather than CA, that tRNACCp> might remain enzymatically bound.

RNase's action on tRNA. They also provided kinetic evidence that A and 2′:3′ cCMP were products of the enzyme's action toward the 3′-CCA terminus of tRNA as has been directly shown by the isolation of these products from incubations.[8]

The mode of inhibition by products can be utilized to establish the order (if any) of release of products.[33] Thus, the kinetic mechanism of the activity of porcine thyroid latent alkaline RNase can be summarized by Scheme 2 which illustrates an apparent ordered uni bi kinetic mechanism. In the diagram the substrate and products used are in bold characters and the inferred substrates and products are in normal print. Frequently such mechanisms for hydrolytic enzymes are in actuality ping pong bi bi mechanisms in which water is the second reactant,[33] however, alkaline RNase is technically not a hydrolase. Instead, it should be considered as a phosphotransferase where the formation of the 2′:3′ cyclic phosphodiester bond displaces the 5′ hydoxyl leaving group of A, 2′:3′ cCMP, or CA.[8]

In contrast to lysosomal acid RNase, which appears to be involved with degradation of cellular RNA,[34] alkaline RNase is generally assumed to play a different and more specific role in cellular RNA metabolism than simply depolymerization. This assumption is supported by the relatively low levels of the enzyme, its intracellular locale, its specificity for pyrimidine residues in single-stranded regions of RNA, and that its effect on protein synthesis is apparently regulated by the levels of endogenous RNase inhibitor.[35] Most efforts have been directed toward examining if mRNA is alkaline RNase's *in vivo* substrate. However, the possibility that tRNA, which also plays a crucial role in protein biosynthesis, is the enzyme's target has not been previously examined. Based on the enzyme's specificity and preference for tRNA shown here, the latter might be considered an oversight.

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