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## *Articles*

# Isolation and Characterization of a Human Colon Carcinoma-Secreted Enzyme with Pancreatic Ribonuclease-like Activity†

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ABSTRACT: A ribonuclease was isolated from serum-free supernatants of the human colon adenocarcinoma cell line HT-29. It was purified by cation-exchange and C18 reversed-phase high-performance liquid chromatography. The protein is basic, has a molecular weight of ~16000, and has an amino acid composition that is significantly different from that of human pancreatic ribonuclease. The amino terminus is blocked, and the carboxyl-terminal residue is glycine. The catalytic properties of this ribonuclease resemble those of the pancreatic ribonucleases in numerous respects. Thus, it exhibits a pH optimum of ~6 for dinucleotide cleavage and employs a two-step mechanism in which transphosphorylation to a cyclic 2′,3′-phosphate is followed by slower hydrolysis to produce a 3′-phosphate. It does not cleave NpN′ substrates in which adenosine or guanosine is at the N position and prefers purines at the N′ position. Like bovine ribonuclease A, the HT-29-derived ribonuclease is inactivated by reductive methylation or by treatment with iodoacetate at pH 5.5 and is strongly inhibited by the human placental ribonuclease inhibitor. However, in contrast, the tumor enzyme does not cleave CpN bonds at an appreciable rate and prefers poly(uridylic acid) as substrate 1000-fold over poly(cytidylic acid). It also hydrolyzes cytidine cyclic 2′,3′-phosphate at least 100 times more slowly than uridine cyclic 2′,3′-phosphate and is inhibited much less strongly by cytidine 2′-monophosphate than by uridine 2′-monophosphate. Other ribonucleases known to prefer poly(uridylic acid) were isolated both from human serum and from liver and were compared with the tumor enzyme. The physical, functional, and chromatographic properties of the serum ribonuclease are essentially identical with those of the tumor enzyme. The liver enzymes, however, differ markedly from the HT-29 ribonuclease. The potential utility of the tumor ribonuclease in the diagnosis of cancer is considered.

We have recently developed a method for maintaining the human colon adenocarcinoma cell line HT-29 in a serum-free and exogenous protein free medium (Alderman et al., 1985). This advance has made possible the detailed examination of several products secreted by these cells, including the blood vessel forming protein angiogenin (Fett et al., 1985b), lysozyme (Fett et al., 1985a), and a vascular permeability factor (Lobb et al., 1985). Determination of the complete amino acid sequence of angiogenin (Strydom et al., 1985; Kurachi et al., 1985) revealed its extensive homology to human pancreatic ribonuclease (RNase),[1] with 35% identity, including virtually

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all of the known active-site residues. Hence, it seemed reasonable to examine the possibility that angiogenin is in fact a ribonucleolytic enzyme. It was determined (Shapiro et al., 1986) that angiogenin does not exhibit activity in a variety of standard assays employed for pancreatic RNase. It does not produce measurable quantities of perchloric acid soluble fragments from wheat germ RNA, poly(C), or poly(U) nor does it act on U>p, C>p, or dinucleotides. Angiogenin does, however, catalyze the limited endonucleolytic cleavage of 28S and 18S ribosomal RNA, generating major products which are 100–500 nucleotides in length.

During the course of the angiogenin study, it was discovered that HT-29 cells also secrete relatively large amounts of an RNase whose activity—in contrast with that of angiogenin—is readily detectable by several of the pancreatic RNase assays. The present report describes the isolation and detailed characterization of this enzyme.

## Experimental Procedures

*Materials.* Dinucleotides, cytidine cyclic 2′,3′-phosphate (C>p), uridine cyclic 2′,3′-phosphate (U>p), cytidine 2′-monophosphate, poly(cytidylic acid), poly(uridylic acid), *p*-nitrophenyl phosphate, bis(*p*-nitrophenyl) phosphate, bovine ribonuclease A (type X-A), and human serum albumin were obtained from Sigma Chemical Co. Poly(adenylic acid), poly(guanylic acid), uridine 2′-monophosphate, and poly(G)–agarose (type 6) were products of P-L Biochemicals. Yeast and wheat germ RNAs (high molecular weight) were purchased from Calbiochem-Behring. Ammonium sulfate (HPLC grade) was obtained from Schwarz/Mann. Human placental ribonuclease inhibitor was isolated by the method of Blackburn (1979). Alkaline phosphatase (type BAPF) was purchased from Cooper Biomedical. CM-cellulose (grade CM-52) was a product of Whatman Ltd. Deionized, sterile water was provided by a Milli RO-20 reverse-osmosis/Milli Q water purification system (Millipore Corp.). All dialyses were performed with 6000–8000 molecular weight cutoff tubing (Spectra/Por).

*Purification of Tumor RNase from HT-29 Cell Conditioned Medium.* Tumor RNase was isolated from medium conditioned by cells from the human colon adenocarcinoma cell line HT-29 (Fogh & Trempe, 1975), maintained under protein-free conditions as described (Alderman et al., 1985). The purification procedure employed was based on that recently described for the purification of the angiogenic protein angiogenin (Fett et al., 1985b; Strydom et al., 1985). Briefly, the conditioned medium was filtered to remove cell debris and then acidified with glacial acetic acid to a final concentration of 5%. It was stored frozen at −20 °C, thawed, clarified by filtration, concentrated by using an Amicon hollow fiber apparatus, dialyzed vs. water, and lyophilized. The lyophilized conditioned medium was dissolved in and dialyzed overnight vs. 0.1 M sodium phosphate, pH 6.6, and applied to a column of CM-52 cation-exchange resin which had previously been equilibrated with the same buffer (starting buffer) at 4 °C. The column was then washed with starting buffer. Material eluting with this buffer is designated as CM 1. Bound material (designated as CM 2) was eluted with 1 M NaCl in starting buffer. The CM 2 fraction, containing the tumor RNase, was dialyzed vs. water and lyophilized. This material was then subjected to reversed-phase HPLC on an octadecylsilane Synchropak RP-P column (Synchrom Inc., Linden, IN) (10-μm particle size, 250 × 4.1 mm) using a Waters Associates liquid chromatography system and an LKB 2138 206-nm detector. Lyophilized CM 2 preparations were reconstituted in 0.1% (v/v) TFA in water (solvent A) (Mahoney & Hermodson, 1980) and applied to the column through a WISP 710A automatic sample injector. The column was eluted with a 90-min linear gradient from 20% to 40% solvent B [0.08% TFA in a 3:2:2 (v/v/v) mixture of 2-propanol, acetonitrile, and water] at a flow rate of 1 mL/min at ambient temperature. One-milliliter fractions were collected in siliconized tubes.

*Purification of Serum RNase 5.* The procedure of Akagi et al. (1976) as modified by Blank and Dekker (1981) was employed for the resolution of serum RNases on phosphocellulose. Fractions corresponding to "RNase 5" were pooled and applied to a Sep-Pak C18 cartridge (Waters Associates). After the cartridge was washed with 10 mL of 0.1% TFA in water, the RNase was eluted with 2.5 mL of 60% acetonitrile/0.1% TFA (v/v) in water and lyophilized. This procedure was performed in order to concentrate the sample and remove any serum proteins which might irreversibly bind to the C18 HPLC column used subsequently. Lyophilized material was dissolved in 0.1% TFA (solvent A) and applied to a Synchropak RP-P column using a Waters U6K injector. Elution was performed with a 45-min linear gradient of 30–40% solvent B (0.09% TFA in a 5:5:4 mixture of 2-propanol, acetonitrile, and water) at a flow rate of 1.0 mL/min. One-milliliter fractions were collected in siliconized tubes.

For some experiments, fractions containing ribonuclease activity were pooled, lyophilized, and further chromatographed on either a phenyl-substituted or a cation-exchange HPLC column. In the former case, a Bio-Gel TSK-phenyl-5-PW column (75 × 7.5 mm; Bio-Rad) was used. Dried material was reconstituted in 0.1 M Mes/0.1 M NaCl, pH 6.0. An equal volume of 2.5 M ammonium sulfate was then added, and the sample was applied to the column. After 10 min of washing with 0.1 M sodium phosphate, pH 7.0, containing 1.7 M ammonium sulfate (buffer A), the RNase was eluted with a linear 90-min gradient of 0–100% buffer B (0.1 M sodium phosphate, pH 7.0) at a flow rate of 0.8 mL/min at room temperature. One-minute fractions were collected. Cation-exchange HPLC was performed with a Synchropak CM-300 column (250 × 4.1 mm; Synchrom, Inc.). Dried material was dissolved in 20 mM sodium phosphate, pH 7.0 (buffer A), and applied to the column. Elution was performed with a 50-min linear gradient from 22% to 68% buffer B (1 M NaCl in buffer A) at a flow rate of 0.8 mL/min. One-minute fractions were collected in tubes containing 20 μg of human serum albumin.

*Isolation of Liver RNases.* Human liver was obtained at autopsy within 12-h postmortem and stored at −70 °C. A section (17.8 g) was homogenized in 18 mL of water by using a Polytron homogenizer (Brinkmann Instruments, Inc.). Cell debris was then removed by centrifugation for 30 min at 12000*g*. The supernatant was acidified to a final concentration of 5% (v/v) acetic acid and frozen at −20 °C. After 3 days, the homogenate was thawed and centrifuged for 30 min at 48000*g*. The supernatant was then dialyzed against 0.1 M sodium phosphate, pH 6.6. Following dialysis, the mixture was centrifuged (30 min, 48000*g*) and the supernatant chromatographed on CM-52 resin as detailed above for the HT-29-conditioned medium. The CM 1 fraction, containing

[1] Abbreviations: RNase(s), ribonuclease(s); C>p, cytidine cyclic 2′,3′-phosphate; U>p, uridine cyclic 2′,3′-phosphate; poly(G), poly(guanylic acid); HPLC, high-performance liquid chromatography; CM, carboxymethyl; TFA, trifluoroacetic acid; C18, octadecylsilane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); poly(C), poly(cytidylic acid); EDTA, ethylenediaminetetraacetic acid; 2′-UMP, uridine 2′-monophosphate; 2′-CMP, cytidine 2′-monophosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

virtually all of the RNase activity, was dialyzed vs. water, lyophilized, and then dissolved in 10 mL of 50 mM sodium phosphate, pH 6.2. It was applied to a 1.0 × 7.0 cm column of poly(G)–agarose (Frank & Levy, 1976) previously equilibrated with the same buffer. After the column was washed with 50 mM sodium phosphate, pH 6.2, RNases were eluted with 1 M KCl in the same buffer. Fractions containing enzymatic activity were pooled, dialyzed vs. water, and lyophilized. This material was dissolved in 0.1% TFA and chromatographed on the same C18 reversed-phase HPLC system described above for serum RNase 5, except that a 100-min linear gradient from 35% to 70% B was employed.

*Gel Electrophoresis.* SDS–PAGE was performed by using 15% gels as described by Laemmli (1970). Gels were either stained with silver [Switzer et al. (1979) as modified by Fett et al. (1985a)] or stained for RNase activity (Blank et al., 1982). Analytical isoelectric focusing was carried out on an LKB 2117 multiphor unit using preformed plates (PAG plates, pH range 3.5–9.5; LKB). The gel was stained with Coomassie Blue by conventional procedures. Standards used were RNase A, cytochrome *c*, and lysozyme.

*RNase Assays.* Activity toward yeast RNA was measured spectrophotometrically by a modification of the method of Kunitz (1946). Sample (5–20 μL) was added to a solution containing 1 mg of RNA in 0.1 M Tris, pH 7.5, and 0.1 M NaCl (final volume 1.0 mL) at 25 °C. The absorbance decrease at 300 nm was continuously monitored by using a Gilford Model 250 spectrophotometer. In some cases, activity toward yeast RNA was also measured by the production of perchloric acid soluble material according to the method of Frank and Levy (1976). Activity toward wheat germ RNA was determined by the precipitation assay of Blank and Dekker (1981).

Activity toward polynucleotides was measured by a modification of the method of Zimmerman and Sandeen (1965). Reaction mixtures contained 100 μL of 0.1 M Tris, pH 8.0, 100 μL of 1 mg/mL substrate in water, 5–20 μL of sample usually diluted in 0.01% human serum albumin, and sufficient 0.01% albumin to bring the total volume to 250 μL. Mixtures were incubated for 30 min at 37 °C, followed by the addition of 250 μL of ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitrate. After incubation on ice for 10–20 min, samples were centrifuged at 15600*g* for 15 min at 4 °C. The absorbance at 260 nm [poly(A), poly(G), and poly(U)] or at 280 nm [poly(C)] was read after dilution with 2 volumes of water. Absorbance in control samples lacking RNase was subtracted. Blanks lacking polynucleotide had no significant absorbance. Acid-soluble nucleotides were quantitated by using molar absorptivities of $1.3 \times 10^4$ and $1.0 \times 10^4$ $M^{-1}$ $cm^{-1}$ for poly(C) and poly(U), respectively (Bock et al., 1956).

Activity toward dinucleotides was measured by two methods: (1) HPLC quantitation of products and starting material and (2) continuous spectrophotometric assay. In the former case, incubation mixtures contained 0.1 mM dinucleotide in 0.1 M Mes, pH 6.0, with 0.1 M NaCl, and 20 nM tumor RNase. After 1 h at 25 °C, 50 μL of the mixture was injected onto a Waters Radial-PAK C18 HPLC column[2] (type 8NVC185, 5-μm particle size, 100 × 8 mm) in an RCM-100 radial compression module, using the automatic sample injector. Elution was achieved with a 35-min linear gradient of 0.1 M potassium phosphate, pH 7.0, to 90 mM potassium phosphate, pH 7.0, in 32.5% (v/v) methanol at a flow rate of 0.8 mL/min at ambient temperature. The absorbance was monitored at 254 nm with a Waters Model 440 detector, in conjunction with a Hewlett Packard 3390A integrator.

In some cases, it was also possible to measure dinucleotide breakdown by the spectrophotometric assay of Witzel and Barnard (1962). This method was employed for all determinations of kinetic parameters and for obtaining pH–activity profiles. Depending on the substrate concentration, an appropriate wavelength between 280 and 293 nm was chosen, and the decrease in absorbance following addition of enzyme was continuously monitored. This absorbance decrease is associated with the first step (transphosphorylation) of the RNase-catalyzed reaction. The second step (hydrolysis of the cyclic phosphate), which produces an increase in absorbance, does not occur at a significant rate during these assays. Initial velocities were measured during the first 5–10% of the reaction. Absorbance changes were converted to reaction velocities (molar per minute) by using values for $\Delta\epsilon$ obtained by allowing the transphosphorylation reaction to proceed to completion. Unless specific otherwise, assays were performed in 0.1 M Mes, pH 6.0, containing 0.1 M NaCl at 25 °C. Where velocities at other pH values were measured, acetate (from pH 4.0 to 5.5), Mes (pH 5.5–7.0), or Hepes (pH 7.0–8.5) buffers were used, all at 0.1 M and containing 0.1 M NaCl. In experiments involving the placental RNase inhibitor, assay mixtures were supplemented with 1 mM EDTA and 10 μg/mL human serum albumin.

Activities toward C>p and U>p were also monitored by both HPLC and spectrophotometric assay. For HPLC measurements, incubation mixtures contained 0.3 mM substrate, 50 nM RNase, and 0.01% human serum albumin in 0.1 M Mes, pH 6.0, with 0.1 M NaCl. After 16 h at 25 °C, 50 μL was loaded onto a Radial-PAK C18 column. For C>p, isocratic elution of substrate and products was achieved with 0.1 M potassium phosphate, pH 7.0. For U>p, the same gradient used for dinucleotides was applied. Activities toward C>p and U>p were also measured by modifications of the spectrophotometric methods of Crook et al. (1960) and Richards (1955), respectively. Absorbance increases at 286 nm (C>p) or 280 nm (U>p) accompanying hydrolysis were continuously monitored at 37 °C. Assay mixtures contained 0.58 mM substrate in 0.1 M Mes, pH 6.5, with 0.1 M NaCl.

Activities toward *p*-nitrophenyl phosphate (Garen & Levinthal, 1960) and bis(*p*-nitrophenyl)phosphate (both 1 mM in 0.1 M Tris, pH 7.5, containing 0.1 M NaCl) were measured by monitoring the absorbance at 405 nm at 25 °C following addition of enzyme.

*Measurement of $K_i$ Values.* These were obtained by using UpA as substrate. For poly(G), poly(A), 2′-CMP, and 2′-UMP, the dependence of $1/v$ on the inhibitor concentration at $[S] \ll K_m$ was plotted. The $-[I]$ intercept of such a plot should closely approximate $K_i$ for simple inhibition mechanisms, regardless of the inhibition mode. A $K_i$ value for the placental RNase inhibitor with the tumor RNase could not be obtained in this manner. This inhibitor binds extremely tightly, with essentially complete loss of RNase activity apparent at concentrations similar to the lowest enzyme concentrations (~2 nM) that can be used in our assay system. Standard kinetic analysis (e.g., $1/v$ vs. [I] plots) cannot be employed under such circumstances because it is based on the assumption that free and total inhibitor concentrations are approximately equal. Alternatively, we used the method of Henderson (1972) which takes into account depletion of in-

[2] After completion of this work, we detected great variation in the capacity of different lots of the Radial-PAK C18 columns to resolve nucleotides and nucleosides. Extensive washing of the column with 0.08% TFA in a 3:2:2 mixture of 2-propanol, acetonitrile, and water improves resolution.

hibitor by enzyme. Here, $[I_T]/(1 - v_i/v_o)$ is plotted against $v_o/v_i$ where $[I_T]$ is the total inhibitor concentration and $v_i$ and $v_o$ represent velocities in the presence and absence, respectively, of inhibitor. The slope of such a plot yields an apparent $K_i$ value, which will closely approximate the true $K_i$ if $[S] \ll K_m$ (again for simple inhibition mechanisms). Reactions were initiated by addition of substrate to mixtures containing enzyme and inhibitor which had been preequilibrated for 10 min. Further equilibration time did not increase the measured inhibition.

*Determination of Nucleotide at 3' Termini of RNase Digests of RNA.* A modification of the method of Farkas and Marks (1968) was used. Yeast RNA (2 mg/mL) in 0.1 M Tris, pH 7.5, containing 0.1 M NaCl was incubated with 10 nM RNase at 25 °C for 2–4.5 h. The reaction was terminated by addition of an equal volume of ice-cold 1.2 M perchloric acid. After 10 min on ice, samples were centrifuged at 15600*g* for 15 min. This treatment is sufficient to hydrolyze the cyclic 2',3'-nucleotides formed at cleavage points. The supernatant was adjusted to pH 8.5 with 3 N ammonium hydroxide and then incubated for 20 min at 37 °C with 25 μg/mL alkaline phosphatase to produce free nucleosides at 3' ends. The reaction was stopped by immersion in a boiling water bath for 5 min. After cooling, the pH was raised to >13 with 3.5 N KOH. The mixture was then incubated for 18 h at 37 °C in order to hydrolyze the remaining phosphodiester bonds. Finally, digests were neutralized with 3.5 N HCl. At the end of this procedure, any nucleotide donating a 3'-phosphate to a cleaved bond should be converted to free nucleoside, while all others (ignoring the small number of nucleotides at 3' ends of the high molecular weight starting material) would be converted to a mixture of 2'- and 3'-nucleotides. Thus, it is possible to determine the percentage of bonds with a particular N component that are cleaved by determining the relative amounts of N nucleoside and nucleotide produced. This can be accomplished by using the same C18 HPLC system employed to examine dinucleotide cleavage. All nucleosides and 2'- and 3'-nucleotides have distinct retention times (with the exception of 2'- and 3'-CMP, which coelute) and can be quantitated by using peak areas at 254 nm obtained for standard nucleosides and nucleotides. Standards themselves were quantitated by using molar absorptivities at 260 nm listed by Beaven et al. (1955). A control sample lacking RNase was also prepared. In this case, identical procedures were followed except that the centrifugation step after addition of perchloric acid was omitted.

*Chemical Modification.* Carboxymethylation of RNases (40 μg/mL) was performed by using 30 mM iodoacetate in 0.1 M sodium acetate, pH 5.5, at 25 °C (Crestfield et al., 1963). Reductive methylation was achieved with 2 mM formaldehyde and 10 mM sodium cyanoborohydride at 25 °C in 50 mM Hepes, pH 7.5 (Jentoft & Dearborn, 1979).

*Protein Assays.* Tumor RNase concentrations were determined by amino acid analysis.

*Chemical Analyses.* Amino acid analysis, carboxyl-terminal amino acid analyses by hydrazinolysis, and Edman N-terminal sequence analyses were performed as described previously (Fett et al., 1985b). Tryptophan analysis was done on a methanesulfonic acid hydrolysate (4 N, Pierce Chemical Co.), prepared according to Liu and Chang (1971), neutralized with sodium hydroxide and derivatized with phenyl isothiocyanate by a procedure that tolerates high amounts of salt (Cohen et al., 1985).

## RESULTS

*Purification of Tumor RNase.* The procedure employed for isolation of tumor RNase was originally developed for the purification of angiogenin. Serum-free medium conditioned by HT-29 cells is fractionated in three steps: acidification, CM-52 cation-exchange chromatography, and C18 reversed-phase HPLC. The HPLC elution profile is shown in Figure 1. When the primary structure of angiogenin was found to display a striking homology to that of human pancreatic RNase, the HPLC column fractions eluting in the vicinity of angiogenin (86.4 min) were assayed for RNase activity. RNase activity was indeed found but not with the angiogenin peak, rather with the one preceding it at 81.9 min. SDS–PAGE, followed by both silver (Figure 2) and RNase activity staining, revealed a single band with an apparent molecular weight of ~16 000. The activity of this protein toward yeast RNA is 2.5-fold lower (per microgram) than that measured with bovine RNase A.

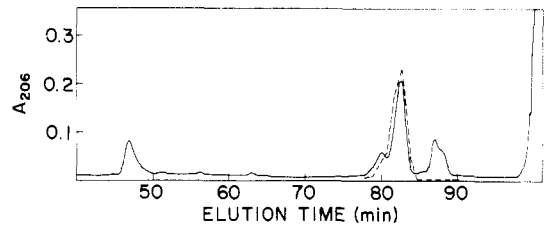


FIGURE 1: Chromatography of 5 mg of CM 2 fraction from acidified serum-free HT-29 cell conditioned medium on a C18 Synchropak RP-P HPLC column. Solvent A is 0.1% TFA in water. Solvent B is 0.08% TFA in a 3:2:2 (v/v/v) mixture of 2-propanol, acetonitrile, and water. Sample was dissolved in solvent A and eluted with a 90-min linear gradient from 20% to 40% solvent B at a flow rate of 1 mL/min. The solid line represents absorbance at 206 nm. The dashed line indicates RNase activity measured by a modification of the Kunitz (1946) assay, with yeast RNA as substrate (see Experimental Procedures).

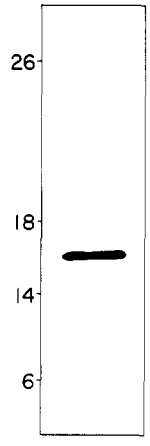


FIGURE 2: SDS–PAGE of tumor RNase in 15% polyacrylamide gels. Positions of molecular weight markers (Bethesda Research Laboratories, low molecular weight standards) are at the left ($\times 10^{-3}$).

The yield of this RNase is typically 2 μg/L acidified HT-29 cell conditioned medium, representing ~0.1% of the total protein secreted (Fett et al., 1985a). There appear to be few other RNases secreted into the medium. The vast majority of RNase activity present in the CM 2 fraction (see Experimental Procedures) loaded onto the HPLC column can be accounted for by this protein. The CM 2 fraction itself contains about twice as much activity as the CM 1 fraction.

*Physicochemical Characterization of Tumor RNase.* Tumor RNase migrates as a single band of $M_r$ ~16 000 by SDS–PAGE both in the presence and in the absence of 2-mercaptoethanol. Analytical isoelectric focusing reveals a p*I* value greater than 9.5, and its amino acid composition is given in Table I. This composition differs markedly from that of

Table I: Amino Acid Composition of Human Tumor Ribonuclease

| amino acid | tumor RNase[a] (mol % ± SD) | human pancreatic RNase[b] (mol %) |
|---|---|---|
| Cys[c] | 6.74 | 6.30 |
| Asp | 11.73 ± 0.49 | 11.81 |
| Thr | 6.54 ± 0.48 | 5.51 |
| Ser | 5.61 ± 0.48 | 12.60 |
| Glu | 9.40 ± 0.19 | 10.24 |
| Pro[d] | 4.01 ± 0.12 | 5.51 |
| Gly | 7.54 ± 0.80 | 3.94 |
| Ala | 3.85 ± 0.23 | 3.15 |
| Val | 5.58 ± 0.66 | 7.87 |
| Met | 3.62 ± 0.53 | 3.94 |
| Ile | 5.21 ± 0.23 | 2.36 |
| Leu | 2.80 ± 0.21 | 1.57 |
| Tyr | 3.31 ± 0.25 | 3.94 |
| Phe | 3.31 ± 0.15 | 3.15 |
| His | 4.66 ± 0.16 | 3.94 |
| Lys | 4.38 ± 0.08 | 6.30 |
| Arg | 10.97 ± 0.34 | 7.87 |
| Trp[e] | 0.93 | 0 |

[a] Average of 19 analyses, 6 of them by the picotag methodology (Waters Associates; Bidlingmeyer et al., 1984). [b] From Beintema et al. (1984). [c] Determined as cysteic acid, average of two analyses. [d] By picotag analysis, average of six analyses. [e] Determined on methanesulfonic acid hydrolysate.

the pancreatic RNase, particularly with respect to the relative abundances of Ser, Gly, Arg, Lys, Ile, and Trp. Edman degradation indicates that the N-terminus is blocked. The C-terminal amino acid is glycine, as determined by hydrazinolysis.

*Substrate Specificity.* Mammalian RNases are typically characterized by their relative activities toward the homopolyribonucleotides poly(C) and poly(U) (Bardon et al., 1976; Sierakowska & Shugar, 1977; Akagi et al. 1976). Pancreatic RNase, and indeed most tissue, serum, and urine RNases studied thus far, is 10–100-fold more active toward poly(C) than poly(U). In contrast, tumor RNase is at least 1000-fold more effective toward poly(U) than poly(C), while exhibiting a specific activity toward poly(U) similar to that of bovine pancreatic RNase A.[3] It does not degrade either poly(G) or poly(A) at a detectable rate.

The specificity of the HT-29 RNase was examined in greater detail by using the 16 common dinucleotides NpN′ as substrates (Table II). The reaction was monitored by C18 reversed-phase HPLC, using a system which separates likely products from starting material in every case (see Experimental Procedures). Only two dinucleotides, UpA and UpG, are cleaved at appreciable rates. UpC, UpU, CpA, and CpG are broken down at slow but measurable rates, while essentially no reaction can be detected with CpU, CpC, and all ApN and GpN dinucleotides. Thus, the tumor RNase strongly prefers uridine in the N position, with cytidine yielding several hundredfold lower $k_{cat}/K_m$ values. In the N′ position, adenosine is the preferred nucleoside, with guanosine and the two pyrimidines yielding ~10-fold and ~1000-fold lower $k_{cat}/K_m$ values, respectively.

In all cases where products could be detected, they appear to be the N cyclic 2′,3′-monophosphate and the free nucleoside N′, on the basis of retention times during HPLC. For example, UpA yields two peaks, at 10.4 and 28.5 min. A U>p standard elutes at 10.4 min, while uridine 3′- and 2′-monophosphates elute at 7.6 and 8.6 min, respectively. An adenosine standard produces a peak at 28.6 min. The product U>p is eventually converted by the enzyme to 3′-UMP, at a rate at least 500 times slower than for production of the cyclic nucleotide from UpA. This hydrolytic reaction can also be monitored by the spectrophotometric method of Richards (1955), which shows its rate to be ~6 times slower than with RNase A. No reaction can be detected when the similar assay of Crook et al. (1960) for C>p hydrolysis is used. The more sensitive HPLC assay reveals a hydrolysis rate at least 400 times slower than that observed with RNase A.

The tumor RNase is inactive toward *p*-nitrophenyl phosphate and bis(*p*-nitrophenyl) phosphate. Thus, general phosphomono- and phosphodiesterase activities are absent.

*Identification of 3′ Termini after Digestion of Yeast RNA.* The specificity of the tumor RNase toward RNA was examined by digesting yeast RNA and then identifying the nucleotides present at the newly formed 3′ termini as described under Experimental Procedures. In addition to the HT-29 RNase digest, two control samples were analyzed. One was

Table II: Activity of Tumor Ribonuclease toward Dinucleotides

| dinucleotide | reaction[a] (%) | $k_{cat}/K_m$[b] ($M^{-1}$ $s^{-1}$) |
|---|---|---|
| UpA | 100 | 246000 |
| UpG | 83 | 26000 |
| CpA | 4.2 | 630 |
| UpC | 3.2 | 480 |
| UpU | 1.9 | 280 |
| CpG | 0.7 | 100 |
| CpC | <0.5 | <75 |
| CpU | <0.5 | <75 |
| all ApN | <0.5 | <75 |
| all GpN | <0.5 | <75 |

[a] Reaction conditions: 0.1 mM substrate, 20 nM tumor RNase, 0.1 M Mes, 0.1 M NaCl, pH 6.0, 25 °C, 1 h. The extent of reaction was determined by HPLC quantitation of starting material and products as described in the text. [b] The $k_{cat}/K_m$ value with UpA was determined by spectrophotometric assay (see text). With the other dinucleotides, $k_{cat}/K_m$ was estimated from the extent of reaction assuming that (1) the substrate concentration is well below $K_m$, (2) product inhibition is negligible, and (3) the reaction is first order with respect to the substrate. All three of these conditions are satisfied with UpA, the best substrate. A value for $k_{cat}/K_m$ can then be calculated from the formula $k_{cat}/K_m = \ln([S_0]/[S])/t[E]$ where $[S_0]$ and $[S]$ are substrate concentrations at the initial time and time $t$, respectively, and $[E]$ is the enzyme concentration.

Table III: Identification of 3′ Termini after Ribonuclease Digestion of RNA[a]

| ribonuclease | base | nmol of product[b] as nucleotide | nmol of product[b] as nucleoside | % cleavage (corrected)[c] |
|---|---|---|---|---|
| HT-29 | C | 3.10 | 0.38 | 5.8 |
| | U | 2.60 | 2.51 | 44.9 |
| | G | 4.72 | 0.09 | <0.5 |
| | A | 4.67 | 0.11 | <0.5 |
| bovine pancreatic | C | 1.39 | 2.57 | 63.1 |
| | U | 1.98 | 3.47 | 60.6 |
| | G | 5.31 | 0.19 | 1.2 |
| | A | 5.18 | 0.18 | 0.7 |

[a] Reaction conditions: 2 mg/mL yeast RNA, 10 nM RNase in 0.1 M Tris/0.1 M NaCl, pH 7.5 at 25 °C, for 2 h (bovine pancreatic RNase) or 4.5 h (HT-29 RNase). [b] Work-up of samples following RNase digestion is detailed under Experimental Procedures. Nucleotides at 3′ termini are converted to free nucleosides, while the remainder are released as 2′- and 3′-nucleotides. Products were separated by HPLC (see Figure 3a,b) and quantitated by using peak areas for standard nucleosides and nucleotides. [c] The percentage of nucleoside product for each base, corrected for cleavage not due to added RNase (Figure 3c). It is assumed that most of this breakdown occurs after digestion by RNases.

[3] Comparisons have been made with bovine rather than human pancreatic RNase since the former has been studied much more extensively (Richards & Wyckoff, 1971; Blackburn & Moore, 1982). The catalytic properties of the two enzymes are reported to be very similar (Weickmann et al., 1981).

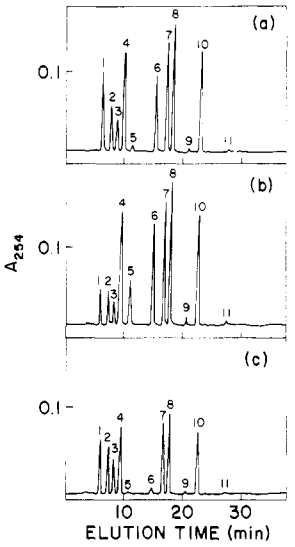


FIGURE 3: HPLC determination of 3′ termini after digestion of yeast RNA by RNases. Samples were prepared by a modification of the method of Farkas and Marks (1968) described under Experimental Procedures. In this procedure, nucleotides at the 3′ termini following digestion are converted to free nucleosides, while the remainder are degraded to 2′- and 3′-nucleoside monophosphates. These products were separated and quantitated on a Waters Radial-PAK C18 HPLC column, using a 35-min linear gradient from 0.1 M potassium phosphate, pH 7.0, to 90 mM potassium phosphate, pH 7.0, in 32.5% methanol at a flow rate of 0.8 mL/min. Absorbance was monitored at 254 nm and recorded with a Hewlett Packard 3390A integrator. Panels a and b show the chromatograms for tumor RNase and RNase A digests, respectively. The sample chromatographed in panel c had no RNase added. On the basis of chromatography of standard nucleosides and nucleotides, the identities of the peaks are as follows: 1, 2′- and 3′-CMP; 2, 3′-UMP; 3, 2′-UMP; 4, 3′-GMP; 5, cytidine; 6, uridine; 7, 2′-GMP; 8, 3′-AMP;9, guanosine; 10, 2′-AMP; and 11, adenosine.

a bovine RNase A digest of RNA. On the basis of the known specificity of the enzyme, it would be expected almost exclusively to yield pyrimidines at the 3′ termini. The second was an RNA sample to which no ribonuclease was added. This should provide an estimate of the inadvertent breakdown of the RNA during the procedure, most likely due to the treatments with perchloric acid and later with alkaline phosphatase.[4]

The results of this experiment (Figure 3 and Table III) are consistent with the pattern observed with dinucleotides. There is essentially no tumor RNase cleavage at ApN or GpN bonds, and cleavage at UpN is much more extensive than at CpN (Figure 3a). With RNase A (Figure 3b), more than half of the CpN and UpN bonds are broken, while almost all GpN and ApN bonds remain intact, as expected. The percentages of RNase-catalyzed cleavage at each nucleotide listed in Table III were adjusted for the relatively small amount of RNA degradation occurring in the control sample lacking added RNase (Figure 3c). In applying this correction, it was assumed that such breakage occurred subsequent to the RNase digestion.

The results obtained also allow the calculation of the average length of the polynucleotides produced, since the chromatograms yield both the base composition of the RNA and the percentage of bonds cleaved on the 3′ side of each nucleotide. For the tumor RNase, under the conditions employed, the average product size is 7.3 nucleotides.

[4] Although the alkaline phosphatase preparation used reportedly contained "minimal nuclease" activity, at high concentrations it generated acid-soluble fragments from both poly(C) and poly(U).

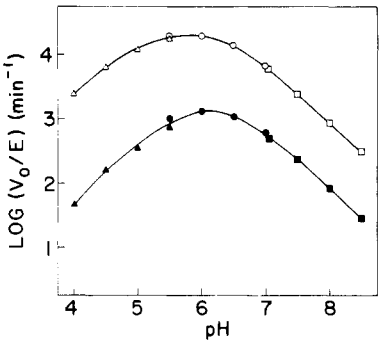


FIGURE 4: pH–activity profiles for cleavage of 0.1 mM UpA by tumor RNase (▲, ●, ■) and RNase A (△, ○, □). Assays were performed in 0.1 M acetate (▲, △), Mes (●, ○), or Hepes (■, □) containing 0.1 M NaCl, at 25 °C.

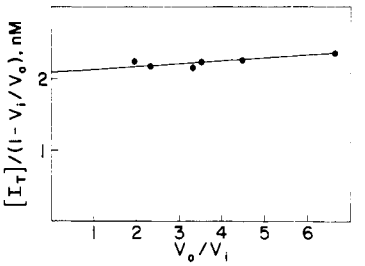


FIGURE 5: Henderson plot for inhibition by human placental RNase inhibitor of tumor RNase catalyzed UpA cleavage. $[I_T]$ represents the total concentration of inhibitor, and $v_o$ and $v_i$ are velocities in the absence and presence, respectively, of inhibitor. Assay mixtures (enzyme and inhibitor in 0.1 M Mes, 0.1 M NaCl, 1 mM EDTA, and 10 μg/mL human serum albumin, pH 6.0) were preincubated for 10 min at 25 °C before the reaction was initiated by addition of substrate. The substrate concentration was 0.3 mM, well below the $K_m$.

*Kinetic Characterization.* The best dinucleotide substrate, UpA, was employed for more detailed kinetic characterization of the tumor RNase. A pH–activity profile was obtained under conditions where [S] ≪ $K_m$ so that initial velocities are directly proportional to $k_{cat}/K_m$. This profile (Figure 4) is quite similar to that found for bovine RNase A, although shifted slightly in the alkaline direction. The profile for RNase A obtained here is in good agreement with that previously reported by del Rosario and Hammes (1969). A Lineweaver–Burk plot at the pH optimum of 6.0 yields $k_{cat}$ and $K_m$ values of 442 $s^{-1}$ and 1.8 mM, respectively, compared with 2690 $s^{-1}$ and 0.7 mM for RNase A.

High chloride concentrations inhibit tumor RNase activity toward UpA (at [S] = 0.1 mM); the velocity at 0.5 M chloride is 65% lower than that measured at 0.1 M. Phosphate markedly inhibits activity; e.g., at 10 mM, 75% inhibition is observed at pH 6.0. Both zinc and copper also inhibit. Five millimolar zinc sulfate lowers activity by 60%, and 1 mM copper sulfate completely eliminates activity.

Although HT-29 RNase degrades neither poly(A) nor poly(G), both are effective inhibitors. The apparent $K_i$ value for poly(G) is $9.6 \times 10^{-8}$ M (in nucleotides). It was not possible to obtain a $K_i$ value with poly(A), since the $1/v$ vs. [I] plot is nonlinear, showing marked downward curvature. This curvature does not indicate that poly(A) is only a partial inhibitor, since complete inhibition is achieved at high concentrations. An inhibition of 50% is observed with $1.5 \times 10^{-7}$ M poly(A). Much higher concentrations of both poly(G) and poly(A) are required to observe appreciable inhibition of RNase A.

The effects of 2′-UMP and 2′-CMP were also examined. At pH 6.0, the former inhibits strongly, with an apparent $K_i$

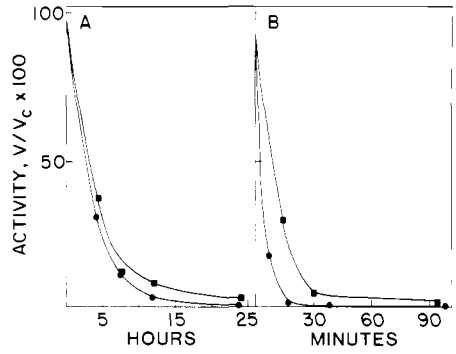


FIGURE 6: Effects of treatment with iodoacetate (panel A) and reductive methylation (panel B) on activities of tumor RNase (■) and RNase A (●). Iodoacetate treatment was in 0.1 M sodium acetate, pH 5.5 at 25 °C, using 30 mM reagent. Reductive methylation was performed by using 2 mM formaldehyde and 10 mM sodium cyanoborohydride in 50 mM Hepes, pH 7.5, at 25 °C. Activities were measured by the spectrometric assay of Kunitz (1946), with 1 mg/mL yeast RNA in 0.1 M Tris and 0.1 M NaCl, pH 7.5.

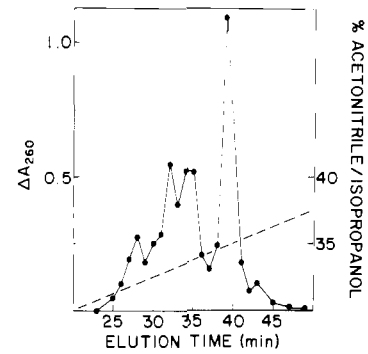


FIGURE 7: Chromatography of liver RNases on a Synchropak RP-P C18 HPLC column. Solvent A is 0.1% TFA in water. Solvent B is 0.09% TFA in a 5:5:4 mixture of 2-propanol, acetonitrile, and water. Liver components binding to poly(G)–agarose (see Experimental Procedures) were dissolved in solvent A, applied to the HPLC column, and eluted with a 100-min linear gradient from 35% to 70% B. The flow rate was 0.8 mL/min. One-minute fractions were collected. RNase activity was measured by using the precipitation assay of Frank and Levy (1976), with yeast RNA as substrate and 10 μL of a 1:100 dilution from each column fraction.

of 40 μM, while the latter binds only weakly, with a $K_i$ too high to measure accurately by the spectrophotometric assay employed, i.e., >500 μM.

The interaction of the tumor RNase with the human placental RNase inhibitor described by Blackburn et al. (1977) was studied also. A Henderson plot (Figure 5) obtained at the lowest enzyme concentration that could be employed, i.e., 2 nM, is virtually flat and sets an upper limit of 40 pM for the $K_i$ value. A competition experiment was performed to obtain an estimate of the relative binding strength of the inhibitor to the tumor RNase as compared with that to RNase A. Assay mixtures contained 200 μM CpG, 3.70 nM RNase A, 2.46 nM inhibitor, sufficient to produce 67% inhibition in the absence of tumor enzyme, and varying amounts of tumor RNase. After 10 min of preequilibration of the other components,[5] the reaction was initiated by addition of substrate. Since CpG is cleaved at least 1000-fold more slowly by the tumor enzyme than by RNase A, the activity observed is determined solely by the concentration of free RNase A. Thus, any increase in activity accompanying addition of tumor RNase reflects binding of the inhibitor by this enzyme. In the presence of 10 nM tumor enzyme, the activity measured is essentially identical with that found in the absence of inhibitor; i.e., all of the inhibitor is bound to the tumor RNase. At a tumor RNase concentration of 1.5 nM, 50% of inhibition is reversed. In this assay mixture, the calculated concentration of free tumor RNase is 9-fold lower than that of free RNase A, suggesting that the inhibitor displays a considerable preference for the tumor enzyme. It should be noted, however, that since the dissociation rates for these RNase–inhibitor complexes are slow (R. Shapiro, unpublished experiments), it is in fact the relative association rates rather than $K_i$ values which are being measured in this experiment.

*Chemical Modification.* Treatment of tumor RNase with iodoacetate at pH 5.5 inactivates the enzyme at a rate slightly slower than that observed for RNase A (Figure 6A). Reductive methylation with formaldehyde and sodium cyanoborohydride also inactivates the tumor enzyme, although in this case at a rate at least 2-fold slower than for RNase A (Figure 6B). These results are consistent with the existence of similar active-site residues (histidine, lysine) for the two RNases.

*Comparison with Other Human RNases.* Several other human RNases have been described that share the capacity of tumor RNase to degrade poly(U) more readily than poly(C), notably the liver/spleen enzyme of Levy and co-workers (Frank & Levy, 1976; Neuwelt et al., 1976) and the serum "RNase 5" described by Akagi et al. (1976). Unfortunately, on the basis of the information thus far reported on these enzymes, it is not possible to determine whether either or both are identical with the tumor RNase. In order to make such a comparison, we have examined the enzymatic and chromatographic properties of the RNases in question.

(*A*) *Liver RNases.* An acidified homogenate of human liver was subjected to cation-exchange chromatography by the same procedure employed for purification of the tumor enzyme. Less than 5% of the RNase activity eluted in the CM 2 fraction. This suggests that the major RNase(s) from this organ is (are) not identical with the tumor enzyme, but the possibility cannot be excluded that the difference is merely in the degree of glycosylation or some other posttranslational modification affecting chromatographic behavior. Further, binding of RNases to anionic molecules present in the liver homogenate but not in tumor-derived conditioned medium could result in elution in the CM 1 rather than CM 2 fraction. Thus, additional purification steps were employed. The preparation was chromatographed on poly(G)–agarose, where essentially all RNase activity was bound and subsequently eluted with 1 M KCl. When this material was subjected to reversed-phase C18 HPLC, at least four peaks of activity were found (Figure 7), all eluting at a much higher percentage of the organic component than required for the tumor RNase. Activity-stained SDS gels on samples from the four major regions in all cases revealed a predominant component at a molecular weight slightly higher than that of the tumor enzyme (data not shown). In two cases (peaks I and III), additional bands of significantly higher molecular weight ($M_r \sim 25000$) were present.

To compare the RNases from the various peaks to each other and to the tumor RNase, activity measurements were performed using yeast RNA, poly(C), poly(U), UpA, and CpA as substrates (Table IV summarizes the results). Materials from all four peaks of liver RNase share an activity toward poly(U) higher than poly(C). However, there are considerable differences as well, and it appears likely that at least two or three distinct enzymatic activities are present. The substrate specificity of all of these liver RNases differs markedly from that of the tumor RNase. This can be seen most clearly in

[5] Inhibitor was added last, after the two RNases were mixed in the assay buffer.

Table IV: Activities of Ribonucleases toward Various Substrates

| substrate | activity per unit[a] tumor RNase | human liver RNases[b] I | II | III | IV | bovine RNase A |
|---|---|---|---|---|---|---|
| yeast RNA | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| UpA[c] | 0.46 | 0.21 | 0.42 | 0.55 | 1.2 | 2.9 |
| CpA[c] | <0.01 | 2.5 | 3.4 | 1.5 | 3.7 | 7.4 |
| poly(U)[d] | 390 | 9.3 | 13.2 | 102 | 135 | 66 |
| poly(C)[d] | <0.6 | 2.8 | 4.3 | 6.0 | 17.6 | 1320 |

[a] One unit is defined as the amount of enzyme required to produce an absorbance change at 300 nm of 1/min in a modified Kunitz (1946) assay system: 1 mg/mL RNA, 0.1 M Tris, and 0.1 M NaCl, pH 7.5, 25 °C. [b] Human liver RNases I, II, III, and IV represent material in fractions 28, 32, 35, and 39, respectively, in Figure 7. [c] Activities with UpA and CpA represent absorbance changes per minute at 280 and 286 nm, respectively, with 0.1 mM substrate in 0.1 M Mes/0.1 M NaCl, pH 6.0 at 25 °C. [d] Activities with poly(U) and poly(C) represent the perchloric acid soluble absorbance at 260 and 280 nm, respectively, generated per minute in a modified assay of Zimmerman and Sandeen (1965). Reaction mixtures contained 100 μg of substrate and 5 μg of human serum albumin in 250 μL of 40 mM Tris, pH 8.0, at 37 °C.

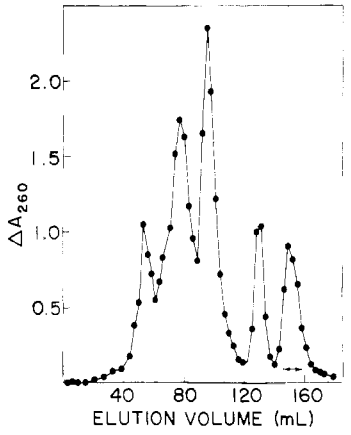


FIGURE 8: Separation of serum RNases by chromatography on phosphocellulose (Akagi et al., 1976; Blank & Dekker, 1981). Serum (23 mL) was dialyzed vs. 10 mM sodium phosphate, pH 6.7, and applied to a 0.9 × 20 cm column of phosphocellulose equilibrated with the same buffer. After the column was loaded, it was washed with 15 mL of equilibration buffer. RNases were then eluted with a 300-mL linear gradient of 0.2–1.8 M NaCl in the same buffer. Fractions were collected in siliconized tubes. RNase activity was measured by the method of Blank & Dekker (1981) using 5-μL aliquots of column fractions and 1-h incubations at 37 °C.

relation to CpA, which is a good substrate for the liver RNases but is cleaved very slowly by the tumor enzyme.

(*B*) *Serum RNase 5.* Serum RNases were separated initially on phosphocellulose by the method of Akagi et al. (1976). The pattern observed (Figure 8) is essentially identical with that found both by them and by Blank and Dekker (1981), with RNase 5 well separated from RNase 4. The ratio of poly(U) to poly(C) activity in the RNase 5 peak is about 2. When this material is fractionated by C18 reversed-phase HPLC (see Experimental Procedures), a peak of absorbance and activity is observed at the same retention time (38 min) as for the tumor RNase. Like the tumor enzyme, this RNase degrades poly(U) at least 500 times faster than poly(C). SDS–PAGE shows a single major band (>90% of the total) at the same position as the tumor enzyme (data not shown). When the purified serum RNase 5 is chromatographed on a phenyl-substituted HPLC column, it again elutes with the same retention time as the tumor RNase; i.e., the peak of activity toward poly(U) in both cases is at 55 min. Moreover, the tumor enzyme and RNase 5 both elute at 30 min during HPLC on a carboxymethyl cation-exchange column. Thus, it seems likely that these two enzymes are identical.

## Discussion

Two major classes of human ribonucleases, i.e., "secretory" and "nonsecretory", have been described (Bardon et al., 1976; Sierakowska & Shugar, 1977) which share numerous physical and catalytic properties but differ in pH optima, relative activities toward poly(C) and poly(U), antigenic behavior (Neuwelt et al., 1977; Weickmann & Glitz, 1982), and several other characteristics. The secretory RNases display a marked preference for poly(C) over poly(U) and, under certain conditions, a pH optimum of ~8.5 for degradation of RNA. This class includes the RNases predominant in pancreas, duodenal contents, serum, and urine (Sierakowska & Shugar, 1977). The nonsecretory class, which prefers poly(U) over poly(C) and has a pH optimum of ~7.0 with RNA as substrate, includes the liver and spleen enzymes, minor components of serum and urine, and several other RNases.

Although it is a secreted enzyme, the HT-29 cell derived RNase shares with the nonsecretory RNases the ability to depolymerize poly(U) more readily than poly(C). Therefore, it was of considerable interest to determine whether the tumor enzyme is identical with any of the poly(U)-preferring RNases described previously. On the basis of published information, it has been possible to answer this question definitively in one instance. The amino acid composition of RNase $U_s$ from urine (Cranston et al., 1980; Niwata et al., 1985) differs markedly from that of the tumor enzyme. In addition, this RNase is reported to have a lysine residue at its N-terminus and an isoleucine at its C-terminus. Since the tumor enzyme has a blocked N-terminus and a C-terminal glycine, it must be a different entity.

For the poly(U)-preferring ribonucleases from liver (Frank & Levy, 1976; Ohta et al., 1982), spleen (Neuwelt et al., 1976), platelets (Reddi, 1977), leukocytes (Reddi, 1976; Akagi et al., 1978), serum (Akagi et al., 1976), and semen (Lee et al., 1983), no sequence information has been published. The amino acid composition of the semen enzyme reported by Lee et al. (1983) does not resemble that of the tumor RNase. It is difficult to assess the relationship between the remainder of these enzymes and the tumor RNase from the combination of chromatographic, electrophoretic, and catalytic properties reported. Differences in both the first and second properties may merely reflect variable extents of glycosylation (Schieven et al., 1982). Apparent differences in kinetic behavior can result from low levels of contamination by other RNases. The latter factor is particularly problematic in view of the multiplicity of RNases found in virtually every source examined, and the low molecular weight and high basicity which the majority share. Nonetheless, it seems likely that the granulocyte RNase of Reddi (1976) is distinct from the tumor enzyme since it does not act on U>p and is not inhibited significantly by phosphate. The leukocyte RNase of Akagi et al. (1978) and the platelet RNase of Reddi (1977) also do not appear to be inhibited strongly by phosphate, as judged by the pH–activity profiles obtained, although phosphate inhibition was not tested specifically.

In addition to degrading poly(U) more readily than poly(C), the human liver and spleen RNases, believed to be similar or identical (Neuwelt et al., 1976), resemble the tumor enzyme in that they prefer purines at the N′ position and are markedly inhibited by poly(A) and poly(G). Although some reported aspects of substrate specificity, e.g., an apparent lack of strong preference at the N position, differ from those observed for the tumor enzyme, the similarities were sufficient to warrant isolation of liver RNase in order to make a more direct and

detailed comparison. We find not one but at least four liver RNase species. In this regard, it should be noted that Frank and Levy (1976) did not claim to have purified their liver enzyme to homogeneity and that Ohta et al. (1982), who did, recovered only 4% of the activity present in their initial homogenate. The substrate specificity data in Table IV demonstrate that all of the major liver RNases differ from the tumor enzyme. The finding that the liver RNases degrade poly(U) more rapidly than poly(C) is consistent with the observations of Frank and Levy (1976). It also demonstrates that these RNases do not represent contaminants from serum present in the liver homogenate (see below).

Akagi et al. (1976) have separated at least five serum RNases by phosphocellulose chromatography. All but one of these show a strong preference for poly(C) over poly(U). With RNase 5, a poly(U) to poly(C) activity ratio of 2.78 was measured. While this ratio is much lower than the one reported here for the tumor RNase (>1000), it would require only a few percent contamination with one of the abundant poly(C)-preferring RNases in serum to change the ratio in this manner. Blank and Dekker (1981) have reported a molecular weight for RNase 5 of 14000, similar to that of the tumor enzyme. The present data provide strong evidence that the two RNases are in fact identical. The ratio of poly(U) to poly(C) activity of virtually homogeneous RNase 5 is actually much higher than that reported by Akagi et al. (1976). RNase 5 has the same mobility as the tumor enzyme on SDS–PAGE, and both of them have identical retention times when subjected to three different types of HPLC, i.e., on C18, phenyl reversed-phase, and cation-exchange columns.

The HT-29 cell derived RNase shares sufficient properties with the well-studied bovine pancreatic RNase A (Richards & Wyckoff, 1971; Blackburn & Moore, 1982) to suggest that they both belong to the same general family of enzymes. Both have similar molecular weights and are very basic. Neither enzyme will cleave a bond containing adenosine or guanosine in the N position at a significant rate. Both show a strong preference for adenosine at the N′ position, with the order of preference among the remaining nucleosides being G > C > U. In both cases, a two-step mechanism is followed, in which initial transphosphorylation to form a cyclic 2′,3′-phosphate is followed by a slower hydrolysis producing a 3′-phosphate. Inactivation of the tumor enzyme by reductive methylation and by treatment with iodoacetate suggests that, like RNase A, it contains essential lysine and histidine residues.

The tumor RNase also resembles RNase A in that it is strongly inhibited by the human placental RNase inhibitor of Blackburn et al. (1977). This inhibitor is a 51000 molecular weight protein, believed to interact with bovine RNase A at lysine-41 as well as several regions outside the active site (Blackburn & Jailkhani, 1979; Blackburn & Gavilanes, 1980, 1982). Its reported $K_i$ value with RNase A is 300 pM (Blackburn et al., 1977), but we have obtained a value that is at least 1 order of magnitude lower (R. Shapiro, unpublished experiments), perhaps because depletion of free inhibitor by enzyme was not considered earlier. Employing Henderson plots, which take such depletion into account, it is evident that the inhibitor binds extremely tightly to the tumor enzyme also. In fact, the results of a competition experiment suggest that the $K_i$ value may be severalfold lower than that measured with RNase A. It is thus likely that interactions contributing to the tightness of binding to RNase A (e.g., involving lysine-41) are also present in the case of the tumor enzyme.

Despite these similarities, the enzymatic properties of the tumor RNase differ from those of the pancreatic enzyme in critical respects. The specificity of the former is more restricted in that only substrates with uridine at the N position are cleaved at appreciable rates. Poly(C) is degraded to acid-soluble nucleotides at least 1000 times more slowly than poly(U). This rate is >50000 times slower than that measured with RNase A in our assay system. The tumor enzyme cleaves UpA, the best dinucleotide substrate, several hundred times faster than CpA. In contrast, with RNase A, the $k_{cat}/K_m$ value for CpA is about 6-fold higher than for UpA (Witzel & Barnard, 1962). It should be noted that the strong preference of the tumor RNase for uridine vs. cytidine at N (as compared with RNase A) results from a marked decrease in the ability to cleave CpN bonds rather than an increase in the rate of UpN breakage; i.e., the two enzymes degrade poly(U) at similar rates, and $k_{cat}/K_m$ with UpA is in fact 14-fold lower for the HT-29 RNase.

This more limited specificity also applies to the second step of the reaction, the opening of the cyclic phosphate, where the rate for the tumor RNase is at least 100-fold higher with U>p than with C>p. In contrast, RNase A hydrolyzes C>p somewhat faster than U>p (Witzel & Barnard, 1962). Furthermore, binding of the inhibitor 2′-UMP to the tumor enzyme is considerably tighter than that of 2′-CMP, while similar $K_i$ values for the two nucleotides are obtained with the bovine enzyme (Anderson et al., 1968). Such inhibitors are thought to bind to RNase A at the same position as N of NpN′ substrates (Richards & Wyckoff, 1973).

The finding of a RNase secreted by a human tumor cell is of considerable interest in relation to the large body of research during the past 25 years exploring the effects of cancer on serum RNase content [see Maor & Mardiney (1979) and Levy & Karpetsky (1981) for reviews]. Clearly, the question arises whether the tumor-derived enzyme could serve as a useful marker for the detection, diagnosis, or monitoring of carcinoma of the colon or cancer in general. In this regard, it may be worthwhile to specifically examine levels of this particular RNase (which is identical with RNase 5) in the serum of cancer patients. Since the tumor RNase is virtually inactive toward poly(C), even a large elevation in this enzyme would not have been detected in the majority of previous studies which used poly(C) as substrate. Some investigators have also employed poly(U) as a substrate (Maor & Mardiney, 1979). In this case, although the RNase acts on this polynucleotide at a substantial rate, it accounts for only 5–10% of the poly(U)-degrading activity in normal serum. Thus, it would require a 10–20-fold increase in this RNase to produce a doubling of serum poly(U) activity. Whether or not levels of this RNase will be of use in the case of neoplasia in general and colon cancer in particular has yet to be determined. Currently, a method for rapid measurement of this enzyme in serum is being developed which will allow such studies to proceed.

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**Registry No.** UpA, 3256-24-4; UpG, 3474-04-2; CpA, 2382-66-3; UpC, 3013-97-6; UpU, 2415-43-2; CpG, 2382-65-2; CpC, 2536-99-4; CpU, 2382-64-1; RNase, 9001-99-4; poly(U), 27416-86-0.

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