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PURIFICATION AND PROPERTIES OF RIBONUCLEASES FROM HUMAN URINE

JOSEPH W. CRANSTON, FULVIO PERINI, ELIZABETH R. CRISP and CATHERINE V. HIXSON

Biological Markers Program, National Cancer Institute, Frederick Cancer Research Center, Frederick, MD 21701 (U.S.A.)

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Summary

The two major ribonucleases (EC 3.1.27.5) present in normal human urine have been highly purified and extensively characterized for their enzymatic, physical, chemical and structural properties. One of the enzymes, RNAase C, is a glycoprotein which exhibits a pH optimum of 8.5 with RNA as the substrate and preferentially degrades the synthetic homoribopolymer poly(C). This enzyme is resolved into multiple components by column electrofocusing. However, prior treatment with neuraminidase results in a single form of RNAase C with an isoelectric point of 10.4, indicating that the charge heterogeneity is the result of variability in sialic acid content. Amino acid composition and NH_2- and COOH-terminal sequence analyses of RNAase C show that this enzyme is very similar to mammalian pancreatic RNAases; the data indicate a peptide chain of 126 amino acid residues and a 33% carbohydrate content. The second enzyme isolated from urine, termed RNAase U, is also a glycoprotein which has a pH optimum of 7.0 with RNA as substrate and is virtually inactive against poly(C). RNAase U lacks sialic acid and focuses as a single component with a highly basic isoelectric point of greater than pH 11.0. The NH_2- and COOH-terminal sequences of RNAase U show little homology with the pancreatic RNAases. However, the amino acid composition of this enzyme indicates it is very similar to human spleen RNAase.

Introduction

The existence of two distinct classes of human RNAases (EC 3.1.27.5), which are present in varying proportion within different tissues and body

fluids, has been established [1,2]. The first type of RNAase, termed pancreas-type, has been isolated from pancreas [1—3], kidney [2], lung [2], brain [2], heart [2], duodenal contents [1], plasma [1,4—6] and urine [1,7—11]. The second type of RNAase, designated liver-spleen type, has been isolated from liver [1,12], spleen [1,7,13], granulocytes [14—16], plasma [1,16] and urine [1,7,17]. Among other differences, these two classes of RNAases can be readily distinguished from one another because only the pancreas-type enzyme exhibits a high degree of preference for the synthetic substrate poly(C) [2,4—6, 9—11,18]. The liver-spleen type RNAase hydrolyzes this homopolymer very poorly [2,12,13]. In the present investigation we have taken advantage of this difference in substrate specificity in order to isolate the two types of human RNAases in highly purified forms from normal urine. These purified RNAases, hereafter to be termed RNAase C and RNAase U for pancreas-type and liver-spleen type enzymes, respectively, were then extensively characterized for their enzymatic, physical, chemical and structural properties in order to extend our knowledge about the nature of these two RNAases.

Experimental procedure

Materials

Human urine was collected from healthy male and female volunteers employed in our laboratory and immediately frozen at —20°C. Synthetic polyribonucleotides were supplied by Miles Laboratories. Yeast RNA, obtained from P-L Biochemicals, was further purified according to Bardoń and Pamula [19]. Highly polymerized calf thymus DNA, *p*-nitrophenyl phosphate, *p*-nitrophenyl thymidine-5′-phosphate and *p*-nitrophenyl thymidine-3′-phosphate were products of Sigma. Amino acid standards were from Pierce and amino, neutral and acidic sugar standards were from Sigma. Neuraminidase (EC 3.2.1.18), isolated from *Clostridium perfringens*, and yeast carboxypeptidase Y (EC 3.4.17.4) were purchased from Miles and Worthington Biochemical Corp., respectively. Additional enzymes or proteins used in these studies were chymotrypsinogen A (Pharmacia), ovalbumin (Pharmacia), cytochrome *c* (Schwarz-Mann) and bovine serum albumin (Sigma). A premixed polyacrylamide gel electrophoresis calibration kit containing phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin was obtained from Pharmacia. The following resins were used: SP Sephadex C-25 (Sigma), Sephadex G-75 (Pharmacia), phosphocellulose P11 (Whatman) prepared for use according to the procedure outlined in the distributor's brochure, and poly(G) Sepharose which was prepared according to the method of Poonian et al. [20], as modified by Schmukler et al. [5], using CNBr-activated Sepharose 4B (Sigma). All reagents employed in polyacrylamide gel electrophoresis experiments were electrophoresis purity grade obtained from BioRad. Ampholytes in various pH ranges were purchased from LKB. Sequencer grade reagents (Beckman) were used in NH_2-terminal sequence studies. Dialysis tubing (Union Carbide) was boiled in 5% $NaHCO_3$ containing 1 mM EDTA prior to use. Sucrose, urea and Tris base were obtained in the ultrapure grade from Schwarz-Mann. All other chemicals and solvents used in this study were of analytical reagent grade.

Enzyme assays

The standard assay for poly(C) hydrolysis was a modification of the method of Reddi [4]. Reaction mixtures (0.25 ml) containing 200 nmol poly(C), 15 μmol sodium phosphate-borate buffer, pH 6.5, 25 μg bovine serum albumin and enzyme were incubated at 37°C for 15 min. Assays were performed in polypropylene reaction vessels because dilute solutions of the RNAases adsorbed to glass surfaces similar to what has been observed for bovine pancreatic RNAase [21]. Bovine serum albumin was also included in reaction mixtures to further stabilize the dilute enzyme solutions. Reactions were terminated by the addition of 0.25 ml of 12% perchloric acid containing 20 mM lanthanum nitrate; the acid-insoluble material was removed by centrifugation at 3200 × *g* for 15 min at 4°C. Aliquots (0.1 ml) of the supernatant fluid were diluted to 1.0 ml with H_2O and the absorbance of the acid-soluble nucleotides was measured at 280 nm. Conversion to moles of acid-soluble nucleotide was made using the extinction coefficient $\epsilon = 13.0 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ reported for CMP at 280 nm and pH 2.0 [22]. 1 unit of enzyme is defined as the activity which renders 1 μmol of poly(C) acid-soluble in 1 min at 37°C.

The standard assay for RNA hydrolysis was a modification of the method of Bardoń et al. [1]. Reaction mixtures (0.5 ml) containing 1 μmol RNA, 0.25 ml Davis buffer [23], pH 7.0, 50 μg bovine serum albumin, and enzyme were incubated at 37°C for 30 min. Reactions were terminated by the addition of 0.5 ml of 1 M HCl in 76% ethanol previously cooled to −15°C; the acid-insoluble material was removed by centrifugation at 3200 × *g* for 15 min at 4°C. Aliquots (0.2 ml) of the supernatant fluid were diluted to 1.2 ml with H_2O and the absorbance of the acid-soluble nucleotides was measured at 260 nm. Conversion to moles of acid-soluble nucleotide was made using the extinction coefficient $\epsilon = 10.0 \cdot 10^3\ M^{-1} \cdot cm^{-1}$. 1 unit of enzyme is defined as the activity which renders 1 μmol RNA acid-soluble in 1 min at 37°C.

DNAase, phosphodiesterase (I and II) and phosphomonoesterase activities were assayed according to the procedures of Kunitz [24], Razzell [25] and Bessey et al. [26], respectively.

Protein determination

Protein concentration was determined by reaction with Amidoschwartz 10B (ICN) as described by Schaffner and Weissmann [27], using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Polyacrylamide (7.5%) gel electrophoresis was performed under nondenaturing conditions by both the anionic system of Davis [28] and the cationic system of Reisfeld et al. [29]. Identical gels were either stained for protein with Coomassie brilliant blue R250 (ICN) as described by Fairbanks et al. [30], or sliced into 1-mm slices and aliquots assayed for enzymatic activity after overnight incubation at 4°C in assay buffer. Stained gels were scanned at 550 nm with a Schoeffel Model SD 3000 spectrodensitometer. The amount of protein present in a given peak was estimated by determining the weight of each peak from the densitometer tracing.

Molecular weight determination

The molecular weights of native RNAases C and U were estimated by gel filtration according to Andrews [31]. A column (1.5 × 95 cm) containing Sephadex G-75 was equilibrated with 0.02 M Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl. The void volume (V_0) was determined using Blue Dextran 2000 (Pharmacia). Distribution coefficients were determined for the following protein standards: bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen A (25 000) and cytochrome *c* (12 400).

The molecular weights of reduced and denatured RNAases C and U were determined by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) according to Laemmli [32]. Separating gels (7 cm) containing either 12% (RNAase C) or 15% (RNAase U) acrylamide and 3% stacking gels (1 cm) were employed. Lyophilized enzymes were diluted with H_2O and mixed with sample buffer such that the final protein concentrations were 100 μg/ml in 0.0625 M Tris-HCl buffer, pH 6.8/2% SDS/10% glycerol/5% 2-mercaptoethanol/0.001% Bromophenol blue dye. After boiling for 2 min, 50–100 μl samples were layered on gels and electrophoresed at room temperature at 2 mA per gel for 30 min and 5 mA per gel until the tracking dye reached the end of the separating gel. Gels were stained for protein with Coomassie blue, scanned and the amount of protein present in a given peak was estimated as described above. The molecular weight of the dissociated protein was determined according to Weber and Osborn [33] using the protein standards phosphorylase *b* (97 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000) and α-lactalbumin (14 400). Duplicate gels were stained for carbohydrate by the procedure of Glossmann and Neville [34].

Column electrofocusing

Electrofocusing experiments were performed at a constant temperature of 4°C in a 110 ml LKB-8100-1 Ampholine electrofocusing column in the pH ranges 3.5–9.5 or 9–11, employing 1% Ampholines in sucrose or sorbitol (5–50%) gradients, respectively. All solutions were prepared and introduced into the column according to the directions in the LKB Instruction Manual. Samples (0.3-ml) were injected directly into gradients via the inlet tubing at approx. 3 cm from the interface between gradient and cathode electrode solution. The cathode was at the top for pH 3.5–9.5 gradients and at the bottom for pH 9–11 gradients. Focusing was performed for 21 h under constant power conditions of 3 W (pH 3.5–9.5) or 15 W (pH 9–11), respectively, with final voltages of 1600 V being attained. Under these conditions the protein standards bovine serum albumin and chymotrypsinogen A focused to their respective isoelectric points of 4.9 and 9.5. Columns were emptied as outlined in the LKB Manual and 1-ml fractions were collected. pH determinations were made at 4°C on every third fraction, and aliquots of each fraction were assayed for either poly(C) or RNA hydrolytic activity.

Amino acid analysis

Samples of proteins were hydrolyzed in 6 M HCl, in sealed evacuated tubes at 110°C for 24, 48 and 72 h (RNAase C) or 21 h (RNAase U). Standard conditions of separation for protein hydrolysates were employed on a Durrum D-500

amino acid analyzer using either ninhydrin or fluorescence [35] detection. Cysteine and cystine were determined as cysteic acid after oxidation with performic acid [36].

Carbohydrate analyses

Amino sugars were liberated from glycoproteins by hydrolysis in 6 M HCl for 3, 7 and 24 h at 100°C in Teflon-lined screw cap tubes. Separation was performed on a Glenco Model MM-70 amino acid analyzer equilibrated with 0.2 N sodium citrate buffer, pH 4.25. Amino sugars were detected by fluorescence after reaction with *o*-phthalaldehyde by methods previously described [37]. Optimum yields of amino sugars were obtained after hydrolysis for 3 h with only minimal destruction at 7 h.

Neutral sugars were determined by a modification of the orcinol/sulfuric acid procedure described by François et al. [38]. To 40 μl of each sample, standard or blank, was added 360 μl of working solution. Capped tubes were incubated at 80°C for 15 min, cooled, briefly centrifuged to bring down condensate which had formed under the cap of the tube and the absorbances read at 505 nm. Under these microassay conditions, absorbance was linear between 1 and 10 μg of a mannose standard.

Sialic acid was measured by a modification of the thiobarbituric acid procedure of Warren [39] in which the volumes of all sample and reagent solutions were decreased 4-fold from the original method. Under these microassay conditions, absorbance was linear between 0.3 and 5 μg of an *N*-acetylneuraminic acid standard.

Sequence determinations

NH_2-terminal sequences were determined automatically with an updated Beckman Model 890B sequenator according to the method described by Oroszlan et al. [40]. The phenylthiohydantoin derivatives of amino acids were identified by high-pressure liquid chromatography as described by Henderson et al. [41].

COOH-terminal sequences were determined by carboxypeptidase Y digestion. Because native RNAases C and U were observed to be resistant to attack by this enzyme, the following conditions were established for the optimum release of COOH-terminal amino acids from these RNAases. RNAase C (60 μg protein) was preincubated in a solution (115 μl) containing 0.1 M sodium acetate buffer, pH 5.5, and 0.17 mM dithiothreitol, for 30 min, at 65°C. Following this preincubation, carboxypeptidase Y (0.1 μg in 5 μl) was added and the incubation continued at 25°C. Aliquots (20-μl) of the reaction were withdrawn at various times, mixed with 40 μl of 0.033 M citric acid-HCl, pH 1.9, centrifuged and the free amino acids in aliquots (50 μl) of supernatants were determined by amino acid analysis as described above. RNAase U (36 μg protein) was preincubated in a solution (30 μl) containing 0.05 M sodium acetate buffer, pH 5.5/0.34 mM dithiothreitol/4 M urea, for 30 min, at 65°C. Carboxypeptidase Y (0.5 μg in 5 μl) was then added and the incubation continued at 25°C. Aliquots (6-μl) were withdrawn at various times, mixed with 54 μl of 0.033 M citric acid-HCl, pH 1.9, centrifuged, and the free amino acids in aliquots (50-μl) of supernatants were determined. The values obtained for both

RNAases were corrected for zero time control reactions and a norleucine standard which was present throughout the experiment. Each RNAase was subjected to two separate analyses which yielded identical results.

Purification of RNAases from human urine

The RNAase purifications were followed using both the poly(C) and RNA hydrolysis assays as described earlier. RNAase C-containing fractions were those which exhibited preferential hydrolysis of poly(C). Fractions which were primarily active against RNA were considered to have RNAase U. All procedures were performed at 4°C. The purification data are summarized in Table I.

RNAase C. Phosphocellulose chromatography. Pooled urine (5 l) was dialyzed against 150 l of 0.01 M sodium phosphate buffer, pH 6.8, containing 0.02% sodium azide, for 72 h and applied to a phosphocellulose (5 × 30 cm) column previously equilibrated with the same buffer. After the column was washed with 2 l of equilibrating buffer, the RNAases were eluted with a 0—0.7 M linear gradient of KCl in 2.8 l of the same buffer. Fractions (10-ml) were collected and assayed for both poly(C) and RNA hydrolysis. RNAase C eluted as a broad, heterogeneous peak between 0.3 and 0.5 M KCl, and those fractions with the highest activity (150—230) were pooled (phosphocellulose).

Poly(G) Sepharose chromatography. Phosphocellulose fraction (850 ml) was dialyzed against 56 l of 0.05 M sodium phosphate buffer, pH 8.0, containing 0.02% sodium azide for 24 h and applied to a poly(G) Sepharose column (50 ml packed resin containing 90 μmol of covalently attached poly(G)) previously equilibrated with the above buffer. After the column was washed with 100 ml of equilibrating buffer, RNAase C was eluted with a 0—1.0 M linear gradient of KCl in 250 ml of buffer and fractions (2-ml) were collected. Those fractions (40—64) which were preferentially active against poly(C) eluted as a single peak at 0.2 M KCl and were pooled (poly(G) Sepharose, pH 8.0).

Sephadex G-75 chromatography. Poly(G) Sepharose fraction (52 ml) was dialyzed against 12 l of 1 mM sodium phosphate buffer, pH 6.2, for 24 h and lyophilized to dryness. The residue was dissolved in 0.5 ml of 0.02 M Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl and 0.02% sodium azide and applied to a Sephadex G-75 column (1.5 × 90 cm) previously equilibrated with the same buffer. RNAase C was eluted with this buffer, and fractions (1 ml) were collected at a flow rate of 10 ml/h. Fractions eluting between 71 and 80 ml were pooled (Sephadex G-75).

RNAase U eluted from the phosphocellulose column at 0.54 M KCl, but was poorly resolved from RNAase C. Fractions containing RNAase U were subjected to additional purification by poly(G) Sepharose chromatography at pH 6.2 and, if necessary, gel filtration chromatography on Sephadex G-75. The resulting preparations were equal in purity to the most highly purified RNAase U obtained by the procedure outlined below. However, yields of RNAase U obtained by this purification scheme were usually very low, i.e., less than 10%.

RNAase U. SP Sephadex C-25 chromatography. Pooled urine (500 ml) was dialyzed against 16 l of 0.05 M sodium acetate buffer, pH 5.2, for 24 h and applied to an SP Sephadex C-25 column (5 × 30 cm) previously equilibrated with the same buffer. After the column was washed with 200 ml of equilibrating buffer, the RNAases were eluted with a 0—0.5 M linear gradient of KCl in

2 l of the same buffer. Fractions (10 ml) were collected and assayed for both poly(C) and RNA hydrolysis. RNAase U was eluted as a single peak from the column between 0.35 and 0.41 M KCl and those fractions with the highest activity (155—198) were pooled (SP Sephadex C-25).

Poly(G) Sepharose chromatography. SP Sephadex C-25 fraction (480 ml) was dialyzed against 12 l of 0.05 M sodium phosphate buffer, pH 6.2, for 24 h and applied to a poly(G) Sepharose column (5 ml of packed resin containing 10 μmol of covalently attached poly(G)) previously equilibrated with the above buffer. After the column was washed with 10 ml of equilibrating buffer, RNAase U was eluted with a 0—1.0 M linear gradient of KCl in 50 ml of buffer, and fractions (1 ml) were collected. Those fractions (20—48) which preferentially hydrolyzed RNA eluted as a single peak at 0.3 M KCl and were pooled (poly(G) Sepharose, pH 6.2).

RNAase C eluted from the SP Sephadex C-25 column as a heterogeneous peak between 0.1 and 0.2 M KCl and was clearly resolved from RNAase U. RNAase C-containing fractions have been further purified by poly(G) Sepharose chromatography at pH 8.0, yielding preparations which were slightly less pure, but otherwise identical, than those obtained by the large-scale procedure described above.

Results

RNAases C and U were purified 94- and 146-fold with recoveries of 52 and 82%, respectively (Table I). Purified enzyme preparations were determined to be free of detectable DNAase, phosphodiesterase, and phosphomonoesterase activities. Both RNAases exhibited marked stability, in that, enzymatic activ-

TABLE I

PURIFICATION OF HUMAN URINE RIBONUCLEASES

Step	Volume (ml)	Protein (mg)	Activity * (units)	Specific activity (units/mg)	Recovery (%)
RNAase C					
Urine	5200	260	203 060	781	100
Phosphocellulose	850	13.6	185 130	13 612	91
Poly(G) Sepharose, pH 8.0	52	4.70	151 857	32 310	75
Sephadex G-75 **	9.6	1.43	105 437	73 732	52
RNAase U					
Urine	500	25.0	1 146 ***	46	100
SP Sephadex C-25	480	0.29	1 281	4 417	112
Poly(G) Sepharose, pH 6.2 **	30	0.14	940	6 714	82

* Activity vs. poly(C) and RNA for RNAases C and U, respectively.

** Dialysis and lyophilization of these fractions as described under Results resulted in a 32-fold concentration of protein, containing 98 975 units of RNAase C with a specific activity of 73 315 units/mg protein and a 100-fold concentration of protein containing 781 units of RNAase U with a specific activity of 6810 units/mg protein, respectively.

*** This value has been corrected for RNAase C activity which is present in the urine. 1 unit of RNAase C activity vs. RNA at pH 7.0 (RNA unit) is equivalent to 100 units poly(C) hydrolytic activity (poly-(C) units).

ities were completely retained after storage in solution at 4°C for longer than 4 months. For a number of the experiments described below it was necessary to concentrate the RNAase preparations. Therefore, these enzymes have been routinely dialyzed against 0.5 mM sodium phosphate buffer, pH 6.2, and lyophilized. The effects of dialysis and lyophilization on the enzymatic activities of RNAases C and U are shown in Table I. RNAase C was essentially unaffected by these procedures, whereas some loss in RNAase U activity was observed.

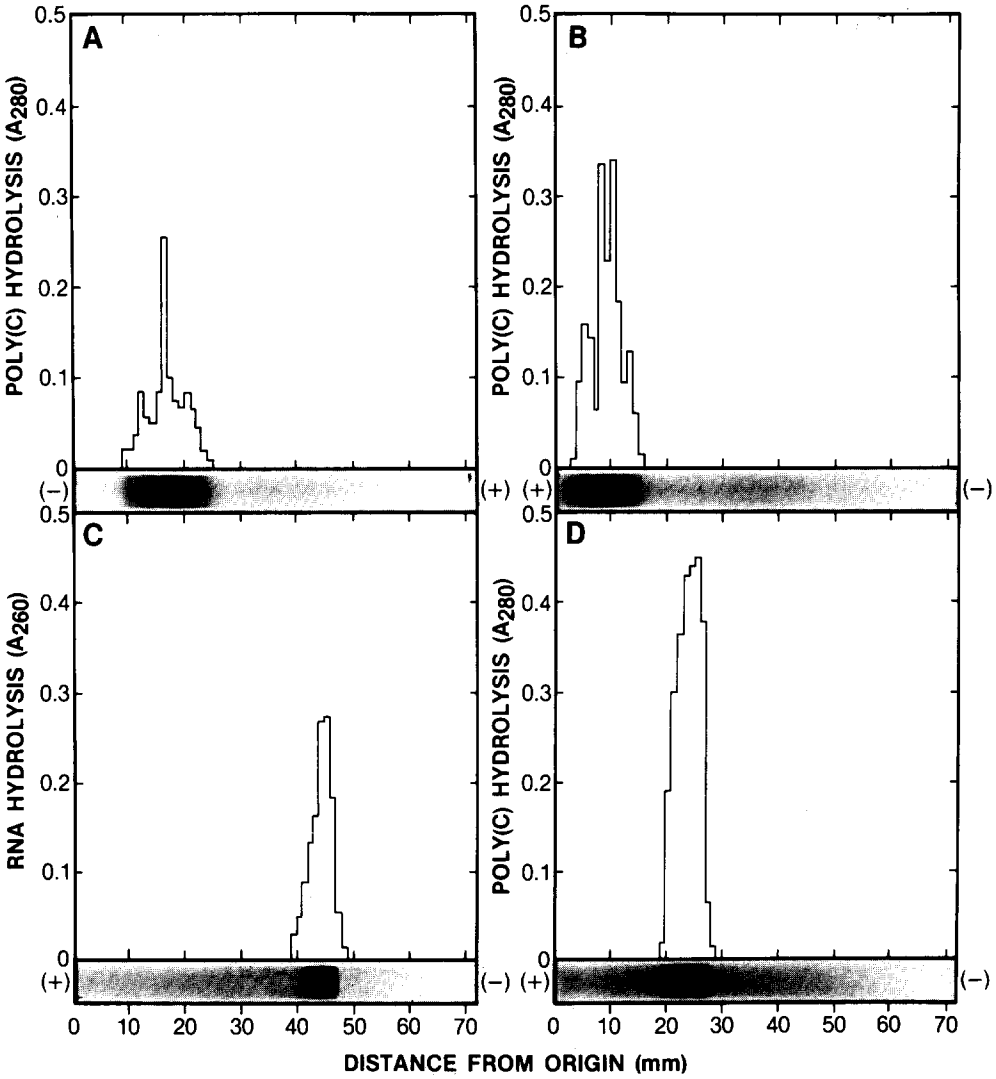

Fig. 1. Polyacrylamide gel electrophoresis of human urine RNAases under nondenaturing conditions. Samples (10 μg) of RNAase C and RNAase U were subjected to polyacrylamide gel electrophoresis in both the standard anionic (pH 9) and cationic (pH 4) systems, and gels were either stained for protein or sliced for enzyme activity measurements (bar graphs) as described under Experimental Procedure. Panel A, RNAase C (anionic); Panel B, RNAase C (cationic); Panel C, RNAase U (cationic) and Panel D, RNAase C previously incubated with 22 munits (1 μg) of neuraminidase for 60 min, at 37°C (cationic).

The purities and electrophoretic mobilities of RNAases C and U were examined by polyacrylamide gel electrophoresis under nondenaturing conditions (Fig. 1). Multiple protein bands were observed for RNAase C in both the anionic (pH 9) and cationic (pH 4) systems. However, peaks of poly(C) hydrolytic activity, as determined on analogous sliced gels, co-migrated with each of these protein bands (Panels A and B). The nature of this observed heterogeneity in RNAase C will be considered later in this section. In contrast to RNAase C, RNAase U migrated as a single band of protein on gels run at pH 4 and RNA hydrolytic activity corresponded in location to this protein band (Panel C). This enzyme did not enter gels run at pH 9, suggesting it was a basic protein. It was concluded that the purities of both RNAases C and U were greater than 95%, based on a comparison of densitometric scans of stained gels with the activity profiles.

Enzymatic properties

Under the standard assay conditions employed (see Experimental Procedure), the rate of poly(C) hydrolysis by RNAase C was linear for at least 15 min. Under these conditions, activity was proportional to enzyme concentration between 1.5 and 10 munits (poly(C)). A linear rate of hydrolysis of RNA by RNAase U was observed for at least 30 min and activity was proportional to enzyme concentration between 1 and 10 munits (RNA).

A number of enzymatic properties of the highly purified RNAases are summarized in Table II. RNAase C exhibited a strong preference for poly(C) as substrate in comparison to RNA or other synthetic polyribonucleotides. This enzyme was 100- and 15-fold less active against RNA and poly(U), respectively, and exhibited no activity against any of the polypurines, e.g. poly(A), poly(I), or poly(G). In contrast, RNAase U hydrolyzed poly(C) very poorly being 30- and 3-fold more active against RNA and poly(U), respectively. This enzyme also failed to hydrolyze polypurines.

pH optima for RNAases C and U were determined under various conditions (Table II). When RNA hydrolysis was assayed in Davis buffer, RNAase U exhibited optimum activity at pH 7.0. In contrast, RNAase C was optimally active at pH 8.5 under these conditions and was 4-fold less active at pH 7.0. The optimum pH for poly(C) hydrolysis by RNAase C in the standard 0.06 M sodium phosphate-borate buffer was pH 6.5. As can be seen in Table II, however, the pH optimum for either RNAase varied with the buffer conditions employed. For example, when RNAase U-catalyzed RNA hydrolysis was assayed in 0.06 M sodium phosphate, the pH optimum was pH 6.5. Similarly, the optimum pH for poly(C) hydrolysis by RNAase C in 0.1 M Tris buffer was pH 7.5. These observations emphasize the importance of defining the reaction conditions employed when assaying these RNAases.

The effects of divalent cations and polynucleotides on poly(C) and RNA hydrolysis by RNAases C and U, respectively, have been examined (Table II). Poly(C) hydrolysis by RNAase C was particularly sensitive to Zn^{2+} and Cu^{2+} being inhibited by 80 and 50%, respectively, at concentrations of 0.1 mM. RNA hydrolysis by this enzyme was also inhibited by these divalent cations. This inhibition could be prevented by EDTA. In contrast, RNAase U-catalyzed RNA hydrolysis was not inhibited by either Zn^{2+} or Cu^{2+} at concentrations as

TABLE II

ENZYMATIC PROPERTIES OF RNAases FROM HUMAN URINE

Reaction conditions for poly(C) and RNA hydrolyses were as described under Experimental Procedure except as noted otherwise in the Table. Other synthetic polyribonucleotides (200 nmol/assay) were assayed under conditions identical to those employed for poly(C). Effects of inhibitors were determined using the standard poly(C) assay for RNAase C and the standard RNA assay for RNAase U. Inhibitor concentrations were Zn^{2+} and Cu^{2+}, 0.1 mM, and poly(A), (G), (C) and (U), 0.8 mM in the poly(C) assays and 0.1 mM in the RNA assays. The inputs of enzymes for the pH optima and inhibitor studies were as follows: RNAase C, 8 munits (poly(C)) in all reactions except the determination of the optimum pH for RNA hydrolysis in Davis buffer where 160 munits (poly(C)) were employed; RNAase U, 4 munits (RNA) in all reactions.

Property	RNAase C	RNAase U
Substrate specificity	Ratio of spec. act.	
Poly(C)/RNA	100	0.03
Poly(C)/Poly(U)	15	0.3
Polypurine/RNA	0	0
pH optima	pH	
RNA as substrate		
a. Davis buffer	8.5	7.0
b. 0.06 M sodium phosphate buffer	N.D. *	6.5
Poly(C) as substrate		
a. 0.06 M sodium phosphate-borate buffer	6.5	N.D.
b. 0.1 M Tris-HCl buffer	7.5	N.D.
Inhibitors	% inhibition	
Divalent cations		
a. Zn^{2+}	80 (25) **	0
b. Cu^{2+}	50 (20)	0
Polyribonucleotides		
a. Poly(A)	88	65
b. Poly(G)	100	85
c. Poly(C)	N.D.	0
d. Poly(U)	100	0

* N.D., not done.

** Numbers in parentheses represent percent inhibition of RNA hydrolysis at pH 8.5, by Zn^{2+} and Cu^{2+}, 0.1 mM.

high as 5 mM. Neither enzyme was inhibited by Mg^{2+} or Ca^{2+}. The simultaneous presence of equimolar concentrations of poly(A), (G) or (U) resulted in essentially complete inhibition of poly(C) hydrolysis by RNAase C. The hydrolysis of RNA by RNAase U was sensitive to poly(A) and (G), but not poly(C) and (U).

Physicochemical properties

When RNAases C and U were subjected to polyacrylamide gel electrophoresis in the presence of SDS under reduced conditions, both enzymes migrated as single, symmetrical bands with apparent molecular weights of 33 000 and 18 000, respectively. Furthermore, both RNAases were concluded to be glycoproteins, because identical gels stained with Schiff's reagent demonstrated the presence of carbohydrate coincident with the protein-stained bands. The native molecular weights of the RNAases have also been estimated by gel filtration

chromatography on Sephadex G-75 as described under Experimental Procedure. By comparison of the relationships between the elution volumes (V_e) and the logarithms of the molecular weights of standard proteins, apparent molecular weights of 33 000 and 18 000 were estimated for native RNAases C and U, respectively, suggesting each enzyme exists as a monomeric form. It must be emphasized, however, that apparent molecular weight values for glycoproteins obtained by either of the above techniques are subject to considerable error. Therefore, the apparent molecular weights observed for RNAases C and U may be only rough estimates of the actual values. The behavior of RNAase C on Sephadex G-75 columns has not been entirely consistent and native molecular weights greater than 33 000, i.e., between 45 000 and 50 000, have also been obtained despite no observed changes in any other property of the enzyme, including its migration in SDS-polyacrylamide gel electrophoresis. The reason for the variable results on Sephadex G-75 is at present unclear, but could reflect either anomalous behavior of RNAase C on this gel due to its glycoprotein structure, as just discussed, or an enzyme aggregation phenomenon despite the relatively high ionic strength that was employed. Bardoń et al. [18] and Schmukler et al. [5] have previously observed aggregation of pancreas-like RNAases under lower ionic strength conditions from both urine and plasma, respectively.

TABLE III

AMINO ACID COMPOSITIONS OF RNAases FROM HUMAN URINE

Hydrolysis and separation conditions were as described under Experimental Procedure. Results are presented as the means ± S.D. for six analyses performed on three separate preparations of RNAase C, with each sample containing 15 μg protein, and for two analyses done on two different preparations of RNAase U with each sample containing 9 μg protein.

Amino acid	RNAase C (mol %)	RNAase U (mol %)
Asp	11.7 ± 0.5	16.8 ± 0.0
Thr	5.6 ± 0.1 *	8.5 ± 0.2
Ser	10.6 ± 0.5 *	5.0 ± 0.2
Glu	10.4 ± 0.5	10.8 ± 0.4
Pro	7.6 ± 1.3	9.1 ± 1.2
Gly	4.5 ± 0.2	3.6 ± 1.1
Ala	3.1 ± 0.2	4.5 ± 0.1
Cys	5.4 **	5.5 **
Val	7.7 ± 0.6	5.9 ± 0.2
Met	4.1 ± 0.7	3.2 ± 0.1
Ile	2.2 ± 0.4	5.0 ± 0.5
Leu	2.2 ± 0.2	4.1 ± 0.3
Tyr	3.5 ± 0.4	2.8 ± 0.1
Phe	3.1 ± 0.2	3.7 ± 0.1
Lys	6.1 ± 0.8	3.1 ± 0.1
His	3.7 ± 0.6	3.7 ± 0.1
Arg	8.0 ± 0.5	6.0 ± 0.1
Trp	N.D. ***	N.D.

* Corrected value based on linear extrapolation to zero time of hydrolysis.

** Determined as cysteic acid after performic acid oxidation; single determinations after 21 h of hydrolysis.

*** N.D., not done.

TABLE IV

CARBOHYDRATE COMPOSITIONS OF RNAases FROM HUMAN URINE

Analyses were performed as described under Experimental Procedure. Sample inputs were 15 μg protein for all analyses on RNAase C, and 9, 36 and 36 μg protein for amino sugar, neutral sugar and sialic acid determinations on RNAase U, respectively. The protein content was estimated by the procedure of Schaffner and Weissmann [27]. Values for the various carbohydrates represent the averages of at least two determinations on different RNAase preparations and were within ± 5% of each other.

Carbohydrate component	RNAase C (μg/100 μg protein)	RNAase U (μg/100 μg protein)
Glucosamine *	16.5	5.5
Galactosamine	0	0
Neutral sugar	26.7	6.7
Sialic acid	7.0	0
Total	50.2	12.2

* The amino sugars were calculated as the *N*-acetyl form; the values shown were obtained on 3 h hydrolysates.

The amino acid analyses for the human urine RNAases are presented in Table III. It is quite evident from these data that RNAase C and RNAase U have different amino acid compositions. The composition of RNAase C, however, appeared to be similar to those previously reported for a number of mammalian pancreatic RNAases, i.e., the pig, cow and rat [42]. Only the high arginine content in RNAase C was considerably different from these other RNAases. The amino acid composition of RNAase U was observed to be quite similar to that previously reported for a human spleen RNAase isolated by Delaney [7].

The carbohydrate compositions of RNAases C and U are shown in Table IV. Although both enzymes were glycosylated, RNAase C contained a much larger proportion of carbohydrate than RNAase U. Furthermore, RNAase C contained sialic acid residues which were not detected in RNAase U. The amino sugar content of both RNAases was entirely in the form of glucosamine with no apparent galactosamine residues. This suggested that the carbohydrate moieties of both enzymes were attached to amide groups of asparagine residues and not to the hydroxyl groups of serine or threonine. Although the neutral sugars were not separated into individual sugars in this study, the presence of sialic acid in RNAase C indicated that at least part of the carbohydrate in this enzyme was present in 'complex'-type chains [43]. However, the exact nature of the carbohydrate structure in both RNAases requires additional investigation.

The purified RNAases from normal human urine have been subjected to column electrofocusing in order to determine their isoelectric points (Fig. 2). When RNAase C was focused in the pH range 3.5—9.5, the enzyme was fractionated into multiple species. The 4 major and 2 minor peaks had apparent isoelectric points of 9.2, 8.7, 8.1, 7.1, 4.3, and 3.7, respectively (Panel A). Recovery of RNAase C activity in this experiment was greater than 95%. That these multiple peaks of RNAase C were, in fact, real and not artifacts of the electrofocusing method was supported by the following experiments. First, the results shown in Panel A were also obtained by both disc and slab gel electro-

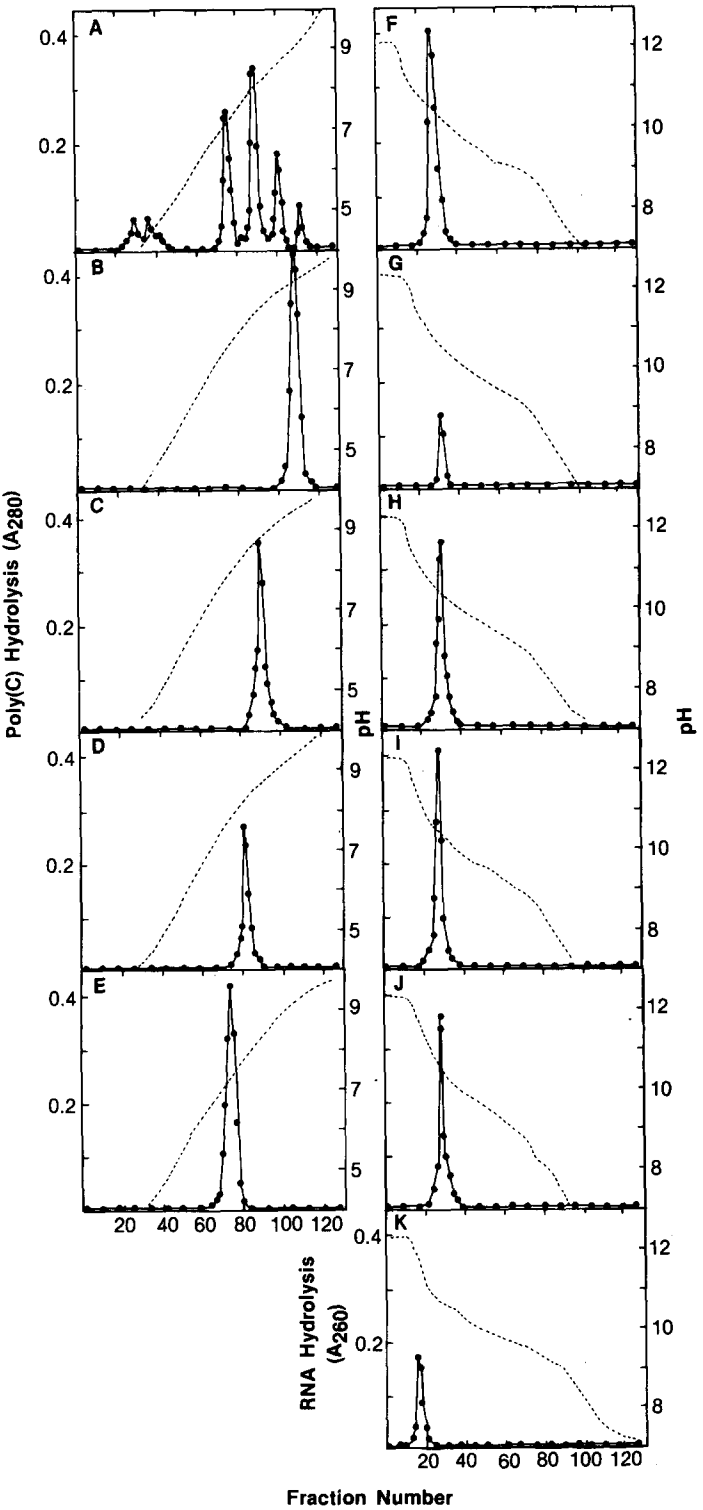

Fig. 2. Column electrofocusing of human urine RNAases. Column electrofocusing experiments in the pH ranges 3.5—9.5 (Panels A—E) or 9—11 (Panels F—K) were performed as described under Experimental Procedure. Samples were as follows: Panel A, RNAase C (58 000 units); Panels B—E, dialyzed and lyophilized peaks I (20 units), II, III, and IV (150 units each) with isoelectric point values of 9.2, 8.7, 8.1, and 7.2, respectively, obtained from the electrofocusing run shown in Panel A; Panel F, RNAase C (400 units) previously incubated with 44 munits (2 μg) of neuraminidase for 2 h at 37°C; Panels G—J, peaks I (20 units), II, III, and IV (150 units each) pretreated with 22 munits (1 μg) of neuraminidase for 2 h at 37°C

focusing methods. Second, the observed profile was not altered when 5% Triton X-100, a nonionic detergent, was included in the gradient solution to prevent aggregation. Third, when the individual peaks were isolated, dialyzed to remove ampholyte [44], lyophilized, and then re-electrofocused under identical conditions, each species focused as a single peak at the same pH (Panels B—E). Finally, using the disc gel electrofocusing method, the profiles observed for RNAase C were the same whether the sample was layered on top of the gel at the cathode end or directly incorporated into the gel. It should be noted, however, that layering the sample on top of the gel at the anode end, or placing the sample in more anodal positions on the electrofocusing column, resulted in a partial or complete loss of the profile shown in Panel A with a concomitant large increase in activity appearing below pH 4 near the anode. The reason for this aggregation at the anode under such loading conditions is unclear, but probably explains why other laboratories [9,10] have reported highly acidic, i.e., pH 3.5—4.0, isoelectric points for this enzyme. The possibility that the charge heterogeneity in highly purified RNAase C was artifactually generated during the purification of the enzyme was also ruled out because pooled urine focused in an identical pattern to that shown in Panel A. Since the charge heterogeneity in RNAase C was concluded to be real, the next step was to determine whether this was due to the existence of genetically distinct isoenzymes or possible variability in the carbohydrate part of the enzyme. Because RNAase C contained sialic acid, the enzyme was treated with neuraminidase prior to electrofocusing. When the asialo form of RNAase C was focused on a pH 3.5—9.5 column, all of the poly(C) hydrolytic activity appeared in the cathode solution. Therefore, this experiment was repeated using a pH 9—11 sorbitol gradient. Under these conditions, a single peak of enzyme activity was observed with an isoelectric point of 10.4 (Panel F). A 90% recovery of enzyme activity was obtained in this experiment. When the individual peaks from the first electrofocusing run were each treated with neuraminidase and then subjected to electrofocusing in the pH 9—11 range, every species had an identical isoelectric point of 10.4 (Panels G—J). Finally, when neuraminidase-treated RNAase C was subjected to polyacrylamide gel electrophoresis in the cationic (pH 4) system, the enzyme no longer exhibited the multiple band heterogeneity previously observed in the absence of neuraminidase. Asialo RNAase C migrated further into the gel as a single, symmetrical protein band with corresponding poly(C) hydrolytic activity (Fig. 1, Panel D). Therefore, it appears that the charge heterogeneity observed in RNAase C is primarily, if not entirely, the result of variability in sialic acid content. In contrast to the heterogeneity observed in RNAase C, urinary RNAase U focused as a single peak in a pH 9—11 electrofocusing column with an extremely basic isoelectric point of greater than pH 11 (Fig. 2, Panel K).

NH_2- and COOH-terminal sequences

The NH_2-terminal sequences of the human urinary RNAases were determined by automated Edman degradation. Results of two analyses, which were performed on different preparations of each enzyme, yielded identical results (Fig. 3). Both RNAases had a lysine residue at the NH_2-terminus and each enzyme contained a single polypeptide chain. Other than a common glutamine

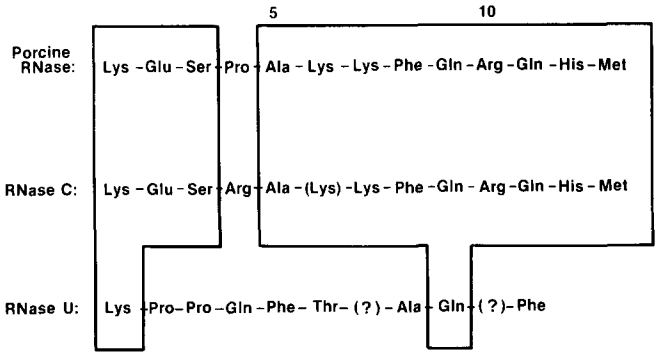

Fig. 3. NH_2-terminal sequences of human urine RNAases. RNAase C (100 μg) and RNAase U (20 μg) were subjected to NH_2-terminal sequence analysis as described under Experimental Procedure. Parentheses indicate unidentified residues or uncertain assignments. The NH_2-terminal sequence of porcine pancreatic RNAase was taken from Wierenga et al. [47].

residue at position 9, however, the remainders of the 13- and 11-residue sequences obtained for RNAases C and U, respectively, showed no homology. The NH_2-terminal sequence of RNAase C exhibited marked homology with all known NH_2-terminal sequences of mammalian pancreatic RNAases (45, 46). The amino acids in positions 2, 5, 7, 8, 10, 11, and 12 of pancreatic RNAases are invariant in 23 mammalian species and are identical to those in RNAase C. As shown in Fig. 3, RNAase C and porcine pancreatic RNAase had identical NH_2-terminal sequences through residue 13 with the exception of position 4. The arginine residue at position 4 in the sequence of RNAase C is the first report of this amino acid at that position. Since the amino acid in this position has often been associated with a particular class of mammals, i.e., alanine in ruminants and dromedary, serine in rodents, and proline in others [46], it is of interest to speculate that arginine in position 4 may be common to primates. To our knowledge no primate pancreatic RNAase has been sequenced.

The COOH-terminal sequences of human urine RNAases were investigated by carboxypeptidase Y digestion and the results clearly showed that RNAase C and RNAase U had different COOH-terminal sequences (Fig. 4). The release of amino acids during the course of digestion of RNAase C indicated that threonine was at the COOH-terminus and that the most likely sequence was -His-Phe-Asp-Ala-Ser-Val-Ser-Thr_{COOH}. This observation was significant because the amino acids which occupy positions 119 through 124 in all known pancreatic RNAases are -His-Phe-Asp-Ala-Ser-Val [48]. Therefore, it appeared that RNAase C contained this invariant sequence (see boxed part of sequence in Fig. 4A) and once again showed a marked similarity to pancreatic RNAases. The presence of two additional amino acids at the COOH-terminus of this enzyme was not without precedent as such additions have been observed in the pancreatic RNAases previously isolated from the horse, coypu and guinea pig [48]. In fact, if the penultimate amino acid at the COOH-terminus of RNAase C is glutamine rather than serine, then the eight residue COOH-terminal sequence shown in Fig. 4A would be identical to that of horse pancreatic RNAase [48]. The release of amino acids during the course of digestion of RNAase U by carboxypeptidase Y clearly indicated that an isoleucine was at the COOH-terminus, but the

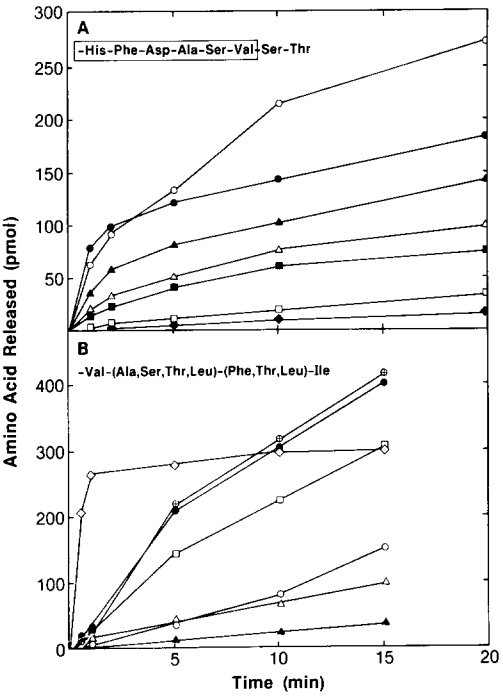

Fig. 4. COOH-terminal sequences of human urine RNAases. The rates of release of amino acids from RNAase C (Panel A) and RNAase U (Panel B) by carboxypeptidase Y were determined as described under Experimental Procedure. Under the conditions of separation, serine could not be distinguished from glutamine or asparagine. ●———●, threonine; ○———○, serine; ▲———▲, valine; △———△, alanine; ■———■, aspartic acid; □———□, phenylalanine; ◆———◆, histidine; ◇———◇, isoleucine; ⊕———⊕, leucine.

sequence of the other amino acids released could not be stated with more certainty than -Val-(Ala, Ser, Thr, Leu)-(Phe, Thr, Leu)-Ile_{COOH}.

Discussion

Employing differences in substrate specificity as a means of discrimination, two RNAases present in normal human urine have been separated and extensively purified. RNAase C and RNAase U were shown to differ in many of their properties including enzymatic, i.e., substrate specificity, pH optima and sensitivity to various inhibitors; physical, i.e., molecular weight and p*I*; chemical, i.e., amino acid and carbohydrate compositions; and structural, i.e., NH_2- and COOH-terminal sequences. Therefore, it must be concluded that RNAase C and RNAase U are, indeed, different enzymes and probably structurally unrelated proteins. Whether additional species of RNAase are also present in normal human urine could not be completely ruled out by the present studies. However, the high yields, i.e., 52%, and more recently 72%, for RNAase C and 82% for RNAase U, of activity obtained using the purification procedures described under Experimental Procedure, and the inability to identify additional RNAases during the course of the purification, support the view that the two RNAases presently being studied are the major species in normal urine.

RNAases C and U were both shown to consist of single polypeptide chains based on the finding of only one NH_2- and COOH-terminal sequence for each enzyme. Combined with the observations that RNAase U and the asialo form of RNAase C migrated as single bands in cationic (pH 4) polyacrylamide gels, and as single peaks in electrofocusing columns, these data suggest that major isoenzymes of either enzyme do not exist in normal urine. The heterogeneity observed for RNAase C is primarily due to variability in the sialic acid content. Renner et al. [49] have suggested the presence of a unique isoenzyme of poly(C)-specific RNAase in the sera of pancreatic cancer patients based on gel electrofocusing experiments. Since the poly(C)-specific RNAase present in human serum has also been shown to be a glycoprotein [4], such a finding may reflect alterations in carbohydrate composition rather than a real isoenzyme.

Other laboratories have previously reported the isolations and partial characterizations of RNAases from human urine [1,7—11,17]. In terms of their enzymatic properties, purified RNAases C and U appear to be very similar, if not identical, to these previously studied enzymes. Preference for poly(C) as a substrate [9—11,18], pH optima of 8.0—8.5 with RNA as substrate [1,7,8,11] or 6.5 with poly(C) as substrate [9,10], and inhibition by Zn^{2+} and Cu^{2+} [1,8,11] or homoribopolymers [10] were consistently observed for one type of urine enzyme. In contrast, the other RNAase showed a lack of preference for poly(C) [18], a pH optimum between 6.5 and 7.0 with RNA as substrate [1,7,17], and no inhibition by Zn^{2+} and Cu^{2+} [1,17].

Physicochemical and structural analyses on RNAases isolated from human urine by other laboratories have been more limited, but comparisons with RNAases C and U can be explored. It is very likely that our RNAase U is identical to an enzyme, also termed RNAase U, previously purified by Delaney [7]. Similar molecular weights, amino acid compositions and the presence of a lysine residue at the NH_2-terminus of each enzyme support this conclusion. Despite striking similarities in catalytic properties, as just discussed, the poly(C)-specific RNAases isolated from urine by different laboratories have varied in their physicochemical properties. Molecular weight differences have been particularly common. The M_r of 33 000 reported for RNAase C in the present study is in agreement with the result of Rabin and Weinberger [9]. Other groups, however, have reported M_r values of 14 000—15 000 [7,18], 21 500 [10] and 45 000 [11]. Although, as noted earlier, poly(C)-specific RNAases have been shown to aggregate at low ionic strength [5,18] and possibly form dimers [18], the present results, and those of Rabin and Weinberger [9], were observed for the reduced and denatured, as well as native, RNAase C which argues against this possibility. Furthermore, it is unlikely that the various laboratories were looking at entirely different protein molecules because the yields of poly(C)-specific RNAases were substantial in most of the studies, which suggests that each group was isolating a major species of enzyme. Although additional studies will be required for final proof, it is our contention that the carbohydrate portion of these purified RNAases has been variable and this has resulted in differences in observed molecular weights. Previously, only Reddi [10] had mentioned the presence of carbohydrate on a poly(C)-specific RNAase isolated from human urine. In the present study, RNAase C, isolated under the mild conditions of phosphocellulose, poly(G) Sepharose and

Sephadex G-75 chromatographies, was shown to contain 33% carbohydrate by weight (Table IV). It is interesting to point out that the laboratories that reported M_r values of 14 000—15 000 for this type of RNAase [7,18] employed acid extraction in their purification procedures. Previous experience with porcine pancreatic RNAase, which also contains a large proportion of carbohydrate, has shown that harsh extraction procedures can result in loss of carbohydrate [42]. In a recent experiment (Cranston, J., unpublished data), RNAase C was treated with a mixture of glycosidases including neuraminidase, β-galactosidase (EC 3.2.1.23), β-*N*-acetylhexosaminidase (EC 3.2.1.52) and the endo-β-*N*-acetylglucosaminidases D and H. RNAase C retained its full activity under these treatment conditions and did not appear to be attacked proteolytically. Following SDS-polyacrylamide gel electrophoresis of the treated enzyme, a disappearance of the diffuse band at M_r of 33 000 with a concomitant appearance of a much sharper protein band at M_r of 17 000 was observed. These data indicate that the removal of carbohydrate from RNAase C causes the enzyme to migrate in SDS-polyacrylamide gels with a molecular weight which is closer to what would be expected for a pancreatic RNAase. The excellent NH_2- and COOH-terminal sequence homologies shared between RNAase C and all known mammalian pancreatic RNAases suggest that the RNAase C apoprotein would contain 126 amino acids and have an M_r of approx. 14 000.

In addition to the molecular weight discrepancies just discussed, the amino acid composition of RNAase C was observed to differ from a pancreas-like RNAase, termed RNAase P, previously purified from human urine by Delaney [7]. RNAase C and RNAase P have similar pH optima for RNA hydrolysis, and both enzymes contain a lysine residue at their NH_2-terminus. However, whereas the amino acid composition of RNAase C is similar to a number of pancreatic RNAases (see Results), that of RNAase P is essentially identical to bovine pancreatic RNAase A. It is unlikely that our purified RNAase C preparations were sufficiently contaminated to give an incorrect result because multiple amino acid analyses on three separate RNAase C preparations, each tested for purity in three gel electrophoresis systems, gave identical compositions. Furthermore, the NH_2-terminal sequence of RNAase C clearly showed only a lysine residue at the NH_2-terminus indicating the lack of another polypeptide. It is possible that Delaney purified a different RNAase, one which we failed to detect, since the yields of RNAase P were extremely low [7]. Finally, variable, albeit similar, amino acid compositions among pancreatic RNAases isolated from different species have been a consistent observation for this rapidly evolving protein [42,46,48].

The sequence homology observed between RNAase C and mammalian pancreatic RNAases [46,48] and the similar amino acid compositions of RNAase U and human spleen RNAase [7], suggest a relationship between these urine enzymes and those present in the pancreas and spleen, respectively. However, the origins of the urine RNAases remains an open question. The kidney, which contains enzymes with properties similar to both RNAases C and U [2], could be a direct source of the urine RNAases. However, Rabin et al. [50] and Humphrey et al. [51] have shown excellent correlations between levels of poly(C)-specific RNAase in serum with the glomerular filtration rate as measured by creatinine clearance. In addition, Rabin et al. [52] found their puri-

fied poly(C)-specific RNAase from urine to be immunologically identical to a partially purified serum enzyme. These studies suggest that RNAase C reaches the urine as a component of filtered plasma. RNAase U-like activity has also been observed in serum [1,16] although a direct relationship to the urine enzyme is less clear. If the urine RNAases are plasma filtration products, a number of tissues could then be the original sources of the enzymes. For example, Peterson [53] has observed poly(C)-specific RNAase activity in the sera of patients after total pancreatectomy suggesting additional, or other, origins of this activity. Using substrate specificity and immunological criteria, Neuwelt et al. [2] have shown the presence of both pancreas-type and liver-spleen type RNAases in many tissues, indicating the ubiquitous nature of these RNAases. It would be of considerable interest to know if all tissue enzymes are structurally identical. Only considerable amino acid sequence and carbohydrate characterization studies can answer this question.

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