Multiple Ribonucleases of Human Urine†

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ABSTRACT: Four major urine ribonuclease (RNase) activities, designated bands A–D, were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and activity staining. Bands A, B, and C have alkaline pH optima and display molecular weights of 31 000, 23 000, and 20 000, respectively, upon sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis and weights of 44 000, 28 000, and 22 000 upon gel filtration. Band D, with a pH optimum slightly below neutrality, has a molecular weight of 16 000 or 15 000, respectively, determined by the above methods. Band A, the most abundant activity in urine, is heterogeneous and resembles serum RNase 1 on electrophoresis and on phosphocellulose and Sephadex chromatography. Band B is similar to a minor, unnamed component of serum RNase activity while band C resembles serum RNase 3. Band D is similar to the leukocyte RNase-like activity of serum [Blank, A., & Dekker, C. A. (1981) *Biochemistry* (preceding paper in this issue)]. Band A is present in urine at a concentration higher than that of RNase 1 in serum. In contrast, urine counterparts of serum RNases 2, 4, and 5 are not apparent upon either phosphocellulose chromatography [see also Yamanaka, M., Akagi, K., Murai, K., Hirao, N., Fujimi, S., & Omae, T. (1977) *Clin. Chim. Acta 78*, 191–201] or $NaDodSO_4$ gel electrophoresis; a urine counterpart of serum RNase 3 can be detected only by the more sensitive electrophoretic method. These results indicate that RNases 2–5 are processed differently by the kidney than RNase 1. After reconciliation of reported differences in their pH optima and molecular weights, five apparently diverse RNase preparations described in the literature can be related to band A activity and three preparations to band D. However, we are unable to confirm a previous report of a human urine enzyme indistinguishable from bovine pancreatic RNase A.

Human urine and serum contain a ribonuclease (RNase) activity which exhibits a molecular weight of 45 000 upon gel filtration and which has catalytic properties similar to those of bovine pancreatic RNase A (M_r 13 690) and its glycosylated homologues ($M_r \leq 22\,400$) (Yamanaka et al., 1977; Akagi et al., 1976; Reinhold et al., 1968). That the high molecular weight observed for the human enzyme is not due solely to aggregation is indicated by the finding that, following heating in sodium dodecyl sulfate ($NaDodSO_4$)[1] and mercaptoethanol, the activity displays a molecular weight of 31 000 on $NaDodSO_4$ gel electrophoresis (Blank & Dekker, 1981). This enzyme is of interest because, though it comprises less than 20% of the RNase activity of serum assayed at alkaline pH, it constitutes more than 60% of the activity of urine. In contrast, most RNases present in serum are excluded from urine. Thus, comparison of the structure of this particular RNase with those of other serum RNases may illuminate determinants involved in the renal processing of small proteins.

As part of our effort to purify and characterize this activity from urine, we have examined the literature on human urine RNases. As summarized in Table I, discrepant values have been reported for the number, molecular weights, and pH optima of RNases present. To add to this collection of apparently diverse enzymes yet another description of a purified RNase seemed of limited value. Therefore, we have also undertaken to (1) clarify the number and nature of RNase species actually occurring in urine and (2) to assess the relationship of the various RNases described in Table I to one another and to the RNase mentioned above. For these purposes, we have utilized conventional chromatographic techniques together with $NaDodSO_4$–polyacrylamide gel electrophoresis and activity staining to resolve and visualize the individual RNase species of normal urine. Exploiting the observation (Rosenthal & Lacks, 1977; Blank, 1978) that many RNases will renature after being heated in $NaDodSO_4$ and mercaptoethanol, we have examined the urine enzymes under conditions which normally preclude self-association and complex formation with other substances. Thus, we have been able to establish the number and to estimate the molecular weights of the major urine RNase species. And, after reconciling reported differences in their pH optima and molecular weights, we have been able to correlate the RNases described in Table I with the activity bands visualized in our $NaDodSO_4$–polyacrylamide gels.

Materials and Methods

Urine and Leukocyte Preparations. Urine was collected from healthy volunteers. For direct electrophoresis, it was cooled on ice and centrifuged at 10000*g* at 2 °C; any precipitate was discarded. For the preparation of "urine RNase concentrate", 2.6 L of urine was cooled to 4 °C, filtered, dialyzed against 5 mM Tris-HCl buffer (pH 7.4), and concentrated 10-fold by dialysis against Carbowax 6000 (Union Carbide). Following further dialysis against 10 mM sodium phosphate (pH 6.7), the RNase solution was loaded onto four phosphocellulose columns (Brown Co., Selectacel type 40, 1.02 mequiv/g), each with dimensions of 0.55 × 6.0 cm, prepared according to Yamanaka et al. (1977). After each column was washed with 2 column volumes of equilibrating buffer, RNase activity was eluted with 3 mL of 10 mM sodium phosphate–2 M NaCl (pH 6.7); eluate containing activity from the first column was used to elute activity from the second column, ad seriatim. RNase activity of the "urine RNase concentrate" thus obtained was concentrated about 400-fold with respect to untreated urine in approximately 70% yield. Electrophoresis and activity staining of "urine RNase concentrate" and of untreated urine revealed no difference between them in the

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[1] Abbreviations used: BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Properties of Normal Human Urinary RNases Reported in the Literature

investigator(s)	designation	mol wt ($\times 10^{-3}$)	pH optimum	investigators' description (relative amounts)
Delaney (1963)	RNase P	13.7[a]	8.0	RNase A-like
	RNase U	18.8[a]	6.5	spleen RNase-like
Naskalski (1972a–c)	fraction I		6.0–6.9	acid (barely detectable)
	fraction II	25.0–22.3[b]	7.8–8.0	alkaline (major)
	fraction III	15.0[b]	6.4–6.9	acid (minor)
Rabin & Weinberger (1975)	urine RNase	33.0[c]	6.5[d]	novel
Bardon et al. (1976a)	fraction A	30.0[e]	8.5	secretory (major)
	fraction B		7.0	nonsecretory (minor)
Yamanaka et al. (1977)	RNase 1	45.0[b]	8.5	alkaline
Reddi (1977)	urine RNase	22.0[f]	6.5[d]	pancreatic

[a] Determined by equilibrium sedimentation and amino acid analysis. [b] Determined by gel filtration. [c] Determined by gel filtration and $NaDodSO_4$–polyacrylamide gel electrophoresis. [d] Poly(C) substrate in sodium phosphate buffer. [e] In their subsequent paper (Bardon et al., 1976b), the authors report that the 30 000 molecular weight enzyme can be *reversibly* converted to a form which displays a molecular weight of 14 000 upon gel filtration under unspecified conditions. [f] Determined by sucrose gradient centrifugation and thin-layer gel chromatography.

number of activity bands and the relative amount of each band.

Homogenates of purified leukocytes, and of lymphocytes isolated from leukocytes by using Lympho-prep (Gallard-Schlesinger) according to the techniques of Boyum (1968), were prepared by M. P. Thelen (Blank & Dekker, 1981).

Analytical Procedures. Ribonuclease activity was assayed as previously described (Blank & Dekker, 1981).

Electrophoresis in 12.5% $NaDodSO_4$–polyacrylamide gels containing embedded RNA, as well as activity staining, was carried out as described by Blank & Dekker (1981) with minor modifications. Samples were prepared for electrophoresis in one of three ways: (1) in 1% $NaDodSO_4$ at room temperature, without heating; (2) in 1% $NaDodSO_4$ with heating for 2 min at 100 °C; (3) in 1% $NaDodSO_4$ and 5% mercaptoethanol with heating for 2 min at 100 °C. Following electrophoresis and removal of $NaDodSO_4$, the resolved and renatured RNases were allowed to digest the embedded RNA by incubating the gel in 0.1 M Tris-HCl (pH 7.4) at 37 °C. Since the length of this incubation depends upon the amount of renatured activity present in the gels, the amount of sample loaded was routinely adjusted so that the incubation period was 1.5 h for gels containing samples prepared in $NaDodSO_4$ alone and 16 h for gels containing samples prepared in $NaDodSO_4$ and mercaptoethanol.

Electrophoresis at high pH under nondenaturing conditions in RNA-cast polyacrylamide gels was carried out as described by Blank & Dekker (1977). After a 1.5-h electrophoretic run at 4 °C, the gel was rinsed with H_2O, incubated for 1.5 h in 0.1 M Tris-HCl, pH 7.4, at 37 °C, and stained in the same manner as for $NaDodSO_4$ gels. Electrophoresis at low pH in polyacrylamide gels was carried out as described by Blank (1978). Since RNA was not embedded in these latter gels, activity staining was performed by diffusing in an oligonucleotide substrate as described by Blank & Dekker (1975). Thus, following electrophoresis at 4 °C, the gel was first rinsed in H_2O and then incubated for 20 min in 0.1 M Tris-HCl (pH 7.4) at 37 °C, and for 15 min in a solution of oligonucleotides (Schwarz Bioresearch yeast sodium ribonucleate, lot NHS 6402, average chain length = 19, A_{260} = 50) in the same buffer at 37 °C. Staining was performed as for a $NaDodSO_4$ gel.

Molecular weights of urine RNase species were determined by gel filtration (Whitaker, 1963; Andrews, 1964). A concentrated solution of urine RNase activity (2 mL of the "urine RNase concentrate" described above) was applied to a Sephadex G-75 column (1.6 × 55 cm) equilibrated with 0.02 M Tris-HCl–0.5 M NaCl (pH 8.5); the buffer was that used by Yamanaka et al. (1977). Fractions (1.02 mL) were collected at a flow rate of 24 mL/h and assayed for RNase activity. Crystalline preparations of ovalbumin, whale skeletal muscle myoglobin, and RNase A were used as protein standards. Elution volumes of ovalbumin (50.2 mL, M_r 43 000) and myoglobin (67.4 mL, M_r 17 800) were determined from the absorbance at 280 and 410 nm, respectively; the elution volume of RNase A (72.1 mL, M_r 13 700) was determined by measurement of activity. Because the urine RNase activities corresponding to bands A–D resolved poorly on Sephadex G-75, the elution volume of each individual band was determined by subjecting equal volumes of column fractions (heated in 1% $NaDodSO_4$) to gel electrophoresis and activity staining; the elution volume of that column fraction displaying the most intense activity for any given band was used to estimate the molecular weight of that band.

Molecular weights of urine RNases were also determined by a modification (Blank & Dekker, 1981) of the gel electrophoretic method described by Weber & Osborn (1969). Crystalline preparations of bovine serum albumin, ovalbumin, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, chymotrypsinogen A, and RNase A were used as molecular weight standards. Reduced samples (i.e., samples heated at 100 °C for 2 min in 1% $NaDodSO_4$ and 5% mercaptoethanol) of protein standards and of urine enzymes were run on the same RNA-cast $NaDodSO_4$–polyacrylamide slab; following electrophoresis, the lane containing protein standards was cut from the gel and stained with Coomassie brilliant blue (Fairbanks et al., 1971) while on the remainder of the slab urine RNases were detected by activity staining.

Phosphocellulose column chromatography of urine (10 mL) was carried out according to Yamanaka et al. (1977). A linear gradient of 0.2–1.8 M NaCl in a total volume of 150 mL was used to elute activity from a 0.8 × 12 cm column. Fractions of 1.32 mL were collected at a flow rate of 30 mL/h.

Results

Detection of Two Types of Urinary Ribonucleases with Different pH Optima. Figure 1A illustrates the pattern of normal urine RNase activity observed following electrophoresis of unreduced samples in an RNA-cast $NaDodSO_4$ gel. As shown, the pattern is altered in a specific and reproducible way when samples which contain 1% $NaDodSO_4$ are heated at 100 °C prior to electrophoresis: Upon being heated, the activity of the band designated D′ (Figure 1B) is greatly reduced while the activity of the band designated D is increased. The same alteration in mobility is characteristic of the RNase activity of leukocyte homogenates [Figure 1A (a, c, and e)].

Our nomenclature for the four major activity bands of urine is indicated in Figure 1B, together with the mobility of serum

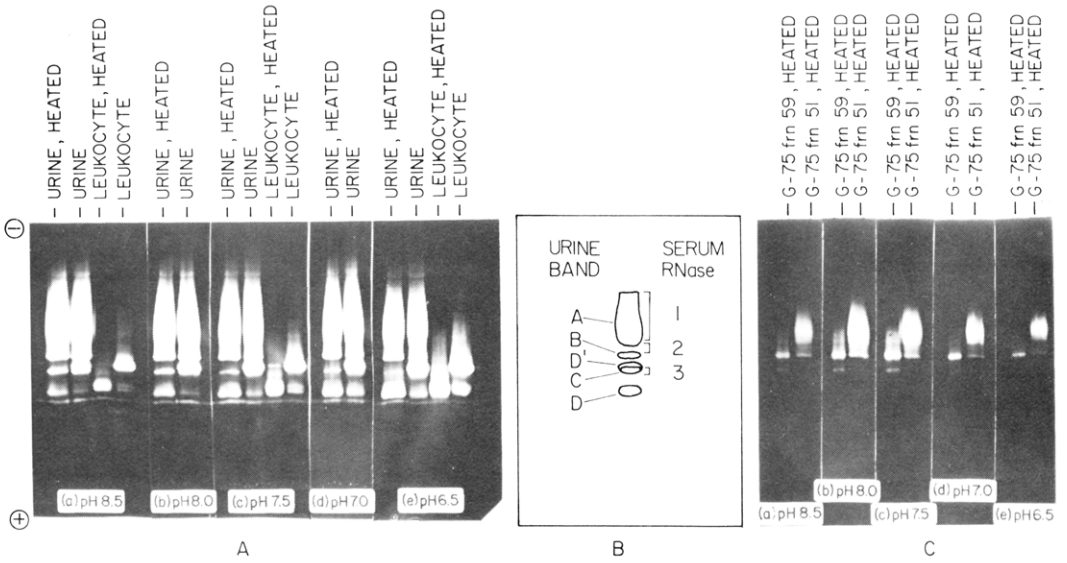

FIGURE 1: Detection by $NaDodSO_4$–polyacrylamide gel electrophoresis and activity staining of two types of urinary RNases with different pH optima. (A) Electrophoresis of unfractionated urine and of a leukocyte homogenate. Urine (3 μL) and a leukocyte homogenate (0.225 μL) were subjected to electrophoresis in a 12.5% gel cast with 0.28 mg/mL RNA. All samples were prepared in 1% $NaDodSO_4$ without mercaptoethanol; those designated "heated" were placed in a 100 °C water bath for 2 min prior to electrophoresis while the remaining samples were held at room temperature. Following electrophoresis, $NaDodSO_4$ was diffused from the gel. The slab was then sliced into five sections. Individual sections were incubated for 1.5 h at 37 °C in 0.02 M Tris-HCl, 0.02 M sodium phosphate, and 0.1 M NaCl at pHs of (a) 8.5, (b) 8.0, (c) 7.5, (d) 7.0, and (e) 6.5. All five sections were then stained with 0.2% toluidine blue O in 0.005 M Tris-HCl and destained as described under Materials and Methods. (B) Diagrammatic representation of activity bands found in urine. The minor, rapidly migrating band traversing the entire slab gel of (A), which is not included in (B), is the frequently observed artifactual activity described in the previous paper. Nomenclature of the urine activity bands, along with the locations of serum RNases 1, 2, and 3 (Blank & Dekker, 1981), is also indicated. (C) Additional electrophoretic patterns of fractionated urine RNase preparations enriched in bands A and B. Sephadex G-75 fractions 51 (0.1 μL) and 59 (0.16 μL) with elution volumes of 52 and 60 mL, respectively (see Results), were heated for 2 min at 100 °C in 1% $NaDodSO_4$ and subjected to electrophoresis and activity staining as described for (A).

RNases 1–3 determined by Blank & Dekker (1981). For establishment of the pH preference of activity bands A, B, C, and D, the single gel slab shown in Figure 1A was cut vertically into five sections following electrophoresis; individual sections were activity stained at different pHs ranging from 6.5 to 8.5. Bands A, B, and C display increased activity at alkaline pH; this observation is confirmed in Figure 1C, showing additional patterns obtained for bands A and B partially purified by gel filtration. Only band D (and D′ in unheated samples) exhibits decreased activity as the pH is increased from 6.5 and 7.0 to 8.5. As expected, the RNase activity of leukocytes is also greater at lower pH; optima of 6.5 (Silber et al., 1967) and 6.8 (Sznajd & Naskalski, 1973) have been reported.

Gel Filtration Chromatography. On Sephadex G-75, the RNase activity of urine chromatographs as a single asymmetric peak (V_e = 50.7 mL), with 80% of the activity eluting between 45 and 56 mL (results not shown). Chromatographic resolution of bands A–D was analyzed by subjecting an aliquot of each individual column fraction to electrophoresis in an RNA-cast $NaDodSO_4$ gel; mercaptoethanol was omitted from samples, which were heated in 1% $NaDodSO_4$, to minimize loss of activity. Visual inspection of a gel (not shown) carefully loaded with a constant volume of each fraction revealed that band A activity elutes slightly ahead of the peak of overall activity (V_e = 49.6 mL), yielding a molecular weight of 44 000. Activity bands B (V_e = 58.7 mL), C (V_e = 63.2 mL), and D (V_e = 71.2 mL) elute on the trailing edge of the peak, yielding molecular weights of 28 000, 22 000, and 15 000, respectively.

Molecular weights of the partially resolved RNase activities recovered from Sephadex G-75 were also determined by electrophoresis of reduced samples according to a modification of the method of Weber & Osborn (1975), as described under Materials and Methods. Results are shown in Figure 2. By comparison of the electrophoretic mobility of each activity band with those of protein standards run in the same gel, band

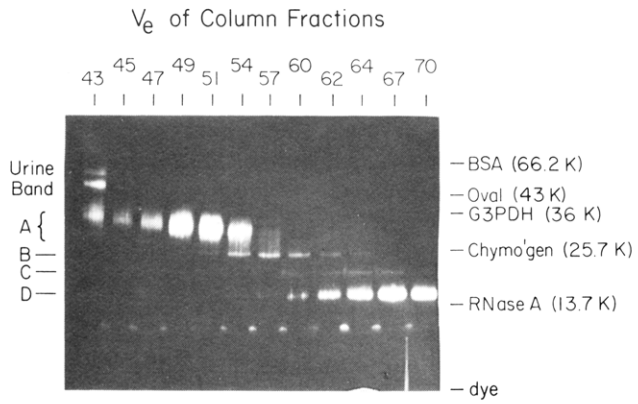

FIGURE 2: Electrophoretic determination of molecular weight of activity bands A–D. Aliquots of selected Sephadex G-75 column fractions enriched in bands A, B, C, or D were heated for 2 min at 100 °C in $NaDodSO_4$ and 5% mercaptoethanol. Reduced, denatured samples were subjected to electrophoresis and stained for activity as described under Materials and Methods. The time allowed for digestion of embedded RNA by the renatured enzymes was 16 h. All lanes in the gel contain 0.7-μL aliquots of fractions except for the leftmost lane which contains 3.3 μL. Elution volumes of column fractions from which aliquots were taken are indicated above each lane in the gel. The positions of molecular weight standards (BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, chymotrypsinogen, and bovine pancreatic RNase A) run on the same slab are indicated at the extreme right.

A species were assigned molecular weights ranging from 35 000 to 27 000, ca. 30 500 being the modal value calculated for the broad bands seen in fractions eluting from Sephadex at 49 and 51 mL (Figure 2). Weights of 23 000, 20 000, and 16 000 were found for bands B, C, and D, respectively. Two minor species with apparent molecular weights of 43 000 and 52 000 were detected in the initial portion of the peak (V_e = 43 mL). Table II summarizes the results of determination by gel filtration and by $NaDodSO_4$ gel electrophoresis of the molecular weights

Table II: Urine Bands A–D: Their Molecular Weights, Classification, and Proposed Relationships to RNases Described in the Literature

urine RNase activity defined electrophoretically	molecular weight: electrophoresis	molecular weight: gel filtration	proposed classification[a]	proposed homologue in serum	urine RNase preparations described in the literature: investigator(s)	urine RNase preparations described in the literature: designation
band A	30 500	44 000	secretory	multiple species eluting in phosphocellulose peak 1 and together designated RNase 1[b,c]	Naskalski (1972) Rabin & Weinberger (1975) Bardon et al. (1976a) Yamanaka et al. (1977) Reddi (1977)	fraction II urine RNase fraction A urine RNase urine RNase
band B	23 000	28 000	secretory	species eluting from phosphocellulose between RNases 1 and 2[b,c]	none	none
band C	20 000	22 000	secretory	RNase 3[b,c]	none	none
band D	16 000	15 000	nonsecretory	leukocyte RNase-like activity[c]	Delaney (1963) Naskalski (1972) Bardon et al. (1976a)	RNase U fraction III fraction B

[a] We have assigned bands A–D to the classes proposed by Sierakowska & Shugar (1977), although our data do not identify the tissue(s) of origin of bands A–D and therefore do not further validate the classification system itself. [b] Akagi et al. (1976). [c] Blank & Dekker (1981).

of the four major bands of urinary RNase activity.

Phosphocellulose Column Chromatography. Chromatography of normal urine on phosphocellulose yields a single asymmetric peak of activity containing more than 98% of the starting activity, confirming the results of Yamanaka et al. 1977). $NaDodSO_4$ gel electrophoresis and activity staining of individual column fractions reveal that band A is the primary activity eluting in the peak tubes (results not shown). Bands B and D elute later, after about 75% of the activity has emerged from the column. Band C appears even later, in fractions at the extreme edge of the peak.

Electrophoresis of Urinary and Leukocytic RNases under Nondenaturing Conditions. Samples of urine, leukocyte and lymphocyte homogenates, and Sephadex G-75 column fractions enriched either in band A or in band D, were subjected to electrophoresis in native polyacrylamide gels. As shown in Figure 3, band D resembles the RNase activity of leukocytes, displaying a characteristic, low-mobility doublet in basic gels and high mobility in acid gels.

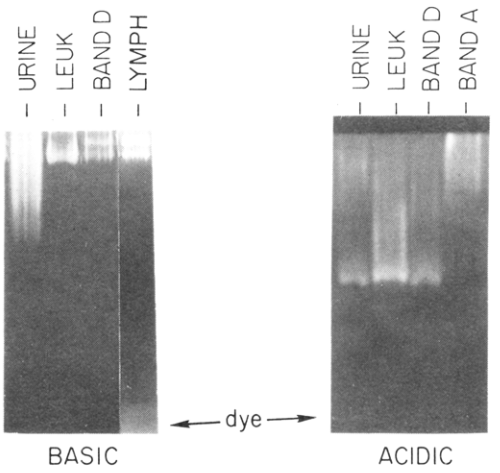

FIGURE 3: (Left) Electrophoresis of urine and leukocyte RNases in a nondenaturing, RNA-cast polyacrylamide gel run at high pH in a Tris–glycine buffer system. Samples of unfractionated urine (0.2 μL), leukoctye homogenate (0.04 μL), dialyzed Sephadex G-75 fraction (V_e = 79 mL) enriched in band D (0.2 μL), and a homogenate of the lymphocyte fraction of leukocytes (10 μL) were run in a 10% gel and stained for activity as described under Materials and Methods. (Right) Electrophoresis of urine and leukocyte RNases in a nondenaturing polyacrylamide gel run at low pH in a β-alanine–acetate buffer system. Samples of "urine RNase concentrate" (0.5 μL), leukocyte homogenate (5 μL), dialyzed Sephadex G-75 fraction (V_e = 79 mL) enriched in band D (18 μL), and G-75 fraction (V_e = 49 mL) enriched in band A (5 μL) were subjected to electrophoresis in a 10% gel. The gel was stained for activity by diffusing an oligonucleotide substrate into the matrix as described under Materials and Methods.

Discussion

Among the limited findings agreed upon by investigators of human urine RNases are the following: (1) Urine contains two types of RNA-degrading activity with pH optima of 7.8–8.5 and 6.5–7.0 [e.g., Stambaugh (1963)], the two types having been termed secretory and nonsecretory,[2] respectively, by Sierakowska & Shugar (1977). (2) Activity with the higher pH optimum elutes earlier from cation-exchange resins (Delaney, 1963; Naskalski, 1972a; Bardon et al., 1976a). Consistent with these points of agreement, we have found band A, the dominant electrophoretic entity with high pH preference, to elute from phosphocellulose ahead of band D, the only electrophoretic species with a lower pH optimum.

As shown in Figure 1, activity with high pH preference is distributed among three electrophoretic entities, here designated bands A–C. Band A, the most prominent among them, is itself heterogeneous, displaying a modal molecular weight of 30 500 in $NaDodSO_4$ gel electrophoresis, while a molecular weight of 44 000 is estimated by gel filtration. The higher value, determined by Sephadex G-75 chromatography in a nondenaturing buffer, could reflect, inter alia, aggregation phenomena precluded under the conditions of $NaDodSO_4$ gel electrophoresis. [A strong tendency to associate has been shown for human serum RNase activity (Schmuckler et al., 1975; Akagi et al., 1976).] Whatever the cause(s), the molecular weight estimated by Sephadex is probably high. Nonetheless, because other investigators have used gel filtration to characterize their urine RNase preparations, our own data were useful in rationalizing the discrepant molecular weights reported in the literature.

The electrophoretic heterogeneity of band A could reflect differences in carbohydrate content of constituent RNases, a hypothesis consistent with the finding that pure band A is a glycoprotein, as determined by the method of DuBois et al. (1956).[3] Since glycoproteins have been observed to behave atypically in $NaDodSO_4$ gels because of impaired $NaDodSO_4$

[2] Though our work does not further validate this classification system, we have used the terms secretory and nonsecretory for ease of reference.

[3] Our unpublished observations.

binding (Weber & Osborn, 1975), the molecular weight of band A was determined at three different acrylamide concentrations (10, 12.5, and 15%). All three concentrations gave a value of 31 000 ± 1000, and neither the Ferguson plot of log R_f vs. gel concentration nor the plot of log R_f vs. gel retardation coefficient (Neville, 1971) revealed any atypical behavior. Nevertheless, since other glycosylated RNases display molecular weights in $NaDodSO_4$ gels which are 13–16% high (Segrest et al., 1971), the possibility remains that the weight of 30 500 found for band A is also an overestimate.

Urine band A and serum RNase 1 (Akagi et al., 1976) behave similarly on phosphocellulose chromatography, exhibit comparable molecular weights (44 000–45 000 in gel filtration and 31 000 in $NaDodSO_4$ gel electrophoresis; Blank & Dekker, 1981), and have similar mobilities in native polyacrylamide gels run at high and low pH.[3] Therefore, we conclude that band A contains RNase species similar to or identical with those comprising serum RNase 1. Our conclusion is simply a refinement of that of Yamanaka et al. (1977), who reported that total urine alkaline RNase activity is essentially identical with serum RNase 1.

Urine band B elutes later than band A from columns of both phosphocellulose and Sephadex G-75. Its molecular weight is estimated to be 23 000 by $NaDodSO_4$ gel electrophoresis and 28 000 by gel filtration. Activity with similar chromatographic and electrophoretic behavior appears in serum, eluting from phosphocellulose between the poorly resolved peaks 1 and 2 [see Blank & Dekker 1981)]. Because this minor activity of serum elutes between the major RNases intrinsic to peaks 1 and 2, it may have been discarded by Akagi et al. during their purification of RNases 1 and 2. Whether discarded or included as a component of purified serum RNases 1 and/or 2, this activity was not designated as a separate entity by the aforementioned authors.

Band C elutes from phosphocellulose and from Sephadex G-75 after band B. Its molecular weight, estimated by $NaDodSO_4$ gel electrophoresis and by gel filtration, is 20 000 and 22 000, respectively. On the basis of its comparable molecular weight and elution position from phosphocellulose, we propose band C to be a urine counterpart of serum RNase 3. Although Yamanaka et al. concluded, on the basis of phosphocellulose chromatography alone, that individuals with normal kidney function do not excrete serum RNase 3 into urine, we have found band C in the urine of all seven healthy adults surveyed and suggest that, while band C is detectable by electrophoresis and activity staining, its activity can easily be overlooked when employing solely chromatographic profiles.

Band D, the nonsecretory RNase activity of urine, displays a molecular weight of 16 000 in $NaDodSO_4$ gel electrophoresis and 15 000 in gel filtration. The electrophoretic behavior of band D closely resembles that of leukocyte RNase activity in native gels (Figure 3) as well as in denaturing gels (Figure 1); it shares with the leukocyte activity and with the leukocyte RNase-like activity of serum (Blank & Dekker, 1981) a characteristic alteration in mobility upon heating in $NaDodSO_4$. Both band D (Sephadex G-75 fraction 78) and the RNase activity of leukocytes exhibit a pH optimum of 6.5 when measured in test tube assays with an RNA substrate and 0.1 M sodium phosphate buffer.[3]

As first reported by Yamanaka et al. (1977) on the basis of phosphocellulose chromatography and confirmed by our electrophoretic data (Blank & Dekker, 1981), urine alkaline RNase activity is less complex than that of serum. Thus, while serum RNase 1-like components (band A) predominate in urine and RNase 3-like activity (band C) is present in a small amount, RNases 2 and 4 are not detected. Of the two nonsecretory RNases of serum, urine contains the leukocyte RNase-like activity (band D) but not RNase 5. Interestingly, band A is present in urine at a concentration exceeding that of RNase 1 in serum. We have assayed at pH 8.5 the plasma RNase activity of 21 healthy adults[4] and have found the activity per milliliter to be about 40% that of urine; other investigators have reported a similar differential [e.g., Levy & Rottino (1960)]. Since at least 60% of the activity of urine is due to band A (this study) and 20% of the activity of serum is due to RNase 1 (Akagi et al., 1976) the concentration of band A in urine is about 7 times the concentration of RNase 1 in serum. In contrast, urine has lower concentrations of RNases 2–5 than does serum. This difference is not related solely to the smaller size of the latter enzymes. Thus, urine contains little, if any, of a serum RNase 2 counterpart, but it does contain a significant quantity of the smaller band B as well as a detectable quantity of a serum RNase 3 counterpart. The bases of this discrimination by the kidney are currently being studied in our laboratory.

Two related objectives of the present study are reconciliation of conflicting descriptions of urine RNases found in the literature (Table I) and correlation of the RNase species described by other investigators with one or more of the electrophoretic entities observed here. These objectives are complicated by the fact that some of the RNases described by other investigators were subjected during isolation to extreme conditions of pH which might well have altered both their physical (Ukita et al., 1964) and their catalytic (Bartholeyns et al., 1974) properties. Nonetheless, as summarized in Table II, we hypothesize that the urine RNase preparations of Yamanaka et al. (1977) (urine RNase 1), of Rabin & Weinberger (1975), and of Reddi (1977), as well as fraction A of Bardon et al. (1976a) and fraction II of Naskalski (1972a,b), contain largely or exclusively species corresponding to our band A. We further propose that fraction B of Bardon et al., fraction III of Naskalski (1972a,c), and RNase U of Delaney (1963) correspond to our band D.

Correlation of band A with urine RNase 1 of Yamanaka et al. is straightforward, as noted earlier. Similarly, band A corresponds to the enzyme of Rabin & Weinberger, who extensively purified in at least 30% yield a urine RNase with a molecular weight of 33 000 in protein-stained $NaDodSO_4$–polyacrylamide gels. An illusory difference between the enzyme of Rabin & Weinberger and our band A concerns pH optima. While the Rabin & Weinberger enzyme exhibits a pH optimum of 6.5, the optimum displayed by band A is close to pH 8 (Figure 1). The apparent discrepancy was resolved when we assayed band A (Sephadex G-75 fraction eluting at 47 mL, Figure 2) with poly(C) and phosphate buffer as did Rabin & Weinberger: The pH optimum of band A does indeed shift to 6.6 when assayed under these conditions. RNases from other human sources likewise display pH optima of 6.5 when both poly(C) and phosphate buffer are employed in assays, but have alkaline optima when RNA is substituted for poly(C) or when Tris-HCl is substituted for sodium phosphate [e.g., Schmuckler et al. (1975)].

That fraction A of Bardon et al. also contains largely band A species is supported by the following facts: (1) like band A, fraction A constitutes a major portion of the RNase activity of urine; (2) its pH optimum for degradation of RNA is 8.5; (3) it hydrolyzes poly(C) 120 times faster than single-stranded RNA in 0.03 M Tris-HCl and 0.1 M NaCl, pH 7.6 (Bardon

[4] A. Blank, W. Wootton, and C. A. Dekker, unpublished observations.

et al., 1976b). With respect to the latter property, we have determined that band A has a similar substrate preference, hydrolyzing poly(C) 60 times faster than ribosomal RNA in 0.1 M Tris-HCl, pH 7.0.

The urine RNase preparation of Reddi, isolated in 29% yield, may also be comprised largely of band A species. Although Reddi records a pH optimum of 6.5 for his preparation, this value was measured with phosphate buffer and a poly(C) substrate, conditions which, as noted above, reduce the pH optimum of band A. Futhermore, the strong poly(C) preference of Reddi's preparation is consistent with those of band A, urine RNase 1 (Yamanaka et al., 1977), and fraction A (Bardon et al., 1976a,b). Moreover, the isoelectric point of 4.1 reported by Reddi is in fair agreement with the value of 3.5 previously reported by Rabin & Weinberger for their $V_{e_{protein}}/V_{e_{cyt\ c}}$ RNase. Although Reddi reports a molecular weight for his RNase preparation of ca. 21 500, determined by both sucrose density gradient centrifugation and thin-layer gel filtration, this finding is subject to question. The sucrose density gradient value is based on a standard curve which includes data for only two proteins (cytochrome *c*, M_r 12 400, and myoglobin, M_r 17 800), both with a molecular weight considerably lower than that found for the urine RNase (20 900). The thin-layer gel filtration measurement (22 000) is an underestimate arising from use of erroneous molecular weights for several protein standards (most importantly, for chymotrypsin) and an incorrectly drawn standard curve.[5] The correct standard curve yields a molecular weight of 28 000 for the author's RNase preparation. Therefore, the molecular weight recorded by Reddi does not preclude the similarity of his urine RNase preparation to our band A.

It is also likely that fraction II of Naskalski, which constitutes a major portion of the total RNase activity of urine and which was reported to have a pH optimum of 7.8, contains band A species. Noting that the molecular weights Naskalski found by gel filtration for the major subcomponents of fraction II (i.e., 25 000 for RNase IIb and 22 300 for RNase IIc) differ significantly from the molecular weight we found for band A (i.e., 44 000), we repeated Sephadex G-75 chromatography under the conditions used by Naskalski: Our purpose was to test the possibility that the difference in molecular weights found by gel filtration is due to buffer effects on the state of aggregation of the RNase(s). However, rechromatography of band A in the buffer used by Naskalski did not dispel the discrepancy. Improved agreement between the molecular weights of band A and fractions IIb and IIc was obtained with an alternative, generalized standard curve constructed by plotting $V_{e_{protein}}/V_{e_{cyt\ c}}$ vs. log $M_{r_{protein}}$ from the classic data of Whitaker (1963) and Andrews (1964).[6] This curve gives the molecular weight of any protein whose V_e from a column of Sephadex G-75 is reported together with that of cytochrome *c*. Thus $V_{e_{RNase}}/V_{e_{cyt\ c}}$, calculated from Naskalski's data, yields a molecular weight of 34 000 for RNase IIb and 29 000 for RNase IIc when the alternative standard curve is used. Comparable treatment of our own data with myoglobin as the reference protein (we did not chromatograph cytochrome *c*) yields a value of 37 000 for band A. In a like manner, we found values of 37 000 and 35 000 for the RNase preparations of Rabin & Weinberger and Yamanaka et al., respectively. On the basis of this analysis, we believe that the apparent discrepancy between the molecular weights reported for Naskalski's fraction II and our band A does not preclude the similarity of these two entities. Moreover, generated in a common way, molecular weights for band A and its proposed equivalents (reported to range from 22 300 to 45 000) converge on intermediate values between 29 000 and 37 000.

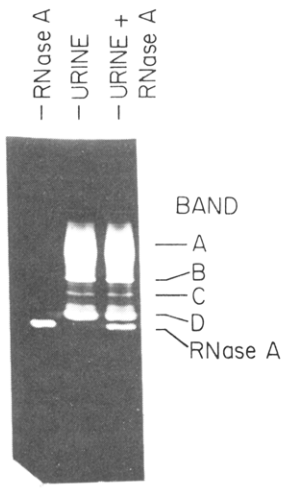

FIGURE 4: Electrophoresis of bovine pancreatic RNase A, human urine, and urine supplemented with RNase A. RNase A [0.2 ng, total activity (ΔA_{260}) = 0.05], urine [5.2 μL, total activity (ΔA_{260}) = 4.4], and RNase A supplemented urine (5.2 μL of urine containing 0.021 ng of RNase A) were subjected to electrophoresis in an RNA-cast $NaDodSO_4$–polyacrylamide gel. All samples were heated in 1% $NaDodSO_4$ without mercaptoethanol. Following electrophoresis and removal of $NaDodSO_4$, the gel was incubated in 0.1 M Tris-HCl (pH 7.4) for 1.5 h and stained with toluidine blue O.

In 1963, Delaney reported that normal human urine contains an enzyme (RNase P) indistinguishable from bovine pancreatic RNase A in amino acid composition, molecular weight, and immunologic and catalytic properties. Our results and those of others [e.g., Dickman et al. (1958)] indicate that normal urine does not contain such an RNase. We repeated the initial purification steps described by Delaney through ammonium sulfate precipitation and found that the electrophoretic pattern of our purified preparation (which had been treated with ethanol, acid, base, and ammonium sulfate) displayed no new bands traveling faster than those of untreated urine which arise as artifacts of purification. To ensure that we are indeed capable of visualizing RNase activity of the kind reported by Delaney, which was isolated from urine in 2% yield, we added RNase A to normal urine so that its activity represented 0.12% of the total activity measured at pH 8.5. Upon electrophoresis of a sample of the supplemented urine heated in 1% $NaDodSO_4$, the RNase A was visualized as a prominent band while normal urine displayed no activity with a comparable R_f (Figure 4). At the present time, Delaney's observations cannot be integrated into the body of published data on human urinary RNases.

The nonsecretory RNase activity of normal urine described by Delaney (1963) (RNase U), by Naskalski (1972a,c) (fraction III), and by Bardon et al. (1976a) (fraction B) elutes from cation-exchange resins after the secretory activity. In this and other properties (see Table I), these RNase preparations are similar to band D, which has a pH optimum of 6.5

[5] The correct molecular weights for ovalbumin, chymotrypsinogen A, α-chymotrypsin, and myoglobin are 43 000, 25 700, 25 200, and 17 800, respectively (Weber & Osborn, 1969; Dayhoff, 1972; Blow, 1971). The appropriate standard curve is a straight line [rather than a biphasic curve (Radola, 1968)] relating migration distance relative to BSA and log M_r.

[6] The alternative standard curve was adopted because Naskalski's own curve is of uncertain validity. Thus, by plotting the V_e values recorded by Naskalski for cytochrome *c*, RNase IIb, and RNase IIc (Figure 7) vs. the logarithm of their molecular weights, one obtains a straight line yielding an unlikely V_e (27% of bed volume) for the ovalbumin standard. The alternative standard curve—a straight line with slope –0.670 passing through the point x = 4.09 (log of 12 400), y = 1.0—is an excellent fit to the data of Whitaker (1963) and Andrews (1964).

and a molecular weight of 15000–16000. While there is thus agreement concerning fundamental properties of urinary nonsecretory or so-called "acid RNase" activity, its tissue(s) of origin is(are) uncertain. Delaney found his RNase U to have an amino acid composition like that of a human spleen RNase preparation. Bardon et al. found their fraction B of urine similar to RNase activity from human liver and spleen in its affinity for SE-Sephadex. Naskalski, on the other hand, demonstrated that fraction III of normal urine is similar in its behavior on CM-Sephadex to fraction III from patients with chronic granulocytic leukemia, the latter in turn resembling the RNase of normal granulocytes (Sznajd & Naskalski, 1973). In the present study, we have demonstrated that band D is electrophoretically similar to the RNase activity of leukocytes; we have observed a comparable leukocyte RNase-like activity in serum (Blank & Dekker, 1977, 1981). We hypothesize that band D activity of urine is synthesized, at least in part, in leukocytes and that it is derived from plasma and perhaps also from leukocytes shed directly into urine (Relman & Levinsky, 1971).

Added in Proof

Since acceptance of this paper, Cranston et al. (1980) have described purification and characterization of the two major RNases of human urine. RNase C, described by these authors, corresponds to our band A (see Table II under Results) and RNase U corresponds to our band D. The amino acid composition of RNase C was observed to differ from that found by Delaney (1963) for RNase P; this difference is consistent with our inability to detect in normal urine an enzyme with the properties reported for RNase P. The heterogeneity of RNase C was observed to be due to variable sialic acid content, confirming results of Thomas & Hodes (1979).

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