Ribonucleases of Human Serum, Urine, Cerebrospinal Fluid, and Leukocytes. Activity Staining following Electrophoresis in Sodium Dodecyl Sulfate–Polyacrylamide Gels†

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ABSTRACT: The ribonucleases (RNases) of human blood serum, urine, cerebrospinal fluid (CSF), and leukocytes were visualized by activity staining after electrophoresis in RNA-cast sodium dodecyl sulfate–polyacrylamide gels. Samples were prepared for electrophoresis by heating for 2 min at 100 °C in 2% sodium dodecyl sulfate ($NaDodSO_4$) and 5% mercaptoethanol, conditions which dissociate proteins into their constituent polypeptide chains and permit estimation of molecular weight. It was found that each of the five peaks of serum alkaline RNase activity separable on phosphocellulose columns, i.e., RNases 1–5 of Akagi et al. [Akagi, K., Murai, K., Hirao, N., & Yamanaka, M. (1976) *Biochim. Biophys. Acta 442*, 368–378], is associated with electrophoretically distinct enzymes. The molecular weights exhibited by these enzymes in $NaDodSO_4$ gels are 31 000 and 28 000 (major species of RNase 1), 25 000 (RNase 2), 20 000 (RNase 3), 16 000 (RNase 4), and 14 000 (RNase 5). The RNase activity of leukocytes displays a molecular weight of 17 000 and exhibits a characteristic dependence of its R_f on the temperature at which samples (in 2% $NaDodSO_4$ without mercaptoethanol) are prepared for electrophoresis. An RNase activity like that of leukocytes, distinct from RNases 1–5, is found in serum. Urine RNase activity is less heterogeneous than that of serum, consisting mainly of species like serum RNase 1 and an enzyme similar to leukocyte RNase. Conversely, CSF RNase activity is more complex and includes enzymes resembling serum RNases 1–5 as well as additional species either not observed in serum or detected in serum as minor components following chromatography. The analytical methods described herein are particularly useful for assessment of heterogeneity of RNase preparations and for direct comparison of the RNases of crude and purified samples.

A number of microbial and mammalian ribonucleases (RNases) have been visualized by activity staining following electrophoresis in $NaDodSO_4$[1]–polyacrylamide gels. After electrophoresis, $NaDodSO_4$ is diffused from gels containing the resolved RNase–$NaDodSO_4$ complexes, permitting renaturation of RNase molecules and subsequent activity staining. In the case of gels cast with RNA in the matrix, activity staining is carried out by simply incubating the gel in a buffer appropriate for enzymatic digestion of embedded RNA (Rosenthal & Lacks, 1977; Blank, 1978). In the case of gels cast without RNA, the gel is incubated with an oligonucleotide substrate which diffuses into the matrix (Sprang et al., 1978; Glanville, 1978). In both cases, RNase activity is ultimately visualized by staining the gel with a dye which binds to RNA, forming either a colored or a fluorescent background in which RNases are revealed as clear zones where substrate has been degraded. Because they employ electrophoresis in the presence of $NaDodSO_4$, these methods permit estimation of the molecular weights of RNases by comparison of the R_f values of activity bands with those of protein standards run in the same gel.

Human blood serum, urine, and cerebrospinal fluid have long been known to contain enzymatic activity depolymerizing RNA (Metais & Mandel, 1955; Laves, 1952; Kovacs, 1953), although biochemical and immunological characterization of the RNase activity is largely an effort of the last 6 years (Schmuckler et al., 1975; Reddi, 1975, 1977; Akagi et al., 1976, 1978a; Bardon et al., 1976a; Rabin & Weinberger, 1975; Yamanaka et al., 1977; Rabin et al., 1977). Recent work on the RNases of these human fluids and of tissues (Bardon et al., 1976a,b; Frank & Levy, 1976; Neuwelt et al., 1976, 1977, 1978), together with important earlier studies (Delaney, 1963; Ukita et al., 1964; Naskalski, 1972a,b), has led to definition of two major classes of human ribonuclease activity. The two classes share such properties as (1) stability to heat and acid, (2) an alkaline or nearly neutral pH optimum for degradation of RNA, (3) a phosphotransferase mechanism, and (4) specificity for pyrimidine residues in RNA. The two classes differ, however, in more detailed catalytic characteristics and in antigenic properties (Bardon et al., 1976a; Neuwelt et al., 1977, 1978; Sierakowska & Shugar, 1977). Activity of the first class predominates in pancreas and in serum, and also appears in urine (Ukita et al., 1964; Reddi, 1975, 1977; Bardon et al., 1976a,b; Neuwelt et al., 1977, 1978). It resembles, catalytically, the exocrine RNases of other mammals (Barnard, 1969; Richards & Wyckoff, 1971; Zan-Kowalczewska et al., 1974; Welling et al., 1975) and has thus been termed secretory[2] (Bardon et al., 1976a; Sierakowska & Shugar, 1977). Among its distinguishing characteristics, demonstrable under appropriate conditions, are a high pH optimum (ca. 8–8.5) for depolymerization of RNA and a large preference for poly(C) over poly(U) as substrate. A second class of human RNase activity, observed in liver and spleen and also in serum and urine (Delaney, 1963; Naskalski, 1972a; Bardon et al., 1976a,b; Frank & Levy, 1976; Neuwelt et al., 1976, 1977, 1978), resembles the intracellular, alkaline or neutral RNases of other mammals (Barnard, 1969; Zan-Kowalczewska et al., 1974) and has thus been termed nonsecretory[2] (Bardon et al., 1976a; Sierakowska & Shugar, 1977). It is distinguished by a more nearly neutral pH optimum (ca. 6.5–7) for depolym-

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

[2] The terms secretory and nonsecretory are used for ease of reference, although our data do not further validate the classification system of Shugar and his colleagues.

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erization of RNA, and a relatively weak preference for poly(C) over poly(U) as substrate, or preference for poly(U), depending upon assay conditions.

Purification of human serum (Schmuckler et al., 1975; Reddi, 1975; Akagi et al., 1976, 1978a; Bardon et al., 1976a), urine (Delaney, 1963; Naskalski, 1972a,b; Rabin & Weinberger, 1975; Bardon et al., 1976a; Yamanaka et al., 1977; Reddi, 1977), and CSF RNases (Rabin et al., 1977) has been undertaken in several laboratories. The RNase activity of all three fluids is chromatographically heterogeneous, though the full extent and physical bases of the heterogeneity are unknown. Serum contains at least six activities separable on phosphocellulose columns. Four of these (RNases 1–4) appear to be secretory while two [RNase 5 and an activity resembling that of leukocytes (Sznajd & Naskalski, 1973; Reddi, 1976; Akagi et al., 1978a)] appear to be nonsecretory (Akagi et al., 1976). The RNase activity of normal urine can be resolved on cation-exchange resins into two major fractions, the first being secretory and the second, more tightly bound fraction, being nonsecretory. The activity of CSF can be resolved by gel filtration into three overlapping peaks. Moreover, several RNase preparations from serum and urine, a few highly purified (Delaney, 1963; Rabin & Weinberger, 1975), have been characterized. Unfortunately, however, correlation of published data on human serum and urine RNases can be difficult because the number and identity of active species present in any particular RNase preparation are frequently indeterminate. Thus, despite recent progress, available data do not yet afford a clear and comprehensive understanding of the RNase complement of either serum, urine, or CSF, and comparison of the RNases of these fluids, with respect both to properties of individual enzymes and to distribution of activity among well defined and perhaps shared species, has yet to be achieved. Such a comparison is essential to eventual understanding of how plasma RNases are processed by the kidney and by the blood–brain barrier.

In the present work, we have exploited the resolving power of $NaDodSO_4$–polyacrylamide gel electrophoresis together with the sensitivity of activity staining to directly visualize the RNases of serum, urine, and CSF. The combination of these two potent techniques provides an enlightening view and a direct comparison of the RNase complements of serum, urine, and CSF, as well as a method for correlating published data on human RNases.

Materials and Methods

Materials. Blood and urine were obtained from normal volunteers. CSF was obtained from untreated, normal volunteers participating in studies of psychiatric disorders. Phosphocellulose (Selectacel phosphate type 40, 1.02 meq/g) was purchased from Brown Co. $NaDodSO_4$ was the product manufactured *as crystalline flakes* by BDH and supplied by Gallard–Schlesinger; readers are cautioned to consult a recent publication (Blank et al., 1980) regarding use of other $NaDodSO_4$ preparations.

Preparation of Serum and Partially Purified Serum RNases. Serum was prepared by permitting blood to stand at room temperature for 45 min prior to centrifugation at 4300*g* at 0 °C. A partially purified preparation of serum RNases (seen in Figure 2) was obtained by adsorbing the RNase activity of dialyzed serum to phosphocellulose as described by Akagi et al. (1976); 1 mL of packed resin was used per 20 mL of serum. The resin was washed with several column volumes of 0.01 M sodium phosphate, pH 6.7, and RNase activity was eluted in a small volume of 2 M NaCl in the same buffer. The RNase activity of serum was concentrated 3.4-fold by this procedure in 75% yield and freed of the bulk of serum albumin.

Preparation of Leukocyte Homogenates. Leukocytes were isolated by using a dextran sedimentation procedure (Gottfried, 1967). Following removal of residual dextran (Bertino et al., 1963), remaining erythrocytes were lysed by the procedure of Fallon et al. (1962). Leukocytes from 10 mL of blood were suspended in 1 mL of 0.1 M Tris-HCl, pH 8.0, containing 10^{-3} M benzamidine and 2.5×10^{-4} M phenylmethanesulfonyl fluoride. The suspension was homogenized for 2 min at 45 000 rpm at 0 °C in a Sorvall Omni-Mixer and centrifuged for 10 min at 14000*g* at 0 °C.

Chromatography of Serum. Serum was chromatographed on phosphocellulose essentially as described by Akagi et al. (1976); the resin was washed with acid and base and freed of fine particles before use (Peterson & Sober, 1962). Serum (23 mL) was dialyzed at ca. 3 °C for 3 h vs. three 600-mL volumes of 0.01 M sodium phosphate buffer, pH 6.7. The dialyzed serum was centrifuged, adjusted to pH 6.7 with dilute HCl, and applied to a 0.95×18.5 cm phosphocellulose column equilibrated with 0.01 M sodium phosphate buffer, pH 6.7. The column was washed first with 24 mL of equilibration buffer and then with a linear gradient of 0.2–1.8 M NaCl in 300 mL of the same buffer. Fractions of 2.9 mL were collected; it was unnecessary to add BSA to protect the activity of peaks 4 and 5 (Akagi et al., 1976) if fractions were collected in siliconized tubes.

RNase Assay. RNase activity was assayed at pH 8.5 in 0.3-mL mixtures containing 0.035 M Tris-HCl, 0.15 M NaCl, 0.05 M sucrose, 100 μg/mL BSA, and 17.25 A_{260} units (ca. 0.8 mg/mL) of wheat germ ribosomal RNA prepared by a modification of the method of Singh & Lane (1964). After incubation at 37 °C for 15 min, 0.7 mL of ice-cold 3.4% perchloric acid was added with vigorous mixing. After the mixtures stood for 10 min in ice, they were centrifuged for 10 min at 14000*g* at 0 °C. Absorbance at 260 nm of the supernatant solution was a linear function of added enzyme up to an A_{260} of at least 1.3; the contribution to A_{260} from acid-soluble material present in enzyme preparations was negligible.

Electrophoresis and Activity Staining. Samples in Figures 1 and 2 were heated for 2 min at 100 °C in 2% $NaDodSO_4$ and 5% mercaptoethanol; samples in Figure 4 were prepared in 2% $NaDodSO_4$ as described in the legend. Electrophoresis in 12.5% slabs was carried out at 4 °C according to Ames (1974) by using an electrophoresis apparatus (constructed in this department) in which the entire surface of both faces of the gel sandwich is in contact with cold running buffer. Ribosomal RNA (7.0 A_{260} units/mL, i.e., ca. 0.3 mg/mL) was added to the separating gel solution along with the customary reagents prior to polymerization. After the 90-min run, $NaDodSO_4$ was diffused from the gel by incubation, with gentle stirring, for a total of 90 min in six successive portions (0.5 L each) of 0.01 M Tris-HCl, pH 7.4. Addition of 25% 2-propanol to the buffer used during the initial part of this process may be required when using $NaDodSO_4$ other than the product employed in this work (Blank et al., 1980). The gel was next incubated in a buffer appropriate for enzymatic digestion of embedded RNA; the time of incubation is specified under Results for each gel shown therein. Digestion of RNA, reflected in the appearance of zones of clearing in the final gel, increases with the amount of sample loaded and with the length of the incubation period.[3] In everyday practice, convenient incubation periods (usually 90 min or ca. 24 h) are

[3] A. Blank and C. A. Dekker, unpublished data.

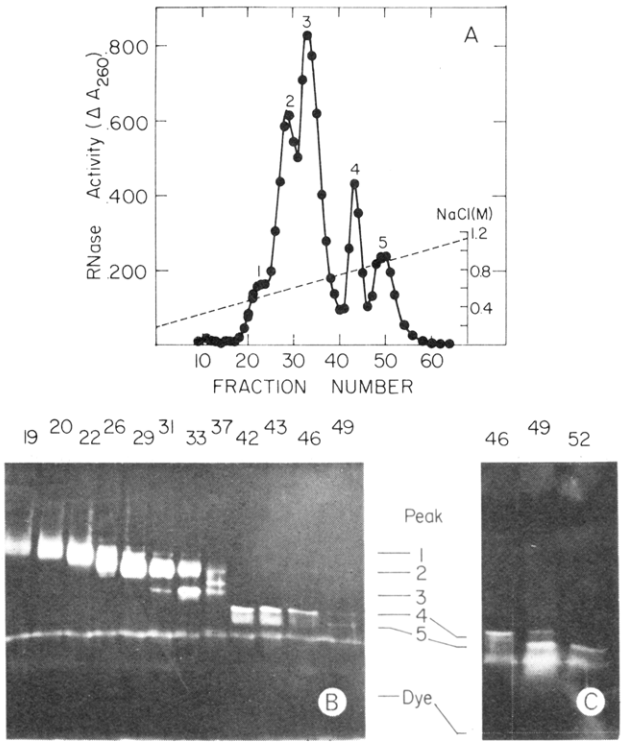

FIGURE 1: Chromatographic profile of human serum ribonuclease activity together with electrophoretic patterns of individual column fractions. (A) Serum was chromatographed on phosphocellulose, and 10-μL samples of column fractions were assayed for RNase activity at pH 8.5 as described in the text. (B) Aliquots of column fractions were heated at 100 °C for 2 min in the presence of 2% $NaDodSO_4$ and 5% mercaptoethanol prior to electrophoresis in a 12.5% RNA-cast $NaDodSO_4$–polyacrylamide slab. Siliconized micropipets and tubes were used for sample preparation. A 20-μL aliquot of fractions 19, 20, and 22, 15 μL of fraction 26, 20 μL of fractions 29, 31, 33, and 37, 25 μL of fractions 42 and 43, and 37 μL of fractions 46 and 49 were applied to the gel; fraction numbers are indicated at the top of the gel. Following electrophoresis and removal of $NaDodSO_4$ and mercaptoethanol, the gel was incubated for 22.5 h at 37 °C in 0.035 M Tris-HCl and 0.15 M NaCl, pH 8.5, and then stained to reveal RNase activity, as described in the text. (C) Additional electrophoretic patterns of 35 μL of fractions 46, 49, and 52 were obtained as described in (B). However, BSA (final concentration 135 μg/mL) was added to the samples together with $NaDodSO_4$ and mercaptoethanol prior to heating, and the gel was incubated 45 h at 37 °C prior to staining, rather than 22.5 h as for the gel in (B).

chosen, and the amount of sample is adjusted empirically so that bands of the desired intensity will be seen. Incubation periods as long as 48 h have been used for detection of small amounts of activity. Following incubation, staining was carried out according to the following protocol: 10–40 min in 0.01 M Tris-HCl, pH 7.4; 5 min in 0.2% toluidine blue O (Eastman) in the same buffer; incubation in the same buffer until the desired degree of destaining is reached (usually 30–60 min).

Results

Chromatographic and Electrophoretic Heterogeneity of Serum RNase Activity. The profile of RNase activity obtained on phosphocellulose chromatography of serum (Figure 1A) closely resembles the profile first found by Akagi et al. (1976). Each of the five peaks of activity (designated RNases 1–5 by Akagi et al.) has been numbered according to these authors. The electrophoretic patterns of individual column fractions are shown in Figure 1B,C; gels were incubated at pH 8.5 to permit digestion of embedded RNA. These patterns demonstrate that each of the five chromatographic peaks is associated with electrophoretically distinct RNases. Assignment of each of the major activity bands in Figure 1B,C to one of the peaks of Figure 1A is indicated beside the gels. The fastest moving band extending all across the gels is an artifact; it is not an RNase intrinsic to the samples loaded.[4]

Electrophoretic Pattern of Serum, Urine, CSF, and Leukocyte RNases. Figure 2A displays the RNases of serum, urine, CSF, and leukocytes visualized after incubation of a gel for 24 h at pH 7.4. Serum RNases 1 and 2 are seen in phosphocellulose fractions 20, 22, 26, and 29 (Figure 1A,B), shown again for ease of reference in wells 1–4. Serum RNases 3, 4, and 5 are shown in a partially purified preparation (well 5); in this preparation, low-mobility activity bands associated with RNases 1 and 2 are partially obscured and displaced downward by the large amount of contaminating protein which coelectrophoreses with them.[5] Molecular weights were estimated for the activity bands of serum by comparison of their R_f values with those of protein standards run in the same gel; visualization of proteins with Coomassie blue (well 13) was unimpaired by the RNA (0.3 mg/mL) embedded in the gel. Also, as shown in Figure 2B, embedding of RNA did not perturb the linear relationship between the mobilities of protein standards and the logarithms of their molecular weights (Weber & Osborn, 1969). The mobilities plotted in Figure 2B, and the molecular weights of activity bands calculated therefrom, are averages of values obtained in multiple gels. Major bands intrinsic to peak 1 (wells 3 and 4) display molecular weights of 28 000 and 31 000. The major band intrinsic to peak 2 (well 1) has a weight of 25 000. The major band of peak 3 displays a weight of 20 000; that of peak 4, 16 000; that of peak 5, 14 000. Reproducibility of molecular weights found in successive gels was excellent. For example, the average and standard deviation calculated for RNase 5 in five gels was 14 300 ± 450.

The electrophoretic pattern of urine RNase activity (wells 9 and 10) is less complex than that of serum. It consists mainly of species like those observed in serum phosphocellulose peak 1 (wells 3 and 4) and an enzyme with mobility similar to that of both serum RNase 4 (well 5) and leukocyte RNase activity (wells 11 and 12). As will be shown in Figure 4, the urine enzyme differs from RNase 4 and resembles the activity of leukocytes.

The electrophoretic pattern of CSF RNase activity (wells 6–8) is representative of numerous spinal fluid samples examined. The pattern includes not only bands with R_f values like those of serum RNases 1–5 but also additional bands which either were not observed in serum or were detected in serum as minor components following chromatography (Figure 1). A prominent triplet headed by an RNase 2-like band

[4] That this activity is an artifact of the electrophoretic and activity staining system is indicated by its appearance in gels loaded only with sample buffer and in gels loaded with nothing at all. That the artifactual activity is enzymatic is indicated by the dependence of its intensity, in carefully controlled experiments, on the duration and temperature of the incubation preceding staining with toluidine blue. The source of the artifactual activity is the ribosomal RNA cast into gels. Since completing the work described here and in an accompanying paper (Sugiyama et al., 1981), we have found that treatment of RNA with 0.5% $NaDodSO_4$ and 2% diethyl pyrocarbonate (Mendelsohn & Young, 1978) eliminates the activity.

[5] When present in very high concentration, contaminating protein can interfere with both electrophoresis of closely migrating RNases and activity staining. The sample in well 5 of Figure 2 contains a large amount of protein with the mobility of RNase 2; this protein displaces RNase 2 downward and also binds toluidine blue in the final staining procedure, giving rise to a dark, crescent-shaped band at the position of RNase 2. Such interference can be eliminated by loading less sample prepared under milder conditions. This expedient increases the sensitivity of activity staining (see Discussion).

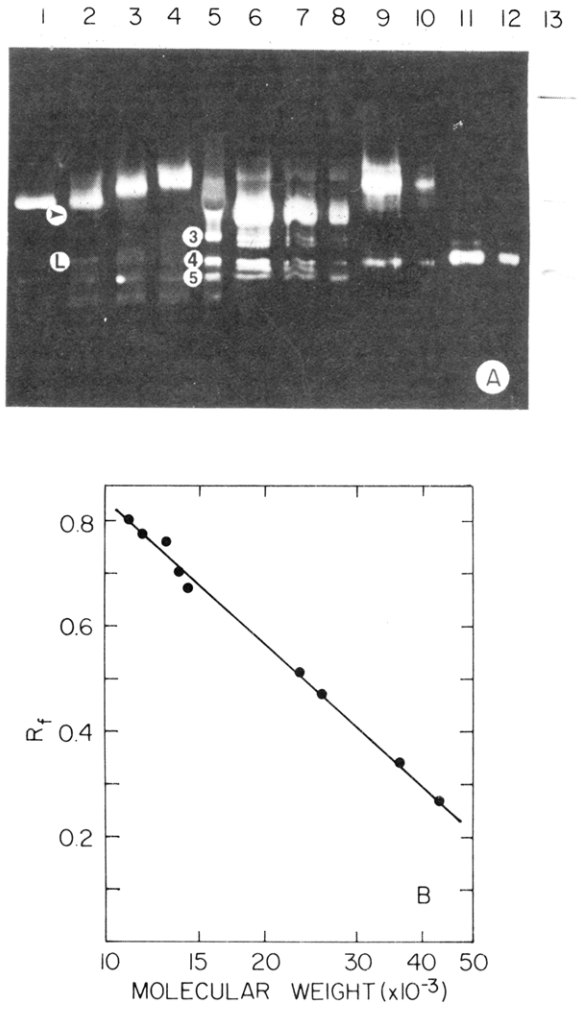

FIGURE 2: Electrophoretic patterns of human serum, CSF, and leukocyte RNases and of molecular weight standards. (A) Samples were heated for 2 min at 100 °C in 2% $NaDodSO_4$ and 5% mercaptoethanol prior to electrophoresis. Wells 1–4 contain 15 μL of phosphocellulose fractions 29, 26, 22, and 20, respectively (see Figure 1); well 5, 1 μL of a partially purified serum RNase preparation (see Materials and Methods); wells 6–8 contain 18, 12, and 6 μL of CSF, respectively; wells 9 and 10, 25 and 10 μL of urine; wells 11 and 12, 0.5 and 0.25 μL of leukocyte homogenate; well 13, 1 μg each of BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, chymotrypsinogen, and RNase A. Following electrophoresis, well 13 was cut from the gel and stained for protein (Fairbanks et al., 1971). The remaining wells were activity stained as described in the text; the gel was incubated for 24 h at 37 °C in 0.1 M Tris-HCl, pH 7.4, to permit digestion of embedded RNA. The symbols 3, 4, 5, L, and → indicate, respectively, RNases 3, 4, 5, leukocyte RNase-like activity, and a minor serum RNase noted under Discussion. (B) Mobilities of protein standards relative to bromophenol blue are plotted against the logarithms of their molecular weights. Proteins used and their molecular weights are as follows: ovalbumin (43 000); glyceraldehyde-3-phosphate dehydrogenase (36 000); chymotrypsinogen (25 700); trypsin (23 300); avidin (14 300); RNase A (13 700); B chain of chymotrypsin (13 000) cytochrome *c* (11 700); C chain of chymotrypsin (11 000) (Dayhoff, 1972; Weber & Osborn, 1969). Trypsin and chymotrypsin were each heated separately at 100 °C for 2 min in sample buffer before being mixed with other protein standards.

dominates the CSF profile; molecular weights of ca. 25 000, 24 000, and 23 000 were estimated for the components. A second triplet with an RNase 3-like band in the center has components with weights of 21 000, 20 000, and 19 000. A third triplet is formed by an RNase 4-like band (M_r 16 000), an RNase 5-like band (M_r 14 000), and a band migrating between them (M_r 15 000).

The electrophoretic pattern of leukocyte RNase activity (wells 11 and 12) includes a single major band (M_r 17 000)

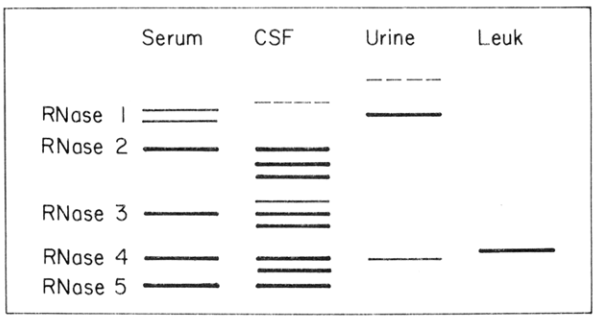

FIGURE 3: Schematic representation of the major RNases of human serum, urine, CSF, and leukocytes visualized after $NaDodSO_4$–polyacrylamide gel electrophoresis and activity staining of reduced and denatured samples.

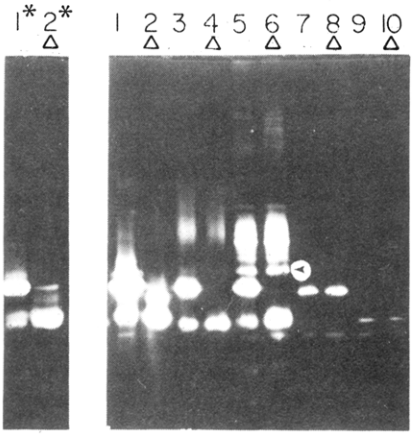

FIGURE 4: Leukocyte RNase-like activity of serum and urine: electrophoresis of unreduced samples containing 2% $NaDodSO_4$. One-half of each sample was held at room temperature while the other half (designated by a triangle at the top of the figure) was heated for 2 min at 100 °C. Wells 1* and 2* contain 0.22 μL of a leukocyte homogenate; the gel was incubated for 90 min in 0.02 M Tris-HCl and 0.05 M NaCl, pH 7.0, prior to staining with toluidine blue. Wells 1 and 2 contain 0.02 μL of leukocyte homogenate; wells 3 and 4, 0.25 μL of urine; wells 5 and 6, 3 μL of serum phosphocellulose fraction 26 (Figure 1); wells 7 and 8, 0.5 μL of serum phosphocellulose fraction 33; wells 9 and 10, 0.3 μL of serum phosphocellulose fraction 43. The gel was incubated 24 h at 37 °C in 0.1 M Tris-HCl, pH 7.4, prior to staining. The arrow indicates a minor serum RNase noted under Discussion.

with a mobility similar to that of serum RNase 4; however, as documented in Figure 4, leukocyte RNase activity and RNase 4 have different properties.

Figure 3 is a schematic representation of the major serum, urine, CSF, and leukocyte RNases revealed by $NaDodSO_4$ gel electrophoresis and activity staining of samples heated in $NaDodSO_4$ and mercaptoethanol.

Presence of a Leukocyte RNase-like Activity in Serum and Urine. The RNase activity of leukocytes has different R_f values, depending upon whether the enzyme is exposed to 2% $NaDodSO_4$ at room temperature (Figure 4, well 1*) or whether it is heated in 2% $NaDodSO_4$ for 2 min at 100 °C (well 2*) prior to electrophoresis. Without heating (well 1*), the leukocyte activity comigrates with serum RNase 3, the latter having the same R_f value both with and without heating (wells 7 and 8). When heated (well 2*), the leukocyte enzyme moves faster, comigrating with serum RNase 4, shown in wells 9 and 10 to be of unchanged R_f on heating. The increment in mobility is an intrinsic property of the leukocyte enzyme as indicated by interconvertibility of its slow (unheated) and fast (heated) forms. Thus, a strip of gel containing the fast form can be excised, washed free of $NaDodSO_4$, crushed, and extracted to recover activity, a significant portion of which runs

as the slow form when applied, in 1% $NaDodSO_4$, to a second gel. Similarly, the slow form can be converted to the fast form by extracting it from the gel, treating it with 1% $NaDodSO_4$ at 100 °C, and rerunning it on a second gel.[6] Conversely, the R_f of serum RNase 3 is unaffected by heating in the presence of, and coelectrophoresis with, leukocyte homogenate.

Activity displaying the change in mobility characteristic of leukocyte RNase (shown again, heavily loaded, in wells 1 and 2 of Figure 4) is present in urine (wells 3 and 4) and in serum phosphocellulose fraction 26 (wells 5 and 6). The activity of serum chromatographs on the leading edge of phosphocellulose peak 2 (Figure 1A); its presence in fraction 26 is noted in Figure 2 by an "L". The leukocyte RNase-like activities of serum and urine appear more prominent (relative to the accompanying bands) in Figure 4 than in Figure 2; as discussed below, the differences in proportion reflect the absence from the samples in Figure 4 of the mercaptoethanol used to prepare the samples of Figure 2.

Discussion

Methods used in this study offer several advantages for analysis of physiological fluids and tissue homogenates. The resolving power of polyacrylamide gel electrophoresis and the sensitivity of activity staining are sufficiently high to permit examination of very crude samples. Inclusion of $NaDodSO_4$ in the electrophoresis buffers eliminates the aggregation to which serum RNases are subject (Schmuckler et al., 1975; Akagi et al., 1976), disrupts noncovalent complexes of RNases with other proteins [including, presumably, RNase inhibitors; e.g., see Blackburn et al. (1977)], causes anodal migration of both acidic and basic RNases, and permits estimation of molecular weight. Thus, the individual RNase species of unfractionated fluids and homogenates can be identified, their molecular weights estimated, their catalytic properties examined, and their response to various physical and chemical treatments ascertained—all without separation of individual RNases from one another or from the bulk of contaminating protein. Assessment of the heterogeneity of RNase preparations and comparison of the RNase complement of diverse samples are therefore simply and rapidly achieved.

Predictably, use of $NaDodSO_4$ in electrophoresis reduces the sensitivity of activity staining relative to that achieved in native gels. Inclusion of mercaptoethanol in the sample preparation further reduces sensitivity. Moreover, because all RNases do not renature at the same rate after exposure to these agents, the relative amounts of any two RNases visualized in a gel may differ from the relative amounts present in the sample(s) before denaturation. The latter fact is exemplified by the gels of Figure 1, displaying serum RNases fractionated on phosphocellulose. These gels, which permit assignment of electrophoretic species to chromatographic peaks, do not reflect the relative amounts of RNases 1–5 in serum, as revealed in the elution profile of Figure 1A. In fact, the amount of any RNase visualized in a gel depends, inter alia, upon how the sample is prepared for electrophoresis. Thus, omitting mercaptoethanol from the samples of Figure 4 allows clear visualization of two serum RNase activities in phosphocellulose fraction 26 which are visible only as trace bands in the reduced sample in Figure 2. One of these bands, labeled "L" in Figure 2 (well 2), is the leukocyte RNase-like activity seen as a prominent band in well 6 of Figure 4. [Because of its low pH optimum (Akagi et al., 1978a), this activity does not appear at all in the gel of Figure 1B, which was incubated at pH 8.5 to digest embedded RNA.] A second activity, indicated by arrows in Figures 2 and 4, is a minor serum RNase noteworthy for having an electrophoretic counterpart in urine designated band B (Sugiyama et al., 1981). This latter activity reproducibly elutes from phosphocellulose columns between the poorly resolved peaks 1 and 2, displays a molecular weight of 23 000, and is conspicuous for not conforming to the generally observed pattern of increasing R_f in $NaDodSO_4$ gels with increasing elution volume from phosphocellulose.

Are there other serum RNases, active at neutral to alkaline pH, which we have not detected because they renature very slowly, if at all, after electrophoresis? Several observations, some not illustrated here, bear on this question. First, for each of the five chromatographic peaks of serum RNase activity observed on phosphocellulose, there appears in gels a discrete band(s) which is (are) most intense in the respective peak tube(s), when equal volumes of sequential column fractions are loaded. [The five peaks together represent 90% of the total serum RNase activity measured at pH 8.5 (Akagi et al., 1976).] Second, the molecular weights estimated for the band(s) assigned to each peak are in reasonable agreement with the values found for RNases 1–5 by gel filtration (Akagi et al., 1976), indicating that the enzymes revealed by phosphocellulose and Sephadex chromatography are in fact seen in gels. Third, when mercaptoethanol is omitted from the column fractions of Figure 1B, thereby increasing the sensitivity of activity staining, the only bands which are conspicuously intensified are the two mentioned above.

Chromatographic heterogeneity of serum alkaline RNase activity was clearly demonstrated in the enlightening work of Akagi et al. (1976). RNases 1–5, separated on phosphocellulose, were shown by gel filtration to have molecular weights of 45 000, 32 000, 20 000, 13 000, and 8500, respectively. RNases 1–4 displayed properties of secretory ribonucleases and appeared catalytically similar to one another as well as to bovine pancreatic RNase A, though differences among them in heat stability, response to metal ions, and degree of preference for poly(C) were noted. The work of Akagi et al. hence raised the possibility that human serum contains secretory RNases having molecular weights significantly higher than any reported for the extensively studied pancreatic RNases of mammals.[7] Crucial to evaluation of this possibility is the question of whether the chromatographically separable RNases 1–4 are intrinsically different molecular entities or whether the high molecular weight RNases 1, 2, and 3 reflect, for example, aggregation of a typical M_r 14 000 RNase, and/or complex formation of such an RNase with other serum proteins. Akagi et al. addressed this question, stating that RNases 1–5 each rechromatographed true on phosphocellulose and that treatment of serum with 7 M urea prior to chromatography did not reduce the number of peaks. Nonetheless, the pronounced tendency of serum RNase activity to aggregate (Schmuckler et al., 1975; Akagi et al., 1976), and the paucity of data documenting the distinctness of RNases 1–4, suggested reexamination of the nature of the putatively high molecular weight activities described by these authors.

The present study, employing $NaDodSO_4$ gel electrophoresis, provides strong evidence that RNases 1–5 are in fact discrete molecular entities. The molecular weights estimated for RNases 1 and 2 in $NaDodSO_4$ gels are 20–30% lower than

[6] R. Sugiyama and C. A. Dekker, unpublished experiments.

[7] The molecular weight of RNase A is 13 690 (Richards & Wyckoff, 1971); glycosylated homologues such as porcine pancreatic RNase have weights as high as 22 400 (Reinhold et al., 1968).

values found earlier with Sephadex columns, suggesting that, for these species, aggregation and/or other factors elevate the gel filtration values. Nevertheless, our data support the finding of Akagi et al. that serum contains enzymes with both catalytic properties of secretory ribonucleases and molecular weights larger than those of known pancreatic RNases. Indeed, RNase 1 displays higher molecular weights in our gels than any of the multiple activity bands observed in crude preparations of porcine pancreas.[6,7] That glycosylation contributes to the large size of some of the serum RNases, and to the multiplicity of electrophoretically separable species, is suggested by the presence of carbohydrate in pure, RNase 1-like activity isolated from human urine.[6] If, in fact, some serum RNase species are glycosylated, their molecular weights may be overestimated in $NaDodSO_4$ gels (Segrest et al., 1971; Gordon, 1975) as well as on Sephadex columns (Whitaker, 1963; Andrews, 1964; Reinhold et al., 1968). Obviously, establishment of accurate molecular weights must await structural analysis. It is noteworthy that RNases 4 and 5 exhibit higher molecular weights in $NaDodSO_4$ gels than on Sephadex columns, suggesting interaction of these enzymes with Sephadex; such an interaction would account for the unlikely weight of 8500 found for RNase 5 by gel filtration.

In addition to RNases 1–5, serum contains an RNase activity eluting on the leading edge of phosphocellulose peak 2 (Figure 2, fraction 26, band designated "L") which is shown in Figure 4 to exhibit a distinctive, temperature-dependent alteration in mobility consequent to heating in 2% $NaDodSO_4$. The same alteration in mobility, which may reflect additional binding of $NaDodSO_4$ at higher temperatures, is displayed by the RNase activity of leukocytes. It is undoubtedly this serum activity which has been partially purified and characterized by Akagi et al. (1978a,b). These authors found their purified serum "acid RNase" to differ in substrate preference from leukocyte RNase and concluded, therefore, that serum "acid RNase" does not originate in white cells. However, it seems likely that their purified "acid RNase" was contaminated with alkaline RNase 2 (the major component of phosphocellulose peak 2), thereby accounting for the observed substrate preference and leaving open the possibility that serum "acid RNase" does derive from leukocytes. As for the data presented here, they complement existing, strong evidence that leukocytes are not the sole source of serum RNases (Houck & Berman, 1958); thus, much of serum RNase activity differs catalytically, chromatographically, and electrophoretically (Ressler et al., 1966; Blank & Dekker, 1977) from that of white cells.

Presumably for lack of availability, the RNases of human CSF have been investigated less extensively than those of serum and urine. Little is known of their physical and catalytic properties, although Rabin et al. (1977) have reported that the three incompletely separated peaks of activity observed on Sephadex G-100 are antigenically related to a purified human urine RNase (Rabin & Weinberger, 1975). The same authors have suggested that CSF RNase activity originates in blood. Some overall similarity in the electrophoretic profiles of serum and CSF RNase activity provides limited support for this hypothesis. Certainly, some activity bands seen in Figure 2 are common to both fluids. Yet, as noted earlier, CSF contains several activity bands which either were not detected in serum or were revealed in serum as minor components following chromatography. These findings suggest that, while CSF RNase activity could be mainly or entirely derived from blood, some mechanism(s) such as selective transport of certain RNases across the blood–brain barrier and/or conversion of plasma-derived RNases to CSF-specific counterparts may alter the composition of CSF RNase activity relative to that of plasma. It is also possible that some RNase activity found in CSF originates in tissue of the central nervous system.

A detailed electrophoretic study of urine ribonuclease activity appears in the following paper (Sugiyama et al., 1981) together with a discussion integrating the often confusing and conflicting literature on human urine RNases.

In conclusion, we emphasize that the high resolution and sensitivity of the methods we have employed, together with their technical simplicity and rapidity, recommend them for assessment of the heterogeneity of crude as well as purified RNase samples. Because the RNase activity of human fluids is highly heterogeneous, purified preparations may contain numerous species in widely varying proportions. It is thus desirable that electrophoretic analysis accompany description of the physical and catalytic properties of such preparations, so that the properties can be ascribed to a well-defined RNase or group of RNases; the difficulty currently inherent in analyzing and correlating existing descriptions of human ribonucleases [e.g., see Sugiyama et al. (1981)] will thereby be reduced.

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