Chemical, Physical, and Enzymic Properties of Several Human Ribonucleases*

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Two highly purified ribonucleases were isolated from urine of human males and a third from human spleen. Urine ribonucleases P and U were resolved on columns of IRC-50 at *p*H 5.8 in acetate-phosphate buffer. Ribonuclease P, which was precipitated by antisera to bovine pancreatic ribonuclease A, preceded ribonuclease U, which was not precipitated by antisera to ribonuclease A, from the column. In physical, chemical, and enzymic properties, ribonuclease P is identical with ribonuclease A except for amide content. In contrast, ribonuclease U differs from ribonucleases A and P in each property studied. Spleen ribonuclease, which has the chromatographic behavior of ribonuclease U on phosphorylated cellulose columns, differs only slightly from ribonuclease U in apparent molecular weight and amino acid composition.

Ribonuclease has often been the subject of investigations because of its high state of purity, stability, size, and known structure (Hirs *et al.*, 1960; Gross and Witkop, 1962; Potts *et al.*, 1962; Smyth *et al.*, 1962). In general, studies on the tissue or intracellular distribution of the enzyme have revealed heterogeneity whenever attempts were made to purify it (Bartos and Uziel, 1961; Maver and Greco, 1962). At present there is insufficient evidence to determine whether these enzymes are a group of closely related molecules or are two or more structurally dissimilar molecules.

The presence of ribonuclease in human urine was first reported by Laves (1952). Although urine was an adequate source, partial purification suggested that two components occurred (Dickman *et al.*, 1958). This report details the purification and partial characterization of these two ribonuclease components.[1] A preliminary report of this work has appeared elsewhere (Delaney and Brown, 1961).

Materials and Methods

Reagents.—All chemicals were reagent grade whenever available. Chromatographic solvents were used without further purification. For the colorimetric determination of tryptophan, glyoxalic acid (C grade) was obtained from California Corporation for Biochemical Research, glacial acetic acid reagent from Fisher Scientific Company (adequately free of glyoxalic acid impurity), and H_2SO_4 "Baker Analyzed" Reagent from J. T. Baker Chemical Company. Yeast nucleic acid (Schwarz Bio-Research, Inc.) was further purified (Vischer and Chargaff, 1948) before use. Cytidine-2′, 3′-phosphate was obtained as the Ba salt (Schwarz Bio-Research, Inc.) and converted to the Na salt by passage through Dowex 50 (Na^+ form) immediately before use.

Protein Purification.—All steps in the purification of the urinary and tissue ribonucleases were carried out at 0–5° except where specifically noted.

Ion-Exchange Resins.—Amberlite IRC-50 (XE-64) resin (Rohm and Haas Company) was screened, cleaned, and regenerated before use (Hirs *et al.*, 1953). Carboxymethylcellulose was prepared from Whatman cellulose powder and acetyl chloride (Peterson and Sober, 1956). The cellulose was regenerated with a solution that was 0.5 M NaOH and 0.5 M NaCl, and then washed with 1 N HCl followed by water before each use. Phosphorylated cellulose, Cellex P (Bio-Rad Laboratories), was used once and discarded. Preliminary experiments with bovine ribonuclease showed that some protein remained on the resin. However, few data were available on the stability of the phosphorylated celluloses under regeneration conditions. Rather than risk changing the properties of the resin, a new sample was used for each chromatography.

Preparative and Analytical Columns.—The use of mixtures of phosphate and acetate buffers with IRC-50 near *p*H 5.8–6.0 necessitated several precautions for reproducibility. The resin was adjusted to the desired *p*H by addition of Na salts of the particular buffer to be used at the same total anion concentration as that of the final buffer. The resin was then cooled to 0–5° and washed with cold buffer until the *p*H of the filtrate and buffer remained within 0.02 units. The resin was transferred to glass columns and equilibrated in the cold by passage of buffer through it until the influent and effluent buffers were within 0.02 *p*H units.

Enzyme Activity Measurements.—The method of Anfinsen *et al.* (1954) was used to assay for ribonuclease through the bulk of the fractionation. Different fractions did not yield superimposable standard curves, owing in part to varying amounts of the two components. When column effluents in strong phosphate acetate solutions were assayed, the procedure was modified by use of a buffer of the same composition and *p*H as those of the particular column effluent to control the inhibitory effects of ionic strength and phosphate ions.

A qualitative assay was used to detect enzyme activity in the carboxymethylcellulose column effluent fractions. A drop of effluent was added to 2 ml of 0.4% ribonucleic acid in Veronal-buffered NaCl, ionic strength 0.1. The mixtures were incubated at 60° for 1 hour. Turbidity was estimated visually after the addition of 0.2 ml of 0.75% uranyl acetate in 25% perchloric acid. The lower limit of detection was 1 μg of ribonuclease A.

* Parts of this work are from a dissertation by R. Delaney to be submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree, Albany Medical College of Union University.

[1] The following names are used for the ribonucleases discussed in this paper: ribonuclease A, the principal chromatographic component of bovine pancreatic ribonuclease; urinary ribonuclease fraction, the mixture of human ribonucleases present in the major chromatographic component at *p*H 6.0; ribonuclease P, the major component of urinary ribonuclease that possesses the immune activity of ribonuclease A; ribonuclease U, the major component of urinary ribonuclease that lacks the immune activity of ribonuclease A; spleen ribonuclease, the ribonuclease of human spleen that has the chromatographic properties of ribonuclease U.

Amino Acid Analyses.—Aliquots were taken from a carefully dialyzed stock solution of each protein. Analyses were made for Kjeldahl N on duplicate aliquots. Another aliquot was lyophilized and dried to constant weight over P_2O_5 at room temperature. The sample, usually 2 mg, was dissolved in 1.0 ml of constant boiling HCl (triple distilled), flushed with nitrogen, evacuated, sealed, and heated to 110° for 22, 70, or 116 hours. The samples were freed of excess HCl on a rotary evaporator, treated to oxidize any cysteine present (Hirs *et al.*, 1954), and brought to 2.5 ml in *p*H 2.19 citrate buffer. Aliquots of this solution were analyzed for Kjeldahl N and the data used in calculating the N and dry weight recoveries. The gradient elution procedure of Piez and Morris (1960) was used for amino acid analysis. Corrections for destruction of labile amino acids during hydrolysis were made, assuming first-order kinetics (Hirs *et al.*, 1954).

Tryptophan was determined by a modification of the glyoxalic acid colorimetric procedure of Shaw and McFarlane (1938). Each sample of dried protein was dissolved in 20 μl of water and mixed with 500 μl of glyoxalic acid in 99% acetic acid (70 mg per liter) followed by 200 μl of concentrated H_2SO_4 with rapid mixing. After 4 minutes, the chromophore was developed by the addition of 20 μl of aqueous $NaNO_2$ (173 mg per liter). The absorbancy was determined at 555 mμ until it reached a maximum. Tryptophan was kept between 1 and 10 μg per determination.

Protein Concentrations.—The protein concentrations of all crude fractions were based on the weight of the dialyzed and lyophilized sample. The final preparations were kept as frozen solutions from which suitable aliquots were dried *in vacuo* to constant weight over P_2O_5 or analyzed by Kjeldahl digestion, ammonia distillation, and Nesslerization.

Ultracentrifugation.—Equilibrium sedimentations were carried out at 25.0° on a Spinco Model E ultracentrifuge fitted with an RITC temperature control. A standard double-sector cell containing a 3-mm column of solution was used (Van Holde and Baldwin, 1958).

Immunologic Tests.—Concentrations of ribonuclease that reacted with rabbit antiribonuclease A were determined by comparing the rate of movement of the band of precipitation in the single diffusion method of Oudin (1948) with that given by known concentrations of ribonuclease A.

Precipitin tests were carried out by the standard procedures described by Kabat and Mayer (1961). The precipitates were measured by the reaction with the Folin-Ciocalteu reagent.

The rabbit antiserum was prepared and tested for homogeneity of the immune system as previously described (Brown *et al.*, 1959). Ribonuclease A was used as the antigen and evoked in this serum a single immune system directed only to ribonuclease.

Results

Isolations of Human Ribonucleases.

1. Preparation of Urinary Ribonucleases.—Approximately 15-liter lots of fresh urine from human males, with thymol as a preservative, were collected and fractionated daily by the method of Dickman *et al.* (1958) through the alcohol and $(NH_4)_2SO_4$ precipitation steps with one modification. The water extract of the alcohol-precipitated protein was adjusted to *p*H 2.5 with 6 N HCl and warmed to room temperature for 15 minutes to activate any uropepsinogen present. The *p*H was adjusted to 8 with 6 N NaOH to denature the activated uropepsin, and the precipitate that formed was centrifuged down and discarded. The solution was returned to *p*H 2.5 and cooled, and the $(NH_4)_2SO_4$ steps were carried out. The final $(NH_4)_2SO_4$ precipitates from 2000 liters of urine were pooled, after dialysis and lyophilization, and stored at −24°.

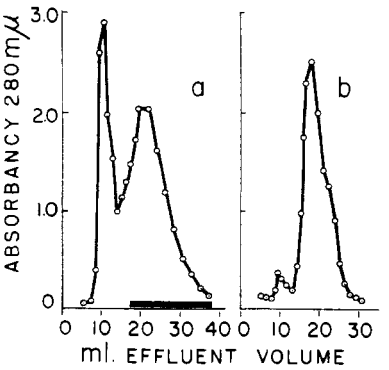

Fig. 1.—Chromatograms of urine ribonuclease on IRC-50 with *p*H 6.0 buffer, 0.2 M in phosphate and 0.2 M in acetate. *a*, Carboxymethylcellulose fractionated pool of urine ribonucleases, 100 mg. *b*, Urine ribonuclease fraction, 25 mg, from a pool of equivalent regions (bar) in *a*.

The pool, in 2- to 4-g amounts, was absorbed on 2.2 × 30 cm columns of carboxymethylcellulose equilibrated with *p*H 5.4 Na acetate buffer, 0.03 M. The column was washed with 10 column volumes of buffer. A solution of 1 M Na acetate was next applied directly to the top of the resin and continued until the ribonuclease activity was completely eluted. This absorption and elution procedure was repeated a second time with the columns equilibrated with *p*H 5.4 Na acetate buffer, 0.2 M, and the activity eluted with the 1 M Na acetate solution.

The active fractions from the second carboxymethylcellulose chromatography were applied to 0.9 × 30 cm columns of IRC-50 equilibrated at *p*H 6.0 with a buffer containing 0.2 M Na acetate and 0.2 M Na phosphate (Fig. 1*a*). The effluent between 17.5 and 38 ml was pooled from each column. This material, referred to as urinary ribonuclease fraction, though apparently homogeneous on rechromatography at *p*H 6 (Fig. 1*b*), was resolved into two major regions of enzyme activity by chromatography at more acid *p*H and lower salt concentrations.

Two preparative columns (0.9 × 30 cm), equilibrated with *p*H 5.80 buffer, 0.12 M in Na acetate and 0.2 M in Na phosphate, were loaded with 78.6 and 94.4 mg of urinary ribonuclease in the same buffer (Fig. 2*a*). The effluent between 2.1 and 3.9 column volumes was pooled from each column and reapplied to another column under the same conditions. The effluents were pooled (Fig. 2*b*) to eliminate the minor impurity just preceding the ribonuclease. This pool, ribonuclease P, contained 27 mg of dry weight.

The ribonuclease (38 mg) from the pool of effluent between 4 to 14 column volumes (Fig. 2*a*) was examined for homogeneity on a phosphorylated cellulose column (0.9 × 30 cm) equilibrated with *p*H 5.08 buffer that was 0.12 M in Na acetate and 0.2 M in Na phosphate (Fig. 3*a*). There was no indication of an impurity in the effluent between 1.66 and 3.73 column volumes as assessed by specific activity determinations. The subpool of 2.9 to 3.73 column volumes had the same specific activity as the earlier fractions and was combined with them. The 18.9 mg of ribonuclease recovered from the phosphorylated cellulose column effluent was called ribonuclease U. The preparation of the two urinary ribonucleases and the data on yields are summarized in Table I.

TABLE I
PREPARATION OF HUMAN URINARY RIBONUCLEASES P AND U

Purification Step	Protein (g)	Activity[a] (mg)	Specific Activity (mg/g)	Yield (%)
Urine		1320		100
0.45-0.85$(NH_4)_2SO_4$ precipitate	88	440	5	33
1st carboxymethylcellulose chrom.	3.5	400	114	30
2nd carboxymethylcellulose chrom.	0.74	200	270	15
IRC-50, *p*H 6.0	0.20	100	500	7
IRC-50, *p*H 5.8, ribonuclease P	0.027	27	1000	2
IRC-50, *p*H 5.8, ribonuclease U	0.078	31	400	2
Phosphorylated cellulose, *p*H 5.08	0.0189	18.9[b]	1000	1

[a] Activity is expressed in mg of ribonuclease A equivalent activity and the specific activity as mg of equivalent activity per g of protein. [b] Assayed at *p*H 6.5, where ribonuclease U possesses maximum activity.

When ribonuclease U was chromatographed on phosphorylated cellulose, only 50% of the activity was elutable from the column. Although 74 mg of protein containing the ribonuclease U was isolated from the *p*H 5.8 effluent (Fig. 2*a*), only 38 mg of it was chromatographed at *p*H 5.08; the remainder was lost in exploratory chromatography on phosphorylated cellulose.

2. PREPARATION OF HUMAN SPLEEN RIBONUCLEASE. —Normal spleens were immersed in cold 0.25 N H_2SO_4 at autopsy, 3 to 15 hours after death. Enzyme assays were performed on the spleen fraction after the heating step (see below). The concentrations of the enzyme in terms of ribonuclease A varied from 3.8 to 5.6 $\times$ 10^{-3} mg of ribonuclease A equivalents per g of spleen wet weight. The yields appeared to bear no relation to the time elapsed before the organ was immersed in the cold acid.

The spleens were weighed, minced, and extracted overnight with a volume of cold 0.25 N H_2SO_4 equal to their weight. The pulp was filtered on S and S No. 520-B fluted paper and resuspended for 4 hours in half the original volume of cold 0.25 N H_2SO_4. The filtration was repeated and the pulp discarded. The pooled filtrates were brought to 0.45 saturation by addition of 260 g of $(NH_4)_2SO_4$ per liter of filtrate, left for 2 hours, and filtered through Whatman No. 3 paper on a Buchner funnel by suction. The chocolate-brown filter cake was discarded and the yellow filtrate brought to 0.85 saturation by addition of another 260 g of $(NH_4)_2SO_4$ per liter. After 16 hours, the precipitate was collected on Whatman No. 50 paper by suction. The filtrate was discarded. The precipitate was weighed and then dissolved in ten times its weight of 0.05 M Na acetate, which was 1 $\times$ 10^{-4} M with respect to ethylenediaminetetraacetic acid. The *p*H was quickly adjusted to 3.5 with 1 N HCl. The samples were placed in Erlenmeyer flasks with about twice the capacity of the sample, a thermometer was inserted through a single-holed stopper in the neck of the flask, and the flask was swirled rapidly with little foaming in an 80° water bath until the temperature of the sample reached 60°. After 10 minutes at 60°, the flask was cooled to 4°, the *p*H adjusted to 6.5–7.0 with 1 N NaOH, and the solution brought to 0.8 saturation by addition of 49.4 g of $(NH_4)_2SO_4$ per 100 ml (Kaplan and Heppel, 1956). After the flask had stood overnight, the precipitate was collected by centrifugation, dissolved in a minimal amount of water, and dialyzed free of sulfate. This fraction was carried through the second carboxymethylcellulose step and the IRC-50, *p*H 5.8, chromatography. The ribonuclease activity was present in the same regions of column effluent as was ribonuclease U. Careful examination of all effluents failed to reveal any trace of immune activity that might correspond to ribonuclease P.

The final purification of this preparation was the same as for ribonuclease U (Fig. 3*b*). The pooled

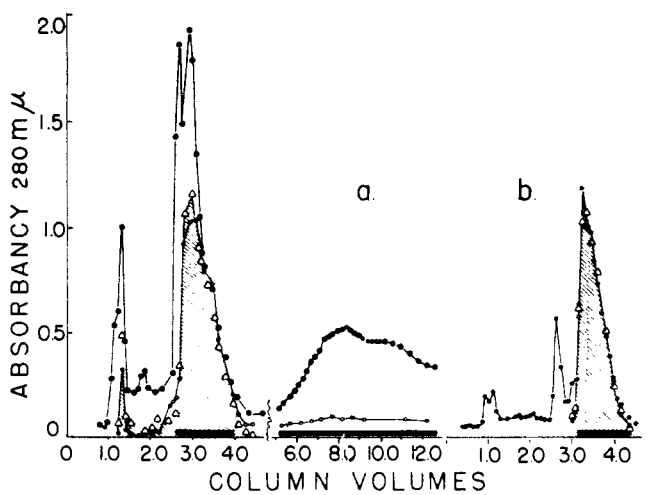

FIG. 2.—Chromatograms on IRC-50 at *p*H 5.80 in buffer, 0.2 M in phosphate and 0.12 M in acetate. *a*, Urine ribonuclease fraction from IRC-50, *p*H 6.0, chromatography, 94.4 mg. *b*, Rechromatography of volumes 2.6 to 3.95. The horizontal bar indicates regions pooled for subsequent study. The hatched area represents normalized immune activity. Experimental points represent immune activity (△), enzyme activity (○), and absorbancy at 280 mμ (●).

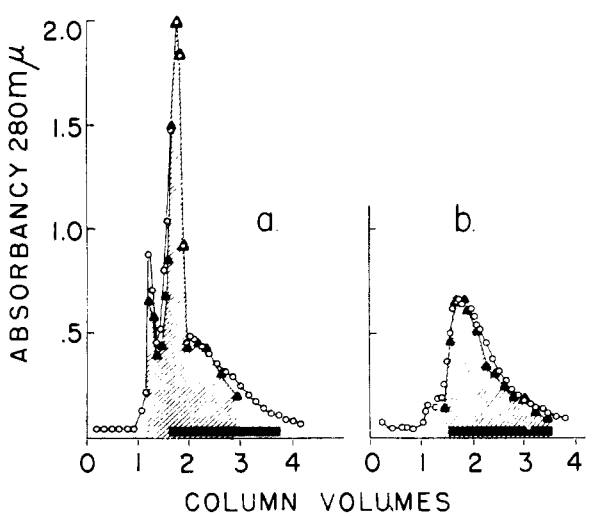

FIG. 3.—Chromatograms of ribonucleases on phosphorylated cellulose at *p*H 5.08 in buffer, 0.2 M in phosphate and 0.12 M in acetate. *a*, Ribonuclease U, 38 mg. *b*, Spleen ribonuclease, 15 mg. Experimental points represent absorbancy at 280 mμ (○) and normalized enzyme activity (▲). Bar represents regions pooled for subsequent study.

TABLE II
MOLECULAR WEIGHTS OF RIBONUCLEASES

	Conc. (g/dl)	$\bar{v}$[a] (ml/g)	M_w	M_z	Av.
Bovine pancreatic ribonuclease A	1	0.692	13,800	13,750	13,780
	0.5		13,560	13,050	13,300
Human urinary ribonuclease P	0.15	0.692	13,800	13,740	13,770
Human urinary ribonuclease U	0.15	0.698	18,300	19,390	18,840
Human spleen ribonuclease	0.3	0.696	18,410	18,230	18,320

[a] Calculated values as explained in the text.

fraction, spleen ribonuclease, totaled 11 mg from 1,868 g of spleen.

3. PREPARATION OF HUMAN PANCREATIC RIBONUCLEASE.—Normal pancreases were immersed and fractionated in the same manner as the spleens up to the heating step. The 0.45–0.85 saturation precipitate was weighed as the filter cake and dissolved in 5 ml of water per g of filter cake, and 4 volumes of 0.2 saturated (room temperature) $(NH_4)_2SO_4$ solution were added (McDonald, 1948). The $(NH_4)_2SO_4$ solution had been adjusted to *p*H 3.5 and preheated to 90° just before addition. The mixture, in a stoppered Erlenmeyer flask fitted with a thermometer, was swirled in a boiling water bath until the sample reached 90–95°. It was kept at this temperature for 5 minutes with rapid swirling and then cooled to room temperature in ice water; after one hour, 420 g of solid $(NH_4)_2SO_4$ was added per liter of solution. The precipitate recovered by centrifugation was dissolved in a small quantity of water, dialyzed, and lyophilized.

Absorption and elution from carboxymethylcellulose of the ribonuclease in this preparation yielded only 0.24 mg of activity from 550 g of pancreas. It was then applied to IRC-50 resin at *p*H 5.8 in the mixed acetate-phosphate buffer. No definite peak of enzyme or immune activity was detected in the effluent, especially between 3 and 4 column volumes.

Chemical and Physical Studies.

1. ELECTROPHORESIS AND ULTRACENTRIFUGATION.—A 1% solution of the urinary ribonuclease fraction in *p*H 8.6 Veronal buffer, ionic strength 0.1, was examined for homogeneity by free boundary electrophoresis. The single symmetric boundary moved toward the cathode.

Apparent molecular weights were determined for each ribonuclease by equilibrium sedimentation. The partial specific volumes were calculated (Cohn and Edsall, 1943) from the amino acid composition of the respective protein and corrected for electrostriction of the charged pairs at the isoelectric point. When a correction for volume change due to the protonation of amino groups (Rasper and Kauzmann, 1962) in the protein at *p*H 7.7 as opposed to its isoelectric point, *i.e.*, 5 H^+, was used, the partial specific volume of ribonuclease A was reduced from 0.694 to 0.688 ml per g. If, however, correction is made for the binding of phosphate to ribonuclease A (Crestfield and Allen, 1954b), the volume correction becomes very small, *i.e.*, 1 H^+, and the partial specific volume would be 0.692 at *p*H 7.7. This value was used for ribonucleases A and P. Spleen ribonuclease and ribonuclease U were treated in the same manner, except that no correction was made for phosphate binding.

The apparent molecular weights were calculated by methods I and II (Van Holde and Baldwin, 1958) to obtain M_w and M_z, respectively. The results of single determinations in Table II indicate a similarity of ribonucleases A and P. The large difference between M_w and M_z for ribonuclease U is not inconsistent with the possible presence of 1% dimer. The value of 18,320 was used as the molecular weight of both spleen ribonuclease and ribonuclease U.

2. THE AMINO-TERMINAL END-GROUPS.—When the urinary ribonuclease fraction (7.6 mg) or ribonuclease A was treated with fluorodinitrobenzene and hydrolyzed, the only amino acid derivatives found were *bis*-dinitrophenyllysine and ϵ-dinitrophenyllysine. The actual recovery of lysine end-group was 0.13 μmoles for urinary ribonuclease and 0.21 μmoles for ribonuclease A. Since the urinary ribonuclease fraction was later resolved into two components of different molecular weights, the μmoles of end-group per μmole of protein could not be calculated.

3. AMINO ACID COMPOSITION.—The composition of ribonuclease P was calculated from the average of two determinations (22-hour hydrolysates), corrected for destruction or incomplete liberation of amino acids from single 70- and 116-hour hydrolysates. The losses of serine, threonine, cystine, and tyrosine and the slow liberation of isoleucine and valine were consistent with previous reports for ribonuclease A (Hirs *et al.*, 1954). The composition of ribonuclease A, determined in this laboratory for comparative purposes, bears a close resemblance to that of ribonuclease P except for amide content (Table III). Ribonuclease U and spleen ribonuclease were calculated on the basis of a single determination on 22-hour hydrolysates and both corrected for destruction or incomplete liberation of amino acids by a single 70-hour hydrolysate of the former. There is a basic similarity in the data for these two enzymes, but the validity of their differences cannot be assessed on this evidence alone. The calculated amide content of spleen ribonuclease exceeded the total number of carboxyl groups available in the protein and most probably resulted from the presence of a nitrogenous, nonamino acid substance that contaminated the hydrolysate.

All compositions are expressed on the basis of 100% recovery of the dry weight as amino acids. The actual nitrogen and dry weight recoveries (Table III) were in good agreement except for the low weight recovery of spleen ribonuclease. An extensive examination of the sample to account for the unrecovered dry weight was precluded by the lack of sample. However, the ultraviolet absorption spectra of spleen and ribonuclease U revealed an abnormally large absorption between 260 and 280 mμ which was consistent with the presence of approximately 1 mole of cytidylic acid per mole of each protein. Not enough material was available to isolate and identify it. The remainder of the missing dry weight was not identified.

Enzyme Studies.—Uridine nucleotides, released from ribonucleic acid by each human ribonuclease at *p*H 7.5 and soluble at *p*H 4.0 in uranyl acetate–perchloric acid (Dickman and Trupin, 1959), were separated by electrophoresis on paper in *p*H 7.5, 0.2 M phosphate. Samples were analyzed after 10-minute, 20-hour, and 96-hour incubations with 20 μg per ml of each enzyme at 36°. The pattern of uridine nucleotides, visualized with an ultraviolet light source at 260 mμ, was similar

TABLE III
THE AMINO ACID COMPOSITION OF RIBONUCLEASES

	Amino Acid Residue (100 g Protein)				Residues (Mole Protein[a])			
	A	P	U	Sp	A	P	U	Sp
Aspartic acid	13.0	13.0	16.4	15.1	15.4	15.4	26.1	24.1
Threonine	7.8	7.6	7.3	6.7	10.6	10.3	13.2	12.1
Serine	9.5	9.7	3.3	4.1	14.9	15.3	7.0	8.6
Glutamic acid	11.4	11.7	13.1	12.4	12.0	12.4	18.6	17.5
Proline	3.0	3.2	7.9	7.8	4.2	4.5	14.9	14.7
Glycine	1.3	1.3	1.2	1.7	3.2	3.1	3.7	5.5
Alanine	6.4	6.2	2.9	3.0	12.3	11.8	7.5	7.8
1/2 Cystine	5.3	5.6	4.6	4.6	7.1	7.5	8.3	8.3
Valine	6.2	6.3	5.7	5.4	8.6	8.7	10.6	10.0
Methionine	4.0	3.6	3.2	2.9	4.1	3.7	4.4	4.0
Isoleucine	2.6	2.8	4.9	5.0	3.1	3.0	8.0	8.1
Leucine	1.7	1.8	3.7	4.1	2.1	2.1	6.0	6.7
Tyrosine	6.8	7.0	4.2	4.8	5.7	5.8	4.8	5.4
Phenylalanine	3.0	3.3	4.8	4.8	2.8	3.0	5.9	6.0
Lysine	9.8	9.0	3.6	4.5	10.5	9.6	5.1	6.4
Histidine	3.8	3.9	4.5	4.4	3.8	3.9	6.0	6.0
Arginine	4.8	4.5	7.8	7.6	4.2	3.9	9.2	8.9
Tryptophan			1.0	1.0			1.0	1.0
Amide					18.1	14.4	32.9	(49.8)
Actual weight recovery	93	92	93	69				
Actual N recovery	96	100	96	92				

[a] The abbreviations used and molecular weights for each ribonuclease are as follows: A, ribonuclease A, and P, ribonuclease P, 13,680; U, ribonuclease U, and Sp, spleen ribonuclease, 18,320.

for each human ribonuclease and ribonuclease A. Uridine-2',3'-phosphate and oligonucleotides were present in the 10-minute and 20-hour digests. In the 96-hour digest, uridine-2' or 3'-phosphate was first detected. The uridine phosphates in the 96-hour digests all had the same mobility as uridine-3'-phosphate upon electrophoresis at *p*H 12.8 (Crestfield and Allen, 1954a). Neither the free base nor the nucleoside was detected in any of the digests.

The *p*H dependence of the ribonucleases was determined with 0.1% ribonucleic acid between *p*H 5.0 and 9.0 by the spectrophotometric method of Kunitz (1946). The relative activities are presented in Figure 4. K_m and V were evaluated for the hydrolysis of ribonucleic acid by the four enzymes at *p*H 8.0. Substrate was varied between 0.2 and 2.0 mg per ml and incubated at 25° with 2×10^{-9} M enzyme. V and K_m for ribonucleases A and P (1.2×10^{-3} mg per ml-sec and 0.14 mg per ml) and for spleen ribonuclease and ribonuclease U (0.37×10^{-3} mg per ml-sec and 0.51 mg per ml) were not expressed in moles per liter because of the nature of the substrate. They were calculated by the method of least squares for the combined data of ribonucleases A and P and for the combined data of spleen ribonuclease and ribonuclease U (Fig. 5).

The hydrolysis of cytidine-2',3'-phosphate was determined as described by Litt (1961) over a substrate concentration of 0.6×10^{-3} M to 6.9×10^{-3} M. K_m and V were calculated by the method of least squares for a Lineweaver-Burk plot of the combined and

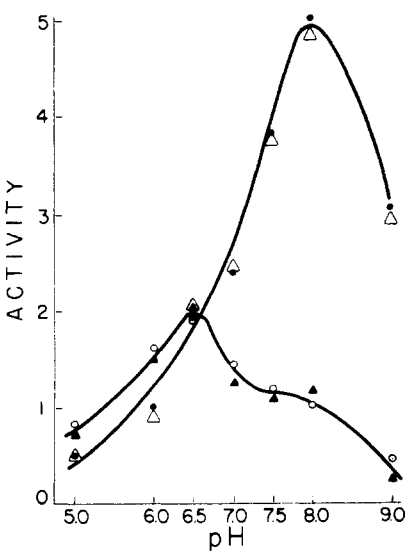

FIG. 4.—The dependence of enzyme activity on *p*H in the hydrolysis of 0.1% ribonucleic acid by: ribonuclease A (●), ribonuclease P (△), ribonuclease U (▲), and spleen ribonuclease (○). The temperature was 25° and ionic strength, 0.1.

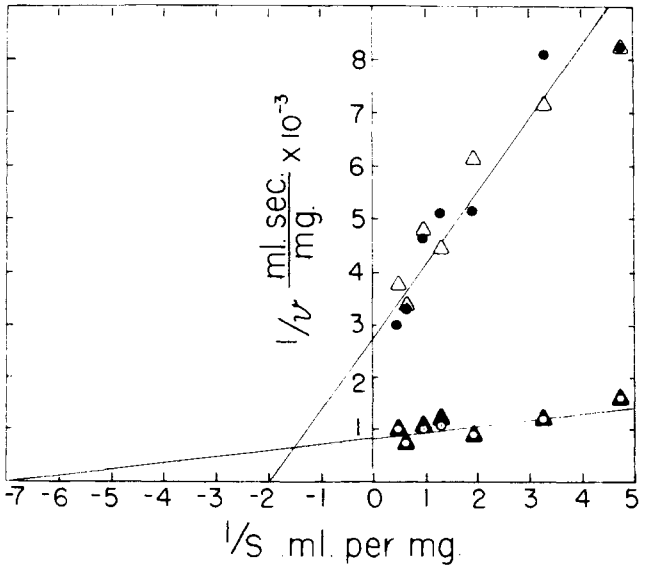

FIG. 5.—The dependence of velocity on ribonucleic acid concentration at *p*H 8.0 and ionic strength 0.1 for the following: ribonuclease A (○), ribonuclease P (▲), ribonuclease U (△), and spleen ribonuclease (●). The lines were calculated from the data by the method of least squares.

individual data from ribonucleases A and P (Table IV). The data represent single determinations for each enzyme, so their kinetic constants are included only for comparative purposes along with the values of Litt (1961), determined at a lower temperature and ionic strength. Attempts to measure initial rates of hydrolysis of cytidine-2',3'-phosphate by either spleen ribonuclease or ribonuclease U were complicated by spectral shifts occurring after the addition of high concentrations of enzyme. Only approximate initial rates could be calculated; these were an order of magnitude lower than with ribonuclease A.

TABLE IV

KINETIC CONSTANTS FOR THE HYDROLYSIS OF CYTIDINE-2',3'-PHOSPHATE

Experiments were performed at 30° in *p*H 7.9 Tris buffer, 0.3 ionic strength. The combined values were calculated from the combined experimental points of both ribonuclease A and ribonuclease P.

	V ($\times 10^{-7}$ M sec.$^{-1}$)	K_m ($\times 10^{-3}$ M)	V/E_0[a] (sec.$^{-1}$)
Bovine pancreatic ribonuclease A	6.5	5.9	5.2
Human urine ribonuclease P	4.1	3.6	3.2
Combined	5.6	5.1	4.4
Bovine pancreatic ribonuclease A[b]	3.0	6.0	2.4

[a] E_0 is the total concentration of enzyme and V/E_0 is the molecular activity. [b] Values reported by Litt (1961) at 25° in *p*H 8.0 Tris buffer, 0.1 ionic strength.

Immunologic Studies.—Varying concentrations of ribonuclease A or ribonuclease P, in 0.2 ml of *p*H 7.5 buffer (0.005 M with respect to Tris and 0.15 M with respect to NaCl) were added to equal volumes of rabbit antiribonuclease A. After incubation for 7 days at 2°, the precipitates were recovered, washed, and measured (Fig. 6). The supernatants from the series were tested for the presence of reactive antigen or antibody. The tube representing equivalence for the reaction of ribonuclease P and antibody was also tested with ribonuclease A for the possible presence of antibody which did not react with the ribonuclease P but would react with ribonuclease A. No precipitation occurred. The amount of antibody precipitated by ribonuclease A was only slightly greater than the amount precipitated by ribonuclease P.

When spleen ribonuclease or ribonuclease U was diffused into agar-antibody mixtures of rabbit antiribonuclease A at concentrations of protein 160 times greater than necessary to produce a band of precipitation with either ribonuclease A or P, there was no visible precipitation. From this, the upper limit for the possible amount of ribonuclease P contamination in ribonuclease U was set at 0.6%.

DISCUSSION

During the procedures used in isolating the ribonuclease from urine, heterogeneity of the enzyme was not apparent until its treatment with IRC-50 at *p*H 5.80 in strong phosphate solutions. In retrospect, poor recoveries in any of the fractionation steps (Table I) could have been due to the loss of a specific ribonuclease with physical characteristics at variance with the ribonucleases isolated. In all cases the isolated activity was the major part of the activity recoverable. The isolation of spleen ribonuclease was purposely intended to parallel the conditions of preparing ribonuclease U. Improvements in yield were not considered. The

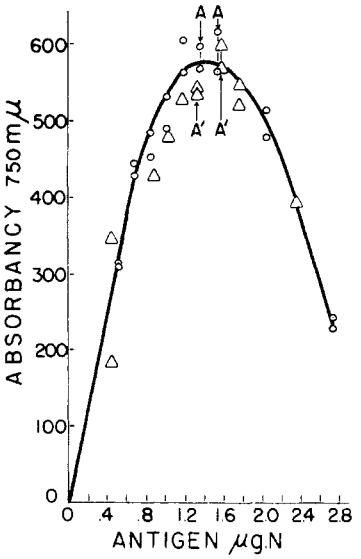

FIG. 6.—Precipitation of rabbit antibody by the homologous antigen, ribonuclease A (○), and the heterologous antigen, ribonuclease P (△). The arrows, A and A', indicate the regions of equivalence for ribonuclease A and ribonuclease P respectively.

failure to isolate suitable ribonuclease from human pancreas may have been due to destruction in the organ, to denaturation during the heating step, or to some unknown factor.

The preparation used for electrophoretic and end-group analysis was the urinary ribonuclease fraction which was later resolved into ribonuclease P and U. From its amino acid composition, it is highly probable that ribonuclease P has an amino-terminal lysine. The nature of the end-group of ribonuclease U is questionable, although the amount of *bis*-dinitrophenyllysine recovered from the urinary ribonuclease fraction was compatible with the presence of lysine as the amino-terminal residue of both ribonuclease P and U.

With respect to the parameters studied, ribonuclease A and ribonuclease P were quite similar. Their molecular weights and amino acid compositions were the same within experimental error. The difference in amide content as calculated from the amino acid analyses was in agreement with the low retention to IRC-50 at *p*H 6.0 under conditions that favor the strong retention of ribonuclease A. Further evidence of their structural similarity was the ability of ribonuclease P to combine with all the antibody in the antiserum capable of reacting with ribonuclease A. Sheep pancreatic ribonuclease, which differs from ribonuclease A at only three residues (Åqvist and Anfinsen, 1959), was not able to precipitate all the antibody from rabbit antiribonuclease A, which has a low molar combining ratio of antibody to antigen (Brown *et al.*, 1960). However, the interchange of any residues at points not involved with reaction with the antibody may not be detectable by this technique. The *p*H dependence of their activity with ribonucleic acid as a substrate, as well as the values of K_m and V for ribonucleic acid and cytidine-2',3'-phosphate, supports the evidence that they are the same enzyme.

Similarly, the investigated parameters of ribonuclease U and spleen ribonuclease emphasized their likeness and set them apart from ribonuclease A and ribonuclease P. Although ribonuclease U and spleen ribonuclease were most active against ribonucleic acid at *p*H 6.5, the values of K_m and V were determined at *p*H 8.0, the optimum *p*H for the pancreatic enzyme, in order that all four enzymes could be compared under like conditions.

Both ribonuclease U and spleen ribonuclease were able to hydrolyze cytidine-2′,3′-phosphate at *p*H 7.0, but the spectrophotometric method did not allow the calculation of accurate rates. The lack of agreement in the molecular weights and amino acid compositions of these two enzymes, although based on single determinations, casts doubts on their identity. The probable contribution to urinary ribonucleases by tissues other than the pancreas and spleen should not be overlooked. The discrepancy in molecular weight and amino acid composition may be due to the presence of other tissue ribonucleases that are not separable from spleen ribonuclease by the procedure employed. If they differed from spleen only by the substitution of several residues and not in enzyme activity, the discrepancy would be explained.

Further evidence for the similarity of ribonuclease A and ribonuclease P and a more complete characterization of ribonuclease U and spleen ribonuclease, obtained from peptide maps and substrate specificity studies, will be presented in a subsequent report.

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