The Effects of Polyamines on a Residue-specific Human Plasma Ribonuclease

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SUMMARY

A ribonuclease, purified some 2700-fold from human plasma, exhibited a strong predilection for the hydrolysis of internucleotide bonds containing cytidylic acid. Analysis of [3'-32P]- and [5'-32P]phosphoryl-terminal fragments obtained after enzymic digestion of rabbit liver and yeast RNA indicated that the nucleotide found at the 3'-terminus of the fragments was invariably cytidylic acid. The nucleotide at the 5'-terminus varied between cytidylic and uridylic acids in a ratio of 9:1. When characterized by DEAE-cellulose chromatography, approximately 70% of the digest consisted of oligonucleotides from 4 to 8 nucleotides in length.

Enzyme activity, when measured in low ionic strength buffer, could be increased severalfold above control levels by the addition of either of the polyamines, spermidine or spermine. These substances also restored nucleolytic activity to preparations inhibited by such ordered synthetic polyribonucleotides as polyguanylic acid. Estimations of the molecular weight of the enzyme, both by Sephadex gel filtration and sucrose density centrifugation, indicate that the weight may vary, depending on the presence or absence of certain cations. Of the cations examined, spermidine and spermine appear to have the greatest effect, causing an alteration in molecular weight from greater than 150,000 to approximately 32,000.

EXPERIMENTAL PROCEDURE

Materials

Human plasma was purchased from a commercial blood bank or was obtained from volunteers. Yeast RNA, purified by the method of Crestfield et al. (7) was prepared in the laboratory. Rabbit liver RNA was purified by the method of Delilhas and Staehelin (8).

The following materials were obtained from commercial sources: synthetic polynucleotides (Miles Laboratories, Kankakee, Ill., or Schwarz-Mann, Orangeburg, N. Y.); Sephadex G-75, G-100, and CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.); active cellulose phosphate (Reeve Angel, Clifton, N. J.) prepared for use according to the procedure outlined in the distributor's brochure; Escherichia coli alkaline phosphatase (Worthington Biochemical Corp., Freehold, N. J.) used according to the method of Garen and Levinthal (9); snake venom phosphodiesterase (Sigma Chemical Co., St. Louis, MO.) freed of 5'-nucleotidase and used as described by Koerner and Sinsheimer (10); E. coli B polynucleotide kinase and Micrococcus luteus polynucleotide phosphorylase (P-L Laboratories, Inc., Milwaukee, Wis.) used according to the procedures of Weiss and Richardson (11) and of Moses and Singer (12), respectively; dialysis tubing (Union Carbide, Chicago, Ill.) freed of impurities by heating at 60° for 30 min in 1 mM EDTA; sodium adenosine 5'-(32P)triphosphate (New England Nuclear, Boston, Mass.); fluorocarbons for protein assays (Hoffmann-La Roche, Inc., Nutley, N. J.); bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill., or Schwarz-Mann); and ECTEOLA-cellulose (epichlorohydrin triethanolamine cellulose) thin layer plates (Analtech, Inc., Newark, Del.).

Methods

Assay of Human Plasma Ribonuclease—The standard assay system contained 1.5 μmol of polymer or 1.0 mg of RNA, 100 μmol of buffer (Tris-HCl, pH 7.5, or (C), or sodium phosphate, pH 8.0, for RNA), and 0.5 mg of bovine serum albumin and enzyme in 1 ml. After incubation for 15 min at 25°, the reaction was stopped by the addition of 1 ml of 2 N perchloric acid and the reaction vessel chilled for 10 min in an ice bath. The cloudy reaction mixture was clarified by centrifugation and the absorb-

It has been shown that the polyamines can exert considerable effects on the activity of a ribonuclease isolated from a soil-living organism, Citrobacter sp. (1–3). The polyanine, spermidine, was found to enhance enzyme activity, to alter the residue specificity exhibited by the enzyme in its attack on RNA, and to reverse the competitive inhibition of enzyme activity caused by highly ordered synthetic polyribonucleotides. Inasmuch as these observations suggested that the polyamines may be playing a significantly different role in RNA metabolism than had hitherto been suspected (4, 5), it was of interest to determine whether these effects were limited to the Citrobacter system or were a reflection of a more general phenomenon in which the polyamines regulated RNA concentration within the cell by controlling RNase activity.

To explore this latter view, the effects of polyamines on a number of ribonucleases were examined. In the earlier paper of this series, the enzymes chosen for study were all highly specific, but were of microbial origin. In the present paper, the ribonuclease from human plasma was selected because of a report suggesting specificity for cytidylic acid (6). The enzyme, purified approximately 2700-fold, exhibited a strong predilection for internucleotide bonds of cytidylic acid. Spermidine and spermine were found to cause as much as a 7-fold increase in enzyme activity.
Molecular Weight of Human Plasma Ribonuclease — The molecular weight of human plasma ribonuclease was estimated by the gel filtration method of Andrews (14), using as protein standards bovine serum albumin (67,000) (15), ovalbumin (45,000) (16), and cytochrome c (12,400) (18).

The ultracentrifugation method of Martin and Ames (19) was also used to estimate the molecular weight of the enzyme, using yeast alcohol dehydrogenase (130,000) (20) and catalase (250,000) (21) as standards. All standards were purchased from the Sigma Chemical Co.

Preparation of [32P]Phosphoryl-terminal Oligonucleotides — To a solution (2 ml) containing 2 mg of rabbit liver RNA and 0.1 M sodium phosphate buffer, pH 8.0, 40 units of human serum ribonuclease were added. After 16 hours of incubation at 37°, the digest was subjected to ultrafiltration through a Centricon membrane cone (CF50, Amicon Corp., Lexington, Mass.) to remove all fragments of molecular weight above 50,000. An aliquot (1.0 ml) then was treated with 0.1 M HCl at 30° for 4 hours to convert the cyclophosphodiester bonds to the monoester form followed by dialysis for 5 hours against five changes (15 liters) of 0.1 M Tris-HCl buffer, pH 7.5. Terminal phosphate groups were removed from the dialyzed oligonucleotides by treatment with alkaline phosphatase (9) for 2 hours at 37°. Mechanical shaking of the reaction mixture with a chloroform-octanol (9:1) solvent was used to remove all protein from the solution. After centrifugation for 20 min, the aqueous phase was separated, extracted three times with equal volumes of ether, and dialyzed for 2 hours against 4 liters of 0.05 M Tris-HCl buffer, pH 8.6. This fraction (0.8 ml) was added to 0.5 ml of a solution containing 0.05 M Tris-HCl buffer, pH 8.6, 0.01 M MgCl2, 0.05 M EDTA, 1 mmol of [γ-32P]ATP (specific activity 2 x 10^14 cpm per mmol), and 5 units of polynucleotide phosphorylase (12).

The reaction mixture was incubated for 2 hours at 37° and extracted with the chloroform-octanol solvent followed by ether as described above. The [32P]phosphoryl-terminal oligonucleotide fraction was dialyzed for 2 hours against 10 liters of water and digested with 1.0 N NaOH for 16 hours at 37°. Ten micromoles of each of the four 3'-mononucleotides were added to the digest which was added to a column (0.9 x 6 cm) of Dowex 1-X8 formate. The mononucleotides were eluted with increasing normality of formic acid as described by Hurlbert et al. (22). Each of the four mononucleotide peaks was identified by absorption spectrum. Each peak was collected, reduced in volume by rotary evaporation and redistillation, and brought to constant specific activity by chromatographic procedures as above, except that an additional system, ascending paper chromatography on Whatman No. 3MM paper with isobutyl alcohol, NH₄OH, and water (66:1:33) as developing solvent, was included as the final step.

Purification of Enzyme

The following operations were performed at 0-5°. The purification data are summarized in Table I.

Step 1: Ammonium Sulfate Fractionation — With the pH maintained at 7.0 by the dropwise addition of 1 N NaOH, solid ammonium sulfate (28 g) was added with stirring to 100 ml of human plasma over a 20-min period. The enzyme solution was clarified by centrifugation and an additional 28 g of ammonium sulfate were added to the supernatant solution in the same manner. The precipitate which formed between 40 and 80% saturation was collected by centrifugation and was dissolved in 0.01 M phosphate buffer, pH 6.8, to a final volume of 36 ml. The yellow, somewhat turbid, solution was dialyzed for 3 hours against 100 volumes of the same low molarity phosphate buffer.

Step 2: Cellulose Phosphatase Chromatography — To a column (2 x 40 cm) of cellulose phosphate, which had been equilibrated previously with 0.01 M phosphate buffer, pH 6.8, 36 ml of the dialyzed enzyme were added. After the column was washed with 150 ml of equilibrating buffer, 250 ml of a linear gradient of elution from 0.1 to 1.5 M KCl in equilibrating buffer was applied. Fractions, 4 ml in volume, were collected (Fig. 1) and those fractions with the highest activity (93 to 94) were combined and were dialyzed for 3 hours against 40 volumes of 0.05 M phosphate buffer, pH 8.

Step 3: Affinity Chromatography — The enzyme solution (110 ml) obtained in the previous step was applied to a column containing 2 g of activated Sepharose 4B (0.9 x 7.0 cm) to which 10 pmol of (G)₄ were covalently bound by the method of Pooni et al. (26). After addition of the entire enzyme solution, the column was washed with 30 ml of 0.05 M phosphate buffer, pH 8.0, to remove completely an initial protein peak which contained a small amount of ribonuclease activity. The ribonuclease was eluted with 0.05 M phosphate buffer, pH 8, containing 1 M KCl (Fig. 2). Fractions, 1 ml in volume, were collected and those (39 to 45) having most of the enzyme activity were combined and used for all subsequent studies.

RESULTS

pH Optima

When the production of acid-soluble nucleotides was used as an index of enzyme activity, considerable variation in pH optima was found, not only between individual substrates, but also between different buffers used in the measurement of hydrolysis of the same substrate. Thus (Fig. 3), at the optimum pH (7.5) in Tris-HCl buffer, hydrolysis of (C)₄ was approximately 1.5 times as great as it was at the optimum pH (6.5) in phosphate buffer. The hydrolysis of RNA showed the same optimum pH in either Tris-HCl or phosphate buffer (pH 8), but the enzyme

<table>
<thead>
<tr>
<th>Table I: Purification of human plasma ribonuclease</th>
</tr>
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<tbody>
<tr>
<td>Step</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>1. Plasma</td>
</tr>
<tr>
<td>2. Ammonium sulfate fractionation</td>
</tr>
<tr>
<td>3. Phosphocellulose chromatography</td>
</tr>
<tr>
<td>4. Affinity chromatography</td>
</tr>
</tbody>
</table>

* Protein concentration was measured by the method of Lowry et al. (24) or at extremely low concentrations by the fluorescent technique of Böhlen et al. (25).
activity in phosphate buffer was approximately twice that found in Tris-HCl.

Estimation of Molecular Weight of Human Plasma Ribonuclease

Sephadex Gel Filtration—On Sephadex G-100 equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, approximately 10 to 20% of the enzyme eluted within the void volume, indicating a molecular weight in excess of 150,000. It was found that approximately 60% of the enzyme was adsorbed to the column but could be eluted by buffer solution containing 5 mM spermine or spermidine. These latter fractions, or fresh enzyme, when applied to a column equilibrated with 0.5 mM spermine or spermidine in the buffer system were eluted at a position corresponding to a molecular weight of 32,000 (Fig. 4). The aggregation phenomenon was reversible by the addition or removal of polyamine from the equilibrating buffer. Neither Ca<sup>2+</sup> nor Mg<sup>2+</sup> at the same concentrations as the polyamines was able to produce these effects.

Sucrose Density Centrifugation—The molecular weight of the plasma ribonuclease also was determined by ultracentrifugation in a sucrose density gradient, both in the presence and absence of spermidine. As seen in Fig. 5, the peak of enzyme activity, in the absence of spermidine, was found at the bottom of the gradient with a gradual reduction in activity as the less dense sucrose concentrations were approached. The pattern seemed to indicate a spectrum of molecular species ranging in weight from well over 150,000 downward. In the presence of the polyamine two sharp peaks of enzyme activity, with estimated molecular weights of 45,120 and 28,050, respectively, were readily differentiated. Centrifugation in the presence of spermidine produced similar results, except considerably greater recovery of enzyme was attained. Although Ca<sup>2+</sup> and Mg<sup>2+</sup> at the same concentration produced an apparent shift in the

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**Fig. 1.** Chromatography of human plasma ribonuclease on cellulose phosphate. Conditions were as described under “Purification of Enzyme.” Hydrolytic activity toward (C)<sub>n</sub> was measured in the standard assay system described under “Experimental Procedure.” Protein concentration was estimated by the absorbance at 280 nm.

**Fig. 2.** Affinity chromatography of human plasma ribonuclease on a column of Sepharose 4B covalently coupled to (G)<sub>n</sub>. Conditions were as given under “Purification of Enzyme.” Enzyme activity toward (C)<sub>n</sub> was measured in the standard assay system as detailed under “Experimental Procedure.” Absorbance at 280 nm was used to estimate protein concentration. Fractions 4 ml in volume were collected through tube 31. At this point 1 M KCl was added to the elution buffer and fractions 1 ml in volume were collected thereafter.

**Fig. 3.** pH Curves. The hydrolysis of polycytidylic acid was measured in Tris-HCl (•—•) and sodium phosphate buffers (○—○) in the standard assay system, except that incubation was for 7½ min and 4 units of enzyme were used. Similarly, the hydrolysis of tRNA was assayed in Tris-HCl (■ ■) and sodium phosphate buffers (□—□) employing the standard assay system with 6 units of enzyme. Buffer concentration was 0.1 M.
FRACTION NUMBER

FIG. 4. Gel filtration chromatography of human plasma ribonuclease on a Sephadex G-100 column (2 X 42 cm). The figure above illustrates results obtained by chromatography under three different sets of conditions. In each case, 700 units of enzyme were applied to a column. In the first experiment (m--m), the column was equilibrated and eluted with 0.05 \text{M} \text{Tris-HCl buffer, pH 7.5}. The other curves show the elution patterns when the column was equilibrated and eluted with the same buffer containing 0.5 \text{mM} spermine (O--O) or spermidine (n--n). Enzyme activity was measured against (C), in the standard system and is shown in terms of increase in absorption at 260 nm.

Fig. 4. Gel filtration chromatography of human plasma ribonuclease on a Sephadex G-100 column (2 X 42 cm). The figure above illustrates results obtained by chromatography under three different sets of conditions. In each case, 700 units of enzyme were applied to a column. In the first experiment (m--m), the column was equilibrated and eluted with 0.05 \text{M} \text{Tris-HCl buffer, pH 7.5}. The other curves show the elution patterns when the column was equilibrated and eluted with the same buffer containing 0.5 \text{mM} spermine (O--O) or spermidine (△--△). Enzyme activity was measured against (C), in the standard system and is shown in terms of increase in absorption at 260 nm.

molecular weight, the recovery of enzyme activity was approximately 40\% of that added to the gradient originally.

Effect of Cations on Enzyme Activity—The hydrolytic activity of human plasma ribonuclease is decreased significantly when the buffer concentration in the reaction medium is reduced from 0.1 \text{M} to 0.01 \text{M}. Activity against RNA could be restored by the addition of various cations to the dilute buffer medium (Table II). The greatest stimulations occurred when either of the polyamines, spermidine or spermine, was added at an optimal concentration of 1 \text{mM}. At the latter concentration, enhancement of activity by Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, or putrescine was considerably less. Raising the concentration of Mg\textsuperscript{2+} or putrescine to 5 \text{mM} resulted in an enhancement of activity which approached that seen with spermidine and spermine. Other cations did not affect activity, with the exception of Zn\textsuperscript{2+} and Ni\textsuperscript{2+} (not shown in Table), which acted as strong inhibitors.

The diminished hydrolysis of (C), in the dilute buffer system was similarly increased by various cations (Table II). At a concentration of 0.5 \text{mM}, spermine and spermidine caused 2- and 3-fold stimulations of activity against (C), respectively, whereas Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and putrescine had lesser or no effect. At a 10-fold greater concentration, all of the cations save spermine produced further enhancement of hydrolysis. Spermine inhibited above 0.5 \text{mM}, possibly because of precipitation of the substrate.

It should be noted that the conditions under which the hydrolysis of the synthetic polymer and that of RNA were studied are quite different. More enzyme was required, as well as a longer incubation time for the digestion of the latter substance than for polycytidylic acid. The enzyme has a considerably greater affinity for the synthetic polymer than for RNA.

Inhibition of Nuclease Activity by Synthetic Polynucleotides—A number of synthetic polynucleotides having ordered secondary structure have been reported to inhibit microbial nucleolytic enzyme activity (1, 27). Although some of these same compounds also inhibit human plasma ribonuclease, the degree of inhibition appears to be dependent on the substrate under study (Table III). The amount of ordered polynucleotide needed to produce 50\% inhibition of RNA hydrolysis can be as
Inhibition of human plasma ribonuclease by synthetic polyribonucleotides

Activity was measured in the standard assay system using either 1.5 µmol of (C)n or 1.0 mg of yeast RNA as substrate. The inhibitor concentrations shown are those which caused 50% inhibition of hydrolytic activity. Enzyme activity was assayed as described under “Methods.”

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G)n</td>
<td>(C)n</td>
</tr>
<tr>
<td>%</td>
<td>0.5</td>
</tr>
<tr>
<td>(C)n</td>
<td>RNA</td>
</tr>
</tbody>
</table>

Effect of addition of cations on inhibition of hydrolysis of (C)n

Reaction conditions were as described in Table II, except that the reaction mixtures were incubated for 7½ min at 25°. When the effects of inhibiting agents and the cations on the reaction were studied, 0.5 µmol of cation and inhibitor (at the concentration indicated below) was added. Hydrolytic activity, measured as described under “Methods,” is expressed as a percentage of control activity.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Hydrolytic activity in inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G)n</td>
<td>(X)n</td>
</tr>
<tr>
<td>(300 µM)</td>
<td>(5 µM)</td>
</tr>
<tr>
<td>% control</td>
<td>14.9</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>112.5</td>
</tr>
<tr>
<td>+ Inhibitor</td>
<td>94.2</td>
</tr>
<tr>
<td>Inhibitor + spermine</td>
<td>36.8</td>
</tr>
<tr>
<td>Inhibitor + Ca2+</td>
<td>33.5</td>
</tr>
</tbody>
</table>

Although the synthetic polynucleotides are not as strong inhibitors of RNA hydrolysis, reversal of this inhibition is less readily accomplished (Table V). This may be related to the fact that much higher inhibitor levels are used. Only spermine appeared to be consistently effective, and it induced only a partial restoration of activity.

Specificity of Enzyme for Synthetic Polyribonucleotides

The purified enzyme has a preference for cytidylic acid residues in RNA. Of the homopolymers examined, only cytidylic acid was attacked (Table VI). (U)n, (A,C)n, (G)n, and (U)n were not hydrolyzed under the conditions described in Table VI. If enzyme concentration was increased 10-fold, (U)n hydrolysis could be demonstrated. Similarly, those co-polymers containing (C)n or (U)n were hydrolyzed but (A,C,G)n, (C,G,U)n, and (G,U)n were not.

Hydrolysis of Nucleic Acids

Analysis of Digestion Products of RNA—No evidence of hydrolysis of either single- or double-stranded DNA was found. When yeast RNA (2.5 mg) was incubated in 0.1 M phosphate buffer, pH 5, with 20 units of enzyme at 37°, the analysis of the digestion products (28) after 16 hours revealed the presence of only one mononucleotide, cyclic 2'-3'-CMP. Analysis of the 3' and 5' termini of the digestion products, large and small, was carried out. Rabbit liver RNA was chosen as a substrate, primarily because it is mammalian in origin and, secondly, because it can be purified in relatively large quantities. Following a 16-hour hydrolysis of this RNA by human plasma ribonuclease, 32P was introduced enzymatically into the 3' and 5' termini of the digest fragments, and their terminal (i.e. radio active) nucleotides were isolated and characterized. Approximately 90% of the total radioactivity at the 5' terminus was in cytidylic acid, whereas 10% was found in uridylic acid (Table VII). Almost 100% of the radioactivity at the 3' terminus was in cytidylic acid (Table VII). Clearly the predominant cleavage of phosphodiester bonds in rabbit liver RNA was between cytidylic acid moieties. Identical results were obtained in the digestion of yeast RNA. In studies in which undigested rabbit liver RNA was used, incorporation of 32P was less than 2% of that occurring in the enzyme-degraded RNA. Thus, corrections for pre-existing end groups would not significantly change the data presented.

Size of Oligonucleotide Fragments—To establish the average
For details of the digestion, formation of the [3'32P] and [5'-32P] phosphoril-terminal mononucleotides, and the chromatographic systems used to separate them see "Methods."

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>CMP</th>
<th>UMP</th>
<th>AMP</th>
<th>GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' Terminus</td>
<td>41,000</td>
<td>6,100</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>3' Terminus</td>
<td>63,000</td>
<td>300</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

Fig. 6. Chromatography of human plasma ribonuclease digest of rabbit liver RNA. To a solution (6 ml) containing 25 mg (400 absorbance units) of rabbit liver RNA and 500 μmol of PO₄ buffer, pH 8, 30 units of human plasma ribonuclease were added. The reaction mixture was incubated at 37° and at the end of 16 hours was placed in a boiling water bath for 5 min. After centrifugation, the supernatant solution (220 absorbance units) was applied to a column (2.5 X 50 cm) of DEAE-cellulose (chloride form) that had been prepared as described by Tomlinson and Tener (29). The column was washed with 500 ml of water and then 3 liters of a linear gradient of elution from 0 to 0.3 M NaCl in 7 M urea and 0.0025 M sodium acetate buffer, pH 4.7, was applied. Fractions 6 ml in volume were collected at a flow rate of approximately 20 ml per hour. The bars at the base of the elution diagram represent the number of fractions which, when combined, were used for phosphorus analysis. The Roman numerals at the top of each peak are used to designate the combined fractions.

The liberation of C-cyclic-P during the course of hydrolysis of polycytidylic acid suggests that the human plasma ribonuclease functions as other ribonucleases in that it cleaves the phosphodiester bond between a nucleoside 3'-phosphate and the 5'-hydroxyl group of the adjacent nucleotide (31). That this method of hydrolytic attack is probably the same when RNA is the substrate is indicated by the finding in RNA digests of C-cyclic-P and oligonucleotides terminating in cyclic phosphates as digestion products. Aside from revealing that the human plasma ribonuclease possesses endonucleolytic activity, analysis of the fragments present in the RNA digest is of considerable interest in another sense as well, primarily because of the apparent high specificity exhibited by the enzyme for ribonucleotide bonds containing cytidylic acid residues. Initially, a strong suggestion of this specificity came from the rather poor hydrolytic activity against synthetic polyribonucleotides that did not contain cytidylic acid. After the introduction, by enzyme means, of 32P into the 3' and 5' termini of oligonucleotide fragments present in the RNA digest, it was possible to show that the enzyme cleaved exclusively between cytidylic acid residues at the 3' terminus and exhibited a preferential specificity for cytidylic over uridylic acid residues in an approximate ratio of 9:1 at the 5' terminus. The high specificity for cytidylic acid residues is of particular moment in view of the recent report of a large tract of cytidylic acid residues within the RNA of an encephalomyocarditis virus (32). This latter observation suggests the possibility that other RNAs with long tracts of cytidylate residues may exist and that it is these substances which are the natural substrates of the human enzyme.

Another aspect of the enzyme's characteristics which is particularly striking is its response to the polyamines, especially spermidine and spermine. The effects of these compounds on

### Table VII

| Size of oligonucleotides obtained after digestion of rabbit liver RNA by human plasma ribonuclease

See "Methods" section for reaction conditions. Each combined nucleotide fraction (designated by Roman numerals) was obtained from a digest of rabbit liver RNA that had been subjected to DEAE-cellulose chromatography (29) (see Fig. 6). The combined fraction, adjusted to pH 8, was freed of urea and salt by rechromatography on separate columns of DEAE-cellulose (carbonate) as described by Tomlinson and Tener (29). Upon completion of this second chromatography the nucleotide-containing fractions were combined, brought to dryness by lyophilization, and the residue redissolved in a small volume of water. When the process had been repeated, the residue was dissolved in 2 ml of water and an aliquot was used to determine the average size of the nucleotides by the total phosphorus to terminal phosphorus ratio (30).

<table>
<thead>
<tr>
<th>Combined fractions</th>
<th>Volume</th>
<th>Absorbance X volume (260 nm)</th>
<th>Per cent of recovered absorbance units</th>
<th>Ratio of total phosphorus terminus phosphorus</th>
<th>Average No. of nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>102</td>
<td>2.9</td>
<td>0.7</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>270</td>
<td>24.2</td>
<td>6.2</td>
<td>1.8</td>
<td>2</td>
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<tr>
<td>III</td>
<td>315</td>
<td>44.1</td>
<td>11.3</td>
<td>3.2</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>190</td>
<td>51.3</td>
<td>13.2</td>
<td>4.1</td>
<td>4</td>
</tr>
<tr>
<td>V</td>
<td>500</td>
<td>135.0</td>
<td>34.8</td>
<td>4.9</td>
<td>5</td>
</tr>
<tr>
<td>VI</td>
<td>287</td>
<td>95.7</td>
<td>24.6</td>
<td>6.8</td>
<td>7</td>
</tr>
<tr>
<td>VII</td>
<td>90</td>
<td>34.8</td>
<td>9.0</td>
<td>8.2</td>
<td>8</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The liberation of C-cyclic-P during the course of hydrolysis of polycytidylic acid suggests that the human plasma ribonuclease functions as other ribonucleases in that it cleaves the phosphodiester bond between a nucleoside 3'-phosphate and the 5'-hydroxyl group of the adjacent nucleotide (31). That this method of hydrolytic attack is probably the same when RNA is the substrate is indicated by the finding in RNA digests of C-cyclic-P and oligonucleotides terminating in cyclic phosphates as digestion products. Aside from revealing that the human plasma ribonuclease possesses endonucleolytic activity, analysis of the fragments present in the RNA digest is of considerable interest in another sense as well, primarily because of the apparent high specificity exhibited by the enzyme for ribonucleotide bonds containing cytidylic acid residues. Initially, a strong suggestion of this specificity came from the rather poor hydrolytic activity against synthetic polyribonucleotides that did not contain cytidylic acid. After the introduction, by enzyme means, of 32P into the 3' and 5' termini of oligonucleotide fragments present in the RNA digest, it was possible to show that the enzyme cleaved exclusively between cytidylic acid residues at the 3' terminus and exhibited a preferential specificity for cytidylic over uridylic acid residues in an approximate ratio of 9:1 at the 5' terminus. The high specificity for cytidylic acid residues is of particular moment in view of the recent report of a large tract of cytidylic acid residues within the RNA of an encephalomyocarditis virus (32). This latter observation suggests the possibility that other RNAs with long tracts of cytidylate residues may exist and that it is these substances which are the natural substrates of the human enzyme.

Another aspect of the enzyme's characteristics which is particularly striking is its response to the polyamines, especially spermidine and spermine. The effects of these compounds on
enzyme activity are in many respects similar to those seen with a microbial enzyme, *Citrobacter* nuclease (1, 2). Enzyme activity, for example, in the presence of these compounds is enhanced severalfold over control levels. The inhibition of hydrolytic activity observed with the ordered polynucleotides can, with some exceptions, be reversed by spermidine and spermine. Other cations which can enhance enzyme activity weren’t as effective in this area.

The most dramatic effect of the polyamines on the human enzyme is their apparent ability to control the degree of aggregation of the enzyme molecule. It was found, for example, that the apparent molecular weight of the enzyme, when determined by gel filtration on Sephadex columns, is decreased from approximately 150,000 to 32,000 in the presence of the polyamines, spermidine and spermine. These compounds similarly cause a change in the position of the enzyme in a sucrose gradient after centrifugation, indicating a shift in molecular weight from well approximately 150,000 to 32,000 in the presence of the polyamines, spermidine and spermine. These compounds similarly cause a change in the position of the enzyme in a sucrose gradient after centrifugation, indicating a shift in molecular weight from well over 150,000 to approximately 29,000. These studies suggest that the enzyme undergoes aggregation-disaggregation reactions under the influence of the polyamines. The previously expressed view (1, 2) that polyamines may represent control factors in the metabolism of nucleic acids thus is further supported by the effects noted upon human plasma ribonuclease.

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