Characterization of Enzymatic Specificity of a Ribonuclease from *Ustilago sphaerogena**

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ABSTRACT: A ribonuclease from *Ustilago sphaerogena* (ATCC 12421) was purified 1100-fold with a yield of 32%. The isolated enzyme was free of contaminating phosphatases, ribonucleases, and deoxyribonucleases. The rate and specificity of enzymatic action were examined by characterization of products from various stages of digestion of high molecular weight RNA in terms of mono-, di-, and oligonucleotide sequence and distribution. After exhaustive digestion, only mono-, di-, and trinucleotide products remained. The ribonuclease was found to act preferentially at purine–purine and purine–pyrimidine phosphodiester bonds. Possible applications of this preferential specificity to the characterization of nucleotide sequences and the preparation of oligonucleotides terminating in Ap are discussed.

Several ribonucleases have been obtained from *Ustilago sphaerogena*. With an RNA medium, Glitz and Dekker (1963, 1964a,b) isolated a guanylic acid specific enzyme of the RNase T_1 type (Sato and Egami, 1957). The same enzyme, designated U_1, was found by Arima *et al.* (1968a,b) using a sample of *U. sphaerogena* obtained from Glitz and Dekker. Arima *et al.* (1968a,b) also isolated three additional ribonucleases named U_2, U_3, and U_4 from this organism. While U_4 was characterized as a nonspecific ribonuclease that hydrolyzed RNA to mononucleotides, U_2 and U_3 were originally reported to have a strict specificity for purines. Thus, these two enzymes were found, by Arima *et al.* (1968b), to release Ap and Gp but no Cp and Up from RNA, and the isolated oligonucleotide products terminated exclusively in Ap and Gp as determined by dephosphorylation and alkaline hydrolysis. This purine specificity was confirmed by Adams *et al.* (1969) by showing that oligonucleotides derived from RNase T_1 digests of viral RNA were split by the U_2 enzyme only next to adenylate residues.

However, Adams *et al.* (1969) also noted that "the enzyme was specific for purines when used in low concentrations." This implies that the apparent enzyme action might have been due to *preferential* cleavage sites rather than to strict purine specificity, a concept in agreement with a later report by Uchida *et al.* (1970), stating that the U_2 enzyme would, indeed, hydrolyze phosphodiester bonds next to pyrimidine when used in 10-fold higher concentrations than the "low concentrations" specified by Arima *et al.* (1968b).

A purine-specific ribonuclease could be useful for the determination of nucleotide sequences of RNA. Furthermore, such a specificity should also permit the large-scale preparation of oligonucleotides terminating in Ap, among which trimers would be most useful as model compounds for various purposes.

For these reasons, another sample of *U. sphaerogena*, obtained from the American Type Culture Collection, was examined for the presence of ribonucleases. Two such enzymes were found. As described in this report, the ribonuclease present in larger amounts exhibits strong preference for Ap and Gp residues upon hydrolysis of RNA with low concentrations of enzyme and Ap- and Gp-terminal oligomers were found as described by Adams *et al.* (1969). Hydrolysis of phosphodiester bonds involves an intermediate 2′,3′-cyclic terminal phosphate stage, followed by further cleavage to a terminal 3′-phosphate form. After exhaustive hydrolysis of RNA with the nuclease preparation described here, all four mononucleotides were found as well as dinucleotides terminating in all four mononucleotides.

Possible applications of the enzyme as well as conditions suitable for its large-scale preparation are described.

Material and Methods

All spectrophotometric measurements were made in cells with a 1-cm light path and are expressed as absorbancy (*A*). For paper chromatography with Whatman No. 3MM paper, two solvents were used, solvent A (1-propanol–concentrated NH_4OH–H_2O, 55:10:35, v/v) and solvent B (40 g of $(NH_4)_2SO_4$ added to 100 ml of 0.1 M Tris·HCl, pH 7.5).

Enzymes. Alkaline phosphatase from *Escherichia coli* (Sigma, Type III) was further purified on DEAE-cellulose to remove nucleases (if present) (Weiss *et al.*, 1968), and the purified phosphatase was used for dephosphorylation as described by Neu and Heppel (1965). Pancreatic ribonuclease (Type IIA) and DNA from sperm were obtained from Sigma. RNase T_1 and T_2 were prepared as described previously (Rushizky and Sober, 1962, 1963).

RNA. High molecular weight RNA from yeast was prepared by the procedure of Crestfield *et al.* (1955) and the fraction insoluble in 1 M NaCl was extracted twice with phenol. RNA of bacteriophage MS2 was obtained essentially as reported (Strauss and Sinsheimer, 1963).

Oligonucleotides. Compounds terminating in Gp (Rushizky and Sober, 1962) and in Cp or Up were isolated by mapping (Rushizky and Knight, 1960).

Adsorbents. DEAE-cellulose (0.8 mequiv/g was obtained

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from Schleicher & Schuell, Keene, N. H. Sephadex G-75 was obtained from Pharmacia.

Assay for RNases were performed at pH 4.5 and 7.5. To 0.8 ml of assay solution (2 mg of yeast RNA/ml, and 0.125 M in either sodium acetate, pH 4.5, or in Tris·Cl, pH 7.5) was added 0.2 ml of diluted enzyme solution and the mixture held at 37° for 15 min. The reaction was stopped by the addition of 1 ml of 6% $HClO_4$. After 15 min at 0°, the precipitate was removed by centrifugation, 0.5 ml of the supernatant was diluted with 4.5 ml of water, and the A_{260} was measured against the appropriate blank. A range of dilutions was tested in each assay. An increase in A_{260} of 1.0 under these conditions was defined as 100 units of enzyme activity. With the same RNA solution, the reproducibility of this assay is better than ±6%.

Purification of RNase. Since similar results were obtained on both the 10- and 300-l. scale only the former will be described in detail.

A freeze-dried preparation of *U. sphaerogena* No. 12421 was obtained from the American Type Culture Collection, and repeatedly subcultured with shaking at 30° in the medium of Arima *et al.* (1968a), to obtain a larger inoculum. Foaming was controlled by the addition of Dow Corning Medical Antifoam, AF Emulsion, diluted 1:5 with water, and sterilized. A 250-ml inoculum was then added to 10 l. of medium in a 14-l. New Brunswick fermentor. After 4 days at 30°, with stirring at 100 rpm and aeration with 3 l. of air/min, the A_{260} at 1:10 dilution was 1.1. Since the level of enzyme activity at pH 4.5 and 7.5 had peaked and was decreasing (Figure 1), the cells were spun out at 4° and solid $(NH_4)_2SO_4$ was added to the supernatant to saturation. Within 15 min, the enzyme activity and other protein rose to the top and the clear solution below was drawn off. The residue was suspended in 100 ml of water, and dialyzed twice against 18 l. of water for 16–24 hr each. A small amount of insoluble material was removed by centrifugation. The clear supernatant (125 ml) was loaded onto a 4 × 30 cm DEAE-cellulose column equilibrated at room temperature with 0.02 M Tris·HCl (pH 8.5) (Arima *et al.*, 1968a). Elution of the column was by a 4-l. linear gradient to 1 M NaCl–0.02 M Tris·HCl (pH 8.5) at a flow rate of 54 ml/hr. Two peaks with ribonuclease activity were observed. The first, eluted at 0.22 M NaCl, contained about one-tenth of the enzymatic activity (pH 4.5) of the second peak which eluted at 0.4 M NaCl. The ratio of enzymatic activities at pH 4.5–7.5 was 1.2 for the first, and about 20 for the second peak. Preliminary results indicated that the activity found in the first peak was nonspecific; *i.e.*, RNA or oligonucleotides were hydrolyzed completely to mononucleotides.

All fractions contained in the second peak were pooled, cooled to 0°, and adjusted to 0.4 N in HCl. After 18 hr at 0°, the solution (325 ml) was neutralized with 0.1 M Tris to pH 7, dialyzed at 4° in three cycles of 18 l. of water for 8–16 hr each, and lyophilized. The material was then suspended in water, a small amount of insoluble matter spun out, and the supernatant (18 ml) was applied to a 4 × 50 cm Sephadex G-75 column equilibrated at room temperature with 0.02 M sodium acetate (pH 5.3). Elution was at 23° with the same buffer, at a flow rate of 51 ml/hr. A single symmetrical peak of ribonuclease activity at pH 4.5 was eluted ahead of a large, inactive A_{280} peak. The fractions containing high specific activity were pooled, dialyzed, and lyophilized. The concentrated

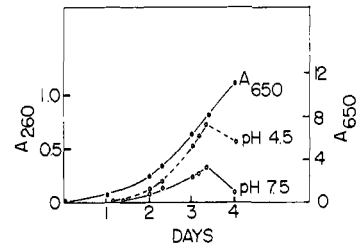

FIGURE 1: Growth of *U. sphaerogena* was measured by A_{650}, and enzymatic activity (after centrifugation to remove cells) at pH 4.5 and pH 7.5 was determined as described in the text.

material was dissolved in 5 ml of 0.02 M Tris·HCl (pH 8.5) and loaded on a 2.2 × 75 cm DEAE-cellulose column equilibrated in the same buffer at room temperature. The column was developed with a 4-l. gradient to 0.05 M Tris·Cl (pH 7.0) plus 0.5 M NaCl at a flow rate of 60 ml/hr. After two inactive A_{280} peaks appeared, a single peak of enzymatic activity was obtained when about 75% of the gradient volume had passed through the column. Fractions containing high specific activity were pooled, dialyzed, and stored at 4° in the presence of chloroform, at an A_{280} of about 0.1/ml. The preparation showed no loss of activity when stored for 3 months. All tests described below were conducted with suitable dilutions of such enzyme preparations.

Linearity of assay, pH optimum, and assay for contaminating enzymes (acid phosphatase, nonspecific phosphodiesterase, and DNase) at pH 4.5 and 7.5 were performed as described previously (Rushizky *et al.*, 1964). The presence of DNase activity was checked by substituting DNA for RNA at pH 4.5 or 7.5, using about 13,000 units of enzyme/mg of DNA, in the standard assay.

Thermal Stability (Arima *et al.*, 1968a). The enzyme (A_{280}/ml of 0.1) was held at 85° for 4 min in 0.002 M Tris·Cl (pH 7.3), chilled in ice, diluted to suitable concentrations, and assayed at once.

Effects of Metals and EDTA (*Arima et al.*, 1968a). Diluted enzyme solutions were incubated for 30 min at 37° with 0.001 M $MnCl_2$, $MgSO_4$, $CaCl_2$, or NH_4–EDTA (pH 7.0) and then tested for activity.

Enzymatic Hydrolysis of RNA and Oligonucleotides. Various diluted aliquots of the purified enzyme were incubated with 1–3 mg of yeast RNA, MS2 RNA, or oligonucleotides for 3–5 hr at 37° and in 0.1 M sodium acetate (pH 4.5). The final volume of the digests was 0.5 ml. The compounds in the digests were then fractionated by mapping. The isolated monomers and oligomers were identified by spectrophotometry at pH 2 and 7; by treatment with pancreatic ribonuclease, RNase T_1, or RNase T_2; by dephosphorylation; remapping or paper chromatography with solvents A or B (Rushizky and Knight, 1960; Rushizky and Sober, 1962, 1963). The proportion of 2′,3′-cyclic terminal phosphate and 3′-phosphate forms of several oligo- and mononucleotides was obtained by paper chromatography at pH 7 with solvent B (Rushizky *et al.*, 1962).

Results

The major ribonuclease of *U. sphaerogena* was purified from 10 l. of medium by treatment with $(NH_4)_2SO_4$ and

TABLE I: Purification of Ribonuclease from 10 l. of *U. sphaerogena* Medium.

	Total Protein as A_{280}	Enzyme Units[a] at pH 4.5	Sp Act.[b]	% Recov
Cell-free supernatant	61,200	688,000	11.2	100
$(NH_4)_2SO_4$ precipitation	4,200	480,000	114	70
First DEAE-cellulose chromatography	1,270	577,000	457	84
Treatment with 0.4 N HCl, 18 hr at 4°	1,120	610,000	545	89
After G-75 Sephadex chromatography	312	432,000	1,380	63
After second DEAE-cellulose chromatography	18	220,000	12,200	32

[a] For a description of enzyme units and enzyme assay, see text. [b] Total units of enzyme/total A_{280}.

TABLE II: Major Mono- and Oligomers Isolated in *U. sphaerogena* RNase Digests of Yeast RNA.[a]

Stage 1	Stage 2	Stage 3	Stage 4	Spot No. (Figure 2)
	Gp	Gp	Gp	6
	Ap	Ap	Ap,Cp	1
			Up	15
CpAp, ApAp[b]	CpAp, ApAp	CpAp, ApAp	CpAp	2
CpGp, ApGp	CpGp, ApGp	CpGp	CpGp	5
UpAp	UpAp, (CpUp)	UpAp, (CpUp)	UpAp, CpUp, UpCp	9
		UpUp	UpUp	20
	UpGp	UpGp	UpGp	11
	GpGp			8
CpApAp, CpCpAp	CpApAp, CpCpAp			3
CpCpGp, CpApGp	CpCpGp, (CpAp)Gp	CpCpGp, (CpAp)Gp		4
CpGpGp	CpGpGp	CpGpGp		7
(UpCp)Ap[c]	UpCpAp, CpUpAp	(UpCp)Ap	UpCpAp, CpUpAp	8
(UpAp)Gp, (CpUp)Gp	UpApGp, (CpUp)Gp	(UpAp)Gp, (CpUp)Gp	UpApGp, (CpUp)Gp	10
UpGpGp	UpGpGp	UpGpGp		12
	UpUpAp, (UpUpCp)	UpUpAp	UpUpAp	13
	UpUpGp	UpUpGp	UpUpGp	14
Tetramers	Tetramers	Tetramers		16, 17, 18
Core material	Core material			19

[a] Digestion conditions: 3.5 mg of yeast RNA in 0.5 ml of 0.1 M sodium acetate (pH 4.5) was digested for constant times of 5 hr at 37° with 1.3, 13.3, 133, and 1070 units of enzyme per mg of RNA. The enzyme was added in 0.1 ml of water. This corresponds to stages 1–4. The extent of digestion was also ascertained by the isolation of 0, 202, 320, and 391 μg of Ap as monomer at stages 1–4. Since the RNA contains 25.5% Ap (Crestfield *et al.*, 1955), this amounts to the release of 0, 23, 37, and 44% of the total Ap, respectively. For a definition of enzyme units, see text. [b] Compounds terminating in 2′,3′-cyclic terminal phosphate and 3′-phosphate are listed together. [c] Parentheses indicate that the base sequence is not known, or was not determined with the material isolated at a particular stage of hydrolysis. A horizontal line indicates (as for Gp in stage 1) that the compound was not found on the maps.

acid, and by chromatography on DEAE-cellulose and Sephadex G-75. Purification was 1100-fold, with a yield of 32% (Table I). When the enzyme was prepared from 300 l. of medium by the same procedure, the yield was 24% and the purification 1600-fold.

The apparent increase in total enzyme activity after steps III and IV (Table I) may be due to the removal of inhibitor(s) concentrated from the cell-free supernatant by saturation with $(NH_4)_2SO_4$. This agrees with a marked decrease in viscosity observed in the partially purified enzyme solution after step III and especially after step IV. A similar increase in total enzyme activity was noted during the purification of *Bacillus subtilis* RNase by an analogous procedure (Rushizky *et al.*, 1963).

The pH optimum in 0.1 M sodium acetate peak was at pH 4.5. A symmetrical peak was obtained with 50% of the maxi-

TABLE III: Hydrolysis of Di- and Trinucleotides by *U. sphaerogena* Ribonuclease.[a]

Compd (mμmoles)	Units of Enzyme/ mμmoles of Compd	Compd Isolated (mμmoles)	Deduced Split	% Hydrolysis
ApCp, 580	0.015	Ap, 561; Cp, 555	Ap/Cp	100
ApUp, 540	0.015	Ap, 523; Up, 518	Ap/Up	100
ApGp, 434	0.014	Ap, 431; Gp, 413	Ap/Gp	100
ApGp, 1970	0.0041	Ap, 800; Gp, 806; ApGp, 1140	Ap/Gp	41
GpCp, 1895	0.0042	Gp, 1260; Cp, 1250; GpCp, 605	Gp/Cp	67
GpUp, 1210	0.0066	Gp, 673; Up, 680; GpUp, 520	Gp/Up	56
CpGp, 2700	0.6	CpGp, 2610; Gp, 7; Cp, 5	CpGp	0.2
UpAp, 1140	1.4	UpAp, 1070		0
UpGp, 1600	1.0	UpGp, 1524		0
CpAp, 1280	1.3	CpAp, 1250; Ap, 2; Cp, 2	Cp/Ap	0.2
UpUpGp, 818	1.13	UpUpGp, 790		0
CpCpAp, 1080	1.5	Cp, 213; Ap, 11; CpAp, 197; CpCp, 6; CpCpAp, 850	Cp/CpAp	19
CpCpGp, 108	0.5	CpCpGp, 96		0
CpCpGp, 539	5.0	Cp, 91; CpGp, 83; CpCpGp, 416	Cp/CpGp	18
ApApCp, 231	0.036	Ap, 317; Cp, 220; ApAp, 51	Ap/Ap/Cp	100
ApApUp, 270	0.0316	Up, 253; Ap, 437; ApAp, 37	Ap/Ap/Up	100
(ApGp)Up, 680	0.003	Ap, 264; Gp, 165; Up, 574; (ApGp), 394; GpUp, 94	(Ap/Gp)/Up	100
(ApGp)Cp, 400	0.0051	Ap, 146; Cp, 392; Gp, 158; (ApGp), 228	(Ap/Gp)/Cp	100
GpGpUp, 274	0.0073	Gp, 148; GpGp, 189; Up, 267	Gp/Gp/Up	100
GpGpCp, 230	0.0091	Gp, 149; GpGp, 144; Cp, 221	Gp/Gp/Cp	100

[a] For a description of the isolation procedure by paper chromatography and mapping, see text. The hydrolysis of a phosphodiester bond is indicated by a slanted line. All digestions were carried out for 3 hr at 37° in a volume of 0.5 ml of 0.1 M sodium acetate (pH 4.5). Hydrolysis was stopped by extraction with phenol (Rushizky *et al.*, 1964) prior to mapping. In all cases, per cent hydrolysis is expressed in terms of the fraction of original compound split.

mal activity remaining at pH 5.3 and 3.8. The enzymatic assay used (release of acid-soluble mono- and oligonucleotides from RNA) was linear up to A_{260} values of about 0.8. Solutions 0.001 M in $MnCl_2$, $MgSO_4$, $CaCl_2$, and NH_4-EDTA at pH 6.5 did not affect enzyme activity. The enzyme was stable to incubation at 85° for 4 min as well as to 0.4 N HCl at 4° for 18 hr. The purified ribonuclease preparation was inactive toward *p*-nitrophenyl phosphate, bis(*p*-nitrophenyl) phosphate, and DNA.

Upon chromatography on Sephadex G-75 the ribonuclease was found to elute with or slightly after RNase T_1, which in turn was retarded considerably more than RNase T_2. This indicated that the size and/or molecular weight of the *Ustilago* enzyme is in the order of that of RNase T_1 (mol wt 11,000) rather than to that of RNase T_2 (mol wt 36,000) (Egami and Nakamura, 1969).

The course of enzymatic action on yeast or MS2 RNA was followed by examining RNA digests prepared with 1.3, 13, 133, and 1070 units of enzyme per mg of substrate (stages 1–4, Table II, Figure 2). At the earliest stage of hydrolysis (stage 1), no mononucleotides, but di-, tri-, and tetranucleotides, and core material (oligomers that did not move on mapping; see spot 19, Figure 2) were found. Further enzymatic action (stage 2) yielded Ap and Gp and caused a gradual decrease in chain length of the oligomers present. At stage 3, no Up or Cp, but UpCp, and UpUp were found. ApAp was present but GpGp was not. After more exhaustive digestions (stage 4), all 4 mononucleotides, dinucleotides, smaller amounts and fewer species of trimers, and no compounds larger than trinucleotides were found.

MS2 RNA, hydrolyzed to stages 1–4, gave qualitative

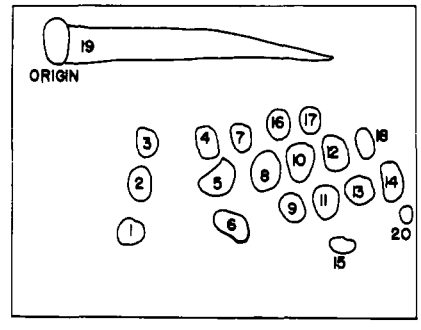

FIGURE 2: Composite drawing of maps showing the fractionation, on Whatman No. 3MM paper, of 3.5 mg of RNA digested by various amounts of *U. sphaerogena* ribonuclease. First dimension: electrophoresis (left to right in 0.75% formic acid for 17 hr at room temperature and 6 V/cm. Second dimension: paper chromatography in descent, for 32 hr with 55:45 (v/v) *tert*-butyl alcohol–0.02 M ammonium formate (pH 3.8). The paper was serrated so that the solvent could run off the paper without edge effects. For an identification of the compounds, see Table II.

results very similar to those obtained with yeast RNA. At stage 2, slight differences were found between the amounts of mono- and oligomers released from both RNAs. For example, MS2 RNA yielded more CpCpGp and (CpAp)Gp than did yeast RNA, in line with the higher Cp content of the viral than of the yeast RNA (26 *vs.* 20%, respectively).

Compounds located on maps of partial digests of yeast RNA were present in both the 2′,3′-cyclic terminal phosphate and 3′-phosphate forms. The proportion of these two forms was not determined except for some compounds at stage 2 (by using the same spots from several maps of the same stage and RNA digest). The per cent of the isolated compounds that existed in the cyclic terminal phosphate form was Ap, 92; Gp, 96; ApAp, 62; ApGp, 60; CpGp, 58; UpGp, 43; CpAp, 72; UpCp and CpUp, 100 (only cyclic forms found); UpUpGp, 43; and (CpUp)Ap, 65.

Additional information about the action of the enzyme was deduced from a comparison of rates of hydrolysis of several small oligomers (Table III). Phosphodiester bonds between purine–pyrimidines (Pu–Py) and Pu–Pu were cleaved at much lower enzyme–substrate ratios than were Py–Pu or Py–Py bonds. Thus, with ApGp, GpCp, and GpUp, 41–67% of the original compound was hydrolyzed by 0.004–0.006 unit of enzyme/mμmole of substrate. By contrast CpGp, UpGp, and CpAp were only barely hydrolyzed (0–0.2%) by 0.6–1.29 units of enzyme per mμmole of substrate. This amounts to a more than 20,000-fold difference in the rate of hydrolysis of the above compounds.

Discussion

The ribonuclease described here exhibits a definite preference for the hydrolysis of Pu–Pu and Pu–Py bonds, in di- and trinucleotides as well as in high molecular weight RNA. Thus, at stage 2 of RNA digestion, most of the oligomer products terminate in Ap or Gp, and these are the only mononucleotides produced. However, not all of the available Pu–Pu or Pu–Py bonds were split, since ApAp, ApGp, GpGp, CpApAp, CpGpGp, and UpGpGp were also found at this stage. Upon further hydrolysis, these compounds disappeared (stages 3 and 4; Table II), but other compounds appeared such as Up and Cp, UpUp, CpUp, and UpCp. No tetra- or higher oligonucleotides are found after exhaustive digestion (stage 4).

The appearance of purine-less compounds may indicate that the ribonuclease is not completely specific for the hydrolysis of Pu–Pu or Pu–Py bonds alone, in the sense in which, for example, pancreatic ribonuclease hydrolyzes bonds next to pyrimidines.

As an alternative, the ribonuclease might really be purine specific, but the preparation was contaminated with another nonspecific nuclease. However, we believe that the presence of a nonspecific enzymatic contaminant may be ruled out. Such an enzyme, if present, would uniformly reduce RNA, di- and trinucleotides to mononucleotides, and would not show the mono-, di-, and trinucleotide patterns obtained.

For example, a nonspecific enzyme should hydrolyze GpUp and UpGp at similar enzyme–substrate ratios but instead GpUp was hydrolyzed to 56% by 0.0066 unit of enzyme/mμmole of substrate, while UpGp was not touched by 1 unit of enzyme/mμmole of substrate (Table III). Even at the most complete stage (stage 4) of RNA hydrolysis (Table II), of the 14 dimers and trimers studied, only three terminated in pyrimidines, and the other eleven compounds contained only one purine located at the 3′-phosphate end. The presence of such a limited number of products, even after exhaustive enzymatic digestion, argues strongly against the presence of a nonspecific, contaminating enzyme.

On the basis of these results, we conclude that the enzyme isolated here has a strong preference, but no absolute specificity for the hydrolysis of Pu–Pu or Pu–Py phosphodiester bonds. Other enzymes known to have preference but no strict specificity for certain bases include micrococcal nuclease (Dirksen and Dekker, 1960) and the extracellular RNase of *B. subtilis* (Whitfeld and Witzel, 1963) which preferentially hydrolyze bonds next to Ap and Up, or next to Gp, respectively.

The nuclease described here resembles the U_2 enzyme first isolated by Egami and his group (Arima *et al.*, 1968a,b) as follows. While both enzymes derive from two different strains of *U. sphaerogena*, the organisms were grown on the same medium. Other similarities between both nucleases include the pH optimum, thermal stability, lack of activation by EDTA, size and/or molecular weight as determined by exclusion chromatography, and elution patterns upon chromatography on DEAE-cellulose.

While the units of enzyme activity used by Arima *et al.* (1968b) differ from ours, comparable amounts of enzyme protein (as A_{280}) are required to hydrolyze RNA to the same stage of digestion. Thus, 0.3 unit of U_2 RNase hydrolyzes 1 mg of yeast RNA in 22 hr at 37° to a stage where 11% of the RNA remains as core material (Arima *et al.*, 1968b). With the enzyme described here, core material is not found beyond stage 2 (Table II). To attain stage 2 hydrolysis, 13.3 units of nuclease/mg of RNA was held at 37° for 5 hr. The U_2 RNase (see above) contained 446 units with a total A_{280} of 0.6, or an A_{280} of 0.0004/0.3 unit of enzyme, while the nuclease preparation used here contained 220,000 units with a total A_{280} of 18, or an A_{280} of 0.0011/13.3 units of enzyme. This amounts to a 0.0011/0.0004 = 2.75-fold difference, indicating that similar quantities of both enzymes are required to hydrolyze RNA to a comparable stage of hydrolysis.

In view of these similarities between both enzymes and the preference but not absolute specificity for phosphodiester bonds adjacent to purines (Uchida *et al.*, 1970; Arima *et al.*, 1968a,b), the two nucleases resemble each other to a large extent or may, in fact, be identical.

At stage 2 of RNA digestion as defined above, the enzyme described here should be useful for the hydrolysis of phosphodiester bonds next to Ap and Gp and thereby aid in the determination of certain nucleotide sequences. Considerable amounts of di- and trinucleotides are also produced at that stage of digestion. Thus, yeast RNA may be hydrolyzed to purine-terminal trinucleotides (stage 2, Table II) with yields of 10%, of which about one-half constitute Ap-terminal compounds. Such trimers may be useful as model compounds.

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Studies on the Secondary Structure of Phenylalanyl Transfer Ribonucleic Acid*

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ABSTRACT: To investigate the secondary structure of *Escherichia coli* Phe tRNA the techniques of spectral analysis and tritium–hydrogen exchange were used on the reaction of 1% HCHO with Phe-tRNA at 35°. The rate and extent of the reaction of HCHO with Phe-tRNA is decreased in the presence of Mg^{2+} or high concentrations of Na^+. The tritium-exchange measurements were made to determine the amount of base pairing before, during, and after reaction. As expected, there is a decreasing number of slowly exchanging hydrogens upon increasing time of exposure to HCHO. Before reaction there were 84 H-bonded hydrogens in a 10^{-3} M Mg^{2+} solution and only 48 after reaction. The tritium exchange-out curve for Phe-tRNA in Mg^{2+}, treated with HCHO, coincided with unreacted Phe-tRNA in 0.03 M PO_4^{2-}. This suggested a stable "core" region of H bonding. The size of this core is that expected on the basis of a cloverleaf-like structure for tRNA.

The thermal denaturation curve of Phe-tRNA in 0.03 M phosphate shows a coincidence with the theoretical curve obtained using a statistical mechanical theory of thermal transitions on the cloverleaf model of Phe-tRNA. Other thermal denaturation and tritium-exchange experiments suggest that the Phe-tRNA molecule may have tertiary folding of the cloverleaf in 0.2 M Na^+ solution.

The primary structures of many tRNAs have been determined, and in all cases, models can be proposed in which the sugar phosphate chain is folded into a cloverleaf configuration (secondary structure). It has also been suggested that the arms of the cloverleaf then arrange themselves into a compact tertiary structure (Henley *et al.*, 1966). X-Ray crystallography of purified tRNAs should eventually deduce this tertiary structure. Kim and Rich (1969) suggest that the molecules are elongated and dimerized in an overlapping antiparallel fashion.

The reaction of formaldehyde with the amino and imino groups of the nucleic acid bases has been extensively used to study the secondary structure of RNAs. Fraenkel-Conrat (1954) showed that while most RNAs reacted with formaldehyde, DNA did not, probably because the bases are inaccessible in the double-helical configuration. The double-helical regions in RNA are thought to be shorter in length and therefore less stable and able to react with formaldehyde. Fasman *et al.* (1965) showed that the thermal denaturation curve of formylated tRNA resembles the broad curves of neutral

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