Studies on Soluble Ribonucleic Acid:
The Action of Snake Venom Phosphodiesterase on Soluble Ribonucleic Acid in Yeast*

T. Nihei and G. L. Cantoni

With the technical assistance of Rachelle Rothenberg

From the Laboratory of Cellular Pharmacology, National Institute of Mental Health, National Institutes of Health, Bethesda 14, Maryland

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Useful information on the structure of soluble ribonucleic acid may be obtained by a study of the action of snake venom diesterase on it, to the extent that the mechanism of action of the enzyme itself is understood.

Snake venom phosphodiesterase has been widely studied and extensively purified (1-5). Two basic conclusions have been reached as to its mechanism of action on ribonucleotide and deoxyribonucleotide polymers: (a) it acts as an exonucleotidase, attacking the polymeric substrate by stepwise removal of 5'-mononucleotide units from the end that bears an unesterified hydroxyl group (1, 2, 4-6), and (b) it acts on different substrate molecules at random, rather than by attacking repeatedly a single substrate molecule until complete digestion. This conclusion follows from the discovery of Berg and Ofengand (7) that S-RNA loses all ability to accept activated amino acids after less than 5% degradation by snake venom diesterase.

S-RNA is a particularly interesting substrate for snake venom diesterase because ultracentrifugal studies from this laboratory revealed that S-RNA is homogeneous with respect to molecular dimensions (8, 9), although it is chemically and functionally heterogeneous since it consists of a mixture of different molecules, each specific for a particular amino acid (10, 11). Conversely, useful information as to the structure of S-RNA may result from a study of its hydrolysis by the enzyme.

In a preliminary study of the hydrolysis of rabbit liver S-RNA by purified snake venom diesterase (12), we indicated that at any degree of digestion the products of the hydrolysis are mononucleotides and a mixture of partially digested polynucleotides of varying chain lengths. We suggested that the theoretical distribution of chain lengths in the polynucleotide fraction may be calculated by applying the model developed by Flory's (13) random attack model. The earlier report on the clustering of uridine, methylated purines, and uridine in the central portion of the average rabbit liver S-RNA chain is confirmed and extended to yeast S-RNA. Finally, observations on the behavior of polynucleotide hypochromicity during enzymatic hydrolysis of S-RNA by snake venom diesterase are correlated with current structural models of S-RNA (14, 15).

EXPERIMENTAL PROCEDURE

Methods and Materials

Phosphodiesterase—Phosphodiesterase from lyophilized Crotaulus adamantus venom (Ross Allen's Reptile Institute, Silver Springs, Florida) was prepared by a method of Razzell and Khourana (2). The acetone fractionation was performed according to Williams, Sung, and Laskowski (5). Chromatographic separation on a diethylaminoethyl Sephadex column was repeated twice to minimize contamination by 5'-nucleotidase. Several enzyme preparations showed approximately a 170-fold purification over the starting material, as measured on a synthetic substrate, di-p-nitrophenyl phosphate (K and K Laboratories, Inc.). The enzyme preparations is still contaminated by a 5'-nucleotidase that attacks 5'-AMP, but is relatively inactive toward 5'-GMP, 5'-CMP, and 5'-UMP. This contaminant, however, is present in small amounts, and its activity is no hindrance to the use of the enzyme for our studies.

S-RNA—Yeast S-RNA was prepared by the procedure of Monier, Stephenson, and Zamecnik (16) as modified by Apgar, Holley, and Merrill (17). Sedimentation homogeneity and chain length were determined as described by Luborsky and Cantoni (9).

Synthetic Polynucleotides—A partially purified polynucleotide phosphorylase preparation from Micrococcus lysodeikticus was kindly supplied by Dr. M. Singer. Ribonucleotide polymers were synthesized from equimolar mixture of ADP, CDP, GDP, and UDP, without addition of primer according to the procedure...
of Singer and Guss (18). Enzymatic synthesis was allowed to approach the point of equilibrium, which was determined in advance by a pilot experiment. Unreacted nucleoside diphosphates were removed by repeated precipitation of the polynucleotides with 2 volumes of ethanol and by dialysis. The average chain length of the synthetic polynucleotide mixture was determined from the ratio of nucleoside to total nucleotides in an alkaline digest (0.3 N KOH for 18 hours at 37°) and was found to be 40 ± 3 monomers per chain. The base composition was determined as described by Cantoni et al. (10) of the method of Volkin and Cohn (11). Polyadenylic acid was purchased from Miles Chemical Company, Elkhart, Indiana, and commercial ribonucleic acid from Worthington Biochemical Corporation, Freehold, New Jersey.

**Assays**—Hydrolysis of di-p-nitrophenyl phosphate was used to compare the activity of different enzyme preparations. The reaction was started by the addition of an aliquot of enzyme to a mixture of 100 μmoles of Tris, pH 8.9, and 0.5 μmole of di-p-nitrophenyl phosphate in 1 ml. The increment of optical density due to the liberation of p-nitrophenylate was measured at 400 nm against a blank identical in all respects except for the presence of S-RNA. At 100% hydrolysis, all of the nucleotide phosphate is soluble in the uranium acetate-perchloric acid reagent, and the absorbance of the solution is 20 to 30% greater than the initial value owing to the hypochromicity of native S-RNA.

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The activity of snake venom phosphodiesterase was determined by a precipitation procedure modeled after the method of Anfinsen et al. (19) (see the legend of Fig. 1). In addition, two procedures were used to study the products formed by action of the enzyme on natural and synthetic polynucleotides. Both are based on the ease with which mononucleosides and mononucleotides may be separated from partially degraded polynucleotides by chromatography on Dowex 1-X2 (formate).

For an estimation of the extent of the digestion, i.e. of the total amount of monomers formed (both mononucleosides and mononucleotides), aliquots of approximately 0.2 to 0.3 ml of the reaction mixture containing about 7 to 20 optical density units of substrate per ml in 0.1 M Tris, pH 8.9, with about 0.01 to 0.03 M in a chloride salt of a divalent cation (see below), were removed at appropriate intervals. Each aliquot was diluted 10-fold with water, brought to pH 3.5 ± 0.5, kept at room temperature for 10 minutes, then charged on a column of Dowex 1-X2 (formate), 1 cm 1 cm, and eluted by 10 ml of water, followed by 60 ml of 1 M formic acid. In this procedure all mononucleotides are eluted in the water fraction; mononucleotides are found in the formic acid fraction. Polynucleotides may be recovered quantitatively from the column by elution with 1.0 N HCl. The amount of monomers formed may be estimated directly from the optical density at 270 μm at pH 2, since the micromolar extinction coefficient of AMP, CMP, GMP, UMP, and ψ UMP at 270 μm is ~10 μM cm⁻¹. For a mixture of the various mononucleotides, one may use a value of 10 optical density units per μmole of nucleotide phosphate and the inaccuracy will not be greater than ±4%.

For a quantitative analysis of the base composition of mononucleotides formed by enzymatic hydrolysis, Cantoni et al.'s modification (10) of the method of Volkin and Cohn (11) was used. Mononucleosides and mononucleotides were recovered by successive elution with water, 0.1 M formic acid, and 1.0 M formic acid. After removal of 1 M formic acid with water, the nondigested portion of the polymer was eluted by 1.0 N HCl.

In both procedures, the total recovery ranged between 97 and 103%.

**Other Methods Used**—DEAE-Sephadex chromatography for separation of S-RNA samples was carried out according to Stachlin and Peterson (20). Sedimentation in the sucrose gradient was performed according to Martin and Ames (21). Phosphatase was determined by the method of Ames and Dubin (22).

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**RESULTS**

**Activity of Enzyme toward S-RNA and Effect of Ionic Conditions**—The conditions under which the enzyme was used were essentially those described by Boman (4). As shown in Fig. 1, in Tris buffer (pH 9.0), 0.05 to 0.1 M, and CaCl₂, 20 mM, complete hydrolysis of S-RNA by the enzyme can be obtained in 20 to 25 hours. The rate of hydrolysis for such a reaction mixture (Fig. 1A) was of the order of 6 to 8 μmoles of mononucleotides liberated per hour per mg of the enzyme. The reaction rate is proportional to enzyme concentration (Fig. 1A, B, and C).

Ca²⁺ is necessary to accomplish complete hydrolysis. If Ca²⁺ is replaced by Ba²⁺, the reaction rate is somewhat faster. On the other hand, when Mg²⁺ is substituted for Ca²⁺, the reaction proceeds at a much slower rate. Na⁺, 0.3 M, is even more inhibitory (Fig. 2).

The relationship between rate of reaction and substrate concentration is shown in Fig. 3. As has been reported by Williams et al. (5), concentrations of substrate above 1 mg per ml inhibit.

**Activity of Enzyme toward Synthetic Polynucleotides**—The reaction rate with mixed synthetic polynucleotides was about 8 to 9 μmoles of mononucleotides liberated per hour per mg of the
enzyme, which is about 10% higher than with S-RNA. With polyadenylic acid, the rate was similar to that with S-RNA but was not influenced by the presence or absence of Ca++. The base composition of a synthetic polymer containing adenosine, guanidine, uridine, and cytidine and the distribution of mononucleotides released from it by partial enzymatic hydrolysis are shown in Table I. The composition of the hydrolyzed fraction corresponds closely to the composition of the polymer. The deviations from theoretical values are close to the experimental error. These data indicate that under our experimental conditions the enzyme is insensitive to the nature of the base linked to the phosphodiester under attack—a conclusion reached earlier by other investigators—and provide support for current concepts of the mode of action of the enzyme.

**Verification of Exonucleolytic Nature of Enzyme Attack**—Utilization of snake venom phosphodiesterase for structural analyses of S-RNA requires that the enzyme be not only insensitive to the base composition of the substrate molecules, but also essentially free of endonucleolytic activity. As illustrated in Fig. 4, two modes of endonucleolytic attack on the phosphodiester ...YpZ... are conceivable: (a) cleavage of the diester bond ...Yp → Z... and formation of two oligonucleotide fragments ending respectively in ...Yp and Z..., 5'-hydroxyl, or (b) cleavage of the bond ...Y → pZ..., which would result in two oligonucleotides ending respectively in ...Y-3'-hydroxyl and ...pZ. As will be shown below, we have established that under the conditions of our experiment there is formation neither of new ...Yp fragments nor of new nucleoside end groups.

Possible cleavage of Type a will be discussed first. The enzyme preparation used does not dephosphorylate 3'-mononucleotides or oligonucleotides; therefore, cleavage of Type a would result in the formation of a resistant core owing to the fact that snake venom diesterase attacks polynucleotide chains ending with a 3'-phosphate either not at all or at a rate much slower than that obtainable when the substrate contains unsubstituted 3'- and 2'-hydroxyl terminus. Fig. 5 shows that when a fraction of the polynucleotide chain ends with a 3'-phosphate group, digestion with snake venom phosphodiesterase stops far short of completion, and a large undigested core ac-

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**TABLE I**

| Mononucleotide composition in partial enzymatic digest of polyribonucleotides containing adenosine, guanidine, uridine, and cytidine |
|---------------|----------------|----------------|
| AMP           | CMP            | UMP            |
| 12.0          | 38.1           | 22.4           |
| 8.4           | 42.5           | 22.6           |
| 10.0          | 41.2           | 24.7           |

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*One hundred per cent hydrolysis is defined as in Fig. 1.*

Fig. 2. Inhibitory effect of MgCl₂ and NaCl on hydrolysis of yeast S-RNA by the enzyme. *Curve A* represents the control, for which conditions were the same as those for *Curve A* of Fig. 1. *Curve B* represents the results obtained when 0.3 M NaCl was added to the solution used in *Curve A*. *Curve C* represents the results obtained when CaCl₂ was replaced in the reaction mixture by MgCl₂, 0.02 M.

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Fig. 4. Possible modes of endonucleolytic attack on a polynucleotide.
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Fig. 5. Relation between rate and extent of reaction and presence of 3'-phosphate terminal groups. Control is yeast S-RNA; NaOH treated is S-RNA after partial hydrolysis by 0.5 m KOH; RNase core is yeast RNA after exhaustive digestion with pancreatic RNase followed by precipitation with 10% trichloroacetic acid, to remove mononucleotides and smaller oligonucleotides, and dissolution of the precipitate in dilute buffer followed by dialysis. Phosphate (phosphatase) treated was prepared by the same procedure as RNase core, with addition of E. coli alkaline phosphatase.

**Fig. 6.** Amount of nucleoside and inorganic phosphate formed in the reaction mixture during digestion by the enzyme. Free nucleoside (□) and inorganic phosphate (△) in the reaction mixture increased simultaneously during the reaction owing to the contaminating 5'-mononucleotidase. The amount of nucleotide produced by alkaline hydrolysis (○) did not show any change during phosphodiesterase digestion. The phosphodiesterase reaction was allowed to proceed under the same conditions as were shown in Fig. 1 with CaCl₂.
necessary when plotting the data to move the origin and change the direction of the abscissa on which the theoretical distribution curves are plotted. When Equation 1 gives a chain length of 0, it is plotted as 70; a value of 10 is plotted as 60; a value of 20, as 50; and so on.

The procedure for determination of the chain length distribution as a function of the degree of enzymatic hydrolysis consisted of two steps: the first was to separate the mixture of polymers obtained by enzymatic digestion of S-RNA into fractions according to their lengths; and the second was to determine the chain length of each fraction. Two procedures were adopted for the first step: chromatography on DEAE-Sephadex, and ultracentrifugation in a sucrose gradient.

After separation by one of these methods, the estimation of chain length was performed by a determination of the ratio of nucleosides to total nucleotides after complete alkaline hydrolysis of the fraction.

1. DEAE-Sephadex—Fig. 7 shows a representative experiment in which, after the enzymatic hydrolysis had been allowed to proceed to 29%, an aliquot of the reaction mixture was chromatographed on a DEAE-Sephadex column. For chain length determination, Fractions 40 to 44 (pooled), 45, 46, 47 and 48, 49 and 50, 51 to 54 (pooled), and 55 to 61 (pooled) were hydrolyzed with 0.33 N KOH. The chain length of these fractions was respectively 35, 40, 44, 47, 51, 54, and 62 ± 1. Fig. 8A shows chain length as a function of the number of chains. The points define a narrow, relatively smooth, bell-shaped curve. Fig. 8B describes an experiment performed exactly in the same manner after enzymatic digestion had been allowed to proceed to 82%. In this case, the curve is much broader and not as symmetrical since, as would be expected, a number of chains have been completely digested (chain length of 1).

2. Sedimentation in Sucrose Gradient—With the use of the sucrose gradient centrifugation technique of Martin and Ames (21), a comparison was made of the sedimentation profiles of undigested yeast S-RNA and the profiles of S-RNA that had been digested to approximately 30% completion with snake venom phosphodiesterase. S values were determined as described by Martin and Ames (21). The sedimentation constant of yeast S-RNA was 4.6 S (or approximately the same as that for rabbit liver S-RNA). The sedimentation constant of S-RNA after 30% enzymatic digestion with snake venom diesterase was 4.8 S. Comparable results were obtained in the Spinco analytical ultracentrifuge with the use of ultraviolet optics. Since the partially digested preparation has a smaller molecular weight, the fact that its S value does not differ greatly from that of the starting material suggests aggregation or, more likely, a change in the hydrodynamic shape of partially digested S-RNA. Further studies of molecular shape, hydration, and ionic interaction will be required before a quantitative estimate of the nature of the change and the degree of heterogeneity can be made.

The sedimentation patterns of S-RNA digested by T1 ribonuclease and 0.5 M NaOH are shown in Fig. 9, C and D. In both cases the cleavage is random and endonucleolytic. The difference between these sedimentation profiles and the ones obtained with snake venom enzyme is striking: a very small amount (3%) of random endonucleolytic cleavage leads to a large increase in sedimentation heterogeneity, in contrast to the very small changes resulting from 30% digestion with snake venom diesterase.

![Fig. 7. Elution pattern of partially hydrolyzed S-RNA (29%) from DEAE-Sephadex column.](http://www.jbc.org)

![Fig. 8. Correlation of the chain length and the number of the polynucleotide chain is expressed as a percentage of the total number of chains. Subfractions separated by DEAE-Sephadex chromatography were digested by 0.3 N KOH at 37° for 18 hours and neutralized by perchloric acid, and the nucleoside to total nucleotide ratio for each subfraction was determined as described in the text. The degree of hydrolysis stated in the figure was also measured by Dowex 1-X2 (see the text). The smooth curves represent the calculated distributions.](http://www.jbc.org)
TABLE III

Liberated mononucleotide composition in partial enzymatic digest of S-RNA

Each component was evaluated from the chromatographic experiment, as shown in Fig. 10. Columns titled A give micromoles of mononucleotides per pmole of S-RNA, where 1 pmole of S-RNA contains 70 mononucleotides. Columns titled B give the percentage of hydrolysis for each component; for example, since there are 11 pmoles of AMP per chain, 3.2 pmoles of AMP are equivalent to 29%.

<table>
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<th></th>
<th>A</th>
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<tr>
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<td>11</td>
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% Hydrolysis

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<td>53</td>
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* The abbreviations used are: MeAMP, methylated adenosine 5'-phosphate; and MeGMP, methylated guanidine 5'-phosphate.

**X**, unknown mononucleotide.

Fig. 10 differs from the elution pattern reported earlier for alkaline digests of S-RNA, but the difference may be ascribed to the fact that in the present instance we were dealing with 5'-mononucleotides rather than with a mixture of 2'- and 3'-mononucleotides.

Table III lists the mononucleotides liberated for partial digestion of yeast S-RNA by the enzyme. These data are similar to the results obtained earlier with rabbit liver S-RNA (12). It is evident that the minor components, especially 5UMP, are predominantly located in the central segment of the S-RNA chains.

Hyperchromicity upon Hydrolysis—Fig. 11 shows the relationship between the increase in optical density of a solution of S-RNA and the degree of hydrolysis.
In Experiment A, S-RNA was digested with snake venom phosphodiesterase. The hyperchromic effect develops rapidly and approaches completion when 40 to 50% of the polynucleotide chains have been digested to mononucleotide residues. Since there is good ground for the concept that the degree of hypochromicity in polynucleotides is dependent upon the nature of the secondary structure and the degree of ordering of the bases (24-26), this finding indicates that almost total loss of ordered secondary structure in S-RNA is attendant upon shortening of the polynucleotide chain by approximately 40 to 50%. In contrast, when S-RNA was digested with 0.5 M KOH, where attack is random and endonucleolytic, the increase in optical density roughly parallels the degree of hydrolysis (Experiment B). Similar results were obtained when random endonucleolytic attack was obtained by use of T1 and pancreatic RNases.

These results are entirely consistent with present concepts of the structure of S-RNA (14, 15), which represent this molecule as consisting of a polynucleotide chain doubled back upon itself to form a double helical region that in different models consists of approximately 25 or 35 base pairs, the two base-paired limbs in this region are joined by a non-base-paired loop structure consisting of either 15 or 3 nucleotides.

As reviewed above, the ionic environment exerts a great influence on the rate and extent of hydrolysis of DNA and S-RNA by the phosphodiesterase. The influence of the ionic environment must be a complex phenomenon since divalent and monovalent cations affect, probably in different ways, the enzyme and the substrate. Thus the enzyme requirement for a divalent cation can be satisfied by Ca++ and Mg++, Mn++ or Zn++. When oligonucleotides are used as substrate (1), whereas there are striking differences when S-RNA is the substrate. It is thus suggested that Ca++ and Mg++ affect the structure of S-RNA in different ways.

DISCUSSION

The concept that snake venom phosphodiesterase acts on S-RNA exclusively as an exonuclease by successive liberation of 5’-mononucleotide units from the end of the chain bearing an unsubstituted 3’-hydroxyl group has been examined and verified. Earlier conclusions on the mode of action of the enzyme on polynucleotides (1-7) are thus confirmed. The results in support of these conclusions are: (a) in its early stages, hydrolysis of S-RNA by phosphodiesterase does not result in the formation of detectable amounts of short oligonucleotides; (b) there is no increase in 3’-hydroxyl terminal groups or 5’-monophosphate end groups in the S-RNA solution during hydrolysis by phosphodiesterase (the small amount of mononucleotides produced during the reaction was found to be a result of contamination with 5’-mononucleotidase); (c) when S-RNA is subjected to limited digestion by ribonuclease T1 or alkali, its ultracentrifuge pattern in a sucrose gradient shows a greater sedimentation heterogeneity than is shown by S-RNA digested to a greater extent by snake venom phosphodiesterase; (d) experiments with synthetic polyribonucleotides show that there is no discrepancy in susceptibility to phosphodiesterase action among four different bases; and (e) venom phosphodiesterase reaction with S-RNA runs a smooth time course till completion. The finding that the chain length distribution of the unhydrolyzed portion of S-RNA is in good agreement with theoretical expectation is another and most significant criterion since it tests the requirements for both exonucleolytic activity and lack of specificity of the bases attacked.

Consequently, we believe that the composition of mononucleotides released during the course of phosphodiesterase digestion reflects the base sequence of S-RNA chains in the portion of the chain that has been hydrolyzed. Data presented in this paper confirm fully for yeast S-RNA earlier deductions on the predominant arrangement of 7 uridine and the methylated purines in clusters in the central portion of the rabbit liver S-RNA chains (12, 27) and provide support for the base sequence model of S-RNA developed by McCully and Cantoni (14). Thus, it appears that the key structural features are common to S-RNAs derived from different biological species.

SUMMARY

The hydrolytic action of snake venom phosphodiesterase on soluble ribonucleic acid in yeast and synthetic polyribonucleotides was examined. It was found that these polynucleotides were digested completely by the enzyme. All of the evidence in our study, as well as in earlier studies, indicates that the action of phosphodiesterase is exonucleolytic and devoid of base specificity. In confirmation of earlier findings with soluble ribonucleic acid in rabbit liver, it was seen that the methylated purines and pyrimidines, and also 7-uridine, of soluble ribonucleic acid in yeast show a tendency to group together in the central segments of the chain.

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