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

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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 exonuclease, 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 (13) for polymers formed by the successive addition of monomers to a fixed number of chains. We concluded therefore that the composition of the mononucleotide fraction released after partial hydrolysis reflects the distribution of bases in the hydrolyzed portion of the average S-RNA chain and is, therefore, helpful in the determination of the base sequence of the average S-RNA molecule.

In this paper, we confirm earlier observations on the exonucleolytic action of snake venom phosphodiesterase and on the indifference of the enzyme to the base composition of its substrate. We show also that the chain length distribution of the nondigested portion of the substrate conforms to that predicted by theoretical calculations based on 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 adamanteus venom (Ross Allen’s Reptile Institute, Silver Springs, Florida) was prepared by a method of Razzell and Kruss (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 preparation 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 Merril (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 diposphates 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 nucleosides to total nucleotides by chromatography on Dowex 1-X2 (formate). The absorbance of the solution was 20 to 30% greater than the initial value owing to the hypochromicity of native S-RNA.

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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 \( \ldots \text{YpZ} \ldots \) are conceivable: (a) cleavage of the diester bond \( \ldots \text{Yp} \rightarrow \text{Z} \ldots \) and formation of two oligonucleotide fragments ending respectively in \( \ldots \text{Yp} \) and \( \ldots 5'-\text{hydroxyl} \), or (b) cleavage of the bond \( \ldots \text{Y} \rightarrow \text{pZ} \ldots \), which would result in two oligonucleotides ending respectively in \( \ldots \text{Y-3'}-\text{hydroxyl} \) and \( \ldots \text{pZ} \). As will be shown below, we have established that under the conditions of our experiment there is formation neither of new \( \ldots \text{Yp} \) 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-

![Fig. 2](http://www.jbc.org/)

**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.

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Relation between reaction rate and concentration of S-RNA. The reaction mixture contained 0.1 M Tris (pH 8.9), 0.02 M CaCl₂, 0.010 mg per ml of the enzyme protein, and various concentrations of S-RNA. Other conditions were as in Fig. 1. In this experiment, the rate of the reaction is expressed as the amount of mononucleotide monomers formed during the first 5 hours.

![Table I](http://www.jbc.org/)

**Table I**

| Mononucleotide composition in partial enzymatic digest of polyribonucleotides containing adenosine, guanidine, uridine, and cytidine |
|---|---|---|
| Phosphodiesterase | A | B |
| AMP | 12.0 | 8.4 | 10.0 |
| CMP | 38.1 | 42.5 | 41.2 |
| UMP | 22.4 | 22.6 | 24.7 |
| GMP | 27.6 | 26.4 | 24.0 |
| Sum | 100.1 | 99.9 | 99.9 |

% Hydrolysis* 29 44 99

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

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Possible modes of endonucleolytic attack on a polynucleotide.

The fact that enzymatic digestion goes to completion under our conditions indicates that endonucleolytic cleavage of Type a, if it occurs at all, is very limited. Even if 3' phosphate-ended chains were not completely resistant, we would expect a progressive decrease of the reaction rate. Figs. 1 and 2 show that this is not the case. The lower limit of endonucleolytic cleavage of Type a was measured directly with the use of Escherichia coli alkaline phosphatase to determine the amount of

![cumulates](http://www.jbc.org/)

The data in Table I indicate that under our 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.
under conditions in which 250 to 650 in 1000 diester phosphate is susceptible to the action of the phosphatase shown in Table II indicate that, at most, only 1 phosphate in a partial digest of S-RNA by snake venom. The results of a partial digest of S-RNA by snake venom phophodiesterase (4.7 pmoles of TP), and the fraction remaining after 66.5 digestion of S-RNA with snake venom phosphodiesterase). * The abbreviation used is: TP, total phosphate.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
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<tr>
<td>Native S-RNA</td>
<td>22.3</td>
</tr>
<tr>
<td>Dephosphorylated S-RNA</td>
<td>0.1</td>
</tr>
<tr>
<td>S-RNA (after 22% digestion with snake venom phosphodiesterase)</td>
<td>0.13</td>
</tr>
<tr>
<td>S-RNA (after 66.5 digestion with snake venom phosphodiesterase)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

<table>
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<th>Subscript</th>
<th>Subscript</th>
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<th>Subscript</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>i</td>
<td>N</td>
<td>N</td>
<td>e</td>
</tr>
</tbody>
</table>

where N_e is the total number of chains; N_0, the number of chains with chain length z; and v, the number of monomers consumed per chain. It may be seen that this treatment is applicable to the case of the snake venom digestion of S-RNA, since the number of chains remains constant (i.e. there is no endonuclease activity) and the rates of all bond-breaking steps are equal (there is no base specificity). Since we are concerned with the degradation of a polymer rather than with its synthesis, it is bonds are cleaved by the exonucleolytic activity of the snake venom phosphodiesterase.

Next, we shall discuss evidence that excludes an endonucleolytic cleavage of Type b. This process was considered more likely since it would resemble the exonucleolytic activity of the enzyme in that the 3'-phosphate bonds would be cleaved. In this case, one new nucleoside end group and one new 5'-monooester phosphate end group would appear for each internal phosphodiester bond cleaved. Fig. 6 shows that the number of nucleoside end groups in S-RNA (determined by alkaline digestion) remains constant during hydrolysis by snake venom phosphodiesterase digestion and thus provides direct experimental evidence against endonucleolysis of Type b.

The absence of endonucleolytic activity under our experimental conditions is indicated also by the experiment described in the next section.

**Analysis of Chain Length Distribution**—Since the enzyme attacks every bond in the substrate without marked specificity and the action is exclusively exonucleolytic, the relative amounts of the mononucleotides liberated at any given time by the action of the enzyme reflect the mononucleotide sequence in the fraction of polymeric substrate that has been digested. The type of information obtainable from this approach is restricted, however, since the enzyme is attacking a mixture of different molecular species at random. The statistical situation resembles that explored by Flory (13). Flory has shown that when a polymer is formed by the successive addition of monomer units at a uniform rate to a constant number of chains, the distribution of species of various chain lengths is given by the expression

\[
N_e = \frac{e^{-vz}}{(x-1)^v}
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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 distribu-
tion 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 accord-
ing 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 ultra-
centrifugation 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 hydroly-
sis of the fraction.

1. **DEAE-Sephadex**—Fig. 7 shows a representative experi-
ment 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 frac-
tions was respectively 35, 40, 44, 47, 51, 54, and 62 f 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 de-
scribed 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 inter-
action 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 T2 ribo-
nuclease 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.
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>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
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<td>6.9</td>
<td>61</td>
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<td>67</td>
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<td>UMP</td>
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<td>70</td>
<td>9.7</td>
<td>88</td>
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<tr>
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<td>&lt;11</td>
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<td>45</td>
<td>0.4</td>
<td>91</td>
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<tr>
<td>MeGMP</td>
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<td>0.7</td>
<td>60</td>
<td>1.1</td>
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<tr>
<td>ψ UMP</td>
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<td>0.6</td>
<td>25</td>
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<tr>
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<td>49.8</td>
<td>72</td>
<td>67.9</td>
<td>72</td>
</tr>
</tbody>
</table>

% Hydrolysis

* The abbreviations used are: MeAMP, methylated adenosine 5'-phosphate; and MeGMP, methylated guanidine 5'-phosphate.

b 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 ψ UMP, 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, this finding indicates that 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 on the enzyme requires 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 the yeast S-RNA earlier deductions on the predominant arrangement of uridine and the methylated purines in clusters in the central portion of the rabbit liver S-RNA (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 exo- or endonucleolytic 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 uridine, of soluble ribonucleic acid in yeast show a tendency to group together in the central segments of the chain.

**References**

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