A major concern in attempts to relate the structure of soluble ribonucleic acid with biological function is whether an entire molecule of the acid is needed for biological function. Specific monophosphatases and exonucleases offer a tool with which to approach this question. Hydrolysis of S-RNA with venom phosphodiesterase (1) destroys the amino acid-accepting ability of S-RNAs (2, 3), presumably through removal of the amino acid-binding site. Treatments with spleen phosphodiesterase (4) designed to remove nucleotides from the amino acid-non-binding end of S-RNAs have yielded conflicting results concerning the essentiality of this end of the molecule (2, 5). Further, these reports are difficult to interpret because of lack of information on the products of the hydrolyses. This paper shows that spleen phosphodiesterase does act as an exonuclease upon the amino acid-nonbinding end of yeast S-RNA and that such action results in rapid loss in the amino acid-accepting ability of yeast S-RNA.

EXPERIMENTAL PROCEDURE

Amino Acid-activating Enzyme of Yeast—An amino acid-activating enzyme preparation active towards a wide variety of L-amino acids was prepared as follows. Equal weights of bakers' yeast (Fleischmann), 150-μ glass beads, and 0.05 M Tris-Cl-0.001 M MgCl₂-0.001 M 2-mercaptoethanol, pH 7.5, were blended in a cooled colloid mill for 10 minutes. Particulate matter was then removed by centrifugation (10,000 × g for 10 minutes, decantation, and then 105,000 × g for 60 minutes), and the clear supernatant (obtained from under the surface lipids) was dialyzed for 24 hours against 0.01 M Tris-Cl-0.0001 M potassium EDTA 0.0002 M 2-mercaptoethanol, pH 7.5. This enzyme preparation is stable for at least 2 months if frozen in a Dry-Ice bath and then stored at −15°C.

Assay Procedures—Yeast S-RNA was prepared by the method of Holley et al. (6) and further purified by gel filtration over Sephadex G-75 equilibrated with 0.01 M ammonium formate. Unless stated otherwise, incubations involving spleen or venom phosphodiesterase (both enzymes as obtained from Worthington Biochemical Corporation) were performed at 37°C with solutions of S-RNA, or S-RNA-AA, in an appropriate buffer. Aliquots (0.5 ml each), containing 2 mg of original S-RNA, were then removed at intervals and heated for 5 minutes at 100°C. These solutions were cooled and assayed directly for their ability to accept amino acids (6) with either enzyme preparations from yeast (above) or the L-tyrosine-activating enzyme of hog pancreas (7). Amino acid-accepting ability was compared with S-RNA similarly treated in the absence of diesterase and expressed as percentage of amino acid-accepting activity retained. Boiling or incubation of S-RNA with heat-denatured diesterase does not alter the ability of S-RNA to accept amino acids.

The percentage of hydrolysis of S-RNA as catalyzed by diesterase was measured by addition of 0.5-ml aliquots of incubations, containing diesterase and S-RNA, to 0.5 ml of 0.6 M HClO₄, sedimentation of the precipitates (10,000 × g for 10 minutes), and comparison of the absorption of the neutralized supernatants with the absorption obtained from a similar neutralized aliquot of S-RNA previously hydrolyzed with KOH.

The percentages of adenosine and cytidine present on the amino acid-accepting ends of intact and hydrolyzed S-RNAs were determined as follows. Two 40-μg samples of S-RNA (one with added spleen phosphodiesterase) were dissolved in 4 ml of 0.1 M potassium succinate, pH 7.4, and taken through the enzymic hydrolysis procedure (see above). After removal of aliquots for determination of percentage of hydrolysis (no hydrolysis occurred in the absence of added diesterase), the remaining boiled samples were passed over Sephadex G-75 to remove small molecules released by diesterase. After lyophilization to dryness, the S-RNA samples were hydrolyzed in 3 ml of 0.3 M KOH for 16 hours at 37°C, back-titrated to pH 9.5 with 0.6 M HClO₄, freed of KClO₄ by centrifugation, and then passed through a water-washed column of Dowex 1-X8 (formate form, 100 to 200 mesh), 0.8 × 22 cm. Under these conditions two separate ultraviolet-absorbing materials are eluted sequentially from the column. These were identified as cytidine followed by adenosine on the basis of their characteristic absorption spectra at different pH values and cochromatography on paper with standards in three different solvent systems. The total absorbance found in each nucleoside was converted to micromoles by using the known extinction coefficients for adenosine and cytidine and a determined extinction coefficient for nucleotides of alkaline hydrolyzed S-RNA based upon a molecular weight, of 27,600 and an average nucleotide chain length of 80.
action of spleen phosphodiesterase upon yeast s-rna

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alanine

tyrosine

leucine

valine

% amino acid retained

% hydrolysis

fig. 1. effect of spleen phosphodiesterase treatment upon amino acid-incorporating ability of yeast s-rna. samples (67 mg each) of yeast s-rna dissolved in 15.5 ml of 0.1 m potassium succinate buffer, ph 6.7, were incubated for 0, 10, 30, 60, and 120 minutes with 10 units of spleen phosphodiesterase. other assays were as described in "experimental procedure."

results and discussion

hydrolysis of yeast s-rna with spleen phosphodiesterase results in a rapid loss in the ability to accept a variety of amino acids (fig. 1). this effect is not limited to the yeast enzyme-yeast s-rna system used, for activating enzymes from other species yield similar results with yeast s-rna (fig. 7). spleen phosphodiesterase acts as an exonuclease on the 5'-hydroxy end of oligoribonucleotides (8). therefore these data suggest that some feature of the amino acid-nonbinding end of s-rna is essential in accepting activated amino acids.

proof of this theory requires elimination of several other explanations for the results of fig. 1. first, the products released from s-rna by hydrolysis catalyzed by spleen phosphodiesterase could be inhibitory to the subsequent amino acid incorporation reaction. this hypothesis is eliminated by the results of fig. 2, in which s-rna and s-rna previously activated by treatment with spleen phosphodiesterase (i.e., containing all products of a 30% hydrolysis) are assayed together. the rate and the extent of amino acid incorporation into the unhydrolyzed s-rna are unaffected by the presence of the hydrolyzed sample.

a second explanation is that the spleen phosphodiesterase preparation contains endonuclease activity which results in internal ruptures of the s-rnas. the various forms of sephadex offer a means of testing this hypothesis, for they will distinguish between molecules having different molecular weights. unhydrolyzed yeast s-rna is eluted as a single narrow width component on all three grades of sephadex (fig. 3). yet when these same samples of yeast s-rna are hydrolyzed to an extent of 4% by spleen phosphodiesterase, varied elution patterns are obtained. the elution pattern on sephadex g-75 trails, indicating a partial inclusion of the hydrolyzed s-rna molecules. (comparison of the elution patterns of intact s-rnas with various large molecule markers shows that s-rnas are just excluded by sephadex g-75.) the appearance of a major narrow peak when this same sample is passed over sephadex g-50 indicates that the majority of the hydrolysis products have molecular weights in excess of 10,000. elution of the same sample over sephadex g-25 yields an included or small molecule peak, equal in size to that observed upon elution over sephadex.
Fig. 4. Effect of spleen phosphodiesterase upon the sedimentation properties of yeast S-RNA. Yeast S-RNA (10 mg per ml of 0.1 M potassium succinate, pH 7.6) was hydrolyzed with spleen phosphodiesterase and subsequently assayed as described in "Experimental Procedure." The hydrolyzed S-RNA and a sample of S-RNA treated similarly in the absence of spleen phosphodiesterase were then centrifuged at the same time in a Spinco model E ultracentrifuge containing both a standard cell and a wedge cell. Centrifugation was performed at 56,100 r.p.m. and 23°C. Sedimentation is from left to right. Pictures were taken at 16-minute intervals with phase plate angles of 55° (first two) and 50° (last two). The calculated Svedberg values at 23°C and 10 mg per ml are 3.78 for the original S-RNA and 3.57 for the 9.9% hydrolyzed S-RNA.

TABLE I

Effect of E. coli alkaline phosphatase treatment upon properties of yeast S-RNA

Gel-filtered yeast S-RNA (135 mg; 30 mg per ml of 0.1 M Tris-Cl, pH 7.5) was treated with 0.38 mg of chromatographically purified E. coli alkaline phosphatase ( Worthington) for 10 minutes at 37°C. Additional phosphatase treatment failed to release further P_i. The phosphatase-treated S-RNA was then reisolated by the phenol method (6) and compared with S-RNA prepared in a similar manner in the absence of phosphatase. Spleen phosphodiesterase hydrolysis (2 units of enzyme; 4 mg of nucleic acid per ml of 0.01 M sodium succinate, pH 6.7) and L-tyrosine incorporation (yeast enzyme) procedures were as described in "Experimental Procedure." Stoichiometry of phosphate release is based upon an S-RNA molecular weight of 30,000.

<table>
<thead>
<tr>
<th>Material</th>
<th>P_i released per mole of S-RNA</th>
<th>Hydrolysis per minute of treatment with spleen phosphodiesterase</th>
<th>L-Tyrosine incorporated per mg of S-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-RNA</td>
<td>0.915</td>
<td>0.5</td>
<td>0.10</td>
</tr>
<tr>
<td>Phosphatase-treated S-RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G-50, indicating that the pieces removed from the S-RNA have molecular weights less than 1,000; i.e. they are most likely nucleotides.

Further proof of the exonuclease action of spleen phosphodiesterase can be obtained from centrifugal analysis of S-RNA before and after treatment with spleen phosphodiesterase. As seen in Fig. 4, the sedimentation properties of S-RNA are only slightly changed, even after 9.9% hydrolysis catalyzed by spleen phosphodiesterase. Since endonuclease action should drastically alter the centrifugal properties of S-RNA, spleen phosphodiesterase must act as an exonuclease upon S-RNA. This exonuclease specificity differs from that reported for spleen phosphodiesterase in other systems (5, 8) in that removal of 5'-terminal phosphate of yeast S-RNA does not enhance the rate of hydrolysis catalyzed by the enzyme (Table I).

A third possible cause for the loss in amino acid-accepting ability shown in Fig. 1 is that, contrary to the reported specificity of spleen phosphodiesterase (8), this enzyme hydrolyzes S-RNA at the amino acid-binding end of the molecule. This possibility is eliminated by the experiment of Fig. 5, in which up to 20% hydrolysis of S-RNA-AA does not release amino acid from the remaining "S-RNA-AA." Since the rates of hydrolysis of S-RNA-AA and S-RNA are similar, introduction of an amino acid does not inhibit this hydrolysis.

Additional proof that spleen phosphodiesterase does not act as an exonuclease on the amino acid-binding end of S-RNA comes from a study of the character of the amino acid-binding ends before and after spleen phosphodiesterase-catalyzed hydrolysis. Even limited exonuclease action upon the amino acid-binding end of S-RNA should result in marked changes in the relative proportion of nucleosides released from the amino acid-binding end by alkaline hydrolysis. Yet, as seen in Table I, extensive hydrolysis of S-RNA by spleen phosphodiesterase does not alter the proportions of the nucleosides present upon the amino acid-binding ends of S-RNA. Thus, the exonuclease action must be at the amino acid-nonbinding end of S-RNA.
**Table II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Hydrolysis observed</th>
<th>S-RNA with adenosine on amino acid-binding end</th>
<th>S-RNA with cytidine on amino acid-binding end</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>I</td>
<td>6.3</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>II</td>
<td>7.2</td>
<td>21</td>
<td>23</td>
</tr>
</tbody>
</table>

**Fig. 6.** Effect of high salt concentration and low temperature upon the rate of loss of amino acid-accepting ability of S-RNAs during spleen phosphodiesterase treatment. Yeast S-RNA (50 mg) was dissolved in 13.5 ml of 0.01 M potassium succinate and incubated with 5 units of spleen phosphodiesterase for 10, 30, and 60 minutes at 37° (○, ●, □). An additional 50 mg of yeast S-RNA, dissolved in 13.5 ml of 0.01 M potassium succinate-0.1 M KCl-0.01 M MgCl₂, pH 6.7, and incubated with 5 units of spleen phosphodiesterase for 20, 60, and 120 minutes at 25° (○, ○, ○). Amino acid-accepting ability was measured with the yeast enzyme preparation.

**Fig. 7.** Effect of spleen and snake venom phosphodiesterase treatment upon L-tyrosine-incorporating ability of yeast S-RNA. Samples (20 mg each) of yeast S-RNA, dissolved in 5 ml of 0.1 M potassium phosphate buffer, pH 6.7, were treated for 0, 10, 20, 60, and 120 minutes with 1.5 mg of snake venom phosphodiesterase (●—●) or 5 units of spleen phosphodiesterase (○—○). Amino acid-accepting ability was measured with the L-tyrosine-activating enzyme of hog pancreas (7).

A fourth possible cause for the rapid loss in amino acid-accepting ability of S-RNAs that appears upon treatment with spleen phosphodiesterase is selective hydrolysis of active S-RNA molecules within the S-RNA preparation. Even though the S-RNA preparation migrates as a single peak in the ultracentrifuge, not all of this material is active in accepting amino acids (9). It is therefore possible that the diesterase preferentially hydrolyzes active S-RNA molecules because of a lack of secondary or tertiary structure. (Spleen phosphodiesterase does not hydrolyze double stranded DNA (4).) If one treats S-RNA with spleen phosphodiesterase under conditions designed to favor secondary structure in S-RNA, i.e., high salt and low temperature, the rate of hydrolysis is reduced, but there is the same rapid loss in amino acid-accepting ability (Fig. 6). This indicates that there is no major preferential hydrolysis of active S-RNAs. Final proof of this point requires diesterase treatment of homogeneous, amino acid-specific S-RNAs.

Elimination of these alternate causes for the rapid loss of amino acid-accepting ability of S-RNAs that develops upon treatment with spleen phosphodiesterase leaves only the conclusion that some feature of the amino acid-nonbinding end of an S-RNA is essential for amino acid incorporation. This feature could be terminal 5'phosphate (10, 11), some portion of the terminal nucleotide other than the 5'phosphate, or some portion of a nucleotide near the 5'phospho end of the molecule. The terminal 5'-phosphate is not the essential site, for exhaustive treatment of yeast S-RNA with the phosphomonoesterase of Escherichia coli (12), with the resultant release of 1 mole of phosphate per mole of S-RNA, does not alter the amino acid-accepting ability of S-RNA (Table I). Two lines of evidence point to the terminal nucleotide as an essential site during amino acid incorporation. First, the data of Fig. 1 fit first order reaction kinetics, indicating that a single hydrolytic event determines activity. Second, the loss in L-tyrosine-accepting ability that occurs when S-RNA is hydrolyzed by spleen phosphodiesterase parallels that which occurs when snake venom phosphodiesterase hydrolyzes S-RNA (Fig. 7). The exonuclease, snake venom phosphodiesterase, initially attacks the amino acid within the S-RNA preparation. Even though the S-RNA preparation migrates as a single peak in the ultracentrifuge, not all of this material is active in accepting amino acids (9). It is therefore possible that the diesterase preferentially hydrolyzes active S-RNA molecules because of a lack of secondary or tertiary structure. (Spleen phosphodiesterase does not hydrolyze double stranded DNA (4).) If one treats S-RNA with spleen phosphodiesterase under conditions designed to favor secondary structure in S-RNA, i.e., high salt and low temperature, the rate of hydrolysis is reduced, but there is the same rapid loss in amino acid-accepting ability (Fig. 6). This indicates that there is no major preferential hydrolysis of active S-RNAs. Final proof of this point requires diesterase treatment of homogeneous, amino acid-specific S-RNAs.

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acid-binding nucleotide of S-RNA (2). Therefore, the agreement of the effects of two phosphodiesterases supports the essentiality of the terminal amino acid-nonbinding nucleotide in the amino acid-accepting reaction. Final proof of this point requires correlation of the loss in amino acid-accepting ability with the spleen phosphodiesterase-dependent release of guanosine 3',5'-diphosphate from an amino acid-specific S-RNA.

This requirement of the amino acid-nonbinding end of S-RNA during amino acid incorporation is particularly pertinent in light of the extensive secondary structure demonstrated by yeast S-RNA in assays of absorbance versus temperature ("melting curves") (13). The existence of extensive secondary structure in S-RNA may also explain the earlier contradictory results concerning the activities of S-RNAs after treatment with spleen phosphodiesterase (2, 5). Under most conditions, spleen phosphodiesterase hydrolyzes yeast ribosomal RNA faster than it hydrolyzes yeast S-RNA (Fig. 8). Therefore, slight contamination of S-RNA preparations with ribosomal RNA would result in an apparently high rate of hydrolysis with little or no hydrolysis of actual S-RNA.

The results presented here can also explain the inability of various S-RNA preparations of apparently equal size to accept their calculated stoichiometric amounts of amino acids (9, 14). Cellular disruption with concomitant exposure of S-RNAs to exonucleases active on either end of the molecules will result in loss in amino acid-accepting ability while not greatly altering the over-all size of the molecules. The recent finding that isolated yeast S-RNA is not homogeneous with respect to its terminal nucleotides (15) supports this hypothesis.

SUMMARY

Spleen phosphodiesterase treatment of yeast soluble ribonucleic acid (S-RNA) results in a rapid loss in L-amino acid-accepting ability within the S-RNA. This loss in activity follows first order kinetics and parallels that which occurs when a nucleotide or nucleotides are removed from the amino acid-binding end of S-RNA. Analyses of the products of the hydrolysis of S-RNA show that spleen phosphodiesterase acts as an exonuclease upon the amino acid-nonbinding end of yeast S-RNA. Accordingly, some feature of the amino acid-nonbinding end of S-RNA is required during amino acid incorporation into S-RNA.

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Action of Spleen Phosphodiesterase upon Yeast Soluble Ribonucleic Acid
J. M. Clark, Jr., Jaime P. Eyzaguirre and Jay K. Gunther


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