

Dedicated to Professor Maciej Wiewiórowski on the occasion of his 80th birthday

Nonenzymatic hydrolysis of oligoribonucleotides. V. The elements affecting the process of self-hydrolysis^{*}

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Chemical instability of some of the phosphodiester bonds, often observed in large RNAs, visualizes the autocatalytic properties of this class of nucleic acids. Unexpectedly, selective hydrolysis occurs also in short oligoribonucleotides (as short as a tetramer or hexamer). Herein, we describe additional experiments which support the conclusion that the hydrolysis is not due to ribonuclease contamination but is of autocatalytic origin and is related to the sequence and structure of single-stranded oligomers. Moreover, we show that the presence in the reaction mixture of polyamines, such as spermidine, is essential for hydrolysis of oligoribonucleotides.

The discovery of the nonenzymatic hydrolysis of the precursor tRNA and self-cleavage of the intron from *Tetrahymena thermophila* changed our understanding of the stability and function of RNA [1, 2]. A similar nonenzymatic processing of RNA was found in viruses, viroides, virosoides and satellite RNA [3]. In general, the nonenzymatic hydrolysis of RNA requires interaction between two

RNA strands (or one for intrastrand hydrolysis) and presence of a bivalent metal cation. One of the strands, the so called ribozyme, acts as an enzyme mediating hydrolysis of the second RNA strand. The products of hydrolysis are oligomers terminated usually with 2',3'-cyclic phosphate on 5'-side and 5'-hydroxyl on 3'-side. The selective and nonenzymatic character of the RNA hydrolysis means

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Abbreviations: PVP, polyvinylpyrrolidone; t_m , melting temperature.

that lower chemical stability of some phosphodiester bonds in RNA is presumably a consequence of the RNA structure surrounding these phosphodiester bonds.

In the past we have described nonenzymatic, selective hydrolysis of oligoribonucleotides as short as a tetramer or hexamer [4, 5]. The hydrolysis was observed in 50 mM Tris/HCl (pH 7.5) in the presence of 1 mM spermidine, 1 mM EDTA and millimolar concentration of some cofactors (see below). The hydrolysis occurred mostly at UA and CA phosphodiester bonds of single stranded oligoribonucleotides.

Several cofactors were tested and found to enhance hydrolysis of oligoribonucleotides. They can be divided into two groups. The first group includes: T4 polynucleotide kinase, T7 RNA polymerase, restriction endonuclease *Eco*R1, reverse transcriptase, bovine serum albumin (BSA), trypsin, pepsin, lysozyme, RNasin and Inhibit-ACE. The last two are ribonuclease inhibitors and commonly used during isolation of various RNAs. Proteinase K was the only one among the tested enzymes which did not affect hydrolysis of oligoribonucleotides. The second group includes: polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), Brij 58 (monoalkyl ether of PEG) and dextrin. Several tested organic compounds did not affect hydrolysis of oligoribonucleotides, e.g. polyglutamic acid, polyproline, (glycine)₆, sucrose, α -cyclodextrine and imidazole.

In this paper we describe experiments strongly supporting the preliminary conclusion that ribonuclease contamination is not responsible for oligoribonucleotide hydrolysis. We present also an analysis of the factors affecting the rate of hydrolysis and demonstrate that single-stranded structure of oligoribonucleotides is required for this reaction. Lastly, we report the results of experiments on the stability of intervening sequence (IVS) from *T. thermophila* under the conditions used for hydrolysis of the oligoribonucleotides.

MATERIALS AND METHODS

Synthesis and purification of oligoribonucleotides. The oligoribonucleotides used in the experiments were synthesized chemically by a phosphoramidite method on polymer support, and deprotected according to a published procedure [6, 7]. Purification was performed on silica gel plates (0.5 mm, Merck) in propanol-1/ammonia/water (55:35:10, by vol.). The purity of oligoribonucleotides was analyzed by C-8 high performance liquid chromatography (HPLC) and 20% polyacrylamide gel electrophoresis (PAGE). Oligoribonucleotides were labeled with ³²P at their 5' termini with T4 polynucleotide kinase; the labeled oligomers were purified by thin-layer chromatography (TLC).

Materials. The reagents used in this work came from the following suppliers: T4 polynucleotide kinase (Biolab), reverse transcriptase (Life Science), T7 RNA polymerase [8], proteinase K (Sigma), Inhibit-ACE (3'-5'), RNasin (from human placenta, Sigma) [9], ribonuclease A and pepsin (Worthington), dextrin (Aldrich), polyvinylpyrrolidone (Sigma), [α -³²P]ATP and [γ -³²P]ATP (New England Nuclear).

The following buffers were used: 50 mM Tris/HCl (pH 7.5), 1 mM spermidine, 1 mM EDTA (buffer A); 50 mM Tris/HCl (pH 7.5), 1 mM spermidine, 1 mM EDTA and 0.1% PVP (buffer B); 50 mM Tris/HCl (pH 7.5), 1 mM spermidine, 1 mM EDTA, 0.1% PVP and 5 mM MgCl₂ (buffer C), and 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂ and 10 mM dithiothreitol (DTT) (buffer D).

Hydrolysis of oligoribonucleotides. In a typical experiment about 0.1 pmol of ³²P-labeled 5'-phosphorylated oligoribonucleotide was incubated in 50 mM Tris/HCl (pH 7.5), 1 mM spermidine and 1 mM EDTA in the presence of 0.1% PVP solution at 37°C. Aliquots were quenched with formamide and analyzed by electrophoresis on 20% polyacrylamide gel.

RESULTS AND DISCUSSION

Nonribonuclease character of hydrolysis of oligoribonucleotides

One of the most important questions connected with spontaneous hydrolysis of oligoribonucleotides is to obtain the proof that this phenomenon is not due to ribonuclease contamination. At the same time it is important to remember that there are no experiments which could prove or disprove this fact unambiguously. The only way to solve this problem is to perform experiments under conditions which would assure that such ribonuclease contamination is highly unlikely.

The experiments which were performed compared hydrolysis in the presence of one of several cofactors with and without preincubation with proteinase K [10] (Fig. 1). The preincubation of RNasin, Inhibit-ACE, lysozyme and pepsin with proteinase K (50 $\mu\text{g}/\text{mL}$ for 12 h at 37°C) abolished the ability of those cofactors to hydrolyze oligoribonucleotides. At the same time, similar treatment of PVP and dextrin did not change their ability to hydrolyze oligomers. These last two cofactors are organic polymers and should be proteinase K resistant. If we speculate that the hydrolysis of oligoribonucleotides was due to ribonuclease contamination then proteinase K should

hydrolyze the ribonuclease contamination possibly present in PVP, dextrin, lysozyme, pepsin, RNasin and Inhibit-ACE. Since PVP and dextrin preincubated with proteinase K retained their hydrolyzing properties this means that PVP and dextrin (and, by extrapolation of this observation, also lysozyme, pepsin, RNasin and Inhibit-ACE) by themselves are responsible for hydrolysis of oligoribonucleotides.

The next set of experiments concerned a comparison of the rate of UCGUAA hydrolysis in the presence of PVP only, PVP contaminated by ribonuclease A, and PVP contaminated by ribonuclease A and preincubated with proteinase K (Fig. 2). For example, the hydrolysis half-times of UCGUAA to UCGUp and AA in the presence of PVP only and in the presence of PVP contaminated with 20 ng/mL of ribonuclease A and preincubated with proteinase K (50 $\mu\text{g}/\text{mL}$ for 12 h at 37°C) differed by a factor of about 5 (hydrolysis half-times were 1.5 h and 7.5 h, respectively). At the same time ribonuclease A present at a concentration of 20 ng/mL in buffer A containing 0.1% PVP hydrolyzed UCGUAA to UCGUp and AA with a half-time of about 10 s. If we assume that PVP used in the experiments was contaminated by ribonuclease A, then the concentration of ribonuclease A should have been around 35 $\mu\text{g}/\text{mL}$ if the hy-

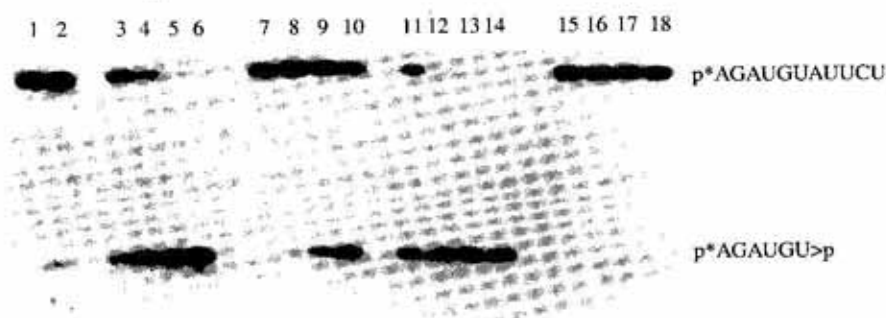


Figure 1. The chemical stability of p*AGAUGUAUUCU in the presence of dextrin and pepsin before and after preincubation of these cofactors with proteinase K.

Lanes 1 and 2: p*AGAUGUAUUCU incubated in buffer A for 0 and 24 h, respectively. Lanes 3-6: p*AGAUGUAUUCU incubated in buffer A containing 0.1% PVP for 1, 4, 8 and 24 h, respectively. Lanes 7-10: p*AGAUGUAUUCU incubated in 0.1% PVP and buffer A which was preincubated with proteinase K (50 $\mu\text{g}/\text{mL}$ for 12 h at 37°C). Lanes 11-14: p*AGAUGUAUUCU incubated in the presence of pepsin (0.4 mg/mL) for 1, 4, 8 and 24 h, respectively. Lanes 15-18: p*AGAUGUAUUCU incubated for 1, 4, 8 and 24 h, respectively, in pepsin (0.4 mg/mL) after the pepsin solution was preincubated with proteinase K (50 $\mu\text{g}/\text{mL}$ for 12 h at 37°C).

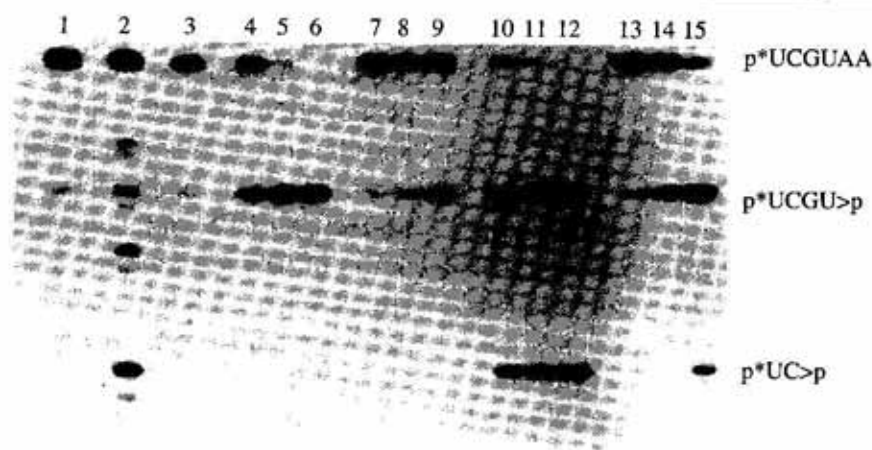


Figure 2. The chemical stability of p*UCGUAA in the presence of PVP and ribonuclease A before and after preincubation with proteinase K.

Lane 1: p*UCGUAA. Lane 2: alkaline hydrolysis of p*UCGUAA (NH_4OH , pH 12, 90°C , 10 min). Lane 3: p*UCGUAA incubated in 50 mM Tris/HCl (pH 7.5) for 25 h. Lanes 4–6: p*UCGUAA incubated in hydrolysis buffer A containing 0.1% PVP for 1, 5 and 25 h, respectively. Lanes 7–9: 0.1% PVP solution was preincubated with proteinase K ($50\ \mu\text{g}/\text{mL}$ for 12 h at 37°C) followed by incubation with p*UCGUAA for 1, 5 and 25 h, respectively. Lanes 10–12: p*UCGUAA incubated with ribonuclease A (20 ng/mL) in the presence of 0.1% solution of PVP for 15 s, 2 and 5 min, respectively. Lanes 13–15: ribonuclease A (20 ng/mL) was preincubated with proteinase K ($50\ \mu\text{g}/\text{mL}$ for 12 h at 37°C) in the presence of 0.1% PVP followed by incubation with p*UCGUAA for 1, 5 and 25 h, respectively.

drolysis half-time similar to that occurring in the presence of PVP only (about 1.5 h) was to be achieved. The fact that proteinase K completely hydrolyzed ribonuclease A means that hydrolysis of UCGUAA in the presence of PVP contaminated with ribonuclease A following preincubation with proteinase K was due to the properties of PVP.

The effect of the reaction conditions on hydrolysis of oligoribonucleotides

For successful observation of specific hydrolysis of the oligoribonucleotides several

conditions have to be fulfilled. They include such requirements as: the presence in the oligoribonucleotide of Y–R (for example UA and CA) or Y–Y (for example UC and CC) phosphodiester bonds (Y – pyrimidine, R – purine), single-stranded structure of oligomer and the appropriate composition of the reaction buffer.

The oligoribonucleotides are the most effectively hydrolyzed in 50 mM Tris/HCl (pH 7.5), 1 mM spermidine, 1 mM EDTA in the presence of a cofactor at 37°C (Fig. 3). To understand which components of the reaction mixture are important, several experiments

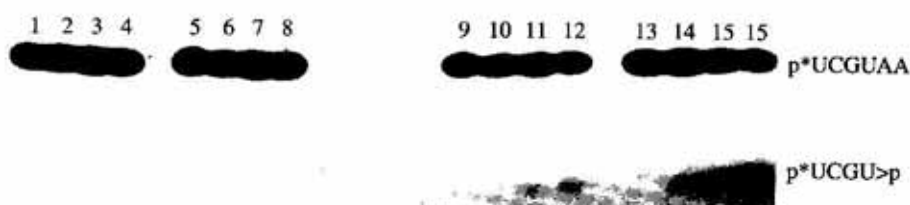


Figure 3. The stability of UCGUAA*pCp in the presence of different components of the buffer at 37°C .

Lanes 1–4: in 50 mM Tris/HCl (pH 7.5), 2 mM EDTA after 0, 10, 24 and 48 h, respectively. Lanes 5–8: in 50 mM Tris/HCl (pH 7.5), 0.1% PVP, 2 mM EDTA after 0, 10, 24 and 48 h, respectively. Lanes 9–12: in 50 mM Tris/HCl (pH 7.5), 1 mM spermidine, 2 mM EDTA after 0, 10, 24 and 48 h, respectively. Lines 13–16: in 50 mM Tris/HCl (pH 7.5), 1 mM spermidine, 0.1% PVP, 2 mM EDTA after 0, 10, 24 and 48 h, respectively.

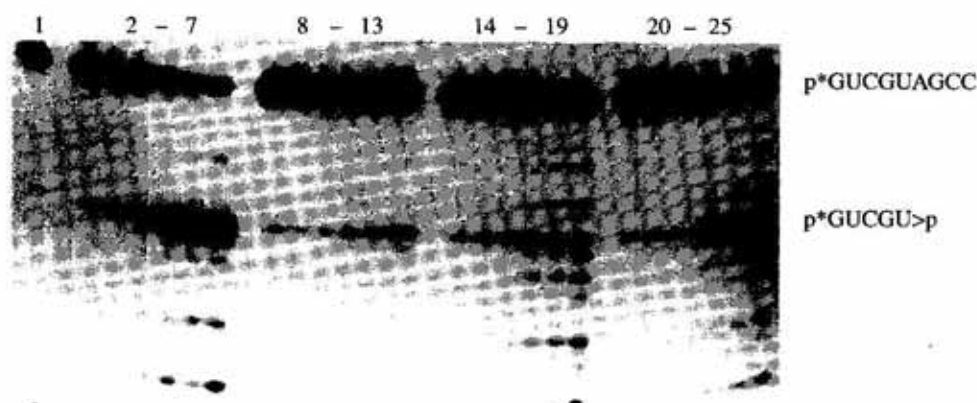


Figure 4. The stability of p*GUCGUAGCC alone and in the presence of the complementary strand – GGCUCGAC.

Lane 1: p*GUCGUAGCC. Lanes 2–7: p*GUCGUAGCC (3.7×10^{-5} M) incubated at 37°C in hydrolysis buffer A in the presence of 0.1% PVP for 0, 1, 5, 24, 48 and 72 h, respectively. Lanes 8–13: p*GUCGUAGCC (3.7×10^{-5} M) and GGCUCGAC (6.8×10^{-5} M) incubated at 37°C in hydrolysis buffer A in the presence of 0.1% PVP for 0, 1, 5, 24, 48 and 72 h, respectively. Lanes 14–19: p*GUCGUAGCC (3.7×10^{-5} M) incubated at 50°C in hydrolysis buffer A in the presence of 0.1% PVP for 0, 1, 5, 24, 48 and 72 h, respectively. Lanes 20–25: p*GUCGUAGCC (3.7×10^{-5} M) and GGCUCGAC (6.8×10^{-5} M) incubated at 50°C in hydrolysis buffer A in the presence of 0.1% PVP for 0, 1, 5, 24, 48 and 72 h, respectively.

with elimination of some components of reaction mixture were performed [11].

The conclusions of these experiments are as follows: (i) the presence of spermidine in the buffer is sufficient for hydrolysis, (ii) the presence of a cofactor, for example PVP, in the buffer containing spermidine significantly enhances the hydrolysis rate (about 7-fold), (iii) the presence of PVP alone in the 50 mM Tris/HCl (pH 7.5) (without spermidine) does not lead to the hydrolysis of oligoribonucleotides, (iv) the replacement of spermidine by Mg^{2+} makes hydrolysis less efficient by a factor of 5, (v) the presence of both spermidine and Mg^{2+} does not increase the hydrolysis rate as compared to that effected by spermidine only.

In order to undergo hydrolysis the oligoribonucleotides must have the single stranded structure

Several experiments have demonstrated that the hydrolysis of oligoribonucleotides containing UA or CA phosphodiester bonds occurs in a single-stranded region and is

structure dependent. For example, it has been found that:

- ◆ self-complementary oligoribonucleotide UCGUACGA does not become hydrolyzed at 37°C at a concentration of 10^{-6} M (calculated t_m at this concentration is 43.8°C) but can be hydrolyzed above t_m of the duplex at 50°C [12];
- ◆ GUCGUAGCC hydrolyzes specifically to GUCGU>p and AGCC at 37°C, however, the addition of the complementary strand – GGCUCGAC – inhibits hydrolysis (Fig. 4). The UV-melting curves for GUCGUAGCC point to its single-stranded character, while in the presence of the complementary oligoribonucleotide (forming a U bulged structure) a curve typical for double-stranded RNA was observed (not shown). Moreover, when GUCGUAGCC was hydrolyzed (the single-stranded structure) at 50°C for 6 h, only a small amount of the products was detected while at an extended time (72 h) random cleavage was observed. At this temperature the presence of the complementary strand did not affect hydrolysis.

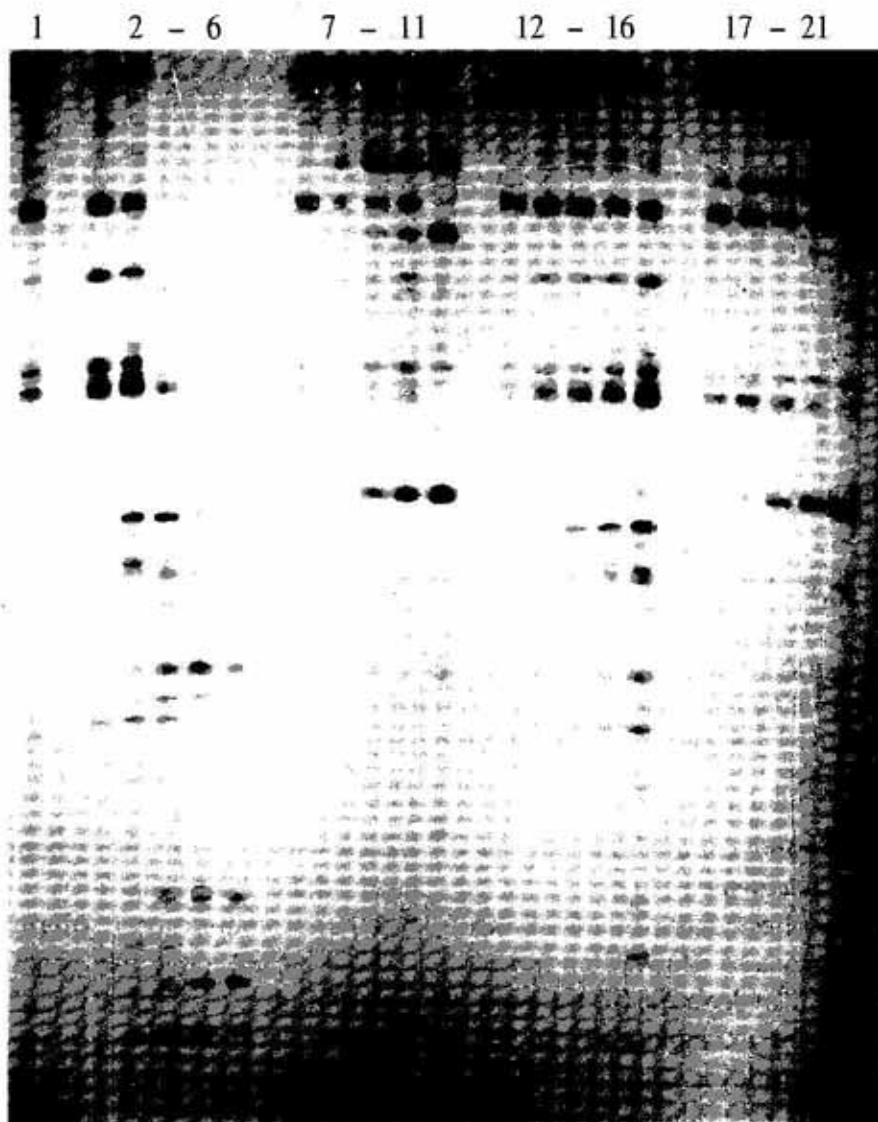


Figure 5. The stability of intervening sequence (IVS) L-21 Sca I of *T. thermophila*.

Lane 1: body labeled L-21 Sca I intron. Lanes 2-6: L-21 Sca I incubated at 37°C in hydrolysis buffer A in the presence of 0.1% PVP. Lanes 7-11: L-21 Sca I incubated at 37°C in hydrolysis buffer A and 5 mM MgCl₂ in the presence of 0.1% PVP. Lanes 12-16: L-21 Sca I incubated at 37°C in hydrolysis buffer A only. Lanes 17-21: L-21 Sca I incubated at 37°C in transcription buffer D in the presence of T7 RNA polymerase. Incubation time in each series was 0, 1, 4, 9 and 24 h, respectively.

The described experiments clearly demonstrated that at 37°C the hydrolysis of the oligoribonucleotides occurred only in single-stranded oligomers, and hybridization with the complementary strand completely inhibited this process. Moreover, for selective cleavage some elements of the single-stranded structure of oligoribonucleotides are necessary. At higher temperature, for example 50°C, the hydrolysis rate was several-fold lower and random hydrolysis was observed.

That was presumably due to lack, under these conditions, of the proper oligoribonucleotide structure.

The stability of an intron from *T. thermophila* in the conditions used during the hydrolysis of oligoribonucleotides

To explore the stability of a large RNA, in the conditions used during selective hydrolysis of the oligoribonucleotides and to compare

hydrolysis requirements of the two RNA classes, an intron from *T. thermophila* [13] was tested. The substrate was L-21 ScaI ribozyme derived from the *T. thermophila* self-splicing large subunit (LSU) ribosomal RNA intron. Plasmid pT7L-21 was linearized by cutting with restriction endonuclease ScaI [14]. T7 RNA polymerase was used to transcribe the cut plasmid in the presence of [α - 32 P]ATP.

The L-21 Sca I ribozyme is a 409 nucleotide long RNA, so it was difficult to determine the position of every phosphodiester bond which was cleaved in the tested conditions. Thus the experiments performed had a qualitative rather than a quantitative character. However, they allowed to evaluate the stability of the entire molecule.

The L-21 Sca I ribozyme was incubated in buffers A–D at 37°C and aliquots of oligomers were analyzed by 10% polyacrylamide gel electrophoresis (Fig. 5). The conclusions from the gel analysis are as follows:

- ◆ L-21 Sca I ribozyme is not stable in buffer B and within 4 h it is completely hydrolyzed to shorter fragments. The hydrolysis is selective and only certain phosphodiester bonds are cleaved. However, some internucleotide bonds are hydrolyzed more intensely and faster than others. In the electrophoretic pattern the primary and secondary cleavage products are present;
- ◆ the presence of 5 mM MgCl₂ in buffer C lowers greatly the hydrolysis rate so that even after 24 h a significant amount of L-21 Sca I ribozyme can be observed. Moreover, the hydrolysis of different phosphodiester bonds other than those seen in the absence of MgCl₂, is observed. The hydrolysis is less intensive and 3–4 predominant cleavage products are present;
- ◆ in buffer A the hydrolysis is much less intensive than in the presence of PVP, the products of hydrolysis are, however, the same;

- ◆ the L-21 Sca I in the presence of T7 RNA polymerase in transcription buffer (buffer D) is cleaved to the same fragments as in buffer C containing 5 mM MgCl₂. This is presumably due to the presence of 3 mM MgCl₂ in the transcription buffer. The presence of Mg²⁺ helps to keep the structure of ribozyme in a more folded form and in this way inhibits hydrolysis of single-stranded fragments.

To test in detail the stability of the phosphodiester bond in the fragment of L-21 Sca I the products of the hydrolysis were analyzed as described by Banerjee *et al.* [15] by using reverse transcriptase and DNA primer (GCGGCTCCAGTGTG) starting at the 350 nucleotide. The analysis of the hydrolysis products is presented in Fig. 6. The cleavage occurred at 5'-side phosphodiester bonds at the following positions: G346, G341, A334, A324, A308, A306, A301 and A290. The arrows (1, 2, 3) mark the increasing efficiency of the cleaved internucleotide bonds. The hydrolysis of L-21 Sca I intron occurred mostly at the internucleotide bond placed in a single-stranded fragment of the intron. However, a few cleavages at UG or UA in single- or double-stranded regions were also observed. This concerns cleavages at G346 (UG at helical fragment), G341 (UG at single-stranded fragment), A324 (UA at helical fragment) and A308 (AU at helical fragment). It is difficult to explain the cleavage of double-stranded fragments. Some of the cleavages can be considered as a consequence of the primary ones. A primary cleavage can change folding of RNA intron and affect single- and double-stranded structure of RNA. Moreover, L-21 Sca I forms a complicated tertiary structure which can influence the RNA cleavage. This observation differs from that concerning the hydrolysis of oligoribonucleotides which are cleaved more efficiently at UA (by 20–50-fold) than at UG phosphodiester bond. Moreover, hybridization of the cleaved oligoribonucleo-

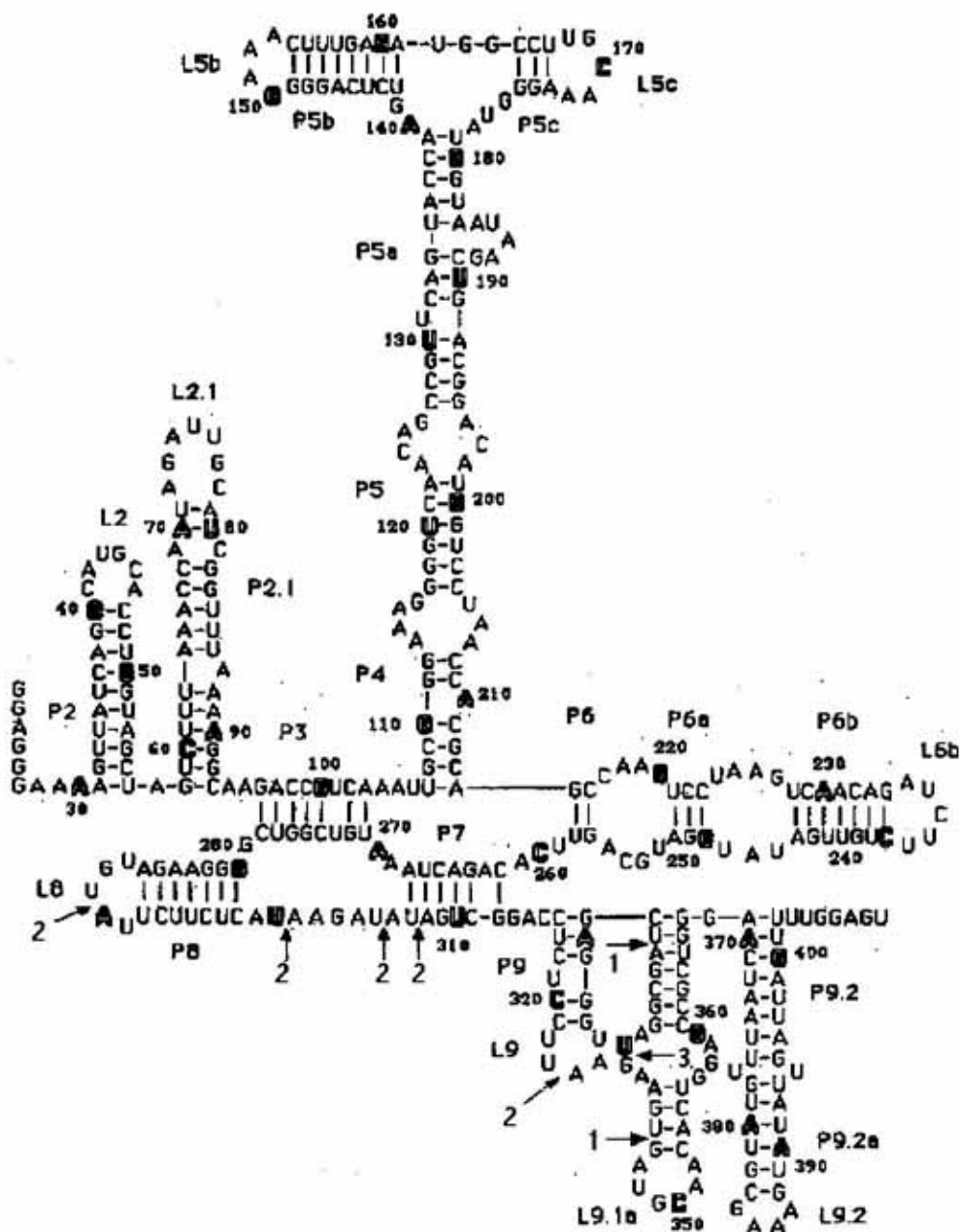


Figure 6. The secondary structure of rRNA intron of *T. thermophila*.

Phylogenetic secondary structure of ribozyme with numbering and nomenclature based on the completely excised intron. The cleavage occurs on 5'-side of the following phosphodiester bonds: G346, G341, A334, A324, A308, A306, A301 and A290. The numbers (1, 2, 3) at the arrows reflect the efficiency of the cleavage.

tides with the complementary strand inhibits hydrolysis.

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