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Hydrolysis of oligoribonucleotides: influence of sequence and length

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ABSTRACT

The chemical stability of phosphodiester bonds of some oligoribonucleotides in the presence of a cofactor like polyvinylpyrolidine (PVP) is sequence dependent. It was found that pyrimidine-A (YA) and pyrimidine-C (YC) are especially susceptible to hydrolysis. The hydrolyzability of this same phosphodiester bond is dependent on its position in the oligomer. The presence of 3′ and 5′-adjacent nucleotides enhances hydrolysis of the UA phosphodiester bond. The acceleration of the hydrolysis of UA by a 5′-adjacent nucleotide is not base dependent. However, a 3′-adjacent purine increases hydrolysis of a UA phosphodiester bond more than a 3′-pyrimidine. The presence of the exoamino group on the 3′-side base (on 6 and 4 position for adenosine and cytidine, respectively) of YA or YZ phosphodiester bond is required for hydrolysis.

INTRODUCTION

The discovery of enzymatic activities of certain RNAs has changed our understanding of the properties and function of this biomolecule[1]. The catalytic activity of RNA includes selective hydrolysis of phosphodiester bonds[2–6]. The products of this cleavage are a 5′-fragment with a 2′,3′-cyclic phosphate and a 3′-fragment with a 5′-hydroxyl group. The mechanism of hydrolysis involves nucleophilic attack by a 2′-hydroxyl group on a neighboring phosphorus atom. Other catalytic activities have also been observed[7]. For example, in the first step of splicing of a group I intron the 3′-hydroxyl group of an external guanosine attacks the phosphorus atom at the 5′-splice site. In the second step the 3′-hydroxyl group of exon-1 attacks the phosphorus atom at the 3′-splice side. The difference between the mechanisms of hydrolysis and splicing reflects differences in structure surrounding the phosphodiester bonds. Most catalytically active natural RNA molecules are large, containing at least 30–40 bp, and form complicated tertiary structures.

In the accompanying paper, we report selective and complete hydrolysis of some oligoribonucleotides in the absence of ribonucleases. In this paper, we show this process can occur on molecules as small as tetramers (for example: GUAA). Additional evidence is presented showing that selectivity of hydrolysis of these oligoribonucleotides is a function of sequence. Data for several analogues of pyrimidine and purine suggest a hydrogen bond interaction may be important for this process.

MATERIALS AND METHODS

Preparation of modified nucleosides

The modified nucleosides were purchased from the following suppliers: purine riboside (Sigma), inosine (Sigma) and 5-bromouridine (Aldrich). The others were synthesized according to the following procedures: 2-aminopurine riboside[8], 6N-methyladenosine[9] and 3N-methyluridine[10]. The 5-methyluridine and 2-thiouridine were obtained by condensation of thymine and 2-thiouracil with beta-D-ribofuranose-1,2,3,5-tetracetate according to published methods[11,12]. All nucleosides were converted to 5′-O-dimethoxytrityl-(N-protected)-2′-Otetrahydropyranylnucleoside according to published procedure[13]. The procedures of the synthesis and purification of oligoribonucleotides[14] as well as hydrolysis of oligoribonucleotides were the same as in the previous paper[15].

RESULTS AND DISCUSSION

Chemical stabilities of different oligoribonucleotides

Stability of a phosphodiester bond is dependent on the nucleosides it connects. For example, UCGUAA is predominantly hydrolyzed between U and A in UCGUAA in 0.1% PVP solution[15]. To get more information on this phenomenon, the following groups of oligoribonucleotides were synthesized and tested for stability: UCRYRA (UCGUAA, UCGCAA, UCGUGA, UCGCGA, UCAUGA, UCACGA, UCAUAA, ACACAA), UCYYRA, (UCUUAA, UCCUAA, UCCCAA, UCCCGA, UCUCAA, UCUCGA, UCCUGA, UCUUGA), and UCGUUA, UCGUCA, UCGAAA, UCGGAA. The last four oligoribonucleotides together with some others, can also be classified in UCGUNA or UCGNAA or UCNUAA groups. The pattern of the hydrolysis of 20 oligoribonucleotides in 0.1% PVP is shown in Figure 1 and quantitative analysis of the hydrolysis of UCGUAA is shown in Figure 2. Beside major hydrolysis between U(4) and A(5) some minor cleavage between U(1) and C(2) as well as C(2) and

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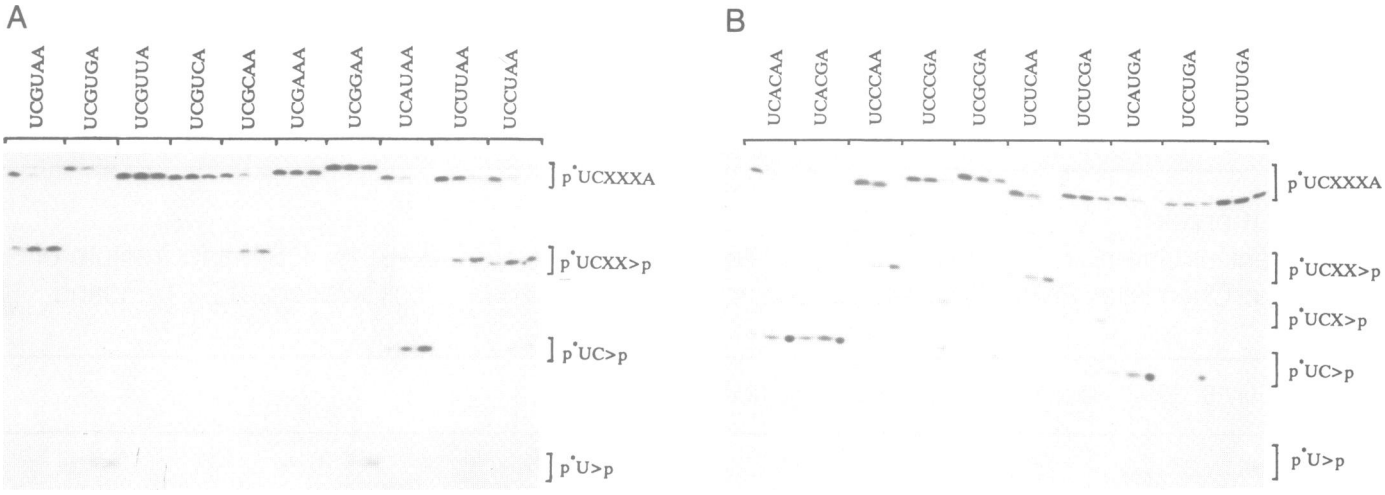

Figure 1.A and **B.** The PAGE of the stability of 20 various hexamers in the presence of a 0.1% PVP solution at 37°C. For every hexamer, lanes correspond to 1, 5 and 25 h hydrolysis times. The brackets on the right side mark the mobility of hydrolysis products.

G(3) was also observed. The hydrolysis hexamers demonstrate that different phosphodiester bonds are hydrolyzed with different rates. The previously reported instability of the UA phosphodiester bond can be generalized to the observation that only YA and YC phosphodiester bonds are hydrolyzed with significant rates over 24 h at 37°C. The phosphodiester bonds in YA are 3–5 fold more sensitive to hydrolysis than YC. UA is 1.5–2.0 fold more sensitive to cleavage than CA. The phosphodiester bonds in YG and YU sequences are at least 20 fold more stable than YA and YC analogues. Phosphodiester bonds in RR and RY are stable under those hydrolysis conditions. Generally, the ability to hydrolyze phosphodiester bonds can be placed in the following order: UA>CA>YC>YG>YU. Moreover, hydrolyzability of a given phosphodiester bond, for example within UA, is dependent on its position in the oligoribonucleotides. For example, UA in UCGUAA is 5–15 fold easier to hydrolyze than in UCGUUA. Also CA in UCAUAA cleaves 5–15 fold faster than in UCGUCA (see Figure 1). Generally the presence of an additional nucleoside on the 3′-site of the hydrolyzed phosphodiester bond enhances hydrolysis.

The above results can be correlated with preferential hydrolysis of YA and YC phosphodiester bonds during decay of the polycistronic lac mRNA of *Escherichia coli*[16] and spontaneous or chemical hydrolysis of t-RNA[17] and dinucleoside monophosphates[18]. Canistrano observed the following distribution in hydrolysis of the phosphodiester bonds during decay of polycistronic lac mRNA: 10 UA, 5 CA, 2 UU and 1 AA and 1 GA[16]. Spontaneous hydrolysis of nine different tRNAs occurs dominantly in YA phosphodiester bonds. Dock-Bregeon and Moras reported that among 49 cuts observed, 41 precede an adenosine and 37 of them follow a pyrimidine[17]. Also alkaline hydrolysis of several dinucleoside monophosphates showed preferential cleavage of phosphodiester bonds in YA and YC dimers[18]. These three cases of hydrolysis all have a preference for YA and YC phosphodiester bonds that is similar to the hydrolysis reported in this paper. This suggests a common origin of this phenomenon.

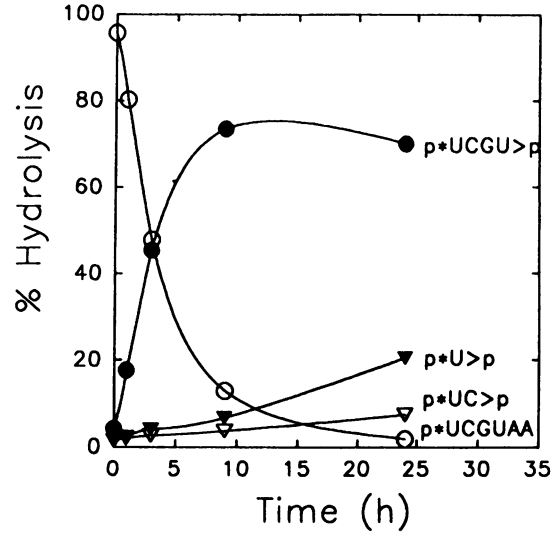

Figure 2. The kinetic of the hydrolysis of p*UCGUAA in the presence 0.1% PVP solution at 37°C. The curves demonstrate disappearance of p*UCGUAA (○) and formation of the products of hydrolysis: p*UCGU>p (●), p*UC>p (▽) and p*U>p(▼).

Influence of adding flanking nucleotides on the rate of hydrolysis

As discussed above, the ability of the YR phosphodiester bond for hydrolysis is dependent on its position in the oligoribonucleotide. The presence of one or more nucleotides on the 5′- and/or 3′-side of the hydrolyzable YR or YY phosphodiester bond increases its rate of cleavage. To make this correlation more clear, two series of UA containing oligoribonucleotides were synthesized and analyzed for their stability in the presence of PVP (Figure 3). The first set includes: UA, GUA, CGUA, and UCGUA. The second set includes: UAA, GUAA, CGUAA, and UCGUAA. Quantitative analysis of the reaction mixture by electrophoresis confirmed the

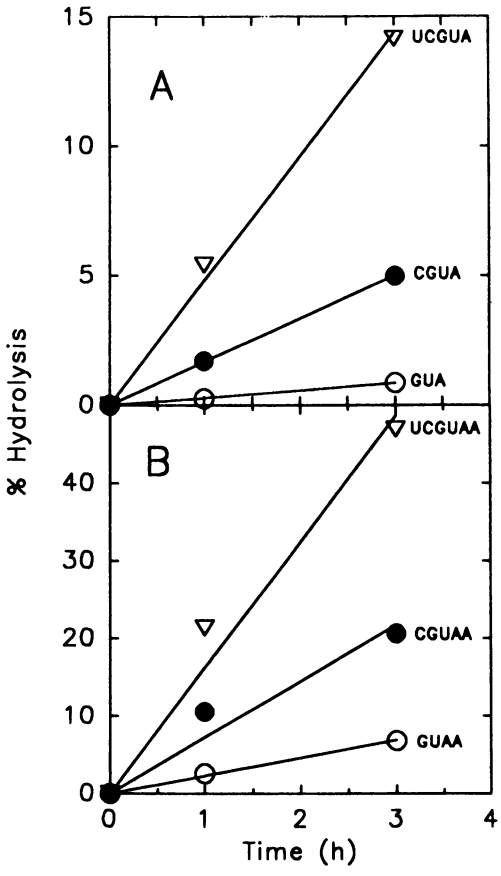

Figure 3.A. The initial rate of the hydrolysis: GUA (○), CGUA (●) and UCGUA(▽). **B.** The initial rate of the hydrolysis: GUAA(○), CGUAA (●) and UCGUAA(▽).

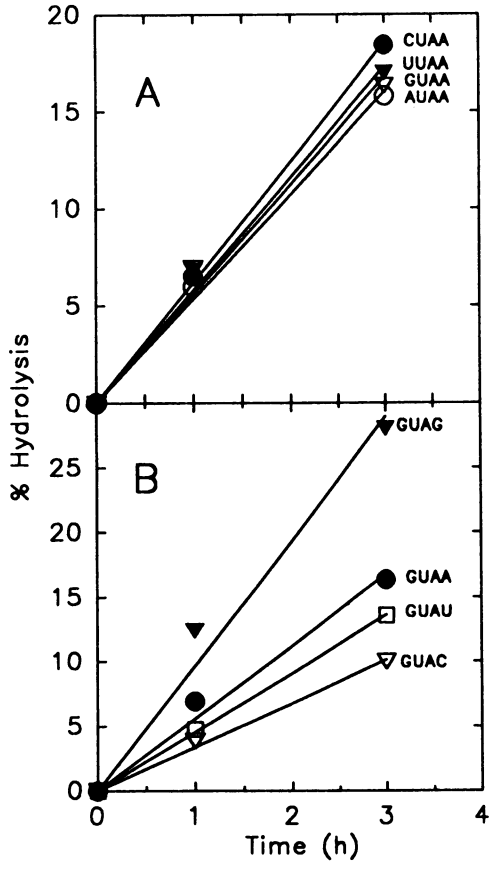

Figure 4.A. The initial rate of the hydrolysis of p*NUAA where N correspond to: A (○), C(●), U (▼) and G (▽). **B.** The initial rate of the hydrolysis of p*GUAN where N correspond to: A (●), C (▽), U (□) and G (▼).

preliminary hypothesis. In both series the shortest oligomers (UA and UAA) were resistant to hydrolysis. For both sets, the initial rate of hydrolysis increases as the length of the oligoribonucleotide increases. The relative rates for GUA, CGUA, and UCGUA are 1:6:18. Similarly, the relative rates for GUAA, CGUAA, and UCGUAA are 1:4:6. Comparison of oligomers between the series shows that an A at the 3′ end enhances cleavage. For GUA vs GUAA, CGUA vs CGUAA and UCGUA vs UCGUAA, the rates of hydrolysis as a function of addition of the 3′ A enhanced hydrolysis rates by 8−10, 6−7 and 3.5 fold, respectively. Thus difference becomes smaller as the oligomer becomes longer. The results show that the presence of a UA (perhaps, generally YR) sequence is not enough for cleavage in the presence of PVP.

Influence of 5′- and 3′-adjacent nucleosides on the rate of hydrolysis of UA phosphodiester bonds

In the previous paragraph, it was demonstrated that the presence of a 5′- and/or 3′-adjacent nucleoside is required for successful hydrolysis of UA phosphodiester bonds. The sequence dependence of this effect was also tested. For this purpose, two types of oligoribonucleotides, NUAA and GUAN, were synthesized and analyzed for stability in the presence of PVP (Figure 4). In the series NUAA, the rate of hydrolysis was similar for all four tetramers. In the series GUAN hydrolysis of the UA, phosphodiester depends on N in the following way: G>A>U>C. In general, hydrolysis of the UA phosphodiester bond is 2−3 fold faster when N is a purine than when N is a pyrimidine.

Hydrolysis of YA phosphodiester bond with different analogues of Y in the presence of PVP

The hydrolysis of different YR sequences of oligoribonucleotides demonstrates that the nature of the pyrimidine base affects the rate of hydrolysis. For example, it was found that among oligoribonucleotides the UA containing oligomer is cleaved 1.5−2.0 fold faster than the CA analogue. Results on hydrolysis of oligoribonucleotides by ribonuclease A suggest that the 2-oxygen of the pyrimidine residue is an acceptor for hydrogen bonding to its own 2′-hydroxyl group[19,20]. This type of interaction increases the nucleophility of this group and its ability to attack the electrophilic phosphorus atom. To analyze the possibility of hydrogen bond interactions between the 2-substituent of the pyrimidine base and the 2′-hydroxyl group, several analogues were synthesized and incorporated into oligoribonucleotides. These nucleosides include: 5-bromouridine, 5- methyluridine, 3N-methyluridine and 2-thiouridine. The different pK_a's for these nucleosides reflect different electrons distribution in the base moiety and in the consequence suggest different proton acceptor ability of the 2-substituent[21]. This

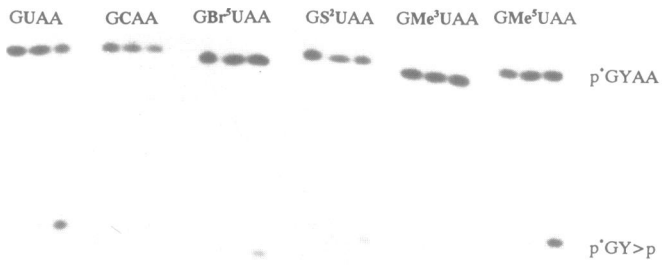

Figure 5. The PAGE analysis of stability of pyrimidine analogues of GYAA in the presence of 0.1% PVP solution at 37°C. The lanes for each tetramer set correspond to 1, 5 and 25 h hydrolysis times.

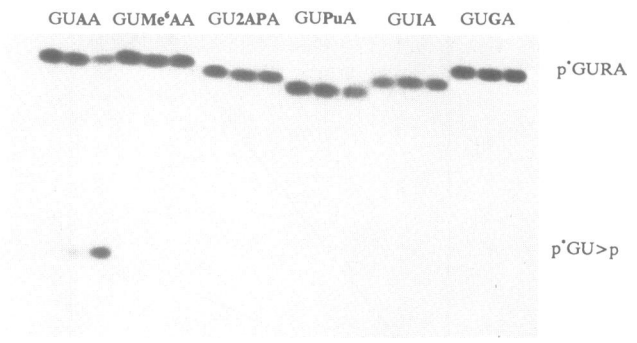

Figure 6. The PAGE analysis of stability purine analogues of GURA in the presence of 0.1% PVP solution at 37°C. The lanes for each tetramer set correspond to 1, 5 and 25 h hydrolysis times.

could affect the rate of hydrolysis. Six tetramers of the form GYAA (Y=pyrimidine nucleoside) were synthesized and tested for stability in 0.1% PVP solution (Figure 5). The results show that GUAA and GMe^{5}UAA hydrolyze the most effectively. The three tetramers containing cytidine, 5-bromouridine and 2-thiouridine hydrolyze with similar rates which are 0.5−0.7 of the hydrolysis rate of GUAA. The tetramer GMe^{3}UAA was resistant to hydrolysis in 0.1% PVP solution. The interpretation of this result is not conclusive. The chemical stability of GMe^{3}UAA could be interpreted as a requirement for the N3 proton of pyrimidine for hydrolysis. In this model, hydrolysis of CA would require protonation at N3 (pK_a= 4.2)[21]. This seems unlikely. Moreover, the similar hydrolyzabilities of GYAA where Y = C, br^{5}U or s^{2}U provide no evidence that the 2′-hydroxyl group hydrogen bonds to the 2-pyrimidine substituent. If presence of a hydrogen bond was important in the rate limiting step, the effect of pyrimidine substituent on electron distribution in pyrimidine would be expected to alter the rate of hydrolysis. An alternative explanation of these results could be based on different base stacking properties of the pyrimidines within the GYAA tetramer. The influence of the modification of the base on stacking ability was reported in the literature for several types of modifications[22−24]. Thus it is difficult to explain the results observed conclusively. It is possible that the susceptibility of GYAA to hydrolysis can reflect both electronic and structural effects of pyrimidine modification.

Hydrolysis of UR phosphodiester bond with different analogues of R in the presence of PVP

The above results demonstrate that cleavage occurs in the sequences NYRN and NYYN. Significant rates of hydrolysis are observed only for NYAN and NYCN, however. The large resistance for cleavage of NYGN and NYUN and the susceptibility to cleavage of NYAN and NYCN suggest that hydrolysis is dependent on the presence of the functional group in position 4 of pyrimidines or position 6 of purines 3′ to the cleavage site. An exoamino group in those positions permits hydrolysis whereas a 'carbonyl' group in that position stops hydrolysis. This can be explained in two ways. One way is the inhibition of the hydrolysis by U and G, perhaps by an inhibiting interaction of a 4- or 6-oxygen with another part of the tetramer. In that model, the presence of 4- or 6-amino functions in adenosine or cytidine is inert for hydrolysis. Alternatively, it is possible that the 4- or 6-amino groups accelerate hydrolysis due to interaction with another part of the tetramer. To distinguish between the two options, several purine analogues of the form GURA (R is a purine nucleoside) were synthesized and tested for hydrolysis in the presence of PVP (Figure 6). These additional analogues were: 6N-methyladenosine, 2-aminopurine riboside, purine riboside and inosine. Among these analogues, only GUAA was hydrolyzed after 24 h in 0.1% PVP at 37°C. Thus the 6-amino group in adenosine is required for hydrolysis of YR phosphodiester bonds. The resistance of tetramers containing purine and 2-aminopurine excludes an inhibitory function of the 6-oxygen in guanosine during YR phosphodiester bond hydrolysis. The experiments with 6N-methyladenosine tetramer, which showed resistance for hydrolysis, also support the importance of the 6-amino group of adenosine. The electron donating character of the methyl group reduces the proton donating ability of the remaining hydrogen of the amino group, thus weakening any hydrogen bonding involving this group. At present, it is not possible to localize the interaction. Model studies suggest the 6-amino group can bond directly or through water to the 2′- or 4′-oxygen of the 5′ pyrimidine or to an oxygen of the phosphate group. The last interaction should be the most favorable since it would increase the electrophilic character of phosphorus and accelerate the rate of hydrolysis

Proposed mechanism for hydrolysis of YR phosphodiester bond

The available results lead to a rough working hypothesis of the oligoribonucleotide properties that are important for hydrolysis. Besides proper sequence of oligoribonucleotides, some cofactor, for example PVP, is required. Spermidine increases the rate of hydrolysis significantly, and Mg^{+2} is not required[15]. The cleavage occurs in single stranded oligoribonucleotides since the presence of a complementary strand limits flexibility of phosphodiester bonds[15]. Moreover, the structure of double helical oligoribonucleotides does not favor 'in line' attack of the 2′-hydroxyl on the phosphorus atom[21,25,26]. A second factor which could determine flexibility of the phosphodiester bond is the limited stacking interaction between pyrimidine and purine bases[17,27−31]. This limited base stacking increases flexibility of YR phosphodiester bonds. The presence of any nucleoside 5′- and 3′-adjacent to the YR dimer could affect the preferred stacking interaction of Y and R due to new stacking interactions introduced. For example, an NMR study of the effect of oligomer length on stacking shows that the stacking of a nucleotide is

dependent on its position relative to the ends of the strands. High flexibility of the YR phosphodiester bond due to limited base stacking interactions is not enough to get hydrolysis, however. The stacking of the bases in UG and UA is similar, but hydrolysis of UA is more than 30 fold higher than UG[32]. The experimental results suggest that hydrogen bonding interactions of the 6-amino group of adenosine (and perhaps the 4-amino of cytidine) are critical for hydrolysis. One possibility for this interaction is a direct or water bridged hydrogen bond to an oxygen in the phosphodiester. This type of interaction should increase electrophilicity of the phosphorus atom. An X-ray crystal structure of UA shows that lengths of the P-O bonds are different, which can mean that one of the bonds is involved in some interaction[31]. In other words, increased electrophilicity of the phosphorus atom and high flexibility of YR phosphodiester bonds make hydrolysis possible.

All control experiments demonstrate that hydrolysis of YR oligoribonucleotides requires presence of cofactors, for example PVP[15]. At the present time it is difficult to determine the function of the cofactor during hydrolysis. The large range of active cofactors, e.g., polymeric organic compounds and proteins, could mean that it is not any specific type of interaction involved in this process. One option is a nonspecific interaction of PVP (or general cofactor) with oligoribonucleotides which can cause conformational changes and in consequence acceleration of phosphodiester bond hydrolysis. The other option would be volume or water exclusion by the cofactor. That process could change the pattern of hydration and hydrogen bond interaction within the RNA molecule[34].

The results in this paper show that hydrolysis is sequence dependent. The large effects of sequence on structure are clearly demonstrated by the comparison of the crystal structure of AU and UA[31,33]. AU forms a small segment of a right-handed antiparallel double helix with Watson-Crick base pairing between adenine and uridine and with strong base stacking[33]. The second dimer UA forms two different structures within a unit cell. The conformation of the phosphodiester bonds is different for both molecules. One molecule of UA has a sharp turn in the single stranded nucleic acid structure. The other form is helical between adenosine and uridine with base stacking involving the ribosyl oxygen atom and the adjacent base[31].

That model of hydrolysis is consistent with the structures of the products of cleavage[35] (pyrimidine nucleoside-2′, 3′-acyclic phosphate and 5′-hydroxyl group) and general knowledge about structure and interaction of RNA[21]. The data demonstrate that chemical stability of some phosphodiester bonds is 'coded' on a level as elementary as tetramer (NYYN or NYRN). The results suggest that some structural motifs, like low base stacking can be responsible for stability of certain phosphodiester bonds.

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ABBREVIATIONS

PVP, polyvinylpyrrolidone; R, purine nucleoside; Y, pyrimidine nucleoside; br^{5}U, 5-bromouridine; s^{2}U, 2-thiouridine; Me^{3}U, 3N-methyluridine; Me^{5}U, 5-methyluridine; Me^{6}A, 6N-methyladenosine; 2AP, 2-aminopurine riboside; Pu, purine riboside.

REFERENCES

1. Cech, T. R. (1990) *Ann. Rev. Biochem.* **59**, 543–568.
2. Kole, R. and Altman, S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3795–3799.
3. Foster, A.C. and Symons, R.H. (1987) *Cell* **49**, 211–220.
4. Uhlenbeck, O.C. (1987) *Nature* **328**, 596–600.
5. Perrotta, A. T. and Been, M. D. (1992) *Biochemistry* **31**, 16–22.
6. Chowrira, B.M. and Burke, J.M. (1991) *Biochemistry* **30**, 8518–8522.
7. Cech, T.R. and Bass, B.L. (1986) *Ann. Rev. Biochem.* **55**, 596–629.
8. SantaLucia, J. Jr., Kierzek, R. and Turner, D.H. (1991) *J. Am. Chem. Soc.* **113**, 4313–4332.
9. Jones, J.W. and Robins R.K. (1963) *J. Am. Chem. Soc.* **85**, 193–201.
10. Hisanaga, Y., Tanabe, T., Yamauchi, K. and Kinoshita, M. (1981) *Bull. Chem. Soc. Jpn.* **54**, 1569–1570.
11. Vorbruggen, H., Krolikiewicz, K. and Bennua, B. (1981) *Chem. Ber.* **114**, 1234–1255.
12. Vorbruggen, H. and Bennua, B. (1981) *Chem. Ber.* **114**, 1279–1286.
13. Markiewicz. W.T., Biala, E. and Kierzek, R. (1984) *Bull. Pol. Acad. Sci.* **32**, 433–450.
14. Kierzek, R., Caruthers, M.H., Longfellow, C.E., Swinton, D., Turner, D.H. and Freier, S.M. (1986) *Biochemistry* **25**, 7840–7846.
15. Kierzek, R. and Turner, D.H., 2B-0185 nos. when known.
16. Cannistraro, V.J , Subbarao, M.N. and Kennell, D. (1986) *J. Mol. Biol.* **192**, 257–274.
17. Dock-Bergeon, A.C. and Moras, D. (1987) Cold Spring Harbor Symposia on Quantitative Biology, Vol. LII, Cold Spring Harbor Laboratory Press.
18. Witzel, H. (1960) *Justus Liebigs Ann. Chem.* **635**, 182–191.
19. Richards, F.M. and Wickoff, H.W. (1971) In Boyer, P.D., (ed.), The Enzymes, Academic Press, New York and London, pp. 647–806.
20. Witzel, H. (1963) In Davidson, J.N. and Cohen, W.E. (ed.), Progress in Nucleic Acids Research, Academic Press, New York and London, pp. 221–258,.
21. Seanger, W. (1984) In Principle of Nucleic Acids Structure, Springer-Verlag, New York, Berlin, Heidelberg, Tokyo.
22. Seela, F., Ott, J. and Franzen, D. (1983) *Nucleic Acids Res.* **11**, 6107–6120.
23. Thedford, R., Fleysher, M.H. and Hall, R.H. (1965) *J. Med. Chem.* **8**, 486–491.
24. Agris, P.F., Sierzputowska-Gracz, H., Smith, W., Malkiewicz, A., Sochacka, E. and Nawrot, B. (1992) *J. Am. Chem. Soc.* **114**, 2652–2656.
25. Kim, S.-H., Quigley, G.J., Suddath, F.L., McPherson, A., Sneden. D., Kim, J.J. and Rich, A. (1973) *Science* **179**, 285–288.
26. Usher, D. A. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 661–667.
27. Lee, C.-H. and Tinoco, I. Jr. (1980) *Biophys. Chem.* **11**, 283–294.
28. Stone, M.P., Winkle, S.A. and Borer, P.N. (1986) *J. Biomolec. Struct. Dyn.* **3**, 767–781.
29. van der Hoogen, Y.Th.–Ph.D. Thesis (Leiden University, 1988).
30. Erza, F.S., Lee. C.-H., Kondo, N.S., Danyluk, S.S. and Sarma, R.H. (1977) *Biochemistry* **16**,1977–1981.
31. Sussman,J.L., Seeman, N.C., Kim, S.-H. and Berman, H.M. (1972) *J. Mol. Biol.* **66**, 403–421.
32. Ezra, F.S., Lee, C.-H., Kondo, N.S., Danyluk, S.S., and Sarma, R. H. (1977) *Biochemistry* **16**, 1977–1987.
33. Seeman, N., Rosenberg, J.M., Suddath, F.L., Kim, J.J.P. and Rich, A. (1976) *J. Mol. Biol.* **104**, 109–144.
34. Ray, J. and Manning, G. S. (1992) *Biopolymers* **32**, 541–549.
35. Markham, R. and Smith, J.D. (1952) *Biochem. J.* **52**, 552–557.