Hydrolysis and Isomerization of the Internucleosidic Phosphodiester Bonds of Polyuridylic Acid: Kinetics and Mechanism

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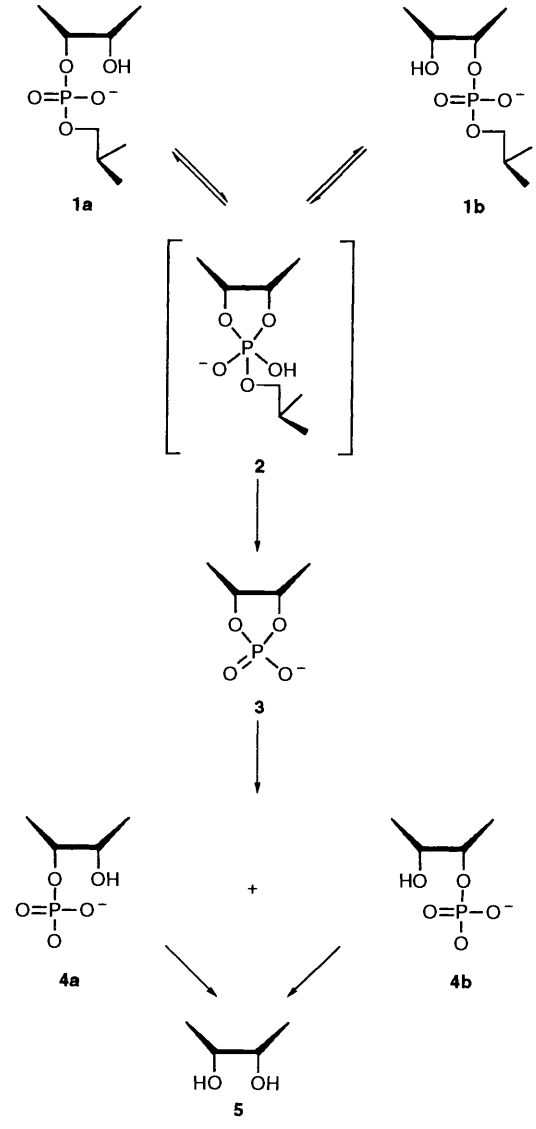
The pH–rate profiles for the hydrolysis of the 3',5'-phosphodiester bonds of poly(U) and their isomerization to the 2',5'-bonds have been determined by following the progress of the reactions by enzymatic digestion with RNAase A and subsequent RP HPLC. The kinetics observed are compared with those of the hydrolysis and interconversion of uridylyl(3',5')uridine and uridylyl(2',5')uridine.

The internucleosidic 3',5'-phosphodiester bond plays a central role in the chemistry and biochemistry of RNA. Its hydrolytic cleavage, for example, is of interest, since it creates nucleic acid fragments that can be religated by enzymes. This, together with the sequence-specific catalysis of hydrolysis, would enable tailoring of the RNA molecule in a pre-designed manner.

The kinetics and mechanism of the hydrolysis have been studied with model compounds, such as dinucleoside monophosphates,[1–4] aryl and alkyl esters of nucleoside monophosphates[5–7] and aryl esters of 3,4-dihydroxytetrahydrofuran monophosphate.[8,9] It has been suggested[1] that a nucleophilic attack of the adjacent 2'-OH on the phosphorus atom of the 3',5'-bond (**1a** in Scheme 1) gives a pentacoordinated phosphorane intermediate (**2**), which may then decompose by two alternative routes: departure of the 5'-linked nucleoside leads to hydrolysis to a 2',3'-cyclic monophosphate (**3**), while cleavage of the P-O3' bond results in phosphate migration, and hence formation of an unnatural 2',5'-phosphodiester bond (**1b**). The cyclic monophosphate (**3**) is subsequently hydrolysed to a mixture of 2'- and 3'-monophosphates (**4a**, **b**). Under alkaline conditions, the attacking nucleophile is the 2'-oxyanion, and the hydrolysis proceeds *via* a pentacoordinated transition state rather than *via* a pentacoordinated intermediate. Accordingly, no phosphate migration takes place.[3]

The model compounds mentioned above typically contain only one internucleosidic phosphodiester bond in an environment that more or less resembles that in RNA. Data on the hydrolytic reactions of the phosphodiester bonds of real polyribonucleotides are scarce. Only the alkaline cleavage has been studied in some detail.[10–12] Kaltreider and Scott[12] have shown that the release of monomeric nucleotides from RNA is first order in hydroxide-ion concentration, the half-lives observed for various nucleotides in 1 mol dm^{-3} aqueous potassium hydroxide at 298 K ranging from 2.3 to 5.6 h. These authors also suggested that the terminal and non-terminal phosphodiesters bonds are cleaved at different rates. Less is known about the hydrolysis under acidic or neutral conditions. According to Bacher and Kauzmann[13] the rate of hydrolysis remains practically constant in the pH range 4–6, while under more acidic conditions the reaction order in hydronium-ion concentration varies from 1 to 2. The half-life estimated from their results is 2 min in 0.48 mol dm^{-3} aqueous hydrogen chloride at 373 K. Since these early studies, the attention has been primarily focused on the possibility to promote the hydrolysis. The effects of metal ions[14,15] and their complexes[16,17] have been studied and many of these appear to be efficient catalysts for the hydrolysis. Oligoamines[18] and polypeptides[19] have also been reported to enhance the hydrolysis of RNA phosphodiester bonds. No data on the phosphate migration at polynucleotide level exist.

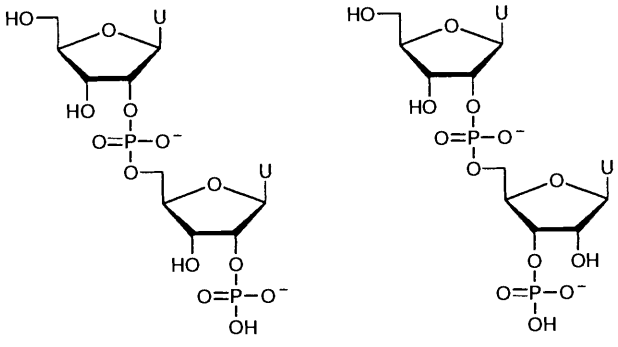
None of the work on polyribonucleotides is based on an experimental method that could provide accurate rate constants for both the hydrolysis and the isomerization. The studies of Breslow[20] concerning the buffer-catalysed hydrolysis of poly(U), are, in this respect, more informative. His method utilizes the enzyme phosphodiesterase I to cleave the unchanged starting material into 5'-monophosphates. If the poly(U) strand has already been cleaved non-enzymatically, a uridine molecule is released by the action of the enzyme. Thus the amount of



Scheme 1

uridine liberated by the enzyme is equal to the number of phosphodiester bonds hydrolysed chemically. As has been mentioned by Breslow himself, the method cannot detect the cleavage of two adjacent bonds, and thus the accurate data can be obtained only during the early stage of the reaction. No attention was paid to the isomerization of the phosphodiester bonds.

We now report an enzymatic method, with which both the hydrolysis and the isomerization of the phosphodiester bonds of poly(U) may be followed. The method resembles that of Breslow but ribonuclease A is used to cleave the unchanged starting material. RNAase A is a pyrimidine-specific endonuclease that produces 3′-mononucleotides from an intact pyrimidine polymer.[21] When the phosphodiester bond (**1a**, **b**) is cleaved non-enzymatically, terminal 2′- and 3′- phosphomonoester (**4a**, **b**) groups are produced in a constant ratio.[3,4] After the enzymatic digestion, the amount of 2′-monophosphate (**4b**) can be determined by HPLC, and the number of phosphodiester bonds cleaved non-enzymatically may thus be calculated. The 2′,5′-phosphodiester bonds (**1b**) are not cleaved by RNAase A. Since the enzyme is an endonuclease, its action on a polymeric strand is not, however, stopped at the 2′,5′-bond, but uridylyl(2′,5′)uridine 2′- or 3′-monophosphate (**6a**, **b**) is



6a **6b**

released, and these products may be detected by HPLC. Accordingly, the rate of isomerization can be estimated. With this method, the hydrolysis and the isomerization of the phosphodiester bonds of poly(U), containing approximately 300–400 uridine residues, have been studied over a wide pH range. The results are compared with those obtained previously[3] with uridylyl(3′,5′)uridine.

Results and Discussion

As mentioned above, the hydrolysis of the phosphodiester bonds of RNA (**1a**) yields a 3′-terminal 2′,3′-cyclic monophosphate group (**3**) and a 5′-terminal free hydroxy function. Under acidic and alkaline conditions the 2′,3′-cyclic phosphodiester is subsequently hydrolysed, leaving the monophosphate group bonded to either the 2′- or 3′-position of the terminal nucleoside, (**4a**, **b**). In neutral solutions a considerable number of terminal 2′,3′-cyclic monophosphate groups are accumulated, as may be detected by the HPLC analysis of the chemically released 3′-terminal nucleotides (Fig. 1). The 2′- and 3′-phosphomonoester groups are produced in a constant ratio ($x_{4a} = 0.6$).[22,23] On prolonged treatment, dephosphorylation to a free 3′-terminal nucleoside (**5**) takes place.[22,24] Isomerization of the 3′,5′-phosphodiester bond (**1a**) to a 2′,5′-bond (**1b**) competes with the hydrolysis (Scheme 1). With poly(U) the products are still mainly polymeric in nature. Only the cleavage of the 3′-or 5′-terminal phosphodiester bonds releases a monomeric product, *viz.*, uridine 2′,3′-cyclic monophosphate (2′,3′-cUMP), uridine 2′- or 3-monophosphate (2′/3′-UMP) or

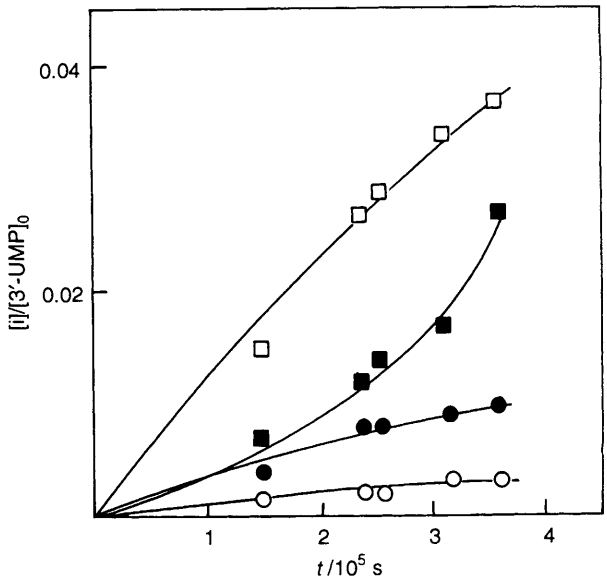


Fig. 1 Time-dependent distribution of the monomeric products of the hydrolysis of poly(U) at pH 5.0 and 363.2 K (I = 0.1 mol dm^{-3}). [i] Stands for the concentration of 2′-UMP (○), 3′-UMP (●), 2′3′-cUMP (□) and uridine (■), and [3′-UMP]$_0$ is the concentration of 3′-UMP obtained by complete enzymic digestion of the unmodified poly(U), *i.e.*, by the digestion of the initial sample.

uridine. The monomers, and thus the number of 3′- and 5′-terminal phosphodiester bonds hydrolysed, can be detected by direct HPLC analysis. Fig. 1 shows as an illustrative example the time-dependent formation of these monomers under neutral conditions.

The total number of the phosphodiester bonds reacted, either hydrolysed or isomerized, may be determined by digesting the polymeric products with RNAase A. This enzyme hydrolyses the 3′,5′-phosphodiester bonds in a stepwise manner. In the first step, the enzyme produces 2′,3′-cUMP, which, in the second step, is cleaved to 3′-UMP. Intact poly(U) is thus cleaved to 3′-UMP, with the exception of the 3′-terminal nucleoside that is released as uridine. If the poly(U) strand has already been hydrolysed or isomerized by the non-enzymatic reaction, several proudcts are obtained by the enzyme digestion: (*i*) cleavage of the 3′-terminal nucleoside 2′-monophosphate (**4b**) yields 2′-UMP, (*ii*) cleavage of the 3′-terminal nucleoside 3′-monophosphate (**4a**) and 2′,3′-cyclic monophosphate (**3**) yields 3′-UMP (as does cleavage of the non-terminal 3′,5′-phosphodiester bonds), (*iii*) cleavage of the dephosphorylated 3′-terminal nucleoside (**5**) yields uridine (as does dephosphorylation of 2′- and 3′-UMP), and (*iv*) the dinucleoside monophosphate fragments bearing a 2′,5′-phosphodiester bond (**1b**) are released as uridylyl(2′,5′)uridine 2′/3′-monophosphate (**6a**, **b**). When the enzymatically digested aliquots are chromatographed, all these products can be separated (Fig. 2). The number of the phosphodiester bonds isomerized may thus be calculated from the amount of **6a** and **6b**, and the number of the phosphodiester bonds hydrolysed may be estimated from the amount of 2′-UMP and uridine as follows. Firstly, the terminal 2′-monophosphate groups (**4b**) are detected as 2′-UMP. Secondly, the dephosphorylated 3′-terminal nucleotides (**5**) appear as uridine. Thirdly, the 3′-terminal 3′-monophosphates (**4a**) and 2′,3′-cyclic monophosphates (**3**) produced by the non-enzymatic hydrolysis cannot be detected, but their amounts may be estimated from the amount of 2′-UMP assuming that the hydrolysis of the non-terminal bonds gives a time-dependent product mixture similar to that of the hydrolysis of the terminal bonds. To accomplish this, each aliquot was chromatographed both before and after the enzymatic digestion, and the ratios

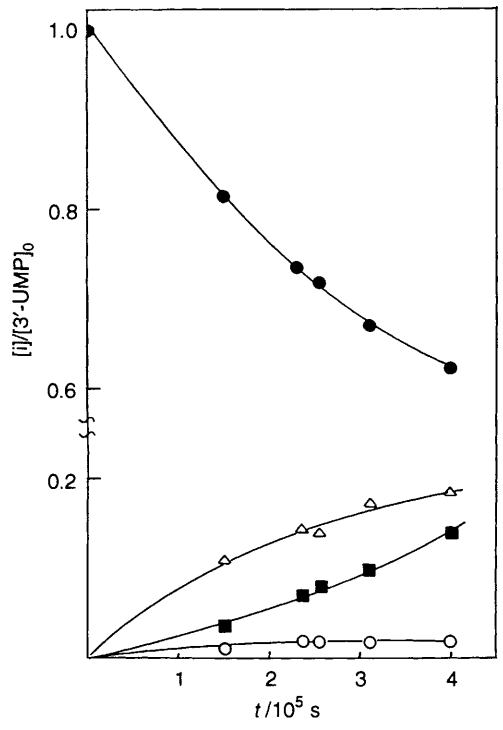


Fig. 2 Time-dependent product distribution observed for the hydrolysis of poly(U) at pH 5.0 and 363.2 K (I = 0.1 mol dm^{-3}) after digestion of the aliquots with RNAase A. [i] Stands for the concentration of 2'-UMP (○), 3'-UMP (●), **6a** + **6b** (△) and uridine (■), and [3'-UMP]$_0$ is the concentration of 3'-UMP obtained by complete enzymic digestion of the unmodified poly(U), *i.e.*, by the digestion of the initial sample.

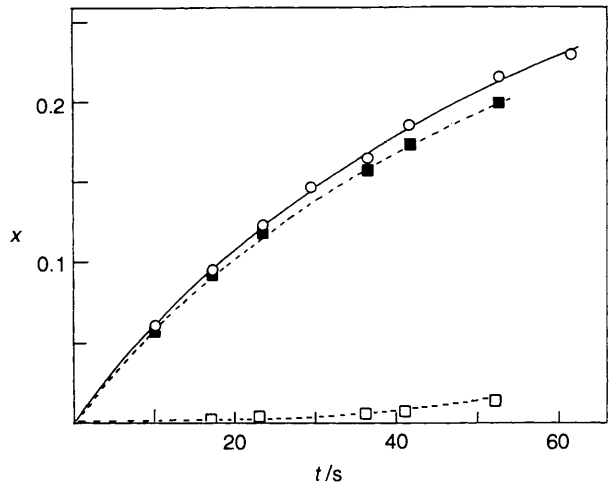


Fig. 3 The progress of the hydrolysis of poly(U) in aqueous hydrogen chloride (0.1 mol dm^{-3}) at 363.2 K: ○, mole fraction of all phosphodiester bonds hydrolysed; □, mole fraction of 3'- and 5'-terminal phosphodiester bonds cleaved; ■, the mole fraction of the non-terminal phosphodiester bonds cleaved

[2',3'-cUMP]/2'-UMP] and [3'-UMP]/[2'-UMP] obtained with the undigested aliquots were then used to calculate the amount of 3'-UMP that was formed during the enzymatic digestion from the terminal 3'-monophosphate groups (**4a**) or 2',3'-cyclic monophosphate groups (**3**), and not from unmodified 3',5'-bonds (**1a**). Accordingly, the total number of phosphodiester bonds hydrolysed chemically may be calculated, and hence the rate constants for the cleavage and isomerization of the phosphodiester bond are obtained. By comparing the amount of 2'-UMP present in the aliquots before and after the enzymic digestion, it is also possible to distinguish the terminal

Table 1 The frequency of terminal bond ruptures as a function of pH of the reaction mixture at 363.2 K[a]

pH	[Terminal bonds cleaved]/ [non-terminal bonds cleaved]
1.0	0.04
2.0	0.03
4.0	0.11
7.0	0.20
8.0	0.18
9.0	0.25
10.0	0.20

[a] 15% of all phosphodiester bonds cleaved.

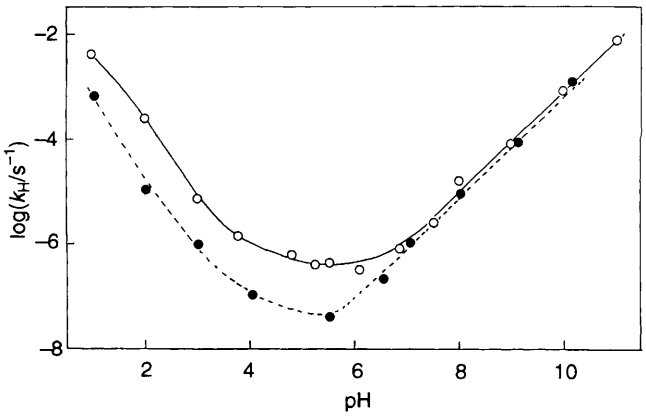


Fig. 4 pH–Rate profile for the hydrolysis of poly(U) at 363.2 K (○). ● Refers to the data reported previously[3] for the hydrolysis of 3',5'-UpU.

bond ruptures from the non-terminal ones. Fig. 3 shows as an example the data obtained under acidic conditions. A similar type of behaviour was observed in neutral and alkaline solutions. It is worth noting that the procedure described above is valid only as long as early stages of the hydrolysis are concerned. Usually less than 20% of all the phosphodiester bonds were cleaved during one kinetic run.

The hydrolysis of poly(U), *i.e.*, the decrease of the number of phosphodiester bonds (**1a** + **1b**), obeys the first-order rate kinetics during the early stages of the reaction, in spite of the fact that the proportion of the terminal bonds is continuously increased. Accordingly, hydrolysis rate of the terminal bonds cannot dramatically differ from that of the non-terminal ones, although one should bear in mind that, during the early stage of the reaction, deviations from the first-order kinetics may be difficult to detect. This argument receives additional support from the observation that the proportion of terminal bonds among all the bonds cleaved was approximately statistical. However, considerable variation with pH may in this respect be observed. As seen from Table 1, at low pH the terminal bond ruptures are even less frequent than statistically expected, while at high pH the terminal phosphodiester bond is cleaved at least as readily as the non-terminal one. In other words, the hydronium-ion-catalysed reaction appears to favour cleavage within the chain, while the hydroxide-ion-catalysed reaction does not exhibit a similar selectivity.

Fig. 4 shows the pH rate profile for the hydrolysis of poly(U). To facilitate the comparisons, the rate profile reported previously[3] for uridylyl(3',5')uridine (3',5'-UpU) is also included. As seen, the profiles are quite similar. Accordingly, dinucleoside monophosphates really seem to constitute an applicable model system for the kinetic and mechanistic studies of RNA, at least as long as metal-ion catalysis is not concerned. In fact, there is only one significant difference in the kinetic behaviour of poly(U) and 3',5'-UpU: the hydrolysis of poly(U), under acidic

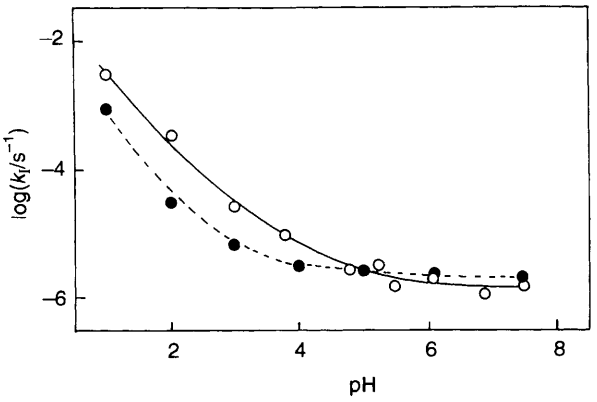


Fig. 5 pH–Rate profile for the mutual isomerization of the 3′,5′- and 2′,5′-phosphodiester bonds of poly(U) at 363.2 K (○). ● Refers to the data reported previously [3] for the interconversion of 2′,5′- and 3′,5′-UpU.

conditions, is about one order of magnitude faster than that of 3′,5′-UpU. Consistent with this, 2′,3′-cUMP is accumulated more markedly during the hydrolysis of poly(U) than during the hydrolysis of 3′,5′-UpU. Under alkaline conditions, the hydrolysis rates of poly(U) and 3′,5′-UpU are equal. One may speculate that the enhanced hydrolysis of poly(U) under acidic conditions results from exceptionally facile protonation of the phosphodiester functions of poly(U). The hydronium-ion-catalysed hydrolysis proceeds by the attack of the 2′-hydroxy group on the protonated phosphodiester function, either a monocationic or neutral one.[3] Hence the rate of the reaction is increased with the increasing basicity of the phosphate group. The hydroxide-ion-catalysed hydrolysis, in turn, proceeds by the attack of the 2′-oxyanion on the monoanionic phosphodiester function, and that is why the basicity of the phosphate group does not play a similar role. Since poly(U) is present as a polyanion under neutral and slightly acidic conditions, the protonation of its phosphodiester functions is electrostatically enhanced. Furthermore, the non-terminal bonds might, for the same reason, cleave more rapidly than the terminal ones.

The pH–rate profile for the isomerization of the poly(U) is shown in Fig. 5. The data obtained previously[3] for the isomerization of 3′,5′-UpU are again presented in the same figure. As with the hydrolysis, the shapes of the profiles are similar, but the acid-catalysed reaction of poly(U) is faster than that of 3′,5′-UpU. Most likely, the explanation for the greater reactivity of poly(U) is the same as for its faster acid-catalysed hydrolysis.

Experimental

Materials.—Poly(U) and all the nucleosides and nucleotides used as reference compounds were Sigma products. They were used as received after purity verification by HPLC. RNAase A was purchased from Aldrich as a lyophilized powder and alkaline phosphatase from Boehringer as a concentrated solution. All the other chemicals were of reagent grade.

Kinetic Measurements.—The kinetic experiments were performed at 363.2 K. The temperature was adjusted with a water bath and kept constant to within 0.1 K. The reactions were carried out in tightly stoppered tubes, containing 0.5 mg poly(U) in 10 cm^3 of reaction solution. The pH of the reaction solutions was adjusted in the pH range 3–9 with formate, acetate, HEPES and glycine buffers. Outside this range aqueous hydrogen chloride and sodium hydroxide were employed. The total buffer concentration varied from 0.01 to 0.1 mol dm^{-3}, and the ionic strength was adjusted to 0.1 mol dm^{-3} with sodium perchlorate. Under these conditions no buffer catalysis was observed. The pH values of the reaction solution were measured both before and after each kinetic run. The values obtained agreed within 0.1 pH unit. The values measured at 298.2 K were extrapolated to 363.2 K by using the known temperature dependences of the pK_a values of the buffer acids.[25–28]

Double aliquots of 500 mm^3 were withdrawn at suitable intervals from the reaction solutions. They were immediately cooled to 273 K to stop the reaction. One of the aliquots was analysed as such by HPLC. The analysis was performed on a Hypersil RP-18 column (250 × 4 mm, 5 μm) using a mixture of acetate buffer (0.025 mol dm^{-3}, pH 4.3, 0.1 mol dm^{-3} NH_4Cl, 99.5% v/v) and acetonitrile (0.5% v/v) as the eluent. Only monomeric products were eluted with this mixture. The pH of the other aliquot was adjusted to 5, and it was treated with RNAase A. The enzyme was added in an acetic acid buffer ([AcOH]/[AcONa] 0.1/0.05 mol dm^{-3}). The volume of the enzyme solution added was 50 mm^3, and it contained 0.025 mg of the enzyme preparate. The digestion was complete within 6 h at room temperature. As an evidence of this, the first sample of each kinetic run always gave only 3′-UMP and about 0.025% uridine, released from the 3′-end of poly(U) chains. No cyclic monophosphate intermediates were detected. The total area of the signals remained constant during each kinetic run. The analyses were performed on the same RP-column described above, but this time gradient elution was applied. Monomeric products were first eluted with the buffer–acetonitrile mixture described above, and after 10 min the acetonitrile content was within 5 min increased to 75%. The monomeric products were identified by spiking the mixture with authentic samples purchased from Sigma. Uridyl(2′,5′)uridine 2′- and 3′-monophosphates (**6a**, **b**) were separated by HPLC from the other products and dephosphorylated with alkaline phosphatase (Boehringer). The dephosphorylated product was identified as uridylyl(2′,5′)uridine by spiking with an authentic sample (Sigma).

Calculation of the Rate Constants.—First-order rate constants, k_H, for the hydrolysis of the phosphodiester bonds were calculated by eqn. (1). Here [3′-UMP]$_0$ is the con-

$$k_H = t^{-1} \ln \{[3'\text{-UMP}]_0/\{[3'\text{-UMP}]_0 - [\text{bonds cleaved}]_t\}\} \quad (1)$$

centration of 3′-UMP obtained by the enzymic digestion of the unmodified poly(U), *i.e.*, by the digestion of the initial sample. Accordingly, this quantity is proportional to the total amount of phosphodiester bonds initially present in the system. [Bonds cleaved]$_t$ stands for the total concentration of phosphodiester bonds cleaved chemically at time t. It was calculated by eqn. (2)

$$[\text{Bonds cleaved}]_t = (1 + a + b)[2'\text{-UMP}]_t + [\text{Urd}]_t \quad (2)$$

from the concentrations of 2′-UMP ([2′-UMP]$_t$) and uridine ([Urd]$_t$) obtained by the enzymatic digestion of the sample withdrawn at time t.

In the latter equation, a and b stand for parameters with which the concentration of 3′-monophosphate and 2′,3′-cyclic monophosphate groups formed during the chemical hydrolysis may be calculated from the amount of 2′-monophosphate groups. Accordingly, a = [3′-UMP]/[2′-UMP] and b = [2′,3′-cUMP]/[2′-UMP] in the non-digested sample withdrawn at time t.

First-order rate constants, k_1, for the isomerization were calculated by eqn. (3). Here x(**1b**)$_t$ is the mole fraction of

$$k_1 = t^{-1} \ln x(\mathbf{1b})_{eq}/[x(\mathbf{1b})_{eq} - x(\mathbf{1b})_t] \quad (3)$$

2′,5′-bonds among all phosphodiester bonds, and it is equal to the ratio {[**6a**] + [**6b**]}/{[3′-UMP]$_0$ − [bonds cleaved]$_t$}. x(**1b**) denotes the same quantity at equilibrium. On the basis of previous studies [3] with dinucleoside monophosphates this value was assumed to be 0.5.

Acknowledgements

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References

1 E. Anslyn and R. Breslow, *J. Am. Chem. Soc.*, 1989, **111**, 4473.
2 R. Breslow and D.-L. Huang, *J. Am. Chem. Soc.*, 1990, **112**, 9621.
3 P. Järvinen, M. Oivanen and H. Lönnberg, *J. Org. Chem.*, 1991, **56**, 5396.
4 R. Breslow and R. Xu, *J. Am. Chem. Soc.*, 1993, **115**, 10705.
5 M. Oivanen, K. Schnell, W. Pfleiderer and H. Lönnberg, *J. Org. Chem.*, 1991, **56**, 3623.
6 M. Oivanen and H. Lönnberg, *Acta Chem. Scand.*, 1991, **45**, 968.
7 T. Shiiba and M. Komiyama, *Tetrahedron Lett.*, 1992, **33**, 5571.
8 D. A. Usher, D. I. Richardson and D. G. Oakenfull, *J. Am. Chem. Soc.*, 1970, **92**, 4699.
9 A. J. Chandler and A. J. Kirby, *J. Chem. Soc., Chem. Commun.*, 1992, 1769.
10 L. F. Cavalieri, *J. Am. Chem. Soc.*, 1951, **73**, 4899.
11 B. G. Lane and G. C. Butler, *Biochim. Biophys. Acta*, 1959, **33**, 281.
12 H. K. Kaltreider and J. F. Scott, *Biochim. Biophys. Acta*, 1962, **55**, 379.
13 J. E. Bacher and W. J. Kauzmann, *J. Am. Chem. Soc.*, 1952, **74**, 3779.
14 J. J. Butzow and G. L. Eichorn, *Biochemistry*, 1971, **10**, 2019.
15 J. W. Huff, K. S. Sastry, M. P. Gordon and W. E. C. Wacker, *Biochemistry*, 1964, **3**, 501.
16 M. K. Stern, J. K. Bashkin and E. D. Sall, *J. Am. Chem. Soc.*, 1990, **112**, 5357.
17 J. R. Morrow, L. A. Buttrey, V. M. Shelton and K. A. Berback, *J. Am. Chem. Soc.*, 1992, **114**, 1903.
18 K. Yoshinari, A. K. Yamazaki and M. J. Komiyama, *J. Am. Chem. Soc.*, 1991, **113**, 5899.
19 B. Barbier and A. Brack, *J. Am. Chem. Soc.*, 1988, **110**, 6880.
20 R. Breslow and M. Labelle, *J. Am. Chem. Soc.*, 1986, **108**, 2655.
21 *Enzyme Nomenclature* (1984) Academic Press, Orlando, p. 304.
22 M. Oivanen and H. Lönnberg, *J. Org. Chem.*, 1989, **54**, 2556.
23 S. N. Mikhailov, M. Oivanen, P. Oksman and H. Lönnberg, *J. Org. Chem.*, 1992, **57**, 4122.
24 M. Oivanen and H. Lönnberg, *Acta Chem. Scand.*, 1990, **44**, 239.
25 H. S. Harned and N. D. Embree, *J. Am. Chem. Soc.*, 1934, **56**, 1042.
26 H. S. Harned and R. W. Ehlers, *J. Am. Chem. Soc.*, 1932, **54**, 1350.
27 N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, *Biochemistry*, 1932, **5**, 467.
28 E. J. King, *J. Am. Chem. Soc.*, 1951, **73**, 155.

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