Metal-ion-promoted Hydrolysis of Polyuridylic Acid

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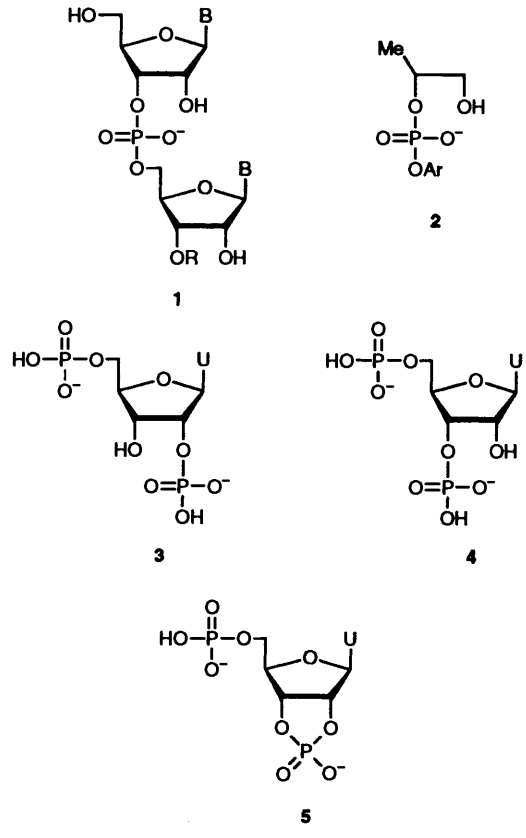
First-order rate constants for the hydrolysis of polyuridylic acid in the absence and presence of various metal ions and their chelates have been determined under neutral and slightly acidic conditions. The hydrolysis has been shown to proceed by cleavage of non-terminal bonds rather than by stepwise release of monomeric nucleotides. Only the hydrolysis of the 3',5'-phosphodiester bonds is accelerated by metal ions, not their isomerization to 2',5'-bonds. The catalytically active species has been shown to be the monohydroxo form of the metal aquo ion. The rate accelerations are parallel, but up to 20 times greater than those obtained with uridylyl(2',5')uridine. The mechanism of the metal-ion action is discussed.

Metal ions and their complexes have received during the past decade considerable interest as research tools for nucleic acid chemistry and molecular biology.[1] One of the central subjects in this field is the development of artificial nucleases that would enable tailoring of nucleic acids in a pre-designed manner. In particular, molecular recognition and sequence-specific cleavage of DNA by synthetic probes have been intensively studied, and several examples of oxidative rupture of the phosphodiester bonds by metal chelates tethered to sequence-recognizing oligonucleotides have been reported.[2] Owing to the extreme hydrolytic stability of the phosphodiester bonds of DNA, no sufficiently efficient artificial catalyst for the hydrolysis of DNA has been introduced. Komiyama *et al.* have reported that cerium(III) salts and chelates rapidly hydrolyse oligodeoxynucleotides,[3,4] but according to Takasaki and Chin even this process is oxidative rather than hydrolytic.[5] Since RNA is hydrolysed, owing to the intramolecular participation of the 2'-hydroxy function, much faster than DNA,[6] and since sequence-specific cleavage of mRNA could be exploited in selective inhibition of gene expression,[7] increasing attention has recently been paid to metal-based hydrolysis of oligoribonucleotides.

Studies on the hydrolysis 3',5'-dinucleoside monophosphates (**1**, R = H)[8-12] and their simple non-nucleotidic analogues, **2**,[8,11,13,14] have given valuable information about the mechanism of the metal-ion action: the metal ion is coordinated to the phosphate group, decreasing the electron density at phosphorus, and its aquo/hydroxo ligand acts as a general acid/base catalyst deprotonating the attacking 2'-hydroxy group and possibly protonating the leaving 5'-oxygen. All these studies, however, suffer from one limitation; the model compounds contain only one phosphodiester bond. According to Butzow and Eichhorn,[15] the metal-ion catalyst does not interact only with the phosphodiester bond that is cleaved, but the adjacent phosphoester groups may also be involved. For example, the Zn^{2+}-promoted hydrolysis of ApAp [**1**, B = Ade, R = PO(O^-)OH] is two orders of magnitude faster than that of ApA (**1**, B = Ade, R = H), and the 5'-terminal phosphodiester bond of ApApA [**1**, B = Ade, R = PO(O^-)O-adenosine(5')] is cleaved seven times as fast as ApA. By contrast, 2'-monophosphate and 2',3'-cyclic monophosphate groups enhance only slightly the hydrolysis of ApA. The works of George *et al.*[16] and Shelton and Morrow[17] have verified these conclusions.

To elucidate the applicability of dinucleoside monophosphates as model compounds in studies aimed at clarifying the action of metal ions on RNA, kinetics of the metal-ion and metal-chelate promoted hydrolysis of polyuridylic [poly(U)] is

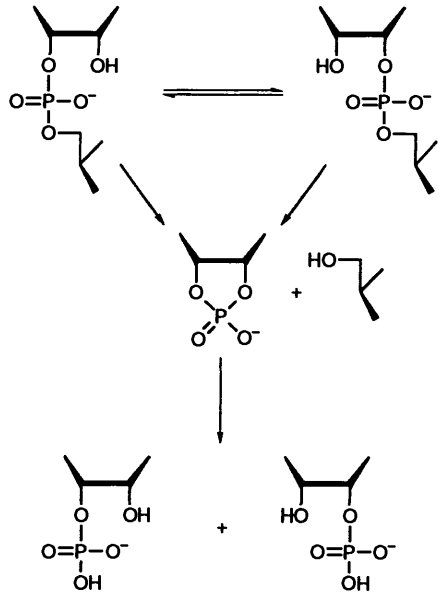


examined in the present work. Previous data on the metal-ion action on ribopolymers are limited to the early qualitative or semiquantitative observations, according to which several metal ions, including Mn^{2+},[18] Ni^{2+},[18] Cu^{2+},[18] Zn^{2+},[18,19] Cd^{2+},[19] Pb^{2+},[19-21] Al^{3+},[19] Bi^{3+} [19] and lanthanide ions,[21-23] hydrolyse RNA to nucleosides and nucleotides. Moreover, several Zn^{2+},[24,25] Cu^{2+} [24,25] and lanthanide[26] chelates derived from bipyridine, terpyridine or macrocyclic Schiff base ligands have recently been shown to exhibit marked catalytic activity towards the hydrolysis of RNA, and Pb^{2+} [27] and lanthanide ions[28] are known to cleave tRNAs site-specifically. The present work, however, describes for the first time the kinetics of the action of various metal ions and metal chelates on the hydrolysis of ribopolymers in a quantitative manner. Additionally, time-dependent accumulation of terminal 2'- and 3'-monophosphate and 2',3'-cyclic monophosphate groups in

the course of the hydrolysis is quantified, and the total rate constants obtained at different pH and metal-ion concentrations are broken down into partial rate constants referring to the cleavage of terminal and non-terminal phosphodiester bonds. In other words, the importance of stepwise cleavage of mononucleotide units from the 3′-terminus is estimated.

Results and Discussion

The phosphodiester bond of poly(U) undergoes in neutral and acidic solutions two concurrent reactions: hydrolysis to a 2′,3′-cyclic monophosphate with release of the 5′-terminal hydroxy function and isomerization to a 2′,5′-phosphodiester bond (Scheme 1). Under neutral conditions the latter reaction



Scheme 1

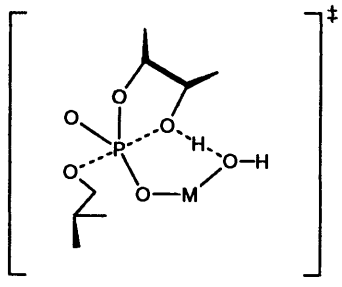
prevails.[30] Previous studies with 3′,5′-UpU (**1**, B = Ura, R = H) have shown that metal ions promote only the hydrolysis of the 3′,5′-bonds, not their isomerization to the 2′,5′-bonds.[12] Application of an RNase A digestion technique[30] that cleaves the 3′,5′-bonds and 2′,3′-cyclic monophosphates to 3′-monophosphates, but leaves the 2′,5′-bonds intact, indicated that, as with UpU, the metal ions accelerate only the hydrolysis of poly(U), not the conversion of 3′,5′-bonds into 2′,5′-bonds. With Co^{2+}, Zn^{2+} and Cd^{2+}, the hydrolysis was accelerated so strongly that no isomerization could be detected as a side reaction. Only with Mg^{2+} and Ni^{2+}, the rate-accelerating effect of which is weak, some isomerization accompanied the hydrolysis, but even then the isomerization represented less than 10% of the total reaction. Bashkin and Jenkins[31] have recently questioned the widely accepted bifunctional role of metal species in the hydrolysis of RNA. According to these authors, metal aquo ions might, instead of being phosphate coordinated intracomplex general acid–base catalysts, act as acids that protonate the phosphodiester bond without coordination to this particular phosphate centre. The absence of isomerization during metal-ion promoted hydrolysis of poly(U) does not, however, support this proposal; unlike protons and organic acids, the metal aquo ions promote only the hydrolysis. Their action more closely resembles that of hydroxide ion: deprotonation of the 2′-hydroxy results in an 'in-line' displacement of the 5′-linked nucleoside without concomitant phosphate migration.[32] According to the bifunctional mechanism, a hydroxo ligand of the phosphate-coordinated metal ion

Table 1 The effect of metal ions (5 mmol dm^{-3}) on the hydrolysis of the phosphodiester bonds of poly(U) at pH 5.6 and 363.2 K[a]

	Poly(U)		UpU[b]	
Metal	$k/10^{-6}$ s^{-1}	k_{rel}	$k/10^{-6}$ s^{-1}	k_{rel}
None	0.30 ± 0.03	1	0.052 ± 0.001	1
Mg^{2+}	3.2 ± 0.3	11	0.104 ± 0.008	2
Co^{2+}	14 ± 2	47	0.455 ± 0.009	9
Ni^{2+}	6.8 ± 0.6	23	0.181 ± 0.011	4
Zn^{2+}	570 ± 60	1900	4.25 ± 0.04	80
Cd^{2+}	110 ± 7	370	2.00 ± 0.01	50

[a] Obtained in a HEPES buffer ([HA]/[A^-] = 0.1/0.01 mol dm^{-3}), the ionic strength of which was adjusted to 0.1 mol dm^{-3} with sodium perchlorate. The pK_a value of HEPES extrapolated to 363.2 K is 6.6 (ref. 29). [b] From ref. 12.

deprotonates the attacking 2′-hydroxy group, and hence an 'in-line' displacement *via* a phosphorane-like transition state could be envisaged (Scheme 2), analogously to the hydroxide-ion catalysed hydrolysis.



Scheme 2

Table 1 records the first-order rate constants obtained by the phosphodiesterase I digestion technique[33] for the hydrolysis of the phosphodiester bonds of poly(U) in the absence and presence of various metal ions (5 mmol dm^{-3}) at pH 5.6 and 363.2 K. As has been indicated by Breslow,[33] the hydrolysis by the phosphodiesterase I gives 5′-UMP from intact poly(U) strands, but a uridine molecule in any place where the strand has already been cleaved by the metal ion. The rate constants obtained previously[12] with 2′,5′-UpU (**1**, B = Ura, R = H) are included for comparison. The rate-accelerating effects that various metal ions exert on the hydrolysis of poly(U) and UpU are parallel, but 5 to 20 times greater with poly(U) than with UpU. A possible explanation for this difference could be that the hydrolysis of poly(U) proceeds in a stepwise manner from the 3′-end of the chain. Cleavage of one phosphodiester bond would give a terminal 2′,3′-cyclic monophosphate, the cleavage of which to a 1:2 mixture of terminal 2′- and 3′-monophosphates is also known[34] to be promoted by metal ions. As mentioned above, a 3′-phosphomonoester function [**1**, R = PO(O^-)(OH)] has been shown[15–17] to increase the susceptibility of the hydrolysis of dinucleoside monophosphates (**1**) to metal-ion acceleration to a much greater extent than a 2′-phosphomonoester function or a 3′-phosphodiester function [**1**, R = PO(O^-)OR′]. Accordingly, the 3′-terminal nucleoside could be rapidly released as a 3′-monophosphate, leaving a new 2′,3′-cyclic monophosphate end group, and hence this stepwise depolymerization might constitute the predominant pathway. The following data, however, indicate that this is not the case, but that the hydrolysis mainly takes place *via* cleavage of the non-terminal phosphodiester bonds.

When an intact poly(U) strand was digested with phosphodiesterase I, all the nucleotidic units were released as uridine 5′-monophosphate (5′-UMP), as detected by RP HPLC. After incubation with Zn^{2+}, several additional products

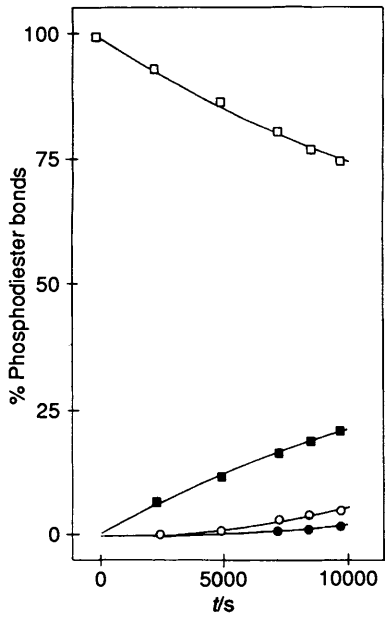


Fig. 1 The progress of the hydrolysis of poly(U) at pH 5.5 (adjusted with HEPES) and 333.2 K in the presence of 5 mmol dm^{-3} Zn^{2+} (I = 0.1 mol dm^{-3} with $NaClO_4$): □, intact phosphodiester bonds; ■, non-terminal phosphodiester bonds cleaved; ○, terminal phosphodiester bonds cleaved; ●, terminal nucleoside 3′-monophosphates cleaved

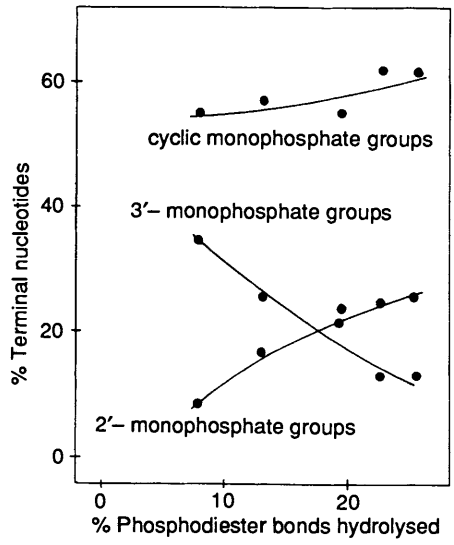


Fig. 2 The distribution of the monophosphate groups at the 3′-terminus of the polymeric products during Zn^{2+}-promoted (5 mmol dm^{-3}) hydrolysis of poly(U) at pH 5.5 (adjusted with HEPES buffer) and 333.2 K (I = 0.1 mol dm^{-3} with $NaClO_4$)

were detected. As described above, the metal-ion-promoted hydrolysis of a phosphodiester bond creates a free 5′-OH terminus and a 3′-terminus bearing either a 2′- or 3′-monophosphate group, or a cyclic 2′,3′-monophosphate group. Upon enzymatic digestion, the newly formed 5′-terminal nucleosides are released as uridine, and the 3′-nucleotides as uridine bisphosphates (**3**–**5**). The amount of uridine, or **3**–**5**, determined by HPLC, is hence proportional to the number of non-terminal phosphodiester bonds cleaved by Zn^{2+}. The Zn^{2+} promoted stepwise depolymerization from either the newly formed 3′-phosphate terminus or 5′-OH terminus, in turn, gives a mixture of 2′-UMP, 3′-UMP and 2′,3′-cUMP, and hence the total amount of these products is proportional to the number of the terminal bond cleavages. Fig. 1 shows the results obtained. On interpreting these data, two points should be noted. Firstly, the metal-ion promoted cleavage of the terminal nucleosides from the intact poly(U), having the 5′-OH phosphorylated and 3′-OH free, gives directly the same products as the non-terminal cleavage upon the enzymatic digestion (uridine + uridine bisphosphates **3**–**5**). Accordingly, these reactions cannot be distinguished from the non-terminal strand scissions. This is, however, of minor importance, since the poly(U) used as the starting material consists of about 300 nucleotides,[30] and hence the proportion of these initial terminal reactions among all the bond ruptures is almost negligible. Secondly, it has been shown previously[12] that Zn^{2+} does not catalyse the dephosphorylation of nucleoside monophosphates to nucleosides. The distinction between the terminal and non-terminal bond ruptures on the basis of uridine and uridine monophosphates is thus valid. As seen from Fig. 1, the predominant reaction still is the hydrolysis of non-terminal phosphodiester bonds. For example, when 10% of all the phosphodiester bonds are cleaved, 90% of these cleavages are non-terminal and only 10% terminal, either 3′- or 5′. It is also worth noting that the amount of 2′-UMP obtained was hardly detectable, while the amount of 3′-UMP was comparable to that of 2′,3′-cUMP. Since the Zn^{2+}-promoted hydrolysis of 2′,3′-cUMP is known to produce a 1:2 mixture of 2′- and 3′-UMP,[34] this means that 3′-UMP is formed exclusively by the release of 3′-terminal 3′-nucleotide, not by the hydrolysis of 2′,3′-cUMP. The proportion of this reaction is also depicted in Fig. 1. 2′,3′-cUMP may, in turn, be released either by a 3′- or 5′-terminal bond rupture.

An obvious explanation for the predominance of non-terminal strand scissions is that the 2′,3′-cyclic monophosphate group formed upon Zn^{2+}-induced cleavage of a phosphodiester bond is hydrolytically quite stable under the experimental conditions, and hence terminal 3′-monophosphates groups are formed only slowly. The first-order rate constant determined previously[34] for the Zn^{2+}-promoted hydrolysis of 2′,3′-cUMP under these conditions is 6.6×10^{-6} s^{-1}, *i.e.*, considerably smaller than that of the hydrolysis of the phosphodiester bonds of poly(U) (2.5×10^{-5} s^{-1}). To verify this argument, the distribution between 2′-, 3′- and cyclic 2′,3′-monophosphate groups at the 3′-termini of polymeric hydrolysis products was determined. For this purpose, two additional enzymatic digestions were carried out in parallel with the phosphodiesterase I digestion: (*i*) digestion with RNAase A was implemented to release the terminal 2′-monophosphates as 2′-UMP (intact 3′,5′-phosphodiester bonds, terminal 3′-monophosphates and terminal 2′,3′-cyclic monophosphates yield 3′-UMP) and (*ii*) digestion with the mixture of phosphodiesterase I and alkaline phosphatase was implemented to release the terminal 2′,3′-cyclic monophosphates as 2′,3′-cUMP (all the other units yield uridine). The total amounts of nucleoside 3′-monophosphates were then obtained by subtracting the amount of terminal 2′-monophosphates [(*i*) above] and 2′,3′-cyclic monophosphates [(*ii*) above] from the total number of the phosphodiester bonds cleaved (determined by phosphodiesterase I digestion). This procedure gives the total amount of each nucleotide species, *i.e.*, the sum of the 3′-terminal nucleotide and the corresponding monomer released by the metal-ion-promoted terminal cleavage. The amount of the 3′-terminal nucleotide may then be calculated by subtracting the amount of monomeric 2′-UMP, 3′-UMP or 2′,3′-cUMP obtained by phosphodiesterase I digestion, from the total amount. The results obtained are depicted in Fig. 2. It is clearly seen that the 2′,3′-cyclic monophosphate group predominates among the 3′-terminal phosphate groups. In fact, its mole fraction is even increased as the reaction proceeds. In other words, new terminal 2′,3′-cyclic monophosphate groups are formed at least as fast as they are hydrolysed to 2′- and 3′-monophosphates, or released as 2′,3′-cUMP. It is also important to note that the amount of terminal 2′-monophosphate groups is continuously increased compared

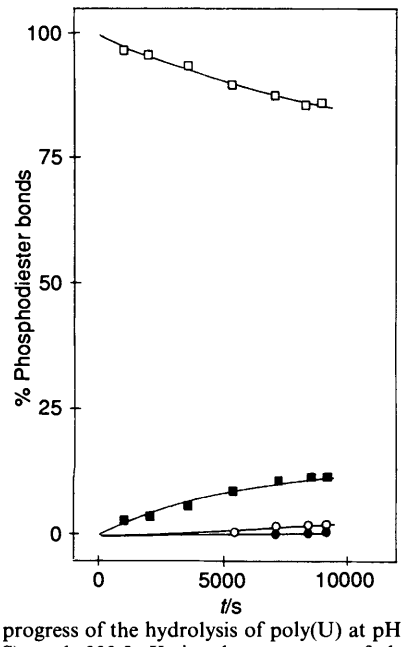


Fig. 3 The progress of the hydrolysis of poly(U) at pH 5.5 (adjusted with HEPES) and 333.2 K in the presence of 1 mmol dm^{-3} Zn^{2+}[12]$aneN_3$ (I = 0.1 mol dm^{-3} with $NaClO_4$): □, intact phosphodiester bonds; ■, non-terminal phosphodiester bonds cleaved; ○, terminal phosphodiester bonds cleaved; ●, terminal nucleotide 3′-monophosphates cleaved

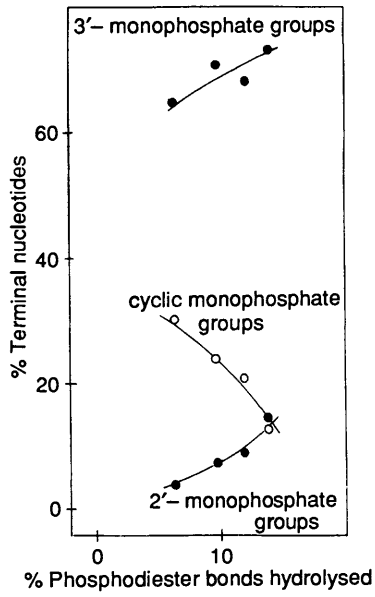


Fig. 4 The distribution of the monophosphate groups at the 3′-terminus of the polymeric products during Zn^{2+}[12]$aneN_3$-promoted (1 mmol dm^{-3}) hydrolysis of poly(U) at pH 6.5 (adjusted with HEPES) and 333.2 K (I = 0.1 mol dm^{-3} with $NaClO_4$)

with that of the 3′-monophosphate groups, consistent with the previous finding that a 3′-monophosphate group, but not the 2′-monophosphate group, induces stepwise depolymerization from the 3′-end of the chain. However, as described above, although this kind of reaction certainly occurs, it does not constitute the main pathway for the metal-ion-promoted hydrolysis of poly(U).

We have shown previously[12] that several tri- and tetra-aza complexes of Zn^{2+} and Ni^{2+} also promote the hydrolysis of 3′,5′-UpU. Among the complexes studied, Zn^{2+}(1,5,9-triazacyclododecane) (Zn^{2+}[12]$aneN_3$) was shown to exhibit the greatest rate-acceleration. When the hydrolysis of poly(U) was studied in the presence of this species by the methods described above, non-terminal strand scissions were found to prevail (Fig. 3), in spite of the fact that the terminal 2′,3′-cyclic monophosphate is now less markedly accumulated than with Zn^{2+} (Fig. 4). As shown previously[34] with 2′,3′-cUMP, Zn^{2+}[12]$aneN_3$ appears to promote the hydrolysis of 2′,3′-cyclic monophosphate more efficiently than Zn^{2+}

As seen from Fig. 5, the hydrolysis of poly(U) is approximately first order in the concentration of both Zn^{2+} and Zn^{2+}[12]$aneN_3$ over the range 0.5 < [Zn^{2+}]/mmol dm^{-3} < 15, suggesting that only one metal-ion species is involved. The slope of the lines log (k/s^{-1}) *vs.* log ([Zn^{2+}]/mol dm^{-3}) is, however, slightly greater than unity, and hence the participation of two metal species in a cooperative manner at high concentrations cannot strictly be excluded. The Zn^{2+}-promoted reaction is also first order in hydroxide-ion concentration at 4 < pH < 6 (Fig. 6), *i.e.*, at pH < pK_a of the Zn^{2+} aquo ion (8.96[35] at T = 298.2 K and I = 0 mol dm^{-3}). At higher pH, Zn^{2+} tends to precipitate as a hydroxo complex. Zn^{2+}[12]$aneN_3$, in turn, remains in solution at pH > pK_a of its aquo ion (7.51[36] at T = 298.2 K and I = 0.1 mol dm^{-3}). Under conditions where the monohydroxo form may be expected to start to prevail at elevated temperature, the hydrolysis rate levels off to a constant value (Fig. 6). This strongly suggests that the catalytically active form is the monohydroxo complex, consistent with the bifunctional mechanism (Scheme 2). Zn^{2+}-[12]$aneN_3$ appears to act as an intracomplex base catalyst

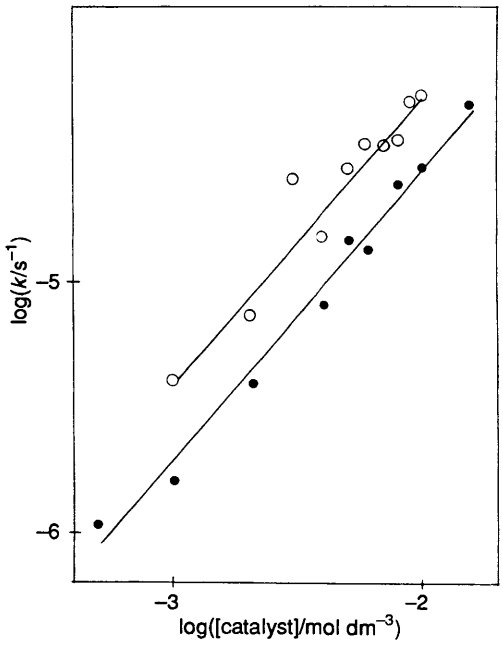


Fig. 5 The effect of the concentration of Zn^{2+} (●) and Zn^{2+}[12]$aneN_3$ (○) on the hydrolysis of poly(U) at pH 5.7 and 333.2 K (the pH was adjusted with a HEPES buffer and the ionic strength to 0.1 mol dm^{-3} with $NaClO_4$). Slopes of the lines: 1.2 and 1.1 for Zn^{2+} and Zn^{2+}[12]$aneN_3$, respectively.

deprotonating the entering 2′-oxygen. Whether it also acts as an acid catalyst protonating the leaving 5′-oxygen is difficult to decide, since the intracomplex proton transfers are kinetically invisible. The lack of phosphate migration, might be accounted for by an 'in-line' mechanism where the bond rupture is facilitated by intracomplex general acid catalysis of the aquo ligand.

Table 2 records the first-order rate constants for the hydrolysis of poly(U) in the presence of various aza complexes of Zn^{2+} and Ni^{2+} (2 mmol dm^{-3}) at pH 6.2 and 363.2 K. For comparison, the effects of the same species on the hydrolysis of 3′,5′-UpU are included. The accelerations are with poly(U) somewhat greater than with 3′,5′-UpU, but the difference is smaller than observed for the metal aquo ions. The catalytic efficiency of various chelates at a fixed pH roughly correlates

Table 2 The effect of metal-ion chelates (2 mmol dm^{-3}) on the hydrolysis of phosphodiester bonds of poly(U) at pH 6.2 and 363.2 K[a]

Metal ion + ligand[b]	pK_a	poly(U) $k/10^{-6}$ s^{-1}	poly(U) k_{rel}	3',5'-UpU[c] $k/10^{-6}$ s^{-1}	3',5'-UpU[c] k_{rel}
None		0.30 ± 0.03[d]	1	0.199 ± 0.004[e]	1
Ni^{2+} + [12]aneN$_3$		11 ± 2	37		
Ni^{2+} + [14]aneN$_4$	13.0[f]	6.4 ± 0.6	21		
Ni^{2+} + [15]aneN$_4$	11.7[g]	0.52 ± 0.06	2		
Ni^{2+} + tren	11.8[h]	2.6 ± 0.2	9	0.78 ± 0.03	4
Zn^{2+} + [12]aneN$_3$	7.5[i]	270 ± 30	900	24.0 ± 0.2	120
Zn^{2+} + [14]aneN$_4$	9.8[i]	3.0 ± 0.3	10	0.9 ± 0.1	5
Zn^{2+} + tren	10.6[k]	3.0 ± 0.2	10	0.44 ± 0.04	2
Zn^{2+} + [12]aneN$_4$	8.0[j]	11 ± 1	370	2.4 ± 0.5	12

[a] Obtained in a HEPES buffer [HA]/[A^-] = 0.065/0.035 mol dm^{-3}, the ionic strength of which was adjusted to 0.1 mol dm^{-3} with $NaClO_4$. The pK_a value of HEPES extrapolated to 363.2 K is 6.6 (ref. 29). [b] Abbreviations: [12]aneN$_3$ = 1,5,9-triazacyclododecane, [14]aneN$_4$ = 1,4,8,11-tetraazacyclotetradecane, [15]aneN$_4$ = 1,4,8,12-tetraazacyclopentadecane, tren = *N*,*N*-bis(2-aminoethyl)ethane-1,2-diamine, [12]aneN$_4$ = 1,4,7,10-tetraazacyclododecane (cyclen). [c] From ref. 34. Original data refer to the catalyst concentration 10 mmol dm^{-3}. To facilitate comparison, these values are divided by 5. [d] From ref. 30. [e] From ref. 37. [f] From ref. 38. [g] From ref. 39. [h] From ref. 40. [i] From ref. 41. [j] From ref. 36. [k] From ref. 42.

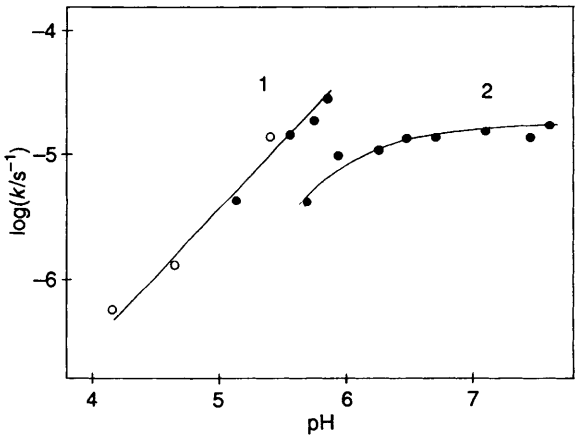


Fig. 6 The effect of pH on the Zn^{2+} (*a*) and Zn^{2+}[12]aneN$_3$ (*b*) promoted hydrolysis of poly(U) at T = 333.2 K and I = 0.1 mol dm^{-3} (adjusted with $NaClO_4$); ○ refers to acetate buffer and ● to HEPES buffer

with the pK_a value of their aquo ion, but, as discussed previously,[34] the coordination geometry also has an effect. For example, the aquo ion of Ni^{2+}[14]aneN$_4$ is catalytically more active although less acidic than that of Ni^{2+}[15]aneN$_4$.

In summary, metal ions and metal chelates promote the hydrolysis of the phosphodiester bonds of poly(U) without accelerating their isomerization to the 2',5'-bonds. The depolymerization takes place by cleavage of the non-terminal phosphodiester bonds rather than by a stepwise release of mononucleotides. The catalytically active species is the hydroxo form of the metal aquo ion. The rate accelerations are up to 20 times greater than those obtained with 3',5'-UpU. Evidently, the neighbouring phosphodiester bonds enhance the metal ion action, but a detailed understanding of this phenomena requires additional studies with short oligonucleotides.

Experimental

Materials.—Poly(U), uridine and 2'-, 3'-, 5'- and 2',3'-cUMP were from Sigma. They were used as received after being checked for purity by HPLC. The ligands were from Aldrich. The metal salts and buffer constituents were all of reagent grade. Phosphodiesterase I (lyophilized powder) was purchased from USB, RNAase A (lyophilized powder) from Sigma and alkaline phosphatase (concentrated solution) from Boehringer Mannheim.

Kinetic Measurements.—The kinetic runs were in principle performed as described earlier.[43] The pH of the reaction solutions was measured at 273.2 K and extrapolated to the temperature of the kinetic measurements with the aid of known temperature dependences.[29,44] After being cooled in an ice bath, aliquots of 750 μl were treated with Chelex to remove the metal ions, which otherwise prevented the action of the enzyme. A typical number of aliquots was 10 to 12. After Chelex treatment, a fixed volume (400 μl) of each aliquot was transferred to another test tube and 50 μl of concentrated TRIS buffer (0.5 mol dm^{-3}) was added to adjust the pH to 9 and to provide Mg^{2+} ions required as enzyme cofactors. The final concentration of the TRIS buffer was 0.05 mol dm^{-3}, that of $MgCl_2$ 0.015 mol dm^{-3} and that of NaCl 0.1 mol dm^{-3}. Phosphodiesterase I was added as a TRIS buffer solution. 0.1 unit of the enzyme (50 μl) was added into each aliquot, and they were incubated for 8 h at room temperature to complete the digestion. The digested aliquots were filtered and the solutions were neutralized with 1 mol dm^{-3} acetic acid. The compositions of the aliquots were analysed by HPLC, using a Hypersil RP-18 column (250 × 4 mm i.d., 5 μm film thickness) and a mixture of acetic acid buffer (pH 4.3, containing 0.1 mol dm^{-3} NH_4Cl) and acetonitrile (99.75%/0.25% v/v) as the eluent.

Calculation of the Rate Constants.—5'-UMP, 2',3'-cUMP, 3'-UMP, 2'-UMP and uridine were completely separated by the chromatographic system described above, the retention times being 3.7, 4.7, 5.5, 6.5 and 8.2 min, respectively. The bisphosphates (**3**–**5**) partially overlapped (t_R 2.7 min). The total signal area of these products (ΣA_0) is proportional to the initial number of phosphodiester bonds in the aliquot, since the absorbing base moieties are not modified during the reaction. In other words ΣA_0 remained constant in all the aliquots of each kinetic run. The sum of the areas of 2',3'-cUMP, 2'-UMP, 3'-UMP and uridine (ΣA_p) is, in turn, proportional to the number of phosphodiester bonds hydrolysed. Accordingly, the mole fraction of the intact phosphodiester bonds was calculated by eqn. (1), and the first-order rate constant for the disappearance of the phosphodiester bonds by eqn. (2).

$$x = (\Sigma A_0 - \Sigma A_p)/\Sigma A_0 \quad (1)$$

$$k = t^{-1}\ln(1/x) \quad (2)$$

Isomerization and End Group Analysis.—RNAase A was employed to study the isomerization of the phosphodiester bonds. Before the RNAase A digestion, the metal ions were removed with Chelex resin, and the analysis was then carried out as described earlier.[30] Digestions with alkaline phosphatase were carried out as described for phosphodiesterase I.

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