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Hydrolytic reactions of diadenosine 5′,5′-triphosphate

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The hydrolysis of diadenosine 5′,5′-triphosphate to AMP and ADP has been studied over a wide pH-range. Under acidic conditions the reaction shows a first-order dependence on the hydronium ion concentration. Below pH 3 the rate-increase begins to level off. From pH 6 to 9 the hydrolysis is slow and pH-independent. Base-catalysed hydrolysis is observed in NaOH-solutions. Under alkaline conditions an intramolecular nucleophilic attack on the phosphate producing 3′,5′-cAMP is also observed, but it is slower than the intermolecular reaction. Depurination of the adenosine moieties competes with the hydrolysis both under acidic and alkaline conditions, but the mechanisms are different. The temperature-dependence of the hydrolysis of Ap_3A and the depurination of adenosine moieties were studied under acidic conditions, and the activation parameters of the reactions were calculated. The results of the work reflect the fact that the negatively charged polyphosphate group is very resistant towards nucleophilic attack. An efficient catalysis is only observed under acidic conditions, where the phosphate group becomes protonated. General acids or bases did not catalyse the hydrolysis. Furthermore, hydroxide ion catalysed cleavage is only observed at high base concentrations and other negatively charged nucleophiles did not attack the phosphate groups of diadenosine polyphosphates.

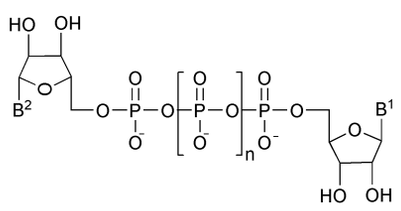
Introduction

Dinucleoside oligophosphates (Scheme 1a) form an interesting class of compounds that are found both in prokaryotes and eukaryotes. They were first discovered in biological systems in the early 1960's,[1] and their biological roles, particularly those of diadenosine triphosphate (**1**) and tetraphosphate (**2**), have since been extensively studied.[2–7] Compounds **1** and **2** have been suggested to be involved in tumour growth suppression, possibly as signal-transducing molecules. They are also substrates of interferon induced 2–5A synthetase and may hence be involved in cellular antiviral defence. In bacteria the level of dinucleoside oligophosphates has been shown to respond to oxidative stress, and they have been speculated to have a role as alarmones. Extracellular dinucleoside oligophosphates have also been suggested to act as signalling molecules and neurotransmitters. Many biological processes where dinucleoside oligophosphates are involved, involve a hydrolysis of the phosphate group,[2,5] and specific enzymes that enhance this reaction *in vivo* have been identified.[8]

A dinucleoside triphosphate moiety is also found in the 5′-*cap* structure of RNA polymerase II synthesised mRNA transcripts (Scheme 1b). The polyphosphate of the 5′-*cap* structure serves in an essential role: the 5′-*cap* is a recognition site of enzymes involved in translation,[9,10] transport[11,12] and maturation[13–17] of the RNA molecule. The 5′-*cap* is of interest as a target for artificial nucleases and it has been shown that an oligonucleotide conjugate, with a lanthanide ion complex as the cleaving agent, recognises its target RNA and significantly decreases the amount of the respective protein by cleaving the triphosphate bridge of the 5′-terminal *cap*.[18] In order to develop efficient catalyst groups, metal ion-promoted hydrolysis of dinucleoside triphosphates has been studied in detail.[19–24] Catalysis by metal ion complexes under neutral conditions is significant: 2 mM Cu^{2+}-bipyridine has been estimated to enhance the hydrolysis by at least 20000-fold at pH 7.5.[24] Bifunctional Zn^{2+} and Cu^{2+} complexes[20,21] and complexes of trivalent lanthanide ions[23] are even better catalysts. The details of the catalysis mechanism are not known, but it is quite clear that a metal bound hydroxide ion attacks the phosphate as a nucleophile and the attack is electrostatically assisted by a phosphate bound metal ion. Recent results from our laboratory have shown that macrocyclic amines also modestly enhance the hydrolysis of the *cap* structure by providing electrostatic activation of the phosphate and an intramolecular nucleophile attacking the phosphate.[25]

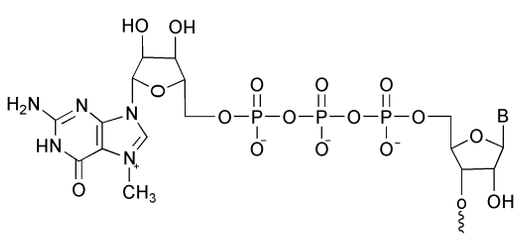
Except for the metal ion promoted hydrolysis, the chemical hydrolysis of dinucleoside oligophosphates has not been studied in detail before. This is in contrast to the reactions of nucleoside phosphomonoesters and phosphodiesters, RNA in particular, whose cleavage mechanisms have been thoroughly studied during the last decades.[26] In order to fill this gap, the present work studies the hydrolysis of diadenosine oligophosphates **1**–**3** in the absence of any metal ion catalysts. The hydrolysis has been studied as a function of pH, temperature and buffer concentration. The aim of the work is to study the reactivity of polyphosphates and the catalysis of the hydrolysis by acids and bases, and thus possibly provide information about the mechanisms utilised by natural enzymes, and assist the development of artificial constructs that hydrolyse mRNA 5′-*cap* structure.

(a)



1: B^1=B^2=Ade, n=1
2: B^1=B^2=Ade, n=2
3: B^1=B^2=Ade, n=0

(b)



Scheme 1

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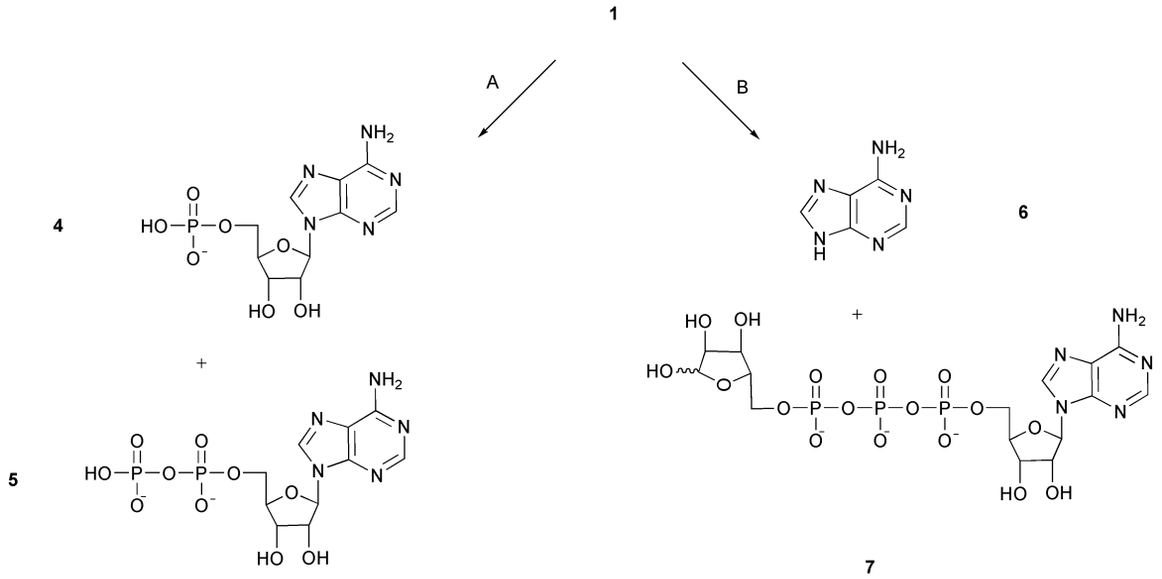


Scheme 2

Results and discussion

The hydrolysis of diadenosine-P^1,P^3-triphosphate (Ap_3A; **1**) to adenosine 5′-monophosphate and diphosphate (**4** and **5**, respectively, in Scheme 2) was followed over a wide pH-range at 90 °C. Reactions were followed by taking samples, typically 10–15, from reaction solutions to cover approximately two half-lives. Under neutral conditions the hydrolysis was very slow and only one half-life or less was covered. Samples were analysed either by capillary zone electrophoresis (CZE) or reversed-phase HPLC (RP-HPLC) as described in more detail in the Experimental section. The choice of the analysis method depended mostly on the composition of the sample. Generally, the CZE method gave better separation, but analysis times were longer. Certain buffers employed, particularly if the concentration was high, affected the CZE separation, and in some cases the peaks were impossible to separate by CZE or the analysis time was long. HPLC analysis gave better results with samples of high buffer concentration, even though the baselines in the chromatograms tended to be poor. In all other cases either method could be utilised. Generally, CZE was used for the analysis of samples from acidic reaction solutions, and RP-HPLC for samples from neutral and basic solutions. As the peak areas in CZE analysis are dependent on the migration time, the peak areas were normalised by dividing the area by the migration time to give the normalised peak areas (A_N).

Acid catalysed reactions

The pH-rate-profile of the triphosphate hydrolysis of Ap_3A (**1**) is shown in Fig. 1. The plot is obtained by fitting the observed first-order rate constants according to the theoretical rate law as explained in the Experimental. As can be seen, the hydrolysis exhibits a first-order dependence on hydronium ion concentration between pH 3 and 6. The second-order rate constant obtained from the fit is 0.11 ± 0.04 dm^{-3} mol^{-1} s^{-1}. Below pH 3 the rate-increase begins to level off. In the most acidic solutions, the hydrolysis (Route A, Scheme 2) competes with acid-catalysed depurination of adenosine moieties (Route B, Scheme 2). AMP (**4**), ADP (**5**) and adenine (**6**) were identified by co-injecting with authentic samples. No attempt was made to characterise the depurinated triphosphate **7**, but consistent with the alleged structure, this compound is initially formed parallel to adenine as is shown by the time-dependent product distribution curves obtained in 0.1 mM HCl by using CZE analysis (Fig. 2a). Rate constants of the hydrolysis and depurination were calculated on the basis of increase of the normalised signal areas (A_N) of the reaction products as a function of

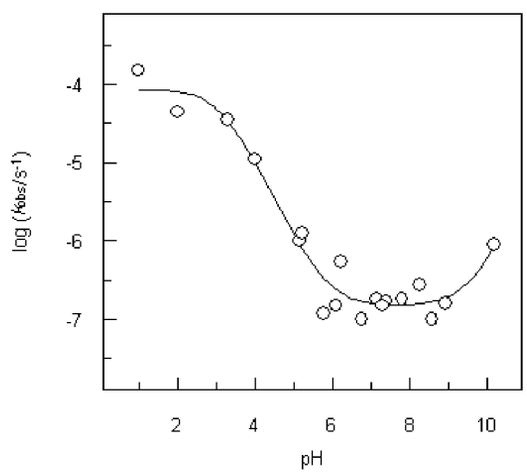


Fig. 1 pH-rate-profile of the hydrolysis of the triphosphate moiety of Ap_3A (**1**) at 90 °C. I = 0.1 M, pH-values refer to 90 °C. The theoretical curve has been obtained by a non-linear fit of eqn. 1 as described in the Experimental. Parameters derived: second-order rate constant of acid catalysed hydrolysis (k_H) 0.11 ± 0.04 dm^{-3} mol^{-1} s^{-1}, first-order rate constant of neutral hydrolysis (k_0) $(1.5 \pm 0.3) \times 10^{-7}$ s^{-1}, second-order rate constant of base catalysed hydrolysis (k_{OH}) $(1.4 \pm 0.6) \times 10^{-4}$ dm^{-3} mol^{-1} s^{-1} and equilibrium constant of protonation of trianionic phosphate (K) 1300 ± 600 dm^3 mol^{-1}.

the reaction time using the integrated rate law of a first-order reaction ($k = 1/t\ln(A_\infty - A_0)/(A_\infty - A_t)$. Using A_N(ADP) + A_N(AMP) or A_N(Ade) + A_N(**7**) as A_t values, and the observed total peak area as the final value A_∞, the calculation gives the rate constant of the triphosphate hydrolysis or depurination, respectively. As products **4**, **5** and **7** also hydrolyse and depurinate under the experimental conditions, only the linear part of the plots was used to calculate the rate constants. The sum of the rate constants thus obtained was consistent with the rate constant calculated on the basis of the decrease of normalised peak area of Ap_3A. As further proof of a correct assignation of the peak assumed to refer to the depurinated triphosphate, the rate constants of formation of the depurination products **6** and **7** are fully consistent with those reported for the hydrolysis of the *N*-glycosidic bond of adenosine and 5′-AMP.[27,28] Values obtained in the present work in 0.1 M and 0.01 M HCl were 4.6×10^{-4} s^{-1} and 5.1×10^{-5} s^{-1}, respectively. Depurination is only observed below pH 3, where the rate of the hydrolysis is levelling off. The rate of depurination, in contrast, is known to show a strict first-order dependence on hydronium ion concentration over a wide pH-range.[28–30] The depurination products of

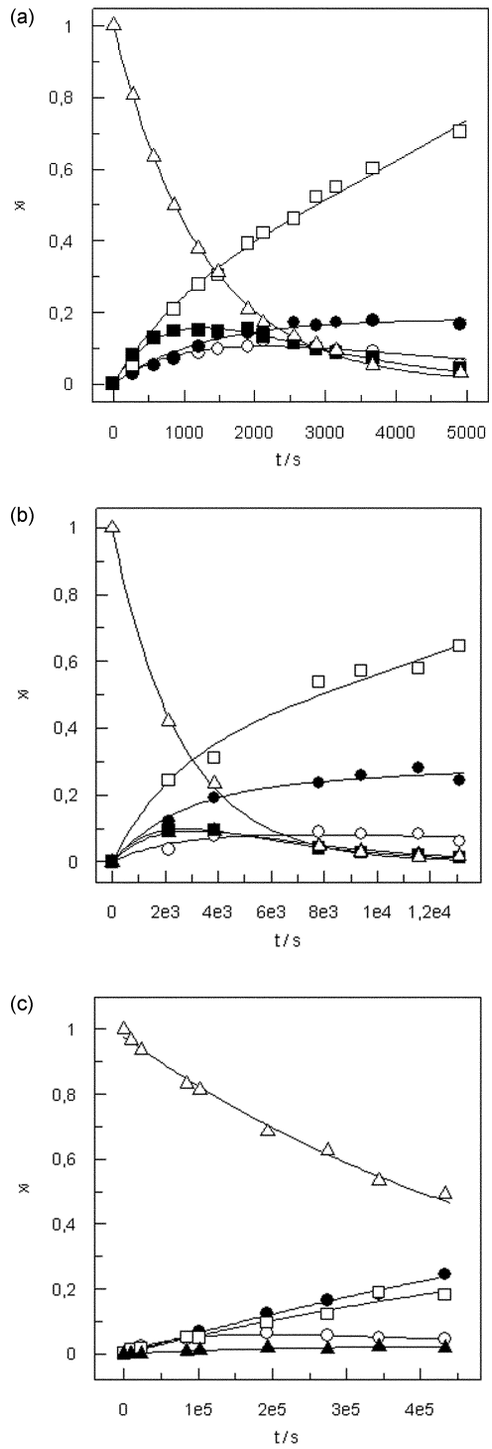


Fig. 2 (a) Product distribution curves observed in the reaction of Ap_3A (**1**) in 0.1 M HCl at 90 °C. Notation: △-Ap_3A (**1**); □-adenine (**6**); ■-depurinated triphosphate **7**; ●-5′-AMP (**4**); ○-ADP (**5**). The data points have been obtained by CZE analysis and the curves are theoretical fits obtained as explained in the Experimental. (b) Product distribution curves observed in the reaction of Ap_4A (**2**) in 10 mM HCl at 90 °C. Notation: △-Ap_4A (**1**); □-adenine (**6**); ●-5′-AMP (**4**); ▲-ATP (**8**); ■-depurinated tetraphosphate **9**; ○-ADP (**5**). The data points have been obtained by CZE analysis and the curves are theoretical fits obtained as explained in the Experimental. (c) Product distribution curves observed in the reaction of Ap_3A (**1**) in 10 mM NaOH at 90 °C. Notation: △-Ap_3A (**1**); ●-5′-AMP (**4**); □-adenine (**6**); ○-ADP (**5**); ▲-3′,5′-cAMP (**10**). The data points have been obtained by RP-HPLC analysis and the curves are theoretical fits obtained as explained in the Experimental.

4, **5** and **7**, which, as mentioned, are likely to be produced over a longer reaction time, do not contain a chromophore, and could not be detected by either analysis method used.

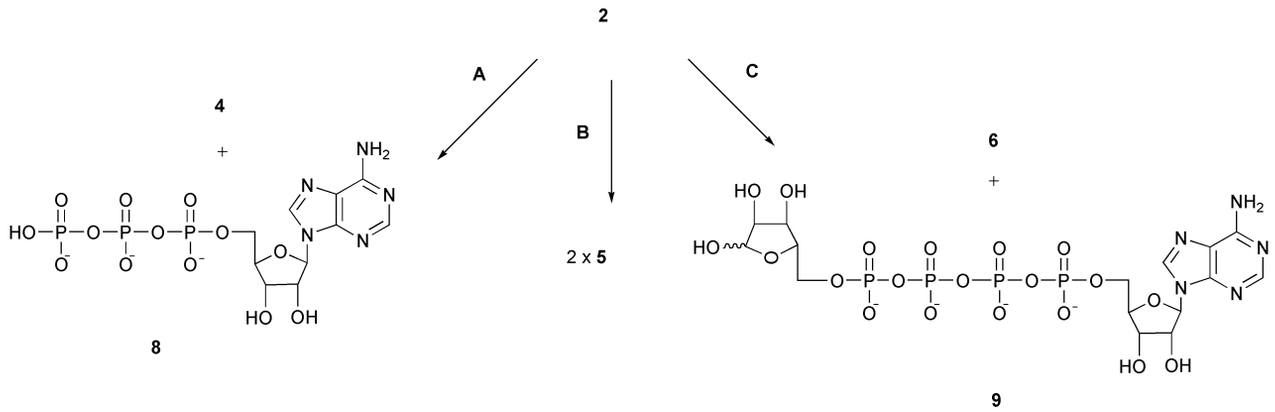
The temperature-dependence of the rate of the acid-catalysed hydrolysis of Ap_3A is large. The rate constants in 10 mM HCl determined at 90 °C, 60 °C, 37 °C and 25 °C were $(3.6 \pm 0.1) \times 10^{-5}$ s^{-1}, $(2.5 \pm 0.2) \times 10^{-6}$ s^{-1}, $(3.8 \pm 0.2) \times 10^{-7}$ s^{-1} and $(4.9 \pm 0.5) \times 10^{-8}$ s^{-1}, respectively. This data allows the calculation of activation parameters of the acid-catalysed hydrolysis in 10 mM HCl solutions. The parameters of the Arrhenius plot calculated from observed first-order rate constants obtained under these conditions were E_a = (97 ± 6) kJ mol^{-1} and ln (A/s^{-1}) = 22 ± 3, and the activation parameters calculated from these values: $\Delta S^{\ddagger} = -71$ J kmol^{-1} and $\Delta H^{\ddagger}$ = 94 kJ mol^{-1}. The entropy and enthalpy of activation of depurination of adenosine moieties calculated using the observed first-order rate constants were $\Delta S^{\ddagger} = -34$ J kmol^{-1} and $\Delta H^{\ddagger}$ = 109 kJ mol^{-1}. The data on the temperature-dependence of the depurination of adenosine moieties in Ap_3A agree very well with the data reported by Hevesi *et al.*[30] for the acidic depurination of adenosine.

The hydronium ion-dependent hydrolysis observed below pH 6 most likely proceeds through nucleophilic attack of water on a protonated phosphate and the levelling off observed below pH 3, is most probably to be attributed to a complete protonation of one of the phosphate groups. The pK_a values of the phosphate groups of **1** are not known, but they could be expected to be close to those of ATP protonated on the γ phosphate. Values reported for the second pK_a of ATP vary from 3.8 to 4.2 at 25 °C and ionic strength 0.1 M, and decrease as the temperature increases.[31] The pK_a value of 3.1 at 90 °C obtained from the non-linear fit of log(k_{obs}/s^{-1}) *vs.* log[H$^+$], as described in the Experimental, is well consistent with these values. The nucleophilic attack most likely results in an S_N(P) type nucleophilic substitution at phosphorous proceeding through a pentacoordinated phosphorane species. The position of the nucleophilic attack cannot be determined on the basis of the present data. There are two non-equivalent phosphate groups in **1**, but nucleophilic attack of water on either of these can result in the formation of ADP and AMP as products. The acidic depurination of adenosine involves a unimolecular heterolysis of the *N*-glycosidic bond,[28] and with no doubt, the depurination of adenosine moieties of **1** under acidic conditions proceeds *via* the same mechanism.

The reactivity of Ap_2A (**3**) and Ap_4A (**2**) were compared to that of Ap_3A (**1**) in 10 mM HCl solutions at 90 °C. The hydrolysis of the diphosphate **3** is slightly slower than that of the triphosphate **1**: While the rate constant of the hydrolysis of **1** under these conditions is $(3.6 \pm 0.1) \times 10^{-5}$ s^{-1}, that of **3** is $(1.0 \pm 0.1) \times 10^{-5}$ s^{-1}. The hydrolysis of the tetraphosphate **2** is faster, and as is shown by the product distribution curves in Fig. 2b obtained by CZE analysis, the cleavage of the tetraphosphate moiety can take place in two different ways: The asymmetric cleavage, which produces AMP and ATP as products (Route A, Scheme 3), and the symmetric, yielding two molecules of ADP (Route B, Scheme 3). The respective observed rate constants of the asymmetric and symmetric cleavages are $(9 \pm 1) \times 10^{-5}$ s^{-1} and $(3.2 \pm 0.4) \times 10^{-5}$ s^{-1}. As the asymmetric cleavage can take place at two different positions, the rate constants show that symmetric and asymmetric cleavages are approximately equally probable. Similarly to the situation with **1**, the triphosphate hydrolysis of **2** and **3** competes with depurination under acidic conditions and the rate constants of the parallel pathways were calculated on the basis of the increase of the signal area of the respective products. The rate constants of depurination of **1**–**3** are approximately the same.

pH-independent hydrolysis

Under neutral and slightly alkaline conditions (pH 6 to 9) the hydrolysis of Ap_3A is pH-independent and very slow (k_{obs} 1.5 × 10^{-7} s^{-1} at 90 °C). Under these conditions **1** reacts solely



Scheme 3

through the triphosphate hydrolysis. However, as the hydrolysis becomes slower on increasing the pH, the subsequent dephosphorylation of the hydrolysis products becomes more significant and therefore different hydrolysis product distributions were observed under slightly acidic and neutral conditions. Under slightly acidic conditions, the amount of ADP remains low and under neutral conditions ADP is not observed at all. This is fully consistent with the data on the reactivity of ADP at 95 °C: below pH 6 the rate constants of the ADP hydrolysis are pH-independent (k_{obs} 1×10^{-4} s^{-1} 95 °C).[32] Above pH 6, the rate constants decrease, but even then the hydrolysis is much faster than the pH-independent hydrolysis of Ap_3A (k_{obs} 1.5×10^{-7} s^{-1}). The dephosphorylation of AMP to adenosine is pH-independent from pH 1–5 (k_{obs} 1×10^{-6} s^{-1} at 90 °C) and shows first-order dependence on hydronium ion concentration above pH 5.[33,34] Dephosphorylation of AMP to adenosine is therefore observed at pH 6–7, but it becomes less significant as the pH increases. Within pH range 6 to 9 the rate constants of the hydrolysis were calculated on the basis of the decrease of the mole fraction of Ap_3A remaining in the reaction mixture. Rate constants calculated directly from the decrease of the signal area of Ap_3A gave similar results, but the standard errors were larger.

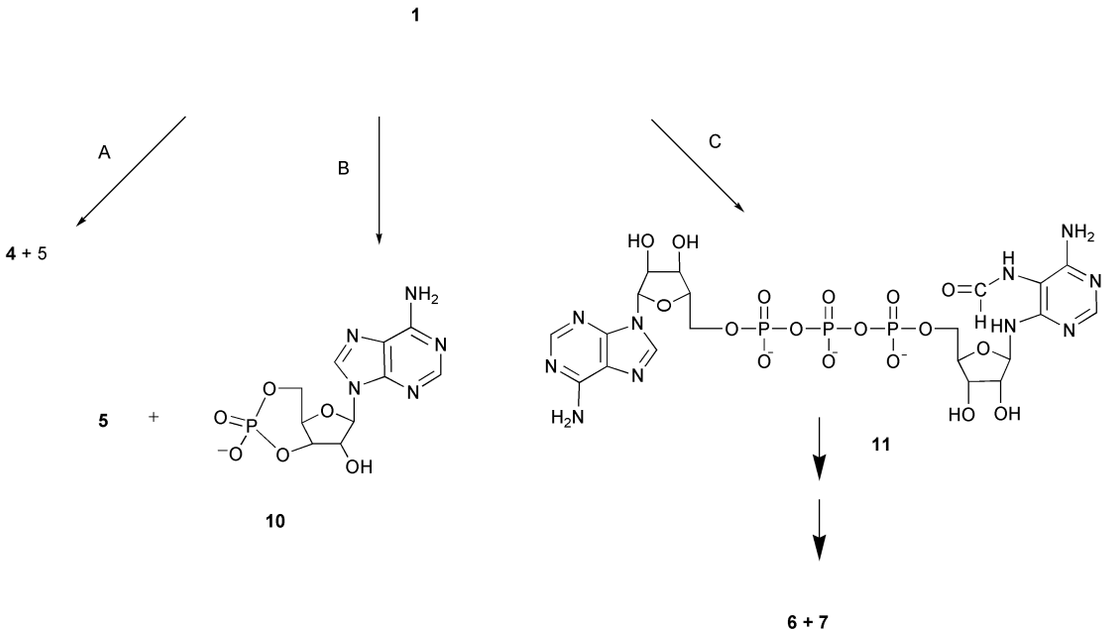
The pH-independent reaction can be accounted for either by a reaction of anionic phosphate group with water as an attacking nucleophile, or by a reaction where a hydroxide ion attacks on a protonated phosphate group. It could also be suggested that the reaction proceeded *via* a similar intramolecular cleavage observed under alkaline conditions (Route B, Scheme 4), but since 3′,5′-cAMP (**10**) was not observed among the reaction products, it seems likely that the intramolecular cleavage does not take place under neutral conditions. 3′,5′-cAMP is very stable under neutral conditions: only 3% of 3′,5′-cAMP was cleaved in 10 days in 0.1 M HEPES buffer (20% free base) at 90 °C. The rate constant of the cleavage can be estimated to be of the order of 3×10^{-8} s^{-1} and therefore 3′,5′-cAMP could be expected to accumulate if it was formed to any significant extent under neutral conditions.

Reactions under alkaline conditions

Under alkaline conditions the predominant products of the Ap_3A cleavage, observed by RP-HPLC-analysis, are AMP, ADP and adenine, with 3′,5′-cAMP and 3′-AMP as the minor products (Fig. 2c). The product analysis suggests that the triphosphate hydrolysis competes with an intramolecular cleavage resulting in the formation of 3′,5′-cyclic monophosphate of adenosine (3′,5′-cAMP; **10**) and ADP, and with alkaline cleavage of the adenine imidazole ring (Routes B and C, respectively, in Scheme 4). Under alkaline conditions adenine is most probably formed as the final product of the adenine base cleavage reaction that involves a nucleophilic attack on the C8 of the base, the ring opening, reclosure after a rearrangement and a hydrolysis of the *N*-glycosidic bond, which is the predominant process adenosine nucleosides undergo under alkaline conditions.[35] Intermediate **11** (Scheme 4) would not be expected to accumulate during the reaction due to its subsequent reactions, which on the basis of data obtained with adenosine[35] can be expected to be much faster than its formation observed in the present work (k_{obs} 6.3×10^{-7} s^{-1}). Surprisingly, the depurinated triphosphate **7** that should be formed as the other stable product of the reaction sequence resulting in a release of adenine, was not observed either under alkaline conditions. It was observed, however, that **7** is very unstable under alkaline conditions. This was shown by an experiment where **7** was produced by hydrolysing Ap_3A for two hours in 10 mM HCl solution. After that the hydroxide ion concentration of the reaction solutions was adjusted to 10 mM with concentrated NaOH solution and the decrease of concentration of **7** was followed by CZE. No rate constant was calculated, but it was observed that **7** disappeared completely in 15 minutes at 90 °C. As is discussed below, the rate constant of formation of **7** is only 6.3×10^{-7} s^{-1}, and since the cleavage is several orders of magnitude faster, **7** does not accumulate under the experimental conditions. The predominant cleavage product of **7** appears to be ADP. As is shown by the product distribution curves in Fig. 2c, the concentration of ADP remains rather low during the reaction of Ap_3A. An independent experiment with ADP as a substrate showed that ADP is hydrolysed *via* two pathways giving either 5′-AMP or adenine as a product. The rate constant of the total disappearance of ADP in 10 mM NaOH at 90 °C is $(5.1 \pm 0.1) \times 10^{-6}$ s^{-1}.

3′,5′-cAMP is most probably formed by an intramolecular nucleophilic attack of the 3′-OH group of an adenosine moiety on the 5′-phosphate. A similar reaction has been observed with Ap_2A under alkaline conditions in the presence of DCC, a condensing agent commonly used in phosphoester synthesis.[36] The concentration of 3′,5′-cAMP remains low during the reaction due to its consequent hydrolysis. In 10 mM NaOH, the rate constant of the disappearance of 3′,5′-cAMP is $(2.0 \pm 0.1) \times 10^{-6}$ s^{-1} at 90 °C. Consistent with reports on the hydrolysis of 3′,5′-cyclic phosphates,[36,37] the predominant product is adenine and only small amount of 3′-AMP and 5′-AMP were detected. 3′,5′-cAMP probably also reacts through the initial cleavage of the adenine base.

To obtain the rate constants of all three reactions of **1** under alkaline conditions, a rate equation of parallel and consecutive reactions[38] was applied to the formation and disappearance of 3′,5′-cAMP. A non-linear fitting of x(3′,5′-cAMP) *vs.* t gave a first-order rate constant of $(1.12 \pm 0.06) \times 10^{-7}$ s^{-1} for the formation of 3′,5′-cAMP. The linear part of the ln [x(AMP) + x(ADP)] *vs.* reaction time gave a rate constant of $(1.04 \pm 0.06) \times 10^{-6}$ s^{-1} for the hydrolysis of Ap_3A to AMP and ADP. As ADP is initially formed *via* two routes, intermolecular and intramolecular triphosphate cleavage, the rate constant calculated consists of contributions of these two processes, and the



Scheme 4

rate constant of the intermolecular process can be obtained by subtracting the proportion of the intramolecular reaction. The subtraction gave a rate constant of 9.0×10^{-7} s^{-1} for the intermolecular triphosphate hydrolysis of **1** in 10 mM NaOH at 90 °C. As the rate constant of total disappearance of **1** is $(1.64 \pm 0.06) \times 10^{-6}$ s^{-1}, a rate constant of 6.3×10^{-7} s^{-1} can be calculated for the cleavage of the imidazole ring of adenine bases of **1**. Similarly to the rate constant of the base cleavage of 3′,5′-cAMP, this value is clearly lower than the rate constant of the alkaline cleavage of adenosine ($k = 4 \times 10^{-5}$ s^{-1} at 90 °C[35]), which most probably results from the negative charge of the phosphate group retarding the nucleophilic attack by a hydroxide ion of C8 of adenine. Such an effect has also been observed with 7-methylguanosine and its 5′-phosphorylated derivatives.[39]

It has to be noted that only the rate constant calculated for the pathway involving the formation of 3′,5′-cAMP is strictly correctly calculated. AMP and ADP as well as adenine may be formed *via* several different routes, and therefore the rate constants calculated from the increase of their signal areas consist not only of the rate constant of the reaction of the starting material Ap_3A, but also of the contributions of other reactions. However, as mentioned above, the rate constant of the triphosphate hydrolysis of Ap_3A was calculated from a linear part of the ln [x(AMP) + x(ADP)] *vs.* reaction time-plot, where approximately equal amounts of ADP and AMP were produced, so this rate constant can most probably be attributed to the triphosphate hydrolysis, even though ADP is also rapidly produced *via* the pathway involving cleavage of the adenine base.

Buffer catalysis

Buffer concentration did not have any significant effect on the hydrolysis of Ap_3A, but the rate constants of the disappearance of Ap_3A were almost independent of the buffer concentration, both under acidic and neutral conditions, suggesting that buffer constituents did not serve as general acid/base catalysts or as nucleophiles attacking the phosphate. In formic acid buffers (50% free base, pH 3.9 at 90 °C) the data obtained was slightly scattered, but no clear correlation between the rate constants and the buffer concentration was observed, even though the experiments were repeated several times. Possibly, these effects resulted from medium effects on the reaction itself or on the pK_a value of formic acid. Ionic strength of the reaction solution, did not, however, have any effect on the rate constants. In HEPES buffers containing 20% or 80% free base (pH 5.9 and 7.1 at 90 °C) no reactivity differences were observed. The ionic strength of the reaction solution did not influence the rate of the pH-independent hydrolysis either. Under acidic conditions, the ionic strength was adjusted with NaCl, under neutral and alkaline conditions with $NaNO_3$. The choice of the electrolyte was not observed to have any effect on the rate constants. The observation that Ap_3A does not react with nucleophiles other than water or hydroxide ion, is in contrast to results obtained with phosphodiesters. Phenyl methyl phosphate, for example, has been shown to react with negatively charged nucleophiles such as formate or acetate ions.[40] This difference most probably reflects the higher negative charge of the triphosphate moiety in Ap_3A, which makes this substrate more resistant towards negatively charged nucleophiles.

Conclusions

The triphosphate bridge of dinucleoside polyphosphates is very resistant towards nucleophilic attack. Efficient hydrolysis is only observed under acidic conditions where the polyphosphate function is protonated (k_H 0.11 ± 0.04 dm^3 mol^{-1} s^{-1}). Only specific acids, however, protonate the phosphate group; general acid catalysis was not observed under acidic or neutral conditions. Under neutral conditions the hydrolysis is slow and pH-independent. Base-catalysed hydrolysis is only observed at a rather high hydroxide ion concentration (k_{OH} $(1.4 \pm 0.6) \times 10^{-4}$ dm^{-3} mol^{-1} s^{-1}), and the hydrolysis competes with an intramolecular cleavage reaction. Furthermore, nucleophiles other than water or hydroxide ion were not observed to react with the polyphosphate function. Depurination of adenosine moieties of Ap_3A competes with the triphosphate hydrolysis both under acidic and alkaline conditions, but the mechanisms of the acidic and alkaline reactions are different. Under neutral conditions Ap_3A reacts solely *via* the triphosphate hydrolysis.

Experimental

Materials and methods

Diadenosine polyphosphates were products of Sigma and they were used as received. Buffer constituents were of reagent grade. Capillary electrophoresis analysis was carried out with a Beckman P/ACE MDQ instrument and HPLC analysis with a Hewlett Packard 1090 instrument.

Preparation of reaction solutions

Reaction solutions between pH 3 and 10 were prepared by adjusting the pH by an appropriate buffer system. The pH of the reaction solutions were checked with a pH meter at room temperature, and the pH at 90 °C were calculated by using the temperature dependencies of the pK_a values found in the literature. Buffers employed and their pK_a values at 90 °C were formate (pK_a 3.9[41]), acetate (pK_a 4.9[41]), MES (morpholino ethanesulfonic acid; pK_a 5.3[42]), HEPES (2-[4-(2-hydroxyethyl)-piperazino]-ethanesulfonic acid; pK_a 6.9[43]), MOBS (4-[*N*-morpholine] butanesulfonic acid; pK_a 6.8[42]), phosphate (pK_a 7.3[41]), CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid; pK_a 9.3 (at 25 °C)) and glycine (pK_a 8.3[44]). The temperature-dependence of CHES buffer was not known, but it was assumed that it is similar to those of other sulfonic acid derivatives. Reaction solutions where pH was below 3, were prepared by adjusting the hydronium ion concentration with a HCl solution of known concentration. Alkaline solutions were prepared from NaOH in doubly distilled water to avoid dissolved CO_2. Hydronium ion concentration of NaOH solutions at 90 °C was calculated using a pK_w value of 12.4.[45] The ionic strength was adjusted with $NaNO_3$ or NaCl.

Kinetic experiments

Kinetic reactions were carried out in stoppered tubes that were immersed in a water bath thermostated at an appropriate temperature. The initial volume of reaction solutions was 2 ml. The substrate was added as a concentrated stock solution after the temperature of the reaction solution had been left to stabilise for at least 15 minutes. The final substrate concentration was 0.1 mM or below. 10–15 150 μl samples were withdrawn at appropriate intervals. Aliquots were cooled down on an ice bath, and samples from acidic and basic reaction solutions were neutralised with a base to quench the reaction. In cases where the reaction was slow, samples were stored in a freezer until analysed.

CZE analysis was carried out by using a fused silica capillary (75 μm i.d., 110 cm total length) with boric acid buffer pH 8.5 as the background electrolyte. The concentration of the buffer was generally 0.15 M, but in cases where the ionic strength of the buffer was high, 0.25 M buffer was used. The run voltage was 30 kV and compounds were detected by UV-detection at 254 nm. Under these conditions, the migration times of the starting materials and products typically varied from 30 to 60 minutes. The composition of the reaction solution (buffer and its concentration and ionic strength), however, had a significant effect on the t_m values. As an example, the migration times of reaction components observed in the reaction of Ap_3A in 0.1 M HCl were as follows: adenine 14.0 min, AMP 36.0 min, Ap_3A 38.0 min, depurinated triphosphate **7** 40.5 min and ADP 43.5 min. Also the base used to neutralise the acidic samples affected the analysis. Samples neutralised with HEPES base, gave good results whereas neutralisation with sodium acetate resulted in a poor baseline in the electropherograms.

HPLC-analysis was carried out using a Hypersil ODS column (250 × 4.6 mm, 5 μm particle size) (Thermo Hypersil-Keystone). The eluent was a mixture of 50 mM phosphate buffer, pH 6.0, and acetonitrile, and the following gradient system was used: 0–5 min 0.2% MeCN, 5–10 min a linear gradient from 0.2–10% MeCN, 10–20 min 10% MeCN. With this elution system, the retention times of reaction components observed in 10 mM NaOH were as follows: ADP 4.8 min, AMP 8.1 min, Ap_3A 11.2 min, 3′-AMP 12.1 min, adenine 12.8 min, and 3′,5′-cAMP 14.8 min. The composition of the samples did not affect the retention times, but the quality of chromatograms grew poorer with increasing buffer concentrations. Alkaline and acidic samples were neutralised. The reaction components were detected with a UV detector at 260 nm.

Curve fitting

The pH-rate profile shown in Fig. 1 was obtained by a non-linear fit of $\log(k_{obs}/s^{-1})$ *vs.* $\log([H^+]/\text{mol dm}^{-3})$. For this purpose, the log *k* values were expressed by eqn. 1, where k_{obs}, k_H, k_0 and k_{OH} are the observed first-order rate constant, the second-order rate constant of the acid catalysed hydrolysis, the first-order rate constant of the neutral hydrolysis and the second-order rate constant of the base catalysed hydrolysis, respectively. *K* is the equilibrium constant of protonation of the trianionic phosphate ($[SH^{2-}]/([S^{3-}][H^+])$. A value of 12.4 was used for the pK_w at 90 C.[45]

$$k_{obs} = k_H[H^+]/(K[H^+] + 1) + k_0 + k_{OH}K_w/[H^+] \quad (1)$$

The product distribution curves in Fig. 2a–c were obtained by fitting the mole fraction (x) of the starting material according to the equation of exponential decay (eqn. 2) and the mole fractions of products according to the rate law of parallel and consecutive first-order reactions.[38] In eqn. 3, x_i is the mole fraction of the product in the reaction mixture, k_1 and k_2 the rate constants of the formation and subsequent disappearance of the product, and a_1 the rate constant of the disappearance of the starting material of the reaction. It has to be noted that a fit according to eqn. 3 is not strictly correct in the case of all of the products, since it assumes that both the formation and disappearance are first-order processes. In some cases this is not true, a product may also be formed *via* a secondary process through the cleavage of a primary product. Such pathways are evidently of minor importance, and the fitted curves are very well consistent with the experimental values.

$$x_t = x_0(\exp(-kt)) \quad (2)$$

$$x_i = (\exp(-a_1t) - \exp(-k_2t))k_1/(k_2 - a_1) \quad (3)$$

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