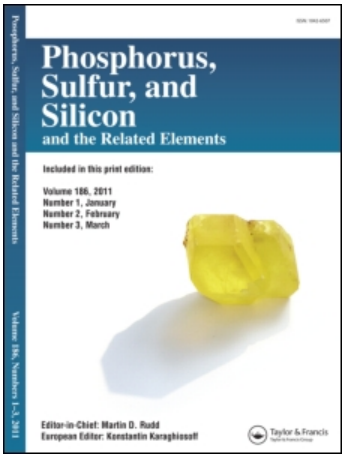

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A COMPARATIVE STUDY OF HYDROLYSIS RATES OF 2′-DEOXYADENOSINE AND ADENOSINE 5′-TRIPHOSPHATES AND 5′-DIPHOSPHATES

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Rates of hydrolysis of 2′-deoxyadenosine 5′-triphosphate and 5′-diphosphate, dATP and dADP, are measured in 0.01 M aqueous solutions at 70°C, in the pH range 0–9, and are compared with previously obtained data for adenosine 5′-triphosphate and 5′-diphosphate, ATP and ADP. Above pH 5 the hydrolysis involves mainly, or exclusively, a cleavage of the polyphosphate chain, probably by an elimination–addition mechanism *via* the monomeric metaphosphate ion, PO_3^-, from the tetraanion, $dATP^{4-}$ and the trianion, $dATPH^{3-}$. The effect of Mg^{2+} and Ca^{2+} ions on the rate of hydrolysis is similar to the effect of these cations on the rate of hydrolysis of ATP. In the pH range 0–4, the hydrolysis involves primarily a cleavage of the *N*-glycosidic bond to adenine and deoxyribose polyphosphate. This reaction is much faster for the 2′-deoxyribonucleotides containing a purine ring than for the ribonucleotide analogs.

INTRODUCTION

Previous papers from this Laboratory have described the mode of coordination of magnesium and calcium ions with adenosine 5′-triphosphate and 5′-diphosphate (ATP and ADP),[2] and the kinetics of the hydrolysis of these compounds in the pH range 0–10.[3,4] We have also established the sites of initial cleavage of the polyphosphate chain in ATP and ADP in the same pH range, from studies on the hydrolysis of [γ-^{18}O]ATP and [β-^{18}O]ADP in unlabeled water, and of ATP and ADP in water enriched with $H_2{}^{18}O$.[5] A recent paper from another Laboratory[6] reported the kinetics of the hydrolysis of ATP in 3 N perchloric acid and of ADP over the acidity range 1–5 N perchloric acid. This information on the basic nonenzymatic chemistry of ATP is supplemented by detailed studies of hydrogen bonding, solvation and self-association of ATP, ADP and their complexes with Mg^{2+} ions, which have been carried out by means of infrared spectrometry.[7,8] The physico-chemical background thus obtained is essential for an eventual understanding of the enzymatic reactions of ATP.[9] It is, therefore, surprising that the same type of physicochemical information is not available for the 2′-deoxy-analogs of these nucleoside polyphosphates dATP and dADP; the present investigation was undertaken to provide information on the kinetics of the hydrolysis of these compounds.

In the absence of enzymes, four different pathways can be written for ATP hydrolysis (Eqs. 1–4; P_i and PP_i = inorganic phosphate and pyrophosphate):

Nucleophile		Electrophile		Nucleophile′		Electrophile′	
H_2O	+	ATP(P_γ)	$\rightleftharpoons$	ADP	+	P_i	(1)
H_2O	+	ATP(P_β)	$\rightleftharpoons$	P_i	+	ADP	(2)

Nucleophile	Electrophile		Nucleophile'	Electrophile'	
H_2O	+ $ATP(P_\beta)$	$\rightleftharpoons$	AMP	+ PP_i	(3)
H_2O	+ $ATP(P_\alpha)$	$\rightleftharpoons$	PP_i	+ AMP	(4)

Utilizing isotopically labeled water, the incorporation of the label into P_i, ADP, PP_i or AMP is to be expected from these four pathways, respectively. A complicating factor is the subsequent hydrolysis of ADP according to Eqs. 5 or 6:

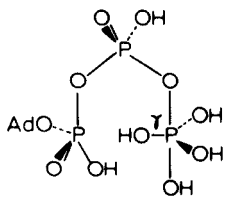
$$H_2O + ADP(P_\beta) \rightleftharpoons AMP + P_i \quad (5)$$

$$H_2O + ADP(P_\alpha) \rightleftharpoons P_i + AMP \quad (6)$$

Again, in labeled water, the label could be introduced at P_i or AMP depending on the direction of the hydrolysis. Finally, hydrolysis of PP_i results in label incorporation from water into one half of the P_i generated (Eq. 7):

$$H_2O + PP_i \rightleftharpoons 2\ P_i \quad (7)$$

Our previous work,[3-5] which was carried out in 0.01 M aqueous solutions of ATP at 70°C, shows that there are different operating mechanisms for the hydrolysis in acidic and basic media. Hydrolysis in 1 N and 0.1 N HCl occurs by an *addition–elimination mechanism* in which intermediates with pentacovalent phosphorus, oxyphosphoranes, are probably generated from the protonated acid, $ATPH_5^+$, the neutral acid, $ATPH_4$, and the monoanion, $ATPH_3^-$. Initial attack by water occurs 93% at P_γ and 7% at P_β. The attack at P_β leads only to P_i and ADP, within the limits of our observations (1–2%). Formulas **1** and **2** illustrate the oxyphosphoranes postulated for the addition of water at the P_γ and P_β atoms of the neutral acid, $ATPH_4$. The results are explained in terms of a combination of steric and electronic factors which favor the formation of these particular oxyphosphoranes in the rate-limiting step of the addition–elimination mechanism.[3-5]



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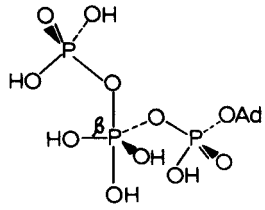

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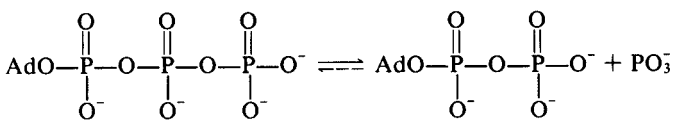
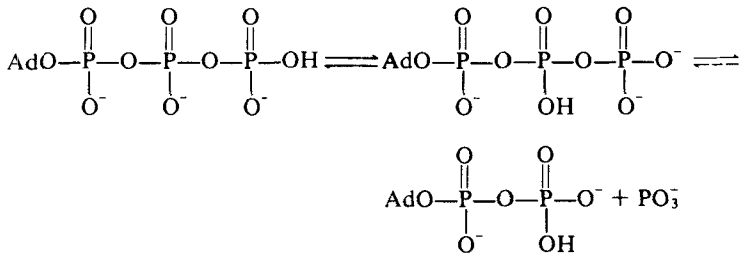
The hydrolysis of ATP at pH 8 occurs by an *elimination–addition mechanism,* in which the monomeric metaphosphate anion, PO_3^-, is formed as an intermediate from the tetraanion, ATP^{4-}, and the trianion, $ATPH^{3-}$, in accord with an earlier suggestion.[10] This type of hydrolysis proceeds exclusively by cleavage of the $P_\beta O$—P_γ bond. Equations (8) and (9) illustrate the elimination–addition mechanism from the tetraanion, ATP^{4-}:



(8)

$$PO_3^- + H_2O \rightleftharpoons H_2PO_4^- \quad (9)$$

Equation (10) depicts this type of mechanism operating on the trianion, $ATPH^{3-}$. Here it is assumed, in accord with a prior suggestion,[11-13] that formation of the metaphosphate ion requires a proton shift of some sort, which allows the loss of the metaphosphate ion rather than of the less stable metaphosphoric acid, $HOPO_2$.



(10)

The pK_a's which govern the various equilibria in ATP are given in Eqs. (11-14)

$$ATPH_4 \rightleftharpoons ATPH_3^- + H^+, \quad pK_a < 2 \tag{11}$$

$$ATPH_3^- \rightleftharpoons ATPH_2^{2-} + H^+, \quad pK_a < 2 \tag{12}$$

$$ATPH_2^{2-} \rightleftharpoons ATPH^{3-} + H^+, \quad pK_a = 4.3 \tag{13}\dagger$$

$$ATPH^{3-} \rightleftharpoons ATPH^{4-} + H^+, \quad pK_a = 7.3 \tag{14}$$

The rate of ATP hydrolysis decreases sharply in the pH range 1 to 4 as the pH of the medium increases. The rate levels off in the pH range 4 to 7 and then decreases rapidly in alkaline medium, leveling off again at about pH 9.3. The rates of the fast hydrolysis in 1 N HCl and the slow hydrolysis at pH 9 differ by a factor of about 4200. This pH-rate profile is consistent with a picture in which species with relatively large rate constants, $ATPH_4$ and $ATPH_3^-$ are being replaced by species with relatively small rate constants, $ATPH_2^{2-}$, $ATPH^{3-}$ and ATP^{4-}. The slow rate of hydrolysis of ATP in basic media appears to be associated with operation of the elimination-addition mechanism. At present there is no information on whether the dianion, $ATPH_2^{2-}$, undergoes hydrolysis by addition-elimination or elimination-addition, or whether the two mechanisms are competitive.

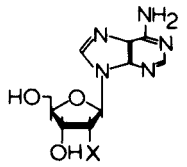
Over the entire pH range of 0 to 10, ATP hydrolysis proceeds in stepwise fashion leading first to ADP and then to AMP, with release of a molecule of P_i at each step. The second step is slower than the first by factors of 2.3 and 3.5, respectively in 1 N and 0.1 N HCl and by a factor of 3 at pH 8.3. ATP and ADP undergo hydrolysis at very similar rates at pH 4. The hydrolysis mechanisms for ATP and ADP appear to be analogous at a given pH; however, the predominance of attack at the terminal phosphorus is somewhat less marked for ADP (83% at P_β *vs* 17% at P_α) than for ATP (93% at P_γ *vs* 7% at P_β).[5]

As would be expected,[8] the effect of divalent cations on the rate of non-enzymatic hydrolysis of ATP depends on the pH of the measurements, since pH affects both the hydrolysis mechanism and the stability of the ATP-divalent cation complexes. Rates of hydrolysis at pH < 3 are similar in the presence and in the absence of divalent cations; however, at higher pH values significant differences are observable. In the absence of divalent cations, the trianion, $ATPH^{3-}$ generates metaphosphate ion faster than the tetraanion, ATP^{4-}, by a factor of approximately 18. Magnesium and calcium ions have little or no effect on the rate of formation of metaphosphate from the trianion. On the other hand, magnesium and calcium ions increase the rate

† Note added in proof. This equilibrium corresponds to protonation of the 6-amino group (*cf.* Ref. 3).

of metaphosphate formation *from the tetraanion* by factors of approximately 10 and 50, respectively.[3,4]

With this background in mind, it is of interest to consider the possible effects on ATP hydrolysis resulting from the replacement of the C2'-hydroxyl group by a hydrogen atom in the adenosine group.



2'-Deoxyadenosine, X = H
Adenosine , X = OH

Most of the earlier research on 2'-deoxyadenosine 5'-triphosphate (dATP) has been carried out in biochemical areas,[14] *e.g.* in the actomyosin system responsible for muscle contraction[15-19] and in other enzymatic hydrolyses.[19-24] Little information is available on the kinetics of nonenzymatic hydrolyses. The nucleotide itself, dATP, has been synthesized by three groups of investigators.[25-27]

A further point of interest concerning the behavior of dATP and dADP in acidic aqueous solution is the *dramatic increase in the rate of hydrolysis of C2'-deoxynucleosides and their 5'-phosphates at the* N-*glycosidic bond relative to the ribonucleoside analogs.*[28] This effect is more pronounced in the purine than in the pyrimidine series.

A final consideration germane to the present research is the actual conformation of the dATP and ATP molecules in aqueous solutions of different concentrations, in the presence and in the absence of divalent cations, with and without reference to the corresponding enzymes. The enzymatic aspect of this problem is outside the scope of this paper.[29,30] Nonenzymatic systems have been studied with varying results. Some investigators have concluded that little interaction exists between the adenine ring and the polyphosphate chain in aqueous solutions in the absence of metal ions.[31] Others have found evidence of ring–chain interactions under certain conditions.[32-36] The literature on complex formation between ATP and divalent cations is extensive and controversial.[2,37-40] Intermolecular self-association among nucleotides in aqueous solutions is known,[41] and could significantly affect the conformation of ATP and dATP above certain concentrations.[7,8]

EXPERIMENTAL

Materials. The salt, $dATPH_2$ $[(CH_3)_4N]_2$, was prepared as follows.[25] The 4-Morpholino-N,N'-dicyclohexylcarboxamidinium salt, $C_6H_{11}{}^{+}NH{=}C[N(CH_2CH_2)_2O]NH \cdot C_6H_{11}$, of 2'-deoxyadenosine 5'-phosphomorpholide (382 mg; 0.5 mmol) was dried by successive evaporations from pyridine (4×, 10 mL) and benzene (2×, 10 mL) in vacuum. Tetrasodium pyrophosphate decahydrate (892 mg; 2 mmol) was dissolved in water (60 ml) and passed through a column of Dowex 50-W resin in its pryidinium form (30 mL). The resin was washed with water and the total effluent was concentrated to *ca.* 15 mL (vacuum). Pyridine (60 mL) and tri-*n*-butylamine (2.0 mL; 8.4 mmol) were added, and the solution was evaporated. The residue was dehydrated by successive evaporations from pyridine (4×, 20 mL) and benezene (2×, 10 mL). The anhydrous pyrophosphate salt was transferred to a flask containing the dAMP-morpholide salt by means of dimethyl sulfoxide (4×, 2 mL). The homogeneous solution was kept at 25°C for three days. Water was added, and the solution was applied to a 2× 35 cm column of DEAE-cellulose in its bicarbonate form. The column was washed with water (250 mL). The products were eluted using a linear gradient of triethylammonium bicarbonate (mixing vessel, 1.5 L of water; reservoir, 1.5 L of 0.35 M triethyl-

ammonium bicarbonate). Of four major fractions (UV detection), the third corresponded to the desired dATP. The water was removed in vacuum, and residual buffer removed by evaporations with methanol (4×). The residue was dissolved in water, and the solution percolated through a column of Dowex 50-W resin in the tetramethylammonium form. The eluate was evaporated in vacuum and the residue dried by evaporation with methanol (2×). The residue was shown to be >99% pure $dATPH_2[(CH_3)_4N]_2$ by liquid chromatography.

The salt dADPH Na_2 was purchased from the Sigma Chemical Company. It was converted to the tetramethyl ammonium salt using Dowex 50-W resin in the tetramethyl ammonium form as described above for the dATP salt.

The buffers used in the experiments were: HCl at pH < 2, formate at pH 2.5–4.0, acetate at pH 5.0–6.0, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate (HEPES) at pH 6.7–8.3, and 4-(2-hydroxyethyl)-1-piperazine-propanesulfonate (EPPS) at pH 8.0–9.3. The buffer solutions were prepared with salts which contained only $(CH_3)_4^+$ as the cation. Ionic strength was adjusted to $\mu = 0.2$ with $(CH_3)_4NCl$.

pK_a Measurements

These were carried out by the procedure of Albert and Serjeant.[42] The titrant was a 0.100 N solution of $(CH_3)_4NOH$. The results are given in Table I. For comparison data for ATP are included.[3]

^{31}P NMR Spectra of the Products of Hydrolysis of 2′-Deoxyadenosine 5′-Triphosphate in 0.1 N HCl

A solution of $dATPH_2[(CH_3)_4N]_2$ in 0.1 N HCl was kept 5 min at 70°C. The pH was adjusted to 8 and the solution was concentrated in vacuum to *ca.* 0.5 mL. This mixture was diluted with 1.5 ml of D_2O. The ^{31}P NMR spectrum of this solution was obtained using a Brucker 400 MHz spectrometer (no ^1H-decoupling). The major product was deoxyribose triphosphate, $ROP_\alpha(O_2^-)OP_\beta(O_2^-)OP_\gamma O_3^{2-}$, as shown by the presence in the spectrum of a multiplet at −5.5 ppm (P_α atom), a doublet at −10.3 ppm (P_γ terminal atom), and a triplet at −21.1 ppm, all to high field of the reference 85% H_3PO_4. There was also evidence of a relatively small amount of inorganic phosphate and the corresponding deoxyribose diphosphate, $ROP_\alpha(O_2^-)OP_\beta O_3^{2-}$. No deoxyribose monophosphate was detected in significant amounts. The spectrum was obtained using a pulse delay of 2 sec to produce reasonably quantitative results.

Kinetic Measurements of Polyphosphate Chain Hydrolysis in dATP and dADP

All experiments were carried out at 70°C ± 0.1°C. The progress of the reactions was monitored by the chromatographic separation technique previously used to follow the hydrolysis of ATP[3].

Kinetic Measurements of N*-Glycosidic Bond Hydrolysis in dATP and dADP*

The same procedure used to follow the polyphosphate chain hydrolysis was used to measure the rate of hydrolysis of the *N*-glycosidic bond.

RESULTS AND DISCUSSION

The ^{31}P NMR spectrum of the products of the hydrolysis of dATP in 0.1N HCl discloses the occurrence of a very rapid cleavage of the *N*-glycosidic bond. Under the

TABLE I

Acid Dissociation Constants of 2′-Deoxyadenosine and Adenosine 5′-Triphosphates at 70°C in 0.01 M Water ($\mu = 0.2$; $(CH_3)_4N^+$)

Equilibria	pK_a
$dATPH_2^{2-} \rightleftharpoons dATPH^{3-} + H^+$	3.9 ± 0.1
$ATPH_2^{2-} \rightleftharpoons ATPH^{3-} + H^+$	4.3 ± 0.1
$dATPH^{3-} \rightleftharpoons dATP^{4-} + H^+$	7.0 ± 0.1
$ATPH^{3-} \rightleftharpoons ATP^{4-} + H^+$	7.32 ± 0.06

TABLE II

Rates of Hydrolysis of the *N*-Glycosidic Bond in 2'-Deoxyadenosine 5'-Triphosphate and 5'-Diphosphate at 70.0 ± 0.1°C in 0.01 M Aqueous Solutions (μ = 0.2; $(CH_3)_4N^+$ as sole cation).[a]

pH	10^2k, min^{-1}	$t_{1/2}$
	dATP	
1.49	151.0	28 sec
2.70	10.1	6.9 min
3.23	2.91	24 min
4.05[b]	0.53	130 min
	dADP	
1.41	109.0	38 sec
2.62	5.80	11.9 min
4.13	0.16	430 min

[a] dATP or dADP → A + deoxyribose polyphosphate.
[b] At this pH some hydrolysis of the polyphosphate chain occurs, dATP → dADP, as a minor pathway.

conditions chosen, the main products of this reaction are adenine and deoxyribose triphosphate. A relatively small amount of deoxyribose diphosphate and P_i are also observed but no deoxyribose monophosphate is detected.

The kinetic results and the corresponding pH-rate profile for the hydrolysis of dATP in the pH range where cleavage of the *N*-glycosidic bond is the main, or the exlusive, reaction are given in Table II and Figure 1, respectively. The reaction is quite rapid, and the pH-rate profile is unexceptional for this type of reaction.[28] Table II and Figure 1 also contain the pertinent data for dADP, which behaves just like the tripolyphosphate analog.

The kinetic results for the hydrolysis of dATP in the pH range 5.12 to 8.54 are given in Table III. The data are plotted in Figure 2, together with the corresponding data for ATP[3]. The main hydrolysis pathway for dATP in this pH range involves the polyphosphate chain, as shown by the analysis of the products by liquid chromatography. *It is evident that the replacement of the C2'-hydroxyl group of ATP by hydrogen to give dATP has no significant effect on the rate of cleavage of the tripoly-*

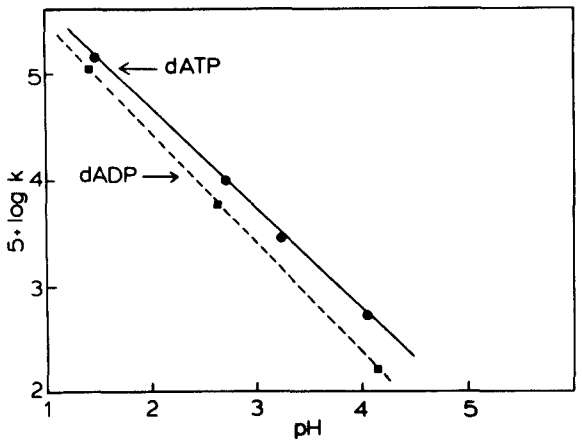


FIGURE 1 pH-rate profiles for hydrolytic cleavage of the *N*-glycosidic bond in 2'-deoxyadenosine 5'-triphosphate (dATP), and 2'-deoxyadenosine 5'-diphosphate (dADP) in 0.01 M aqueous solution at 70°C [μ = 0.2; $(CH_3)_4N^+$].

TABLE III

Rates of Hydrolysis of the Polyphosphate Chain in 2'-Deoxyadenosine 5'-Triphosphate and 5'-Diphosphate, at 70.0 ± 0.1°C in 0.01 M Aqueous Solutions (μ = 0.2; $(CH_3)_4N^+$ as sole cation).[a]

pH	10^4k, min^{-1}	$t_{1/2}$
	dATP	
5.12[b]	6.21	18.7 h
6.65	3.62	32 h
7.00	3.35	34.5 h
7.83	1.12	103 h
8.54	0.56	205 h
	dADP	
5.02[b]	6.42	18 h
6.82	2.33	49.5 h
8.45	0.21	545 h

[a] dATP → dADP → dAMP.
[b] At this pH some depurination, dATP or dADP → A + deoxyribose polyphosphate occurs as a minor reaction pathway.

phosphate chain of these nucleotides. For reasons discussed in our previous paper,[3] we assume that these hydrolyses involve the loss of monomeric metaphosphate ion, PO_3^-, from the tetraanion, $dATP_{4-}$, and the trianion, $dATPH^{3-}$, in the rate-determining step, as suggested in Eqs. 8, 9 and 10, above.

Experiments were also carried out with the magnesium and calcium salts, $MgdATPH_2$ and $CadATPH_2$, as had been done for ATP[3]. The effects of these divalent cations on the rate of hydrolysis of dATP and ATP *via* the elimination–addition mechanism (metaphosphate intermediate) are comparable, and therefore, the data are not reproduced here.

Table III includes data for the hydrolysis of dADP in the pH range 5.02–8.45. As shown in Figure 3, this reaction involves the cleavage of the pyrophosphate group, and the rate of reaction is comparable to that observed for ADP[3]. Again, the replacement of hydroxyl by hydrogen at C2' has no significant effect on the rate of hydrolysis of a dipolyphosphate chain in a nucleotide. We assume that these hydro-

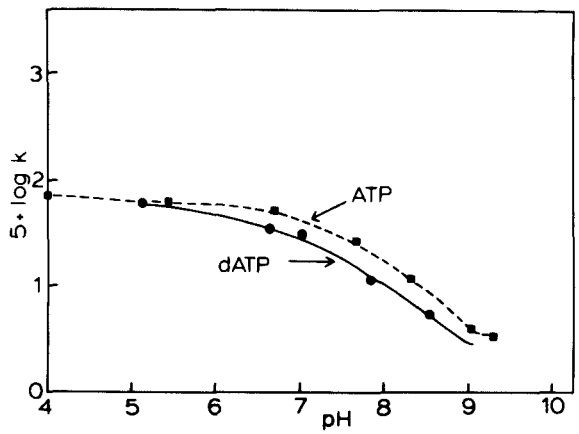


FIGURE 2 pH-rate profiles for hydrolysis of the polyphosphate chain in 2'-deoxyadenosine 5'-triphosphate (dATP) and adenosine 5'-triphosphate (ATP) in 0.01 M aqueous solution at 70°C [μ = 0.2; $(CH_3)_4N^+$].

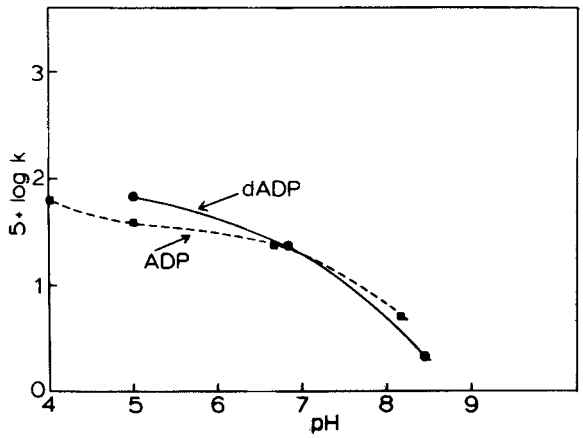


FIGURE 3 pH-rate profiles for hydrolysis of the polyphosphate chain in 2′-deoxyadenosine 5′-diphosphate (dADP) and adenosine 5′-diphosphate (ADP) in 0.01 M aqueous solution at 70°C [$\mu = 0.2$; $(CH_3)_4N^+$].

lyses occur from the trianion, $dADP^{3-}$, and the dianion, $dADPH^{2-}$, by the elimination–addition mechanism *via* a monomeric metaphosphate ion.

Plots of the rate of hydrolyses of dATP *vs.* dADP, and of ATP *vs.* ADP in the pH region 5–9 confirm that neither the length of the polyphosphate chain, nor the type of substituent at the C2′-position, H or OH, plays a significant role in the transfer of the phosphoryl group from the nucleotide to the nucleophile in the elimination–addition mechanism.

The *N*-glycosidic bond of 2′-deoxyribonucleosides containing a purine base undergoes acid-catalyzed hydrolytic cleavage at a much faster rate than the *N*-glycosidic bond of the ribonucleoside analogs.[28] This effect is not significantly enhanced by the presence of a phosphate or pyrophosphate group at the C5′-position. Hence, the phenomenon appears to be an inherent property of the nucleoside molecule, and is not affected by the polyphosphate chain in nucleotides. This is consistent with the results of this investigation. The structural modification of the nucleoside (H *vs.* OH at C2′) does not significantly affect the hydrolytic behavior of the polyphosphate chain. Conversely, the presence or the length of the polyphosphate chain does not influence the acid-lability of the *N*-glycosidic bond of nucleosides.

Several suggestions have been made concerning the mechanism of the acid-catalyzed cleavage of the *N*-glycosidic bond in nucleosides.[28] The marked differences in rates of hydrolysis between deoxyribonucleosides and ribonucleosides are attributed to a negative inductive effect of the hydroxyl group at C2′ in the ribo-derivatives, which hinders protonation of the cyclic oxygen of the sugar. Although the magnitude of this inductive effect is perhaps surprising, two observations have been offered in support of this mechanism. (a) Substitution of the hydrogen atoms of the hydroxyl groups in the carbohydrate residues of nucleosides by more strongly electronegative groups, such as tosyl or 2,4-dinitrobenzoyl, causes an appreciable decrease in the rate of hydrolysis. (b) 2′,3′-dideoxynucleosides are hydrolyzed even more rapidly than the corresponding 2′-deoxynucleosides.[28] Whatever the reasons for the observed differences in the stability of dATP *vs.* ATP and dADP *vs.* ADP in aqueous solutions at certain pH values, the present work demonstrates that those differences are not reflected in the reactivity of the polyphosphate chain. While in retrospect this conclusion is not startling, its experimental verification is desirable

since the absence of a hydroxyl function from the C2'-position of a ribonucleoside could have introduced significant alterations of intra- and intermolecular associations in dATP and dADP.[7,8]

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