Research Article

Yoke-Leng Sim*, Beljit Kaur

Kinetics and Mechanistic Study of Hydrolysis of Adenosine Monophosphate Disodium Salt (AMPNa₂) in Acidic and Alkaline Media

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Abstract: Phosphate ester hydrolysis is essential in signal transduction, energy storage and production, information storage and DNA repair. In this investigation, hydrolysis of adenosine monophosphate disodium salt (AMPNa₂) was carried out in acidic, neutral and alkaline conditions of pH ranging between 0.30–12.71 at 60°C. The reaction was monitored spectrophotometrically. The rate ranged between $(1.20 \pm 0.10) \times 10^{-7} \text{ s}^{-1}$ to $(4.44 \pm 0.05) \times 10^{-6} \text{ s}^{-1}$ at [NaOH] from 0.0008 M to 1.00M recorded a second-order base-catalyzed rate constant, k_{OH} as $4.32 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$. In acidic conditions, the rate ranged between $(1.32 \pm 0.06) \times 10^{-7} \text{ s}^{-1}$ to $(1.67 \pm 0.10) \times 10^{-6} \text{ s}^{-1}$ at [HCl] from 0.01 M to 1.00 M. Second-order acid-catalyzed rate constant, k_{H} obtained was $1.62 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$. Rate of reaction for neutral region, k_0 was obtained from graphical method to be 10^{-7} s^{-1} . Mechanisms were proposed to involve P-O bond cleavage in basic medium while competition between P-O bond and N-glycosidic cleavage was observed in acidic medium. In conclusion, this study has provided comprehensive information on the kinetic parameters and mechanism of cleavage of AMPNa₂ which mimicked natural AMP cleavage and the action of enzymes that facilitate its cleavage.

Keywords: Phosphate ester hydrolysis; kinetics; acid base catalysis; P-O cleavage.

1 Introduction

Phosphate esters are the most common chemical functional group in our body as it involves many processes in the human body. Among some of the important processes are the production of cellular energy which involves ATP and phosphoenol pyruvate, essential part of nucleic acids, as an important component of cell membrane, and most importantly storage of genetic information [1]. It is often very hard for scientists to study the mechanism of phosphate esters such as monoesters, diesters or triesters, as the cleavage rates are extremely slow in neutral conditions and also due to its complicated mechanism [1]. These phosphate esters are highly stable as they have an estimated half-life of 3×10^9 years at pH 6.8 and 25°C and only selected nucleases and phosphatases can accelerate the cleavage rate by factors up to 10^{16} and 10^{21} [2].

To understand their cleavage activity, non-natural substrates have often been employed as mimics of phosphate ester linkages [2]. Among other non-natural mimics of phosphate ester linkages include bis (4-nitrophenyl) phosphate (BNPP), 2-hydroxypropyl-4-nitrophenyl phosphate and so on [2,3]. In this study, adenosine monophosphate disodium salt (AMPNa₂) was chosen as a model substrate to mimic the phosphate ester bond in phosphate monoesters. There is extensive research in the recent years on adenosine and its corresponding nucleotides as they are biomolecules that are involved in energy production and substrates for various cellular biochemical processes [4]. Adenosine Monophosphate (AMP) is also known as 5'-adenylic acid. AMP is a nucleotide and performs the role of a monomer in RNA. The structures of AMP and adenosine are shown in Figure 1 and Figure 2 respectively.

Adenosine monophosphate is present in the human body and it is broken down to adenosine by Alkaline Phosphatase and ecto-5'-AMPases and endo-5'-AMPases. Natural adenosine monophosphate can also be hydrolysed into adenine and ribose 5-phosphate by

*Corresponding author: Yoke-Leng Sim, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900, Kampar, Perak, Malaysia, E-mail: simyl@utar.edu.my

Beljit Kaur: Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900, Kampar, Perak, Malaysia

Figure 1: Structure of adenosine monophosphate(AMP).

Figure 2: Structure of adenosine.

Figure 3: Structure of AMPNa₂.

adenosine monophosphate nucleosidase [5]. Carrying out studies on the adenosine monophosphate disodium salt provides useful knowledge on how this enzyme carries out its function in terms of mechanism. Kinetic studies on the adenosine monophosphate disodium salt using acid-base catalysis also allows the confirmation of the principles used by these enzymes in breaking down adenosine monophosphate and therefore allowing the development of synthetic enzymes in the future to mimic the actions of these enzymes. The enzymatic cleavage of phosphate esters always employs general acid-base catalysis or metal-ion assisted catalysis where protons are accepted and donated by enzyme functional groups [2,6]. General acid-base catalysis stabilizes unfavorable changes that develop in the structure during the transition state, activates weak nucleophiles and stabilizes poor leaving groups [6].

In light of this, it was decided to investigate the hydrolysis of adenosine monophosphate disodium salt (AMPNa₂) (Figure 3) in alkaline and acidic conditions to study the relationship between pH and the bond cleavage reaction of the adenosine monophosphate disodium salt. This study is expected to provide insights on the effect of pH on the kinetics and the mechanism of the phosphate monoester bond cleavage.

2 Materials and Methods

2.1 Part 1: Chemicals

Adenosine 5'-monophosphate disodium salt of $\geq 99.0\%$ was purchased from Sigma Aldrich. NaCl and NaOH of the highest available purity were obtained from QRéc. TRIS and glycine of highest available purity were obtained from Fisher Scientific and R&M Chemicals, respectively. NaCl served to control the ionic strength of the solutions. NaOH, HCl, TRIS, and glycine were used to vary the pH of the solutions. Infrared spectrum of AMPNa₂ was obtained to ensure the purity of AMPNa₂ before the investigation as well as to identify the structure of hydrolytic product during the kinetic studies.

2.2 Part 2: Kinetic Study of Nucleotide Analogue, AMPNa₂

0.01M AMPNa₂ was prepared in distilled water for the kinetic studies. Sample solutions of 20 mL each consisting of NaCl (to maintain ionic strength) under different buffer conditions were prepared and thermostated at 60°C prior to kinetic runs. A small amount of AMPNa₂ (0.0001M) was injected into the reaction mixture and it was immediately measured by UV-Vis Spectrophotometer. Subsequent samplings were taken until the reaction went into completion of more than eight half-lives. The pH value of each sample solution was taken before and after the reaction completion with an EL20-Mettler Toledo pH meter. In the kinetic runs in alkaline region, the ionic strength was maintained at 0.2M for [NaOH] less than 0.2M, while for [NaOH] more than 0.2M, ionic strength was increased to 1.0M. The rate of reaction was calculated according to Equation 1 or Equation 2 depending on the increase or decrease in absorbance against time for all the sample solutions. In Equation 1 or Equation 2, E_{app} is apparent molar extinction coefficient of the reaction mixture, A_{∞} is absorbance at reaction time, $t = \infty$, A_0 is absorbance at

reaction time, $t = 0$, k_{obs} is pseudo-first-order rate constant and $[X_0]$ represents the initial concentration of substrate, AMPNa_2 . Product characterization was carried out by using Fourier Transform Infrared Spectroscopy (Perkin Elmer Spectrum ex1) and Liquid Chromatography Mass Spectrometry.

$$A_{\text{obs}} = E_{\text{app}} [X_0] \exp(-k_{\text{obs}} t) + A_{\infty} \quad (1)$$

$$A_{\text{obs}} = E_{\text{app}} [X_0] \{1 - \exp(-k_{\text{obs}} t)\} + A_0 \quad (2)$$

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

3.1 Part 1: Hydrolysis of AMPNa_2 in Alkaline Conditions at 60°C

The hydrolysis of AMPNa_2 was carried out in alkaline solutions ranging from pH 9.95–12.71 at 60°C. The reaction was monitored spectrophotometrically at different time intervals until the reaction proceeded for more than 8 half-lives. Figure 4 shows typical absorption spectra of alkaline hydrolysis of AMPNa_2 at $[\text{NaOH}]$ 1.0 M and 60°C. AMPNa_2 in basic solution displayed a maximum absorption peak at 260 nm which is responsible for the absorption of adenosine [7]. A reduction in the absorbance value was observed along the reaction indicating the decrease in the concentration of the reactant with time (Figure 5).

The alkaline hydrolysis of AMPNa_2 is following a pseudo-first-order kinetic model. Absorbance change with time was observed and the three parameters: k_{obs} , E_{app} and A_{∞} were calculated using Equation 1 for all the sample solutions covering pH 9.95–12.71. Rate constants of alkaline hydrolysis of AMPNa_2 in 60°C were determined where the rate ranged from $(1.20 \pm 0.10) \times 10^{-7} \text{ s}^{-1}$ to $(4.44 \pm 0.05) \times 10^{-6} \text{ s}^{-1}$ at $[\text{NaOH}]$ from 0.0008 M to 1.0000 M. A table consisting of $[\text{NaOH}]$, pH, and all the kinetic parameters (k_{obs} , E_{app} , A_{∞} and $\sum \text{di}^2$) of this investigation is provided in SI-1 of Supporting Information.

The pseudo-first-order rate constants, k_{obs} were plotted against $[\text{NaOH}]$ as shown in Figure 6. The solid line is drawn through the calculated data points using Equation 3 with the k_{b} and k_0 obtained were $4.32 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$ and $6.30 \times 10^{-8} \text{ s}^{-1}$, respectively. In Equation 3, k_{obs}

Figure 4: UV-Vis absorption spectra of alkaline hydrolysis of AMPNa_2 at $[\text{NaOH}]$ 1M at 60°C.

Figure 5: Alkaline hydrolysis of AMPNa_2 in the presence of $[\text{NaOH}]$ 1.0 M at 60°C. The solid line was drawn through the calculated data points using Equation (1) with $k_{\text{obs}} = 4.44 \times 10^{-6} \text{ s}^{-1}$, $E_{\text{app}} = 8466 \pm 44 \text{ M}^{-1} \text{ cm}^{-1}$, and $A_{\infty} = 0.294 \pm 0.003$.

Figure 6: Pseudo-first-order rate constant, k_{obs} versus $[\text{NaOH}]$ for alkaline hydrolysis of 0.0001M AMPNa_2 at 60°C. The solid line is drawn through the calculated data points using Equation 3.

is the observed pseudo-first-order rate constant of the reaction, k_b represents second-order base-catalyzed rate constant and k_0 represents uncatalyzed rate constant for the cleavage of P-O bond in AMPNa₂.

$$k_{\text{obs}} = k_b[\text{OH}^-] + k_0 \quad (3)$$

Equation 3 allows estimation on the contribution of specific base catalysis on the P-O bond hydrolysis of AMPNa₂ in basic condition. The linear relationship between k_{obs} and $[\text{OH}^-]$ will also allow us to determine the theoretical rate constants of AMPNa₂ hydrolysis due to extremely slow rate of reaction at lower pH. It was observed that the rate constant, k_{obs} increased with the increase of pH under basic conditions. As the pH increases, the concentration of hydroxide ion increases as well. The hydrolysis of AMPNa₂ was catalyzed by hydroxide ions, whereby hydroxide ion acts as a nucleophile to attack the phosphorus center. The base-catalyzed hydrolysis of the phosphate ester proceeds through a B_{p2} reaction, which is a base-catalyzed, phosphoryl-oxygen fission, bimolecular reaction analogous to an S_N2 reaction. The attack of hydroxide ion on the phosphorus atom is known as the rate-limiting step of this hydrolysis [8]. As happens in most S_N2 reactions, increasing concentration of the nucleophile increases the rate of the reaction [9]. This explains the pH dependency in the hydrolysis of AMPNa₂.

Table 1 summarizes the second-order base catalyzed rate constant, k_b for diesters and triesters at 25°C for phosphate ester bond cleavage that have been previously reported as a comparison to the k_b of AMPNa₂ at 60°C and 25°C in the current study. As can be seen in Table 1, the rate of P-O hydrolysis is the slowest for AMPNa₂ at 25°C.

The mechanism of hydrolysis of AMPNa₂ in basic conditions is shown in Scheme 1. The hydroxide ion serves as a nucleophile to attack the phosphorus center. This provides an insight into the role of hydroxide ion in catalyzing the reaction and phosphate ester cleavage proceed through specific base catalysis. The role of hydroxide ion as a nucleophile was proposed by previous studies involving *p*-nitrophenyl phosphate and benzoyl methyl phosphate [12-14]. The mechanism of AMPNa₂ cleavage in the presence of specific base catalyst is proposed to be initiated with a nucleophilic attack on the phosphorus center as depicted in Scheme 1. This in turn results in the cleavage of P-O bond followed by the production of Na₂PO₄H and deprotonated adenosine. The adenosine undergoes further deprotonation, due to the presence of OH⁻ and Cl⁻ which act as deprotonating agents and forms a deprotonated adenosine product [15,16]. Abstraction of a hydrogen atom from adenosine

Table 1: Second-order base-catalyzed rate constant values of phosphate ester hydrolysis in previous study [10,11] and present study.

Ester	k_b at 25°C, M ⁻¹ s ⁻¹
bis-3-(4-carboxyphenyl)-neopentyl phosphate	1.00×10^{-6}
Trimethylphosphate	1.60×10^{-4}
Triethylphosphate	8.20×10^{-6}
Triphenylphosphate	2.5×10^{-1}
Benzoyl Methyl Phosphate	3.4×10^{-1}
AMPNa ₂ (this study)	4.32×10^{-6} (60°C) 2.81×10^{-8} (25°C)

is favourable as the lone pair electrons of oxygen in the aromatic ring can be delocalized. Hydrogen atoms can also be abstracted from C-H bonds [17].

Positive ion LC-MS spectra of the product of hydrolysis was obtained as shown in Figure 7, which helped to verify the proposed mechanism. From the mechanism in Scheme 1, the peaks corresponded to the products were expected to have m/z 141.9588+1 and m/z 261.1937+1. The peaks in the positive-ion LC-MS spectrum (Figure 7) that correspond to these values are $m/z = 142.9658$ and $m/z = 262.9397$. The peak with $m/z = 262$ corresponds to adenosine that has gone through hydrogen abstraction in the presence of hydroxide [20,21] as its theoretical m/z is 268.2.

The hydrolysis of AMPNa₂ in 1.0 M [NaOH] produced an insoluble white precipitate which was isolated and analyzed by FTIR in support of the proposed products formation. The IR spectra of AMPNa₂, the filtrate and the residue (powder) were obtained and compared as shown in Figure 8. Specific peak assignments of IR spectra given to AMPNa₂, the filtrate, as well as the residue from alkaline hydrolysis of AMPNa₂ are presented in Table 2. The LCMS spectra of the alkaline hydrolysis products can be obtained in Supporting Information as SI-4

As we can see from Table 2, hydroxy, adenine, phosphate and phosphate ester group presence can be confirmed from the alkaline hydrolysis of AMPNa₂. In previous studies, where the FTIR spectra of adenosine monophosphate disodium salt was obtained, the presence of phosphate group was justified by the presence of absorption band at 1091 cm⁻¹ [22]. Absorption band at 976 cm⁻¹ corresponds to phosphate ester or the ribose phosphate skeletal motions [22,23]. These absorption bands are similar to the ones obtained for AMPNa₂ in this investigation. The FTIR spectra of AMPNa₂ obtained in this investigation had absorption bands at 978 cm⁻¹ and 1093

Scheme 1(a): Proposed mechanism of alkaline-hydrolysis of AMPNa_2 under basic condition with OH^- acting as a nucleophile [12,18,19].

Scheme 1(b): Proposed mechanism for the formation of the final product in the concerted $\text{B}_{\text{p}2}$ reaction [15-17].

Figure 7: Positive-ion LC-MS spectrum of the final product of alkaline hydrolysis of 0.0001 M AMPNa_2 in at 60°C.

Figure 8: Comparison of FTIR spectra of the substrate, residue and the filtrate for alkaline hydrolysis of 0.0001 M AMPNa₂ in the presence of [NaOH] 1.0 M at 60°C.

Table 2: Peak assignments of IR absorbance spectra for the substrate, residue and the filtrate obtained in alkaline hydrolysis of AMPNa₂ at 60°C.

Absorption (cm ⁻¹)			Assignment
Filtrate	Residue	Adenosine 5'-Monophosphate disodium salt, AMPNa ₂	
3238	3456	3424	Hydroxy group [23,24]
1635	1654	1649	Adenine [23,24]
	1016	1093	Phosphate [22]
		978	Phosphate ester, ribose phosphate skeletal motions [22,23]

cm⁻¹ representing phosphate ester band and phosphate group band respectively. In the filtrate and the residue, there was no absorption at around 970 cm⁻¹, indicating disappearance of reactant after hydrolysis. This confirms that phosphate ester bond was cleaved producing a phosphate salt (residue) and an adenosine salt (filtrate). The filtrate had absorption bands at 3238 cm⁻¹ and 1635 cm⁻¹ representing a hydroxy group and an adenine group

respectively, which indicates the presence of adenosine [23,24]. The spectrum of the filtrate was also compared with a literature spectrum of adenosine and similarity was noticed [25]. An absorption band at 1396 cm⁻¹ indicated that *N*-glycosidic bond was still present in the product [26] to show that *N*-glycosidic bond cleavage did not take place in the alkaline hydrolysis of AMPNa₂.

In a previous investigation of UV-photodissociation of AMP, similar products were obtained whereby phosphate-based fragments were found in high abundance especially PO₃⁻. Marcum and his co-workers proposed a mechanism whereby the phosphate ester bond was broken and producing PO₃⁻ and adenosine [19]. A metal complex study aiming to cleave AMP also resulted in the formation of adenosine [18]. Enzymatic hydrolysis of AMP by enzymes such as alkaline phosphatase (AP) also produced the same products as proposed in the mechanism in Scheme 1 [27].

3.2 Part 2: Hydrolysis of AMPNa₂ in Acidic Conditions at 60°C

Acidic hydrolysis of AMPNa₂ was also carried out at pH ranged between 0.30 to 1.83 with 0.01-1.00 M [HCl]. The UV-Vis absorption spectrum of acidic hydrolysis of AMPNa₂ at [HCl] 1.0 M at 60°C as shown in Figure 9 indicated the

Figure 9: UV-Vis absorption spectra of acidic hydrolysis of AMPNa_2 at $[\text{HCl}]$ 1M at 60°C .

Figure 10: Pseudo-first-order rate constant, k_{obs} versus $[\text{HCl}]$ for acidic hydrolysis of 0.0001 M AMPNa_2 at 60°C . The solid line is drawn through the calculated data points following Equation 4.

existence of a transition state in the acidic hydrolysis of AMPNa_2 . There is a shift of absorption maximum to the right from 259.5 nm to 260.5 nm due to the breakdown of adenosine and the production of adenine [7]. This shift to the right was also demonstrated by Stockbridge [28].

The increase in absorbance with time was observed and the three kinetic parameters: k_{obs} , E_{app} and A_{∞} were calculated using Equation 2 for all the sample solutions covering pH 0.30-1.83. The rate constants of hydrolysis of AMPNa_2 in acidic medium at 60°C were determined where the rate ranged from $(1.32 \pm 0.06) \times 10^{-7} \text{ s}^{-1}$ to $(16.7 \pm 1.0) \times 10^{-7} \text{ s}^{-1}$ at $[\text{HCl}]$ from 0.01 M to 1.00 M. SI-2 in Supporting Information consists of $[\text{HCl}]$, pH, and all the kinetic parameters of this investigation.

The pseudo-first-order rate constants, k_{obs} were plotted against $[\text{HCl}]$ as shown in Figure 10. The solid line

Scheme 2: Proposed mechanism of acidic hydrolysis of AMPNa₂ under acidic condition with H⁺ ion acting as a protonating agent [8,30,31].

Scheme 3: The mechanism of *N*-glycosidic bond cleavage of AMPNa₂ in acidic conditions at 60°C [32,33].

Figure 11: Comparison of FTIR spectra of AMPNa₂ and product of acidic hydrolysis of 0.0001 M AMPNa₂ in the presence of [HCl] 1.0 M at 60°C.

Figure 12: Positive-ion LC-MS spectrum of the product of acidic hydrolysis of 0.0001 M AMPNa₂ in [HCl] 1.0 M at 60°C.

is drawn though the calculated data points using Equation 4. From Equation 4, the k_a and k_0 obtained were $1.62 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$ and $1.03 \times 10^{-8} \text{ s}^{-1}$ respectively, where, k_{obs} represents pseudo-first-order rate constant of the reaction, k_a and k_0 are second-order acid catalyzed-rate constant and uncatalyzed rate constant for the cleavage of P-O bond in AMPNa₂.

$$k_{\text{obs}} = k_a [\text{H}^+] + k_0 \quad (4)$$

It was observed that the rate constant, k_{obs} increased with the decrease of pH under acidic conditions. In specific acid catalysis, the H^+ ion enhanced the rate of the reaction by providing an alternative mechanism, which was more favourable energetically. A hydronium ion did this by withdrawing electron density from the phosphorus atom that held the adenosine leaving group making the phosphorus atom more susceptible to nucleophilic attack by the chloride ion during the phosphate ester cleavage [29]. The mechanism of hydrolysis of AMPNa₂ in acidic conditions is shown in Scheme 2 and Scheme 3 respectively.

AMPNa₂ undergoes two different cleavage reactions in acidic medium, a phosphate ester cleavage and followed by *N*-glycosidic bond cleavage. The phosphate ester cleavage begins with protonation of the oxygen from the leaving group due to high concentration of H^+ ions in acidic medium. Cl^- acts as a nucleophile and attacks the phosphorus center. This results in the cleavage of the phosphate ester bond [29,34,35]. The evidence of the phosphate ester cleavage can be obtained from the comparison of the infrared spectra of AMPNa₂ and product of acidic hydrolysis as shown in Figure 11. The LCMS Spectra of AMPNa₂ and the acidic hydrolysis products are attached in Supporting Information as SI-5.

In the infrared spectrum of AMPNa₂, an absorption band at 978 cm^{-1} represents ribose phosphate skeletal motions and this absorption band is not present in the acidic product [22, 23]. This indicates phosphate ester bond cleavage has occurred in AMPNa₂ under acidic conditions. Next, acid-catalyzed depurination of adenosine begins with the attack of H^+ on N7 of adenine and this leads to the formation of a monoprotonated intermediate due to high concentration of H^+ under highly acidic conditions. This causes a series of charge redistribution and in turn causes *N*-glycosidic bond cleavage of the C'1 from the ribose ring and N7 of the adenine ring. This provides an insight into the role of the hydrogen ion in catalyzing the depurination of adenosine as H^+ protonated the leaving group as depicted in Scheme 4. This resulted in the formation of protonated ribose compound and double protonated adenine ($\text{C}_5\text{H}_7\text{N}_5$) [32,33]. At highly strong acidic conditions, it is possible that the double protonation of the adenine had accelerated the hydrolysis. This double protonation occurs at N3, where hydrogen ions are abstracted towards nitrogen and results in the formation of a double protonated adenine as shown in Scheme 4 [32]. Positive-ion LC-MS spectrum of the product has been carried out to support the proposed mechanisms in Scheme 3 and Scheme 4, as shown in Figure 12.

The $m/z = 136.0622$ and $m/z = 138.9065$ values both correspond to protonated ribose ($m/z = 135.1379 + 1$) and protonated adenine ($m/z = 137.1426 + 1$) respectively. Theoretically, adenine corresponds to $m/z = 136.1$ and according to a previous study, protonated adenine corresponds to a peak with $m/z = 137$ [20]. Since in the present study, due to high concentration of H^+ ions, adenine gets double protonated, therefore the peak can be expected around $m/z = 138.1426$ [36]. The appearance of adenine in the LC-MS spectrum indicates *N*-glycosidic

Figure 13: A plot of $\log k_{\text{obs}}$ against pH for the hydrolysis of AMPNa₂ at 60°C.

Scheme 4: The possible mechanism of anomerization of adenosine at pH 7.

bond cleavage. This proposal is in agreement with the UV-Vis absorption spectrum of hydrolysis of AMPNa₂ throughout the reaction with a shift of absorption towards the right as shown in Figure 9, in accordance with absorption maximum values of adenosine and adenine at 259.5 nm and 260.5 nm, respectively [7]. As the reaction progressed, more adenosine was broken down to produce more adenine.

3.3 Part 3: General Acid and Base Hydrolysis of AMPNa₂ at 60 °C and pH rate profile

In order to study the effect of general acid and base catalysis on the rate of hydrolysis of adenosine monophosphate disodium salt, glycine and TRIS buffers were prepared to cover pH from 1.82-2.00 and from 8.03-8.42. A table consisting of [TRIS], [glycine], pH, and all the kinetic parameters (k_{obs} , E_{app} , A_{∞} and Σdi^2) of this investigation is provided in SI-3 of Supporting Information. Therefore, theoretical values for the rate constant close to neutral pH (around pH 6) were determined by extrapolating the lines from the acidic and the basic region. Figure 13 depicts the $\log k_{\text{obs}}$ against pH for the hydrolysis of AMPNa₂.

A linear relationship was observed from specific base catalysis (pH 11.81-12.71) and specific acid catalysis (pH 0.30-1.83) respectively. Unfortunately, experimental determination of k_{obs} from pH 2-10 does not fit to the linear lines. The rates of reaction from pH in this region were pH independent, which are scattered along the dotted line with an uncatalyzed rate constant around 10^{-7} s^{-1} . As can be seen from Figure 13, theoretical value for the rate constant for close to neutral pH can be determined by extrapolating the lines from the acidic and the basic regions [28]. These two lines intersect each other to obtain a theoretical uncatalyzed rate constant, k_{p} , at about 10^{-11} s^{-1} . Therefore, there was a rate enhancement of 10^4 s^{-1} from the theoretical rate constant at neutral region of hydrolysis of AMPNa₂. The rate enhancement is mostly attributed to the buffer catalysis provided by glycine and TRIS buffers. TRIS buffer usually acts as a nucleophile, which attacks the phosphorus center while glycine acts as a protonating agent for the leaving group [37-39]. These rate enhancements provided by TRIS and glycine have been noticed in previous cleavage of P-O bonds in phosphate monoesters in the presence of alkaline phosphatase [37].

The rates of reaction at pH close to physiological rates were not able to be determined from pH 3 and pH 8. At these pH values, the ribose ring of the adenosine could open and re-close to yield other adenosine anomers such as furanoside and pyranosides namely adenine α -ribofuranoside, adenine β -ribofuranoside and adenine α -ribofuranoside. These ribose ring openings were not observed in acidic or basic hydrolysis. The opening and closing of the ribose ring forms anomers and also could form adenine making it difficult to determine the rates of the reaction at neutral conditions. This anomerization was also reported in previous studies [28]. Scheme 4 shows the mechanism for the possible mechanism of adenosine anomerization at pH 7.

4 Conclusion

It was found that phosphate ester bond cleavage can occur in specific, as well as, general acid-base conditions. A pH dependency of the rate of hydrolysis of AMPNa₂ was observed in highly alkaline and highly acidic environments. This correlation is due to the increasing concentration of hydroxide and hydronium ions at these pH values. It was noticed that the rate of reaction increased linearly with the increase of [NaOH] and [HCl]. This shows that acid and base catalysis can accelerate the AMPNa₂ hydrolysis. A second-order base-catalyzed rate constant, k_{OH} and acid-catalyzed rate constant, k_H obtained were $4.32 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$ and $1.62 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$, respectively. Competition between P-O cleavage and N-glycosidic cleavage was noticed in acidic hydrolysis of AMPNa₂. A rate constant at neutral pH was determined by extrapolating from acidic and alkaline regions, which was determined to be k_0 as 10^{-7} s^{-1} .

This nucleotide analogue has gained attention mainly due to their importance in clinical and food analyses [40,41]. The AMPNa₂ used as subject in this research could be further studied for its use as a cancer markers, a diagnostic marker for human immunodeficiency virus (HIV), and a therapeutic agent due to its antiviral, antitumoral, and antiimmunostimulatory properties [42,43]. In order to employ these analogues in clinical analysis, the kinetics and mechanism of hydrolysis of these analogues has to be extensively studied to further understand how these analogues work in our body. AMPNa₂ can be employed for future kinetic and mechanistic studies as a phosphate ester model, as well as, in enzymatic and non-enzymatic studies. Information on the adenosine production will also benefit to create artificial enzymes that are capable of catabolizing natural AMP to adenosine [44].

Supporting Information

SI-1: Table consisting of [NaOH], pH, and the kinetic parameters (k_{obs} , E_{app} , A_{∞} and Σdi^2) for alkaline hydrolysis of AMPNa₂ of this investigation.

SI-2: Table consisting of [HCl], pH, and the kinetic parameters (k_{obs} , E_{app} , A_{∞} and Σdi^2) for acidic hydrolysis of AMPNa₂ of this investigation.

SI-3: Table consisting of [TRIS], [glycine], pH, and the kinetic parameters (k_{obs} , E_{app} , A_{∞} and Σdi^2) for hydrolysis of AMPNa₂ of this investigation.

SI-4: LCMS Spectrum of the alkaline hydrolysis products of AMPNa₂ in 1.0 M NaOH.

SI-5: LCMS Spectrum of the acidic hydrolysis products of AMPNa₂ in 1.0 M HCl.

This material is available free of charge via the Internet at http://_____

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