The Methyl Ester of α-Aminophenylacetic Acid: pH-Dependence and Phosphate Catalysis of Hydrolysis

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The dependence of the rate of spontaneous (non-enzymic) hydrolysis of α-aminophenylacetic acid methyl ester on the acidity of a medium was studied over the pH range 0.95—11.6. The mono- and di-anion of phosphate was found to have a catalytic effect on this reaction, according to the mechanism of general base catalysis. Catalysis of the protonated substrate form hydrolysis by different phosphate ions, the second molecule of water, and the hydroxide ion follows the Brönsted catalysis law with the slope 0.60. At a strong alkaline pH, phosphate slows down the ester hydrolysis, probably due to the formation of an ester–phosphate complex; the calculated dissociation constant is 4.2×10^{-3}M, while the ratio of the hydrolysis rate constants for free ester and its phosphate complex is 7.7.

The esters of α-aminophenylacetic acid are known to be the best acyl-group donors in the enzymatic synthesis of such β-lactam antibiotics as ampicillin, cephalexin, and cephaloglycine.[1] Spontaneous (non-enzymic) hydrolysis is a concomitant process which lowers the yield of the end product. It is thus essential to find optimal reaction conditions under which there is a sufficient rate of the biocatalytic reaction and a relatively low rate of spontaneous hydrolysis. The most sensitive parameter of such optimization is pH. The study and quantitative description of the pH dependence of spontaneous hydrolysis is therefore of theoretical interest, since, over a wide pH range, the methyl ester of α-aminophenylacetic acid is present in two equilibrium forms—protonated and unprotonated, differing in the charge near the reaction centre, and in reactivity.

Experimental

D-(−)-α-Aminophenylacetic acid was obtained from Sigma, USA, and KOH was from Chemapol, Czechoslovakia; all other salts and reagents were from Reachim, USSR. The methyl ester of D-(−)-α-aminophenylacetic acid was obtained by literature methods.[2] Concentrations of the methyl ester and the free α-aminophenylacetic acid in the reaction mixture was determined by h.p.l.c. on a 2.2 × 250 mm column with a strong anion-exchanger, Aminex A-28 (Bio-Rad, USA), at pH 7 in 0.25M ammonium phosphate–ethanol (80:20 v/v) at 50 °C. A liquid chromatograph Varian-8500 (Switzerland) with a u.v.-detector (254 nm) was used. In a typical experiment, 3—4 ml of a solution of the methyl ester of α-aminophenylacetic acid at a concentration of 5×10^{-3}—0.1M was incubated in the thermostatted cell of an automatic titrator RTS-622 (Radiometer A/S, Copenhagen) at 25 °C and the required pH value; the ionic strength was sustained by KCl. 10 μl Samples were taken at definite time intervals and loaded onto the h.p.l.c. column.

The order of the reaction was determined as a plot of log v *versus* log c, where v is the initial velocity of the hydrolysis and c is the initial substrate concentration. In all cases, the hydrolysis proceeded according to a first-order rate equation. The results of the kinetic experiments were optimized by the least-squares method on a PDP-11/45 computer.

The ionization constant of the ester amino group at 25 °C was determined by titration with a KOH solution (5×10^{-2}M) in an automatic RTS-622 titrator.

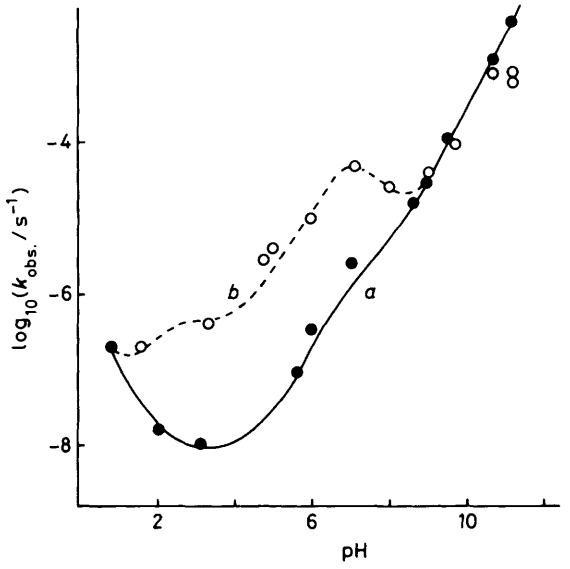


Figure 1. pH Dependence of the hydrolysis rate constant for methyl ester of α-aminophenylacetic acid in the absence (curve *a*) and in the presence (curve *b*) of 1M potassium phosphate. Optimal curves (*a*) and (*b*) were calculated according to equations (6) and (10). Temp. 25 °C, ionic strength 2.9M except: in the presence of phosphate at pH 9.7, 3.0M; pH 10.5, 3.1M; pH 11.2, 3.3M.

Results and Discussion

Hydrolysis of the methyl ester of aminophenylacetic acid, over the pH range investigated (0.95—11.6), with or without phosphate, obeys first-order reaction kinetics [equation (1)]

$$v = k_{obs.}[E]_0 \quad (1)$$

where $k_{obs.}$ is the rate constant observed for the first-order reaction and $[E]_0$ is the total concentration of the amino acid ester in the solution.

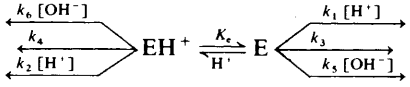
The pH dependence of the rate constant of the first-order reaction in the absence (curve *a*) and in the presence (curve *b*) of phosphate is shown in Figure 1.

The following general kinetic scheme (2) applies for the hydrolysis of the α-amino acid ester:

Table. Alkaline hydrolysis rate constants for amino acid esters (25 °C)

Methyl ester	k_{alk}·M·s[a]	Reference
gly	1.28	4
trimethylgly	61.0	5
α-ala	1.1	4
	80 (k_+)	6
	1.1 (k)	6
β-ala	6.9 (k_+)	6
	0.14 (k)	6
α-aminobutyric acid	45 (k_+)	6
	0.39 (k)	6
val	0.076	4
ile	0.067	4
leu	0.45	4
ser	1.0	4
met	0.77	4
β-phe	0.55	4
trp	0.29	4
tyr	0.27	4
his	26 250 (k_{2+})	7
	56 (k_+)	
	0.65 (k)	
cys	23 (k_+)	7
	1.6 (k)	
	0.041 (k_-)	
N-acetylphe	1.9	8
N-benzoylgly	2.7	9

[a] k with the index +, 2+, − denotes a constant for the amino acid ester charged form. k without index denotes a constant for the amino acid ester neutral form. These values were computed by the authors postulating a complete absence of neutral hydrolysis of the amino acid ester.



(2)

and this gives the hydrolysis rate equation:

$$V = k_1[E][H^+] + k_2[EH^+][H^+] + k_3[E] + k_4[EH^+] + k_5[E][OH^-] + k_6[EH^+][OH^-] \quad (3)$$

where EH^+ and E are the amino acid ester, protonated and unprotonated at the α-amino group; k_1, k_2, k_3, k_4, k_5, and k_6 are the reaction rate constants of their acid, neutral, and alkaline hydrolysis respectively; $K_e = [H^+][E]/[EH^+]$, the ionization constant of the methyl ester of α-aminophenylacetic acid (pK_e at 25 °C in 1M KCl solution = 7.12). Taking into account the mass balance:

$$[E]_0 = [E] + [EH^+] \quad (4)$$

it follows from equations (1) and (3) that

$$k_{obs.} = \frac{(k_2/K_e)[H^+]^2 + (k_1 + k_4/K_e)[H^+] + k_3 + k_6K_w/K_e + k_5K_w/[H^+]}{I + [H^+]/K_e} \quad (5)$$

or

$$k_{obs.} = \frac{A[H^+]^2 + B[H^+] + C + D/[H^+]}{I + [H^+]/K_e} \quad (6)$$

where $A = k_2/K_e$, $B = k_1 + k_4/K_e$, $C = k_3 + k_6K_w/K_e$, $D = k_5K_w$ (7)

$$\Delta v = k'_1[EH^+][H_2PO_4^-] + k'_2[E][H_2PO_4^-] + k'_3[EH^+][HPO_4^{2-}] + k'_4[E][HPO_4^{2-}] + k'_5[EH^+][H_3PO_4] + k'_6[E][H_3PO_4] \quad (8)$$

and K_w is the ionic product of water under the experimental conditions, equal to 1.75×10^{-14} (ref. 3).

The optimal A, B, C, and D values [equation (5)—(7)] for the dependence obtained (Figure 1*a*) are $20M^{-2}\ s^{-1}$, $0.125M^{-1}\ s^{-1}$, $2.8 \times 10^{-6}\ s^{-1}$, and $2.8 \times 10^{-14}M\ s^{-1}$, respectively. Hence only the k_2 and k_5 values can be determined directly, equal to $1.5 \times 10^{-6}M^{-1}\ s^{-1}$ and $1.6M^{-1}\ s^{-1}$, respectively. The k_5 value correlates well with the alkaline hydrolysis constants of *N*-alkylated amino acid esters (see Table).

The k_2 value is close to the acid hydrolysis rate constants of *N*-alkylated amino acid esters with a charge in the α-position, specifically, to the second order rate constant for acid hydrolysis of trimethylglycine equal to $1 \times 10^{-6}M^{-1}\ s^{-1}$ (ref. 10).

Unfortunately, conventional kinetic methods of evaluating pH-dependence do not make it possible to separate kinetic constants in the equation coefficient at one and the same degree of hydrogen ion concentration. To estimate these constants separately, one should apply theoretical premises or selective experimental techniques for determining definite kinetic parameters. Comparing the B and C values with the hydrolysis rate constants for carboxylic and *N*-substituted amino acid esters, we can estimate the k_4 value and determine the upper limits for k_3 and k_6 consistent with experimental data. Since k_1 must be close to the rate-constant values of carboxylic and *N*-substituted amino acid ester hydrolysis, equal at 25 °C ($I - 7.5 \times 10^{-5}M^{-1}\ s^{-1}$ ref. 11), it follows that $k_4/K_e \gg k_1$, $B \approx k_4/K_e$, while the k_4 value is $9.5 \times 10^{-9}\ s^{-1}$. By assuming that $k_3 \gg k_6\ K_w/K_e$, the upper level for k_3 must be $2.8 \times 10^{-6}\ s^{-1}$. Yet this estimate can hardly be acceptable for two reasons. Firstly, the reactivity of the substrate protonated form under neutral hydrolysis will in this case be nearly 300 times less than that of the unprotonated ester form. Secondly, by supposing that k_3 has a significantly lower value and consequently that $k_6K_w/K_e \gg k_3$, we obtain results more consistent with literature data. In this case, the k_6 value, $12.1M^{-1}\ s^{-1}$, accords with the alkaline hydrolysis rate constants for trimethylglycine (a substrate which is a model of the amino acid ester protonated form), equal to $61M^{-1}\ s^{-1}$ (see Table). Also, the reactivity of the protonated form of α-aminophenylacetic acid methyl ester in an alkaline hydrolysis reaction becomes nearly 8 times as high as that of the unprotonated form (the second order rate constants k_6 and k_5 equal to $12.1M^{-1}\ s^{-1}$ and $1.6M^{-1}\ s^{-1}$, respectively).

Hydrolysis of the methyl ester of α-aminophenylacetic acid in 1M potassium phosphate at pH 2—8 is remarkable for the appreciable phosphate-induced catalysis, most effective in the pH range 3—7. At pH 6.0, the hydrolysis rate is linearly dependent on the phosphate concentration, the ionic strength of the solution being constant (Figure 2). Considering the dissociation of phosphate in the pH range investigated ($pK_1 = 2.15$ and $pK_2 = 7.21$), we may evolve the following equation for the difference in the rates of α-aminophenylacetic acid ester hydrolysis, catalysed and not catalysed by phosphate equation (8).

where k'_1, k'_2, k'_3, k'_4, k'_5, and k'_6 are the rate constants of respective reactions. It follows from equation (8) and the mass balance that

$$\frac{\Delta k_{obs.}}{[P]_0} = \frac{\frac{k'_5}{K_1K_e}[H^+]^2 + \left(\frac{k'_1}{K_e} + \frac{k'_6}{K_1}\right)[H^+] + k'_2 + k'_3\frac{K_2}{K_e} + \frac{k'_4K_2}{[H^+]}}{\left(1 + \frac{[H^+]}{K_e}\right)\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right)} \quad (9)$$

or

$$\frac{\Delta k_{obs.}}{[P]_0} = \frac{A'[H^+]^2 + B'[H^+] + C' + D'/[H^+]}{\left(1 + \frac{[H^+]}{K_e}\right)\left(1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right)} \quad (10)$$

$$A' = k'_5/K_eK_1;\ B' = k'_1/K_e + k'_6/K_1;\ C' = k'_2 + k'_3K_2/K_e;\ D' = k'_4K_2$$

where $\Delta k_{obs.}$ is the difference in the constants of the phosphate-catalysed first-order rate reaction and the non-catalysed hydrolysis of α-aminophenylacetic acid ester.

The optimal A', B', C', and D' values for the dependence obtained experimentally (Figure 1*b*) are, respectively, 0, 6.6M^{-2} s^{-1}, 1.84×10^{-4}M^{-1} s^{-1}, and 0. Consequently, k'_5 and k'_4 are equal to 0; the following equations hold for the other constants.

It is hardly possible for the H_3PO_4-catalysed hydrolysis of the unprotonated form to proceed five orders of magnitude faster than the $H_2PO_4^-$-catalysed hydrolysis of the protonated form; so, one may assume that k'_1 is responsible for the main contribution in equation (11), and then $k'_1 = 5.0 \times 10^{-7}$M^{-1} s^{-1}. The magnitude of k'_3 undoubtedly accounts for the main contribution to equation (12); it reflects the rate constant of the

$$k'_1 + 1.1 \times 10^{-5}\,k'_6 = 5.0 \times 10^{-7}\text{M}^{-1}\text{ s}^{-1} \quad (11)$$

$$k'_2 + 0.81\,k'_3 = 1.84 \times 10^{-4}\text{M}^{-1}\text{ s}^{-1} \quad (12)$$

hydrolysis of the more active (protonated) form of the substrate, as well as catalysis by a more active base.

General base catalysis is the expected mechanism for the hydrolysis of acyl-activated esters[12] (the protonated form of the methyl ester of α-aminophenylacetic acid is related to them). As anticipated, in the case of phosphate-catalysed ester hydrolysis, the ester disappearance correlates with the accumulation of α-aminophenylacetic acid; no intermediate product of acid residue transfer to the catalyst is detectable. General base catalysis of the hydrolysis of α-aminophenylacetic acid methyl ester (protonated form) reveals the involvement of various phosphate forms and obeys the Brönsted equation with a slope of 0.60. As seen from the plot (Figure 3), neutral hydrolysis of the substrate (protonated form), phosphate mono- and di-anion-catalysed hydrolysis, and alkaline hydrolysis follow the same pattern.

It is remarkable that at pH 11.2 phosphate slows down the rate of ester hydrolysis (Figure 4*a*), and the rate constant of the first-order rate reaction plotted *versus* the phosphate concentration is a saturation curve. This kind of dependence could be explained by the formation of a complex of the ester and PO_4^{3-}, present in a significant amount at the given pH value (the pK_3 value of phosphoric acid being 12.17):

$$\xleftarrow{\alpha k''} E + PO_4^{3-} \rightleftharpoons E{\cdot}PO_4^{3-} \xrightarrow{k''} \quad (13)$$

with $\alpha k''$ and k'' being the hydrolysis rate constants for the free ester and its complex with phosphate, and K'' the dissociation constant of this complex.

The complex is more resistant to hydrolysis than the free ester. The calculated dissociation constant of the given complex, K'' (Figure 4*b*), is 4.2×10^{-3}M, while the ratio α of the hydrolysis rate constants for free ester and its phosphate complex is 7.7. We have found no literature data concerning phosphate complexes with amino acids, but there are indications of similar complexes forming on the hydrolysis of *N*-substituted acetimidate esters and 4-hydroxybutyranilide hydrolysis, with the intermediate products; this complexation affects the course of the reaction.[13]

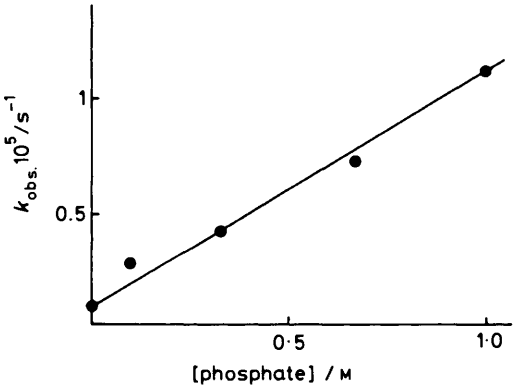


Figure 2. Dependence of the hydrolysis rate constant for methyl ester of α-aminophenylacetic acid on the phosphate concentration at pH 6.0. Temp. 25 °C, ionic strength 2.9M (sustained by KCl)

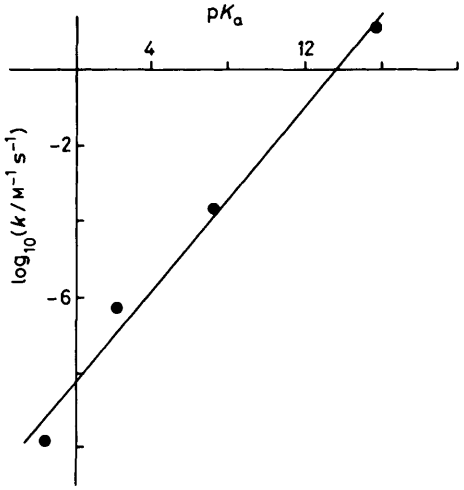


Figure 3. Brönsted plot of the catalytic rate constants for general-base catalysed hydrolysis of the protonated form of α-aminophenylacetic acid methyl ester. Bases: water, mono- and di-anion of phosphate, hydroxide ion

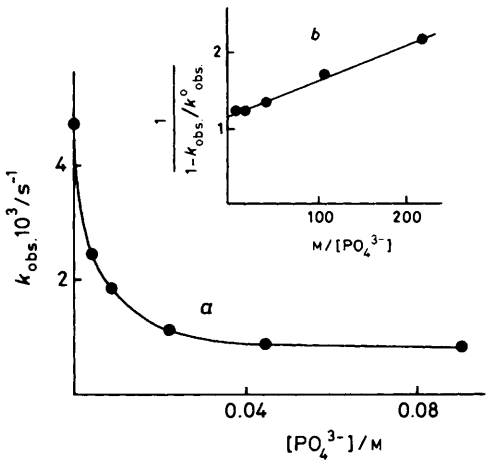


Figure 4. Phosphate-induced inhibition of methyl ester of α-aminophenylacetic acid hydrolysis at pH 11.2. (*a*), Dependence of the observed first-order rate constant $k_{obs.}$ on $[PO_4^{3-}]$ (*b*), PO_4^{3-} and ester binding analysis; $k^0_{obs.}$ = rate constant in the absence of phosphate. Temp. 25 °C, ionic strength 3.3M (sustained by KCl)

As shown above, spontaneous hydrolysis of α-aminophenylacetic acid (and, most probably, of all the esters of amino acids with a free amino group) differs significantly from the hydrolysis of *N*-acylated amino acid esters. To select conditions for maximal stability of these compounds in water solutions, one should bear in mind that with the presence of a free amino group in an ester its hydrolysis is subject to general base catalysis. This factor accentuates the importance of the control of the composition of the reaction medium; spontaneous hydrolysis may be the cause of an enhanced expenditure of the ester in the synthesis of β-lactam antibiotics *via* an enzymatic transfer of its acyl group to the 'nuclei' of these compounds.

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