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Kinetic Approach to the Development in β-Lactam Antibiotics. I. Comparative Stability of Semisynthetic Penicillins and 6-Aminopenicillanic Acid in Aqueous Solution[1]

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The kinetics of the hydrolyses of carbenicillin, sulbenicillin, propicillin, cyclacillin, 6-(α-toluenesulfonamido)-penicillanic acid (TSPA), the substituted phenylpenicillins and 6-aminopenicillanic acid (6-APA) were studied over wide pH ranges at 35° and $\mu=0.5$.

For the hydrolysis of penicillins, the psuedo first-order rate constants, k_{pH} at zero buffer concentration were formulated as the equation: $k_{\text{pH}}=(k_{\text{H}}[\text{H}_3\text{O}^+]+k_0)f_{\text{HP}}+(k'_0+k'_{\text{OH}}[\text{OH}^-])f_{\text{P}^-}$, where f_{HP} and f_{P^-} are the fractions of free-acid penicillin (HP) and ionized penicillin (P^-), respectively. These catalytic rate constants were compared with those reported previously for other penicillins. The activation parameters were also determined for some penicillins. In acidic solution, penicillins except TSPA were confirmed to be hydrolyzed to the penicillenic acids *via* hydronium ion-catalyzed rearrangement of HP the (k_{H}) and spontaneous rearrangement of HP (k_0). The Hammett plots for k_{H} and k_0 of the substituted phenylpenicillin series gave excellent linear correlations with both $\rho=-1.60$. The magnitude and sign of the ρ values were consistent with the intramolecular catalyzed mechanism by the neighboring side-chain amido groups.

The hydrolysis of 6-APA was found to follow first-order kinetics at low initial concentration in the range of 0.005—0.01 M. From the log k_{pH} −pH profile and the pertinent primary salt effects, the possible mechanisms for the acidic hydrolysis of 6-APA were discussed. The activation parameters were evaluated at each reaction region. At pH 5, 6-APA was found to be hydrolyzed 3—6 times faster than relatively acid-stable penicillins, which may be attributed to the inductive effect by the protonated 6-amino group.

The k'_0 and k'_{OH} values were nearly the same in the hydrolyses of all penicillins and 6-APA. It can be concluded that the basic and neutral hydrolyses of these compounds are, respectively, simple hydroxide catalyzed and water catalyzed reactions.

The bicyclic β-lactam thiazolidine structure in penicillin is more susceptible to base- or acid-catalyzed hydrolysis than simple β-lactam and linear amide structure.[3] Pharmaceutical importance of penicillin-stabilization has prompted to study the hydrolysis kinetics of these antibiotics in our systematic works[4] on the stability of amide linkage.

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[3] *a*) M.S. Manhas and A.K. Bose, "Beta-Lactams. Natural and Synthetic," Part 1, Wiley Interscience, New York, 1971, p. 61; *b*) M.A. Schwartz and F.H. Buckwalter, *J. Pharm. Sci.*, **51**, 1119 (1962); *c*) R.J. Washkuhn and J.R. Robinson, *ibid.*, **60**, 1168 (1969).

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Since 1947, excellent kinetic studies on the stability of penicillins in solution have been performed in penicillin G,[5–8] phenethicillin,[9] methicillin,[10,11] ampicillin,[12] oxacillin[13] and cloxacillin.[14] As noted in referring to these kinetic studies, the side chain structure of the various penicillins endows each with different stability characteristics. Unfortunately, none of penicillin available at present is sufficiently stable to allow marketing as liquid dosage form. With regard to the stablization of penicillins by chemical modification, further study on the mechanisms of the hydrolyses and the effects of structural changes upon these should be required.

The present study on therapeutically useful penicillins, some semisynthetic penicillins and 6-aminopenicillanic acid (6-APA) was begun with the expectation that a systematic quantitative approach to the stabilization would help to produce much better penicillins in future.

Experimental

Materials——The penicillins except cyclacillin and 6-APA were available as the sodium or potassium salt. Cyclacillin and 6-APA were supplied from Takeda Chemical Ind., Ltd. (Takeda), and penicillin G and cloxacillin from Meiji Seika Kaisha, Ltd. Samples of sulbenicillin (Takeda), propicillin (Takeda), ampicillin (Takeda), carbenicillin (Taito Pfizer Co., Ltd.) and oxacillin (Banyu Pharmaceutical Co. Ltd) were normal commercial materials. Sodium salt of 6-(α-toluenesulfonamido)-penicillanic acid (TSPA) was prepared as follows: 6-APA (4.7 g) was dissolved in 150 ml of acetone and 150 ml of two equimolar $NaHCO_3$ solution. Then benzylsulfonyl chloride (4.1 g) in 40 ml of acetone was added at room temperature with stirring and keeping pH 7—8 over 10 min period. The solution was stirred for 2 hr at 0° and filtered, and the acetone was removed from the filtrate under reduced pressure. After extracting the residue with ether, the aqueous phase was freeze-dried to give the sodium salt. Substituted phenylpenicillin potassium salts were prepared from 6-APA and the corresponding acyl chlorides in $KHCO_3$ aqueous solution in the same manner with TSPA. Infrared (IR) spectra of these penicillins confirmed the presence of an intact β-lactam ring.

All other chemicals were commercially available reagent grade.

Procedure——In most cases, the kinetic studies were carried out at 35°. The penicillin (5×10^{-3}M) was dissolved in 100 ml of an appropriate buffer solution preheated at desired temperature. In the case where the half-life was more than a day, the solution was filled into 5 ml ampules. The ampules were sealed and placed in a thermostatically controlled bath (±0.1°). At appropriate intervals, samples were taken from the reaction mixture and cooled on ice. The residual penicillin concentration was determined by a modified iodometric titration procedure,[7] but for the hydrolysis of oxacillin, the spectrophotometrical method reported by Bundgaard and Ilver[14] was employed. The hydrolyses were also followed by a modified colorimetric assay with hydroxylamine.[15] The change of ultraviolet (UV) spectra of the reaction solution in an appropriate buffer was automatically recorded by repetitive scan technique with Shimadzu multipurpose recording spectrophotometer, Model MPS-5000.

The pH of the solution was maintained at the desired value by using appropriate buffer systems or by pH-stat technique with Radiometer titrator, TTT-2 and autoburet, ABU 12. The buffer systems used were HCl–KCl, H_3PO_4–NaH_2PO_4, CH_3COOH–CH_3COONa, Na_2 citrate–Na_3 citrate, NaH_2PO_4–Na_2HPO_4, $NaHCO_3$–Na_2CO_3 and Na_2HPO_4–Na_3PO_4. The ionic strength of each solution was adjusted to 0.5 (except for investigation concerned with primary salt effect) by the addition of potassium chloride. The pH of buffer solution was determined at experimental temperature initially and at the end of the experiment by a Hitachi-Horiba pH meter, Model F-5 scale expander. No significant changes in pH were observed.

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Result

At constant pH and in the presence of a great excess of buffer over penicillins and 6-APA, all iodometrically or spectrophotometrically determined rate constants (k_{obs}) were found to be pseudo-first-order. In every case, the values of k_{obs} were in fairly good agreement with those obtained by hydroxamate assay method. Plots of the experimentally determined k_{obs} *vs.* total buffer concentration were found to be linear. Extrapolation to zero buffer concentration provides as intercept the pseudo-first-order rate constant, k_{pH}, for the nonbuffer-catalyzed reactions. The most significant buffer concentration effect was observed with NaH_2PO_4–Na_2HPO_4 buffer system in all hydrolyses.

Semisynthetic Penicillins

a) log k_{pH}-pH Profile——In Fig. 1 is plotted log k_{pH} *vs.* pH for sulbenicillin, propicillin, carbenicillin, cyclacillin and TSPA. The shape of the log k_{pH}–pH profiles, which are fairly similar to those reported in other penicillins,[5–14] can be formulated as Eq. 1

$$k_{pH} = (k_H[H_3O^+]+k_0)\frac{[H_3O^+]}{Ka+[H_3O^+]} + (k_0'+k_{OH}'[OH^-])\frac{Ka}{Ka+[H_3O^+]} \qquad (1)$$

where Ka is the dissociation constant for penicillin. k_H represents for the acid-catalyzed hydrolysis of undissociated acid penicillin (HP). k_0 and k'_0 represent, respectively, the spontaneous (*e.g.*, water-catalyzed) hydrolysis rates of HP and ionized penicillin (P^-). k'_{OH} is the second-order rate constant for the hydroxide ion-catalyzed hydrolysis of P^-. The concentration of hydronium ion, $[H_3O^+]$ and of hydroxide ion, $[OH^-]$ at $\mu=0.5$ and 35° can be calculated from the pH of the solution according to the following equations.[12]

$$\log[H_3O^+] = -pH + 0.12 \qquad (2)$$

and

$$\log[OH^-] = pH - 13.56 \qquad (3)$$

The four rate constants and one dissociation constant in Eq. 1 were computed from the experimental data by weighting least-squares method on the base of Eq. 1. These parameters are listed in Tables I and II together with those reported previously for other penicillins. The points of Fig. 1 are experimental values and lines are calculated from Eq. 1 using the parameters thus determined.

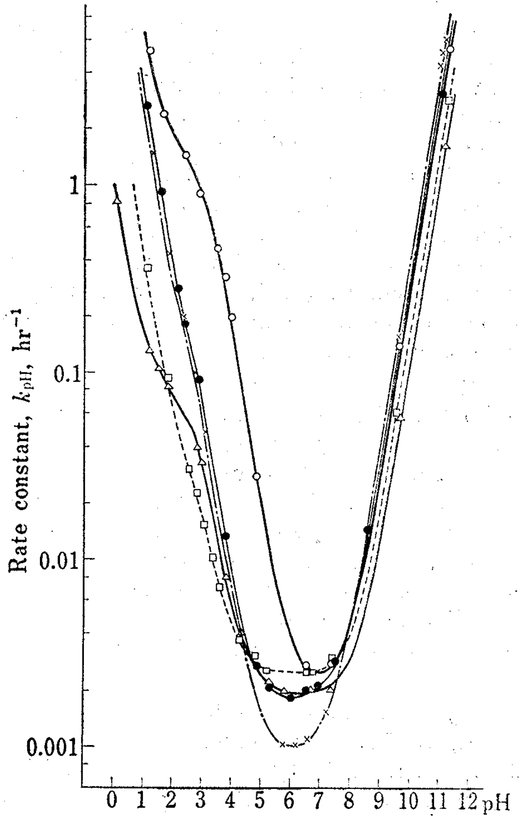


Fig. 1. log k_{pH}–pH Profile for the Hydrolysis of Penicillins at 35° and $\mu=0.5$

The drawn lines are calculated from Eq. 1 using the catalytic rate constants listed in Table I. The points are experimental values.

—○—: carbenicillin
—●—: sulbenicillin
--×--: propicillin
—□—: cyclacillin
—△—: TSPA

b) Effect of Temperature——The temperature effects on the reaction rates for some penicillin hydrolyses were determined at each reaction region. The activation enthalpies ($\Delta H^{\neq}$) and entropies ($\Delta S^{\neq}$) for rate constants (k_H, k_0, k'_0, and k'_{OH}) of five penicillins were calculated by analysis of data available in the literatures and those obtained in the present work. These parameters are listed in Table III, and the typical Arrhenius plots are given in Fig. 2.

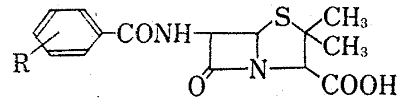
c) **Primary Salt Effect**——The rates of nonbuffer-catalyzed hydrolyses of some penicillins were determined at various ionic strength and pH 3—4 which was maintained by a pH-stat. The observed k_{pH} was plotted as a function of the square root of ionic strength in Fig. 3. In any case, no primary salt effects were observed in the pH region where penicillins exist as P^-. Similar observation of the ionic strength effect was noted by Finholt, *et al.*[7] in the hy-

Table I. Catalytic Rate Constants[a] for the Hydrolysis and Dissociation Constant of Penicillins at 35° and $\mu=0.5$

Penicillin	Rate constant					p*K*a
	k_H $M^{-1}hr^{-1}$	10^2k_0 hr^{-1}	$10^3k'_0$ hr^{-1}	$10^{-3}k'_{OH}$ $M^{-1}hr^{-1}$	$10^2k_{HPO_4^=}$ $M^{-1}hr^{-1}$	
Penicillin G[b]	456[c]	259	0.90	1.09	7.8	2.80[c]
Penicillin G methylester[d]	340[e]	—	480	6.72	—	—
Methicillin[f]	600[g]	382	0.74	2.01	8.7	2.74[g]
Carbenicillin[b]	39.6	139	2.04	0.92	6.4	3.06[h]
Oxacillin[i]	30.0	31.4	1.10	1.05	7.3	2.68[i]
Cloxacillin[j]	27.0	15.9	0.94	1.02	9.2	2.68[j]
Sulbenicillin[b]	24.9	19.1	1.77	0.97	7.8	2.45[h]
Propicillin[b]	23.3	10.1	0.89	1.31	12.8	2.72[k]
Phenethicillin[l]	12.5	20.4	3.25	0.76	9.4	2.90[l]
Cyclacillin[b]	3.50	3.29	2.49	0.50	35.5	2.68[m]
Ampicillin[n]	1.38	4.22	0.75	1.95	16.6	2.60[n]
TSPA[b]	0.69	8.17	1.92	0.36	3.0	2.61[h]

a) Various rate constants can be defined in Eqs. 1 and 8.
b) present work
c) ref. 5
d) The hydrolysis was carried out in 13.3% acetone-water at 31.3° ($\mu=0.2$). ref. 25
e) The hydrolysis was carried out at 30° ($\mu=0.2$). H. Bundgaard, *J. Pharm. Sci.*, **60**, 1273 (1971)
f) ref. 11
g) ref. 10
h) kinetically determined
i) ref. 13
j) ref. 14
k) ref. 16
l) ref. 9
m) ref. 12*b*
n) ref. 12*a*

Table II. Catalytic Rate Constants[a] for the Hydrolysis of Substituted Phenylpenicillins at 35° and $\mu=0.5$



R	Rate constant			
	k_H $M^{-1}hr^{-1}$	10^2k_0[b] hr^{-1}	$10^3k'_0$ hr^{-1}	$10^{-3}k'_{OH}$ $M^{-1}hr^{-1}$
4-CH_3	—	1660	—	1.29
H	—	741	—	—
4-Cl	—	303	—	1.39
3,4-$(Cl)_2$	228	78.7	1.45	1.23
4-NO_2	96.4	37.8	1.45	1.36
3,5-$(NO_2)_2$	11.4	4.54	1.55	1.32

a) Various rate constants can be defined in Eq. 1.
b) calculated using p*K*a=2.70

Table III. Enthalpies of Activation, $\Delta H^{\ddagger}$ and Entropies of Activation, $\Delta S^{\ddagger}$ for the Hydrolyses of Penicillins

Penicillin	$\Delta H^{\ddagger}$ (kcal/mole) at 35°				$\Delta S^{\ddagger}$ (e.u.)[a] at 35°			
	k_H	k_0	k'_0	k'_{OH}	k_H[b]	k_0[c]	k'_0[c]	k'_{OH}[b]
Penicillin G[d]	17.6	21.0	—	9.8	−7.6	−6.8	—	−32.2
Methicillin[e]	—	20.6	22.0	13.4	—	−7.3	−19.8	−18.3
Oxacillin[f]	17.2[g]	17.5	22.8	11.9	−14.3[g]	−22.4	−16.4	−24.4
Cloxacillin[h]	17.4	18.4[g]	19.0	10.8	−13.8	−20.8[g]	−29.0	−28.0
Ampicillin[i]	16.4	21.3[g]	—	9.2	−23.0	−14.0[g]	—	−31.9

[a] These activation parameters were calculated from: $k=(kT/h)\exp(\Delta S^{\ddagger}/R)\exp(-\Delta H^{\ddagger}/RT)$ and $\Delta H^{\ddagger}=\Delta E_a-0.6$, where T is the absolute temperature and ΔE_a is obtained from the slopes of the Arrhenius plots of log k *vs.* $1/T$.
[b] based on M^{-1} sec^{-1}
[c] based on sec^{-1}
[d] ref. 5
[e] ref. 11
[f] ref. 13
[g] present work
[h] ref. 14
[i] ref. 12

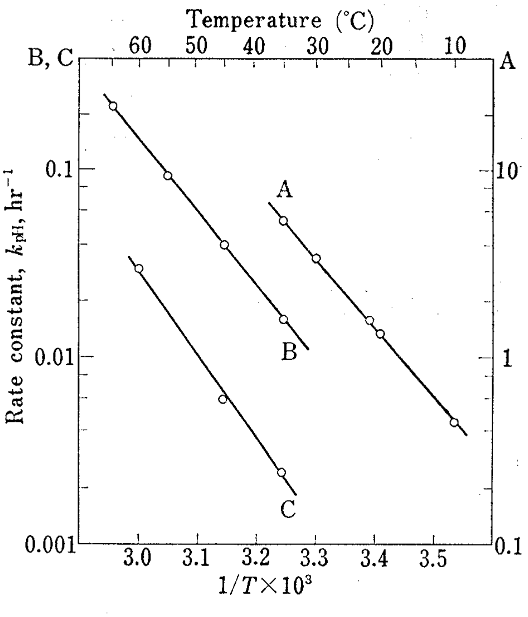


Fig. 2. Typical Arrhenius Plots of the Pseudo-First-Order Rate Constants, k_{pH}, for the Hydrolysis of Penicillins at μ=0.5

A: oxacillin at pH 1.15, rate constant in 0.1N HCl
B: cloxacillin at pH 3.81, Rate constant extraporated to zero acetate buffer concentration.
C: ampicillin at pH 3.95, Rate constant extraporated to zero acetate buffer concentration.

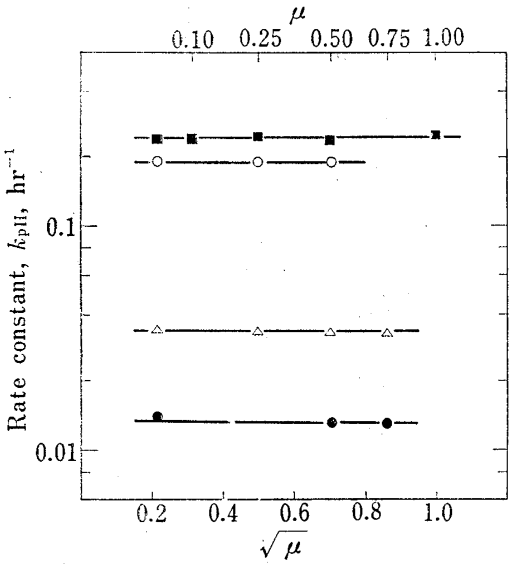


Fig. 3. Effect of the Ionic Strength (μ) on the Pseudo-First-Order Rate Constant, k_{pH} of the Hydrolysis of Penicillins in Nonbuffered Solution[a] at 35°

—■—: penicillin G at pH 4.00
—○—: carbenicillin at pH 4.00
—△—: TSPA at pH 3.00
—●—: sulbenicillin at pH 3.80
[a] pH of the reaction solution was maintained by a pH-stat.

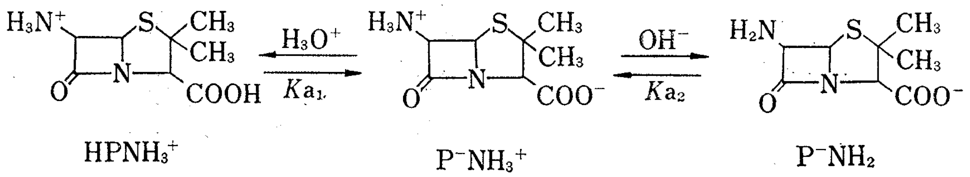


Chart 1. Dissociation Equilibrium in Aqueous Solution of 6-APA

drolysis of penicillin G at 60° in acetate buffer solution of pH 4.5 where the buffer has no catalytic effect.

6-APA

a) Determination of Dissociation Constants——The dissociation constants of 6-APA were determined[12b] potentiometrically at 35° and $\mu=0.5$. The titration with acid is a process of proton association (proton gain) from zwitterion ($P^-NH_3^+$) to cation ($HPNH_3^+$), while that with base is a process of proton dissociation (proton lost) from zwitterion to anion (P^-NH_2), as shown in Chart 1. $pKa_1=2.86$ (±0.04) and $pKa_2=4.72$ (±0.05) were obtained from these titration of 6-APA (5.00×10^{-3}M) with 0.10N HCl and 0.10N NaOH, respectively.

Previously reported values were $pKa_1=2.30$ and $pKa_2=4.90$ at 25° (ionic strength was not given) by Rapson and Bird.[16] Recently, $pKa_1=2.67$ and $pKa_2=4.72$ were reported by Lubinson,[17] although experimental conditions of temperature and ionic strength were not described.

b) Effect of 6-APA Concentration on Reaction Rate——The rate constants at 35° and $\mu=0.5$ were determined using three different concentrations of 6-APA (2.50×10^{-3}, 5.00×10^{-3} and 1.00×10^{-2}M) in carbonate buffer of pH 9.62, phosphate buffer of pH 7.06 and in acetate buffer of pH 3.76. No significant change in k_{obs} at three different pH values was observed, as shown in Table IV.

TABLE IV. Effect of Initial Concentration on Reaction Rate Constant of the Hydrolysis of 6-APA at Various pH and 35° ($\mu=0.5$)

Initial concentration of 6-APA M	Rate constant, k_{obs}, hr^{-1}		
	pH 3.76[a]	pH 7.06[b]	pH 9.62[c]
2.50×10^{-3}	2.62×10^{-2}	9.00×10^{-3}	4.00×10^{-3}
5.00	2.57	8.93	4.03
10.00	2.60	8.95	4.05

a) determined in 0.15 M acetate buffer
b) determined in 0.15 M phosphate buffer
c) determined in 0.15 M carbonate buffer

Dennen[18] reported the semi-quantitative study on the hydrolysis of 6-APA with initial concentration of 0.05—0.92M in the pH range 5—10 and found both hydrolysis and dimerization taking place. Below pH 7.5, the hydrolysis reaction predominated while above this pH the dimerization was more significance. The present results indicate that the hydrolysis of 6-APA follows first-order kinetics in whole pH ranges and that the dimerization or further polymerization of 6-APA could not occur significantly with the initial concentration below 0.01M.

c) log k_{pH}-pH Profile——The kinetic result as shown in Fig. 4 for the hydrolysis of 6-APA could be described with Eqs. 4, 5, 6 and 7

$$k_{pH} = (k_H[H_3O^+]+k_0)f_1 + k'_{SH}f_2 + (k'_0+k'_{OH}[OH^-])f_3 \tag{4}$$

$$f_1 = [H_3O^+]^2/([H_3O^+]^2+Ka_1[H_3O^+]+Ka_1Ka_2) \tag{5}$$

$$f_2 = Ka_1[H_3O^+]/([H_3O^+]^2+Ka_1[H_3O^+]+Ka_1Ka_2) \tag{6}$$

and

$$f_1+f_2+f_3=1 \tag{7}$$

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[16] H.D.C. Rapson and A.E. Bird, *J. Pharm. Pharmacol.*, **15**, 222T (1963).
[17] G.S. Lubinson, *Khim. Parm. Zh.*, **5**, 37 (1971).
[18] D.W. Dennen, *J. Pharm. Sci.*, **56**, 1273 (1967).

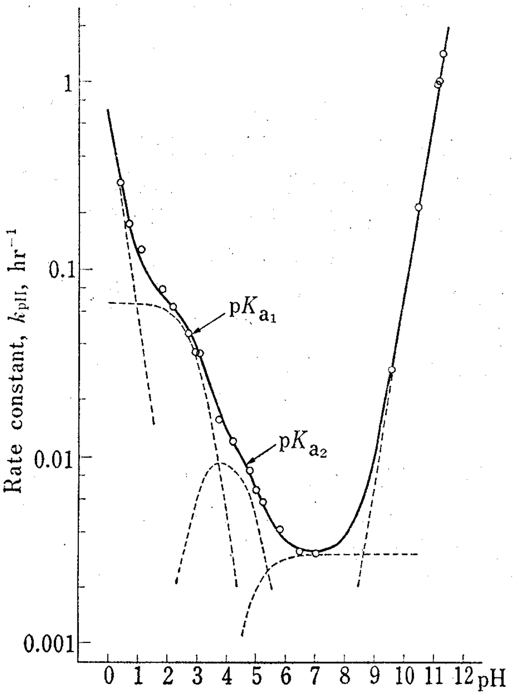

Fig. 4. log k-pH Profile for the Hydrolysis of 6-APA at 35° and $\mu=0.5$

The drawn line is calculated from Eqs. 4—7 using the catalytic rate constants listed in Table V. The points are experimental values. The dashed lines are the separated log k_{pH}–pH profiles.

where f_1, f_2 and f_3 are the fractions as the cationic ($HPNH_3^+$), zwitterionic ($P^-NH_3^+$) and anionic (P^-NH_2) species of 6-APA, respectively. k_H, k_0, k'_0 and k'_{OH} are defined in Eq. 1.

k'_{SH} represents the spontaneous hydrolysis of $P^-NH_3^+$. These parameters were computed by a weighting least-squares analysis and are listed in Table V. The curve in Fig. 4 was calculated from these constants, while the points are experimental results.

d) **Effect of Temperature**——The temperature dependence of the hydrolysis of 6-APA was determined by measuring the pseudo-first-order rate constants at various temperature and at constant ionic strength ($\mu=0.5$). A constant pH was maintained by a pH-stat. A list of the observed first-order rate constants, k_{pH}, under various conditions (pH 3.00, 5.00 and pH 10.50) is given in Table V, and the corresponding Arrhenius type plots are shown in Fig. 5. Resultant activation parameters for k_0, k'_{SH} and k'_{OH} at 35° are also listed in Table V.

e) **Primary Salt Effect**——A series of runs was made keeping pH by a pH-stat, but varying the ionic strength by addition of different amount of potassium chloride. The resultant primary salt effects for 6-APA were shown in Fig. 6. A linear positive salt effects were observed at pH 0.70 and pH 10.50, indicating

TABLE V. Rate Constants, k_{pH} and Activation Parameters for the Various Catalytic Rate Constants of the Hydrolysis of 6-APA

pH	Temp. (°C)	Rate constant[a] k_{pH}, hr^{-1}	Catalytic rate constant at 35°	$\Delta H^{\ddagger}$ at 35° kcal/mole	$\Delta S^{\ddagger}$[b] at 35° e.u.
0.70	35	0.175	$k_H=0.461$ M^{-1}hr^{-1}		
3.00	25	1.94×10^{-2}	$k_0=6.66\times10^{-2}$hr^{-1}	13.9	−35.2
	35	3.70			
	40	5.51			
	50	10.9			
5.00	25	3.37×10^{-3}	$k'_{SH}=1.14\times10^{-2}$hr^{-1}	14.1	−38.0
	35	6.80			
	40	12.0			
	50	24.5			
	60	39.2			
7.00	35	3.05×10^{-3}	$k'_0=3.03\times10^{-3}$hr^{-1}		
10.50	35	0.214	$k'_{OH}=2.39\times10^{2}$M^{-1}hr^{-1}	12.0[c]	−25.1
	40	0.431			
	45	0.792			
	50	1.60			

a) Determined in nonbuffered solution of $\mu=0.5$ at various pH which were maintained by a pH-stat.
b) based on M^{-1} sec^{-1} or sec^{-1}
c) The apparent activation energy ΔE_a at pH 10.50 from the Arrhenius plot was 25.7 kcal/mole. By substructing the value of 13.1 kcal/mole[d] as the heat of ionization of water, the heat ΔE_a of 6-APA hydrolysis in basic solution, 12.6 kcal/mole, was obtained.
d) H.S. Harned and W.J. Hammer, *J. Am. Chem. Soc.*, **55**, 2194 (1933)

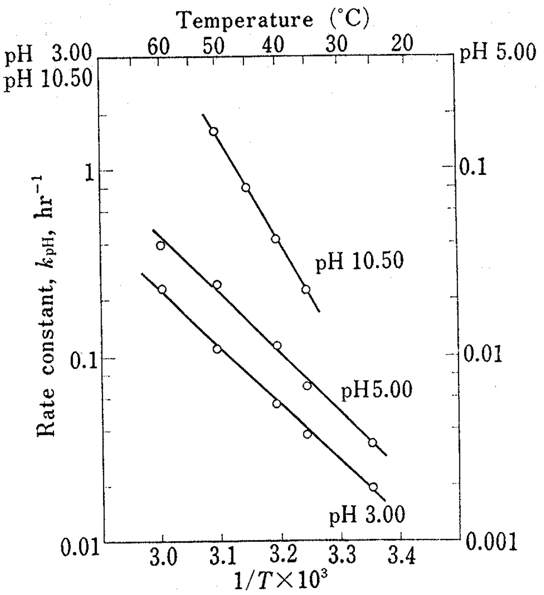

Fig. 5. Arrhenius Plots of the Rate Constants, k_{pH} for the Hydrolysis of 6-APA at Various pH[a] and $\mu=0.5$

[a] pH of the reaction solution was maintained by a pH-stat.

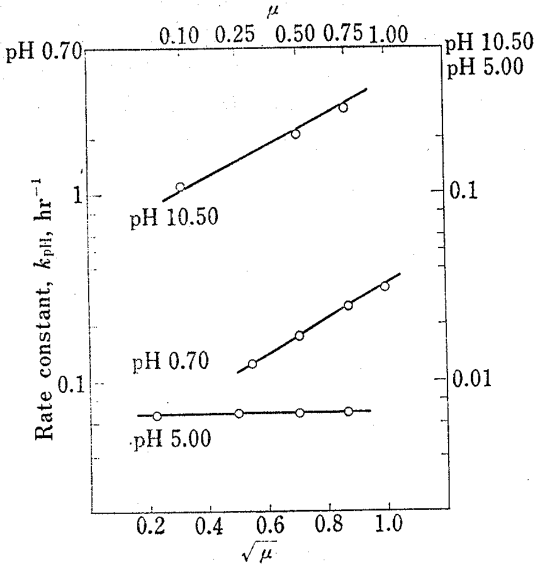

Fig. 6. Effect of the Ionic Strength (μ) on the Pseudo-First-Order Rate Constant, k_{pH} of the Hydrolysis of 6-APA at Different pH Values[a] and 35°

[a] pH of the reaction solution was maintained by a pH-stat.

that the dominating processes at these pH solutions are reactions between two species with the same charge. No significant primary salt effect was observed at pH 5.00.

Discussion

Acidic Hydrolysis

a) Semisynthetic Penicillins——In the log k_{pH}–pH profiles of penicillins, the shoulder-type breaks depending on p*K*a are due to the differential rates between the reaction of hydronium ion-catalyzed hydrolysis of free-acid penicillins (reaction 1) and that of ionized penicillin (reaction 2) or kinetically equivalent spontaneous (*e.g.*, water-catalyzed) hydrolysis of free-acid penicillin (reaction 3). Many investigators[5,6,8–14] have accounted the shoulder-type break appeared in log k_{pH}–pH profile by the reactions 1 and 2. The negative unity slope of pH–rate profile in the acidic site below pH 1 supports the reaction 1 taking place. Although reactions 2 and 3 are kinetically indistiguishable, but a choice between the two is possible on the basis that reaction 2 but not reaction 3 should be very sensitive to primary salt effect. The lacks of ionic strength effect on the rate constants of some penicillins in the partinent pH region, pH 3—4, (see Fig. 3) indicate that reaction 3 is predominantly operative.

A comparison of several penicillins illustrates the large dependence of acidic hydrolysis rate on side-chain structure. It can be seen that methicillin and penicillin G are the most acid-unstable, whereas cyclacillin, ampicillin and new synthesyzed TSPA are fairly acid-stable. Carbenicillin, sulbenicillin, oxacillin, cloxacillin and propicillin show intermediate reactivity in acidic solution. Among these penicillins, the variation in acid stability at pH 1 is over about 600-fold (see Table I). This remarkable effect by side-chain structure is known to be attributed to the rearrangement initiated by attack of the side-chain amido-carbonyl upon the β-lactam as shown in Chart 2.[19] In acidic solution of pH 1—5 at 35°, penicillins studied except

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[19] *a*) F.P. Doyle, J.H.C. Nayler, H. Smith, and E.R. Stove, *Nature*, **191**, 1091 (1961); *b*) for the review see ref. 3*b* and J.P. Hou and J.W. Poole, *J. Pharm. Sci.*, **60**, 503 (1971).

TSPA and 6-APA gave UV absorption maximum at 320—360 nm. During hydrolysis of these penicillins, the absorption at λ_{max} increased and then decreased with time, indicating the presence of the corresponding penicillenic acids.[3b,19b] These are known to be acidic hydrolysis intermediate formed by the intramolecular rearrangement, and to be rappidly hydrolyzed to other products.[19b,20]

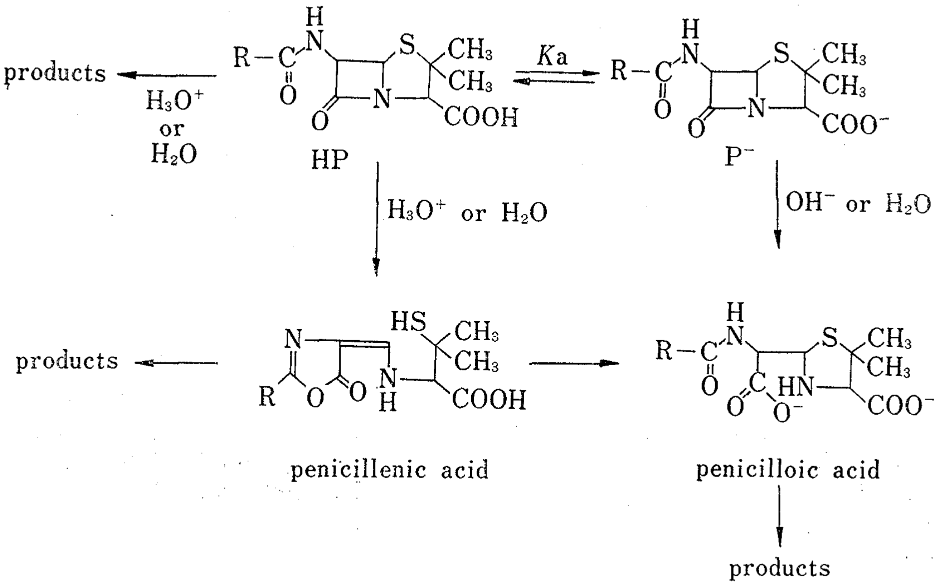

Chart 2. Hydrolysis Pathway of Penicillin in Aqueous Solution

Recently, such a intramolecular catalyzed hydrolysis of amide-bond by a neighboring amide carbonyl-oxygen has been found in the acidic hydrolyses of desthiopenicillins[21] and 2-acylamidobenzamides.[4f,22] Desthiophenoxymethylpenicillin was found to be 7 times stable as desthiobenzylpenicillin in 0.1 N H_2SO_4 at 56.7°.[21] The reactivity of 2-arylamidobenzamides is markedly decreased by electron-withdrawing substituents and correlates well with the Hammett σ-constant to give negative ρ value.[4f] Doyle, *et al.*[19a] have previously found the qualitative correlation between p*K*a of the side-chain acid and the acid-stability of α-substituted benzylpenicillins.

log k_{pH}–pH profiles for the hydrolyses of the substituted phenylpenicillins are shown in Fig. 7. There is a clear dependence of reactivity on substitution in their acidic hydrolyses. The introduction of a electron-withdrawing groups sgnificantly increases the acid-stability, whereas electron-donating groups decrease it. Hammett plots for the rate constants, k_H and k_0 of these hydrolyses are shown in Fig. 8. The values of ρ were -1.60 ($r=0.998$) for both reactions. The magnitude and sign of ρ values in the acidic hydrolyses of the penicillins are consistent with the intramolecular catalyzed mechanism by neighboring side-chain amido groups, as discussed in the acidic hydrolysis of 2-acylamidobenzamides.[4f] The present structure-reactivity correlation is widely applicable for quantitative prediction of acid-stability of newly synthesizing penicillins.

b) 6-APA——Since 6-APA contains no side-chain, it cannot undergo intramolecular rearrangement to form a penicillenic acid type of product. It is, therefore, the most acid stable in strong acid solution (see Table I, II and V).

The log k_{pH}–pH profile at zero buffer concentration gave two shoulder-type breaks in acidic region. This complicated pH dependence is not surprising, as 6-APA can exist in complex

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20) J.L. Longridge and D. Timms, *J. Chem. Soc.* (B), **1971**, 852.
21) A.S. Khokhlov and E.V. Kachalina, *Antibiotiki*, **4**, 3 (1959).
22) T. Cohen and J. Lipowitz, *J. Am. Chem. Soc.*, **86**, 5611 (1964).

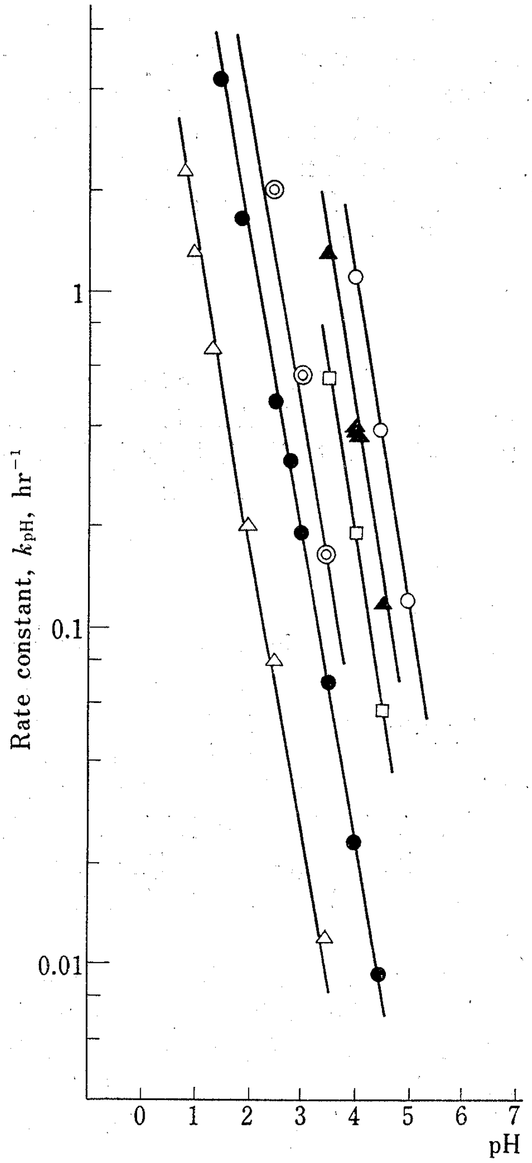

Fig. 7. log k_{pH}–pH Profile for the Hydrolysis of Substituted Phenylpenicillins at 35° and μ=0.5

The drawn lines are calculated from Eq. 1 using the catalytic rate constants listed in Table II. The points are experimental values.

—○—: R=4-CH$_3$ —▲—: R=H
—□—: R=4-Cl —◎—: R=3,4-(Cl)$_2$
—●—: R=4-NO$_2$ —△—: R=3,5-(NO$_2$)$_2$

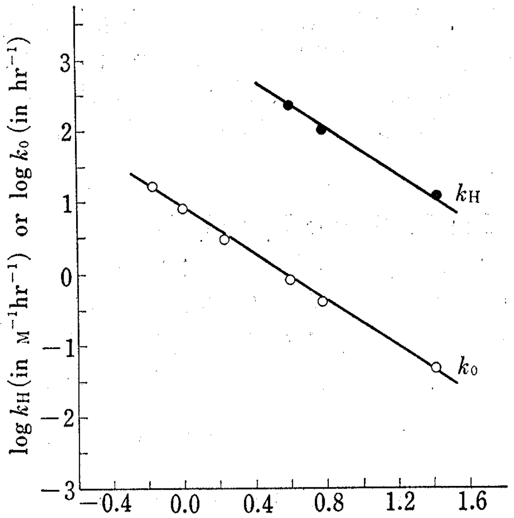

Fig. 8. Hammett Plots for the Catalytic Rate Constants, k_H and k_0 of the Hydrolysis of Substituted Phenylpenicillins at 35° and μ=0.5

$HPNH_3^+ \xrightarrow{H_3O^+}$ products reaction 4
$HPNH_3^+ \xrightarrow{H_2O}$ products reaction 5
$P^-NH_3^+ \xrightarrow{H_3O^+}$ products reaction 6
$P^-NH_3^+ \xrightarrow{H_2O}$ products reaction 7
$P^-NH_2 \xrightarrow{H_3O^+}$ products reaction 8
$P^-NH_2 \xrightarrow{H_2O}$ products reaction 9
$P^-NH_2 \xrightarrow{OH^-}$ products reaction 10

Chart 3. Possible Hydrolytic Reaction of 6-APA in Aqueous Solution

equilibrium of cationic ($HPNH_3^+$), zwitterionic ($P^-NH_3^+$) and anionic (P^-NH_2) forms capable of reactions at different rates *via* differing mechanisms. Possible reactions are given in Chart 3.

The hydrolysis of 6-APA below pH 1 exclusively consists of a specific hydronium ion-catalyzed reaction of $HPNH_3^+$ (reaction 4), which is strongly supported by the positive primary salt effect at pH 0.70 (see Fig. 6).

The shape of the log k_{pH}–pH profile between pH 1 and 3 is thought to be the result of the spontaneous (*e.g.*, water-catalyzed) hydrolysis of $HPNH_3^+$ (reaction 5) or the kinetically equivalent hydronium ion-catalyzed hydrolysis of $P^-NH_3^+$ (reaction 6). It seems likely that reaction 5 is predominantly operative, because the lack of the primary salt effect at pH 3.00 (Fig. 3) was observed in the hydrolysis of TSPA which does not undergo intramolecular rearrangement as well as 6-APA.

The log k_{pH}–pH dependency in the pH region 4—6 may be accounted for by either water-catalyzed hydrolysis of $HPNH_3^+$ (reaction 7) or alternatively by kinetically equivalent specific acid-catalyzed hydrolysis of P^-NH_2 (reaction 8). The lack of the primary salt effect at pH 5.00 (Fig. 6) indicates that the dominating reaction cannot be reaction 8, but reaction 7.

At pH 5, 6-APA is hydrolyzed 3—6 times faster than relatively acid-stable penicillins. This rate-enhancement may be attributed to the inductive effect of the protonated amino group at α-position, which can increase the electrophilic nature of the β-lactam carbonyl center.

Neutral and Alkaline Hydrolysis

It is well known that penicillin hydrolyzes in neutral and basic solutions to form penicilloic acid (see Chart 2).[3,19b,23] The principal hydrolysis product of 6-APA in the low concentration has been found to be penicic acid (open-lactam 6-APA).[18] Their hydrolytic rate constants, k_{obs}, in aqueous buffer of pH greater than *ca.* 6 can be formulated as:

$$k_{obs} = k'_0 + k'_{OH}[OH^-] + k'_B[B] \qquad (8)$$

where k'_B is the catalytic rate constant for general base, B.

All penicillins are remarkably susceptible to the attack by nucleophiles such as hydroxide ion or several amines.[3a,b] The fused β-lactam thiazolidine structure in penicillin molecule is much more sensitive to such reagents than simple β-lactams, ordinary amides and unstrained lactams.[3c] The k_{OH} values (*ca.* $10^3 M^{-1}hr^{-1}$) of penicillins and 6-APA are approximately 10^4 times greater than those of N-substituted amides (*ca.* $10^{-1} M^{-1}hr^{-1}$)[3c,4a] and β-lactams (*ca.* $10^{-1} M^{-1}hr^{-1}$)[3c] under the same condition (at 35°). This high reactivities of penicillins and 6-APA seem to be reflected in the low heat of activation (9—13 kcal/mole) compared with high values of activation enthalpies (14—17 kcal/mole) for the basic hydrolyses of above cited amides[3c,4a] and β-lactams.[3c]

Some investigators[24] have stated that participation of the neighboring acylamido groups as observed in their acidic hydrolyses contribute partially to the lability of penicillin β-lactam hydrolysis in neutral and basic regions. If the enhanced reactivity of spontaneous and alkaline hydrolyses of penicillin would be attributed not only to the structural characterlistics of penicillin nucleus, but significantly to such a intramolecular catalysis by the neighboring side-chain amido group, it may be possible to make, by chemical modification, penicillins which will be sufficiently stable to allow marketing as liquid dosage form. Table I shows that the influences of the structure of the side-chains upon k'_{OH} and k'_B (*e.g.*, for $HPO_4^=$) are comparatively small. No significantes in reactivity of various penicillins were also observed in their spontaneous hydrolyses (k'_0). The reactivity of substituted phenylpenicillin series, which is expected to probably give a remarkable polarization effect on the reaction rates, is almost the same within the experimental error (see Table II). It may be said, therefore, that the enhanced reactivities of alkaline and spontaneous hydrolyses of penicillins are not primary due to the intramolecular catalysis suspected previously,[24] and that spontaneous reactions observed in all penicillin hydrolyses are probably resultant of kinetically equivalent nucleophilicor general base-catalysis by water molecule.

As noted in the hydrolyses of TSPA and 6-APA, water catalyzed hydrolysis of undissociated penicillin (HP) observed in acidic region is *ca.* 8—40 times faster than water-catalyzed hydrolysis of penicillinate ion (P^-). Such a difference in the two spontaneous reactions may be reflected in the reactivity of penicillin ester. The k'_0 and k'_{OH} values of benzylpenicillin

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23) J.R. Johnson, R.B. Woodward, and R. Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, 1949, p. 440.

24) *a*) F.P. Doyle and J.H.C. Nayler in "Advances in Drug Research," Vol. 1., ed. by N.J. Harpter and A.B. Simmonds, Academic Press, New York, 1964, p. 1; *b*) F. Moll, *Arch. Pharm.*, **301**, 272 (1968); *c*) ref. 3*a*.

methyl ester[25] are, respectively, *ca.* 10^2 and *ca.* 10 times greater than those of benzylpenicillin under the same condition, but the acidic hydrolyses of above both types of penicillin have almost the same reactivity (see Table I).

Pharmaceutical Considerations

From the pH profile in nonbuffered solution, it can be observed that the pH of the maximum stability is about 6—7 for all penicillins. The fact that the optimum pH lies in the neutral region is quite suitable for formulation of pharmaceuticals. Table VI gives the calculated time of 10% loss of activity ($t_{0.90}$) at maximum stability pH in nonbuffered solution at 35°, 25° and 4° of several penicillins. Relatively stable penicillins are ampicillin, cloxacillin, oxacillin and propicillin, which exhibit predicted $t_{0.90}$ to be about 4 days at 35°, 12 days at 25° and 100—200 days at 4°.

TABLE VI. Predicted 10% Loss-Period ($t_{0.90}$) for the Hydrolysis in Nonbuffered Solution of Penicillins at Maximum-Stability pH ($\mathrm{pH_{min}}$) and at Various Temperatures

Penicillin	Temp. (°C)	$\mathrm{pH_{min}}$[a)]	$10^4 k_{\mathrm{pHmin}}$[b)] ($\mathrm{hr^{-1}}$)	$t_{0.90}$[c)] (day)
Penicillin G	35	6.9	13.4	3.3
Methicillin	35	6.8	14.0	3.1
	25	6.9	3.9	11
	4	7.1	0.22	199
Carbenicillin	35	6.9	24.4	1.8
Oxacillin	35	6.4	12.0	3.6
	25	6.5	3.6	12
	4	6.7	0.20	219
Cloxacillin	35	6.3	10.3	4.2
	25	6.3	3.6	12
	4	6.5	0.31	141
Sulbenicillin	35	6.2	18.5	2.4
Propicillin	35	6.1	9.8	4.5
Cyclacillin	35	6.0	25.2	1.7
Ampicillin	35	5.8	8.2	5.3

a) calculated from the equation: $\mathrm{pH_{min}} = (\log k_0/Ka - \log k'_{\mathrm{OH}} + \mathrm{p}K_{\mathrm{w}}/2)$

b) k_{pHmin} is the apparent first-order rate constant at $\mathrm{pH_{min}}$ and can be calculated from the equation: $k_{\mathrm{pHmin}} = k_0[\mathrm{H_3O^+}]/Ka + k'_0 + k'_{\mathrm{OH}}[\mathrm{OH^-}]$. The catalytic rate constants at desired temperature were calculated from those at 35° and ΔE_{a} (from Table I and III).

c) calculated from the equation: $t_{0.90} = 0.105/k_{\mathrm{pHmin}}$ (hr)

The present accerelated stability study indicates that the pharmaceutical products of penicillins intended for administration in liquid dosage forms must be marketed as dry preparations and reconstituted before use. To maintain the pH of these penicillin solutions at their optimum pH, citrate buffer rather than phosphate buffer may be suitable for use because citrate ions are found, in the present and the earlier works[7,12,14] on the penicillin hydrolyses, to have negligible catalytic effect on the hydrolysis at pH 6—6.5 in contrast with the large catalytic effect by phosphate buffer components in this pH region.

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25) M.A. Schwartz, *J. Pharm. Sci.*, **54**, 1308 (1965).