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Stabilization of Ampicillin Analogs in Aqueous Solution. II.[1] Kinetic Analysis of the Mechanism of Degradation of Ampicillin with Benzaldehyde in Aqueous Solution

Hiroshi Fujiwara, Susumu Kawashima,* and Masako Ohhashi

School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa 920-11, Japan

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Ampicillin, buffered to pH 8.00, in the presence of benzaldehyde was found to degrade according to pseudo-first-order kinetics at 35°C, and its rate constant was smaller than that of ampicillin alone. This stabilization was assumed to be attributable to the formation of ampicillin–benzaldehyde complex. Therefore, pseudo-first-order rate constants of ampicillin in the presence of various amounts of benzaldehyde were determined. It was concluded that a 1:1 molar complex was formed between ampicillin and benzaldehyde, and that the reversible complexation reaction was very fast compared to the degradation reaction of ampicillin or the complex.

From the degradation behavior in various concentrations of buffer at the same pH, it was clear that the stability constant of the complex was independent of buffer concentration, and that the degradation of complexed ampicillin was affected by general acid–base catalysis in the same way as that of uncomplexed ampicillin. The complexation was not observed at pH <6.00, while the degradation of ampicillin was inhibited by the addition of benzaldehyde at pH >7.00.

The suppresion of the degradation of complexed ampicillin seems to be due to the inhibition of hydroxy ion attack on the β-lactam carbonyl group of the complex. Furthermore, it was shown that only the ampicillin anion (uncharged α-amino group) participates in the complexation, because the stability constant of the complex increased with increase of pH.

Keywords——ampicillin; benzaldehyde; ampicillin–benzaldehyde complex; stability of complex; stability constant; double reciprocal plot; general acid-base catalysis; pH–rate profile; catalytic rate constant; equilibrium state

In a previous paper,[1] we reported that the degradation of ampicillin follows apparent first-order kinetics at pH 8.00 and 35°C in the presence of benzaldehyde and was slower than that in the absence of benzaldehyde. This stabilization seemed to be attributable to complexation between these compounds.

In this paper, this interaction of ampicillin and benzaldehyde was investigated by determination of its kinetic parameters.

Experimental

Materials——Ampicillin sodium (Sigma Chemical Company) was of guaranteed reagent grade. Benzaldehyde and all other chemicals were of the highest commercial grade available and were used without further purification.

Reagents——All reagents used for I_2-colorimetry were ones described in the previous paper.

Buffer Solutions——The buffer systems used were as follows: at pH 4.00, phthalate–HCl; at pH 5.00, CH_3COONa–HCl; at pH 6.00—8.00, NaH_2PO_4–Na_2HPO_4 and at pH 8.50—9.00, H_3BO_3–NaOH. The ionic strength of these buffers was adjusted to 0.5 by the addition of KCl. The pH of buffers was measured at the experimental temperature with a Toa pH meter, model HM-18ET.

Determination of Benzaldehyde——High performance liquid chromatography (HPLC) was used to determine benzaldehyde concentration in aqueous solution. The apparatus was a Waters HPLC system, model 6000A/U6K, equipped with a UV detector, SOMA s-310A, set at 240 nm and a stainless steel column, 4 mm i.d. × 300 mm. μ-Bondapak C_{18} and 50% aqueous methanol were used as the stationary and mobile phases, respectively. The sample was eluted at a flow rate of 1 ml/min at room temperature. The amount of benzaldehyde was determined by comparing the peak area with that of the similarly chromatographed standard. A fresh calibration curve for the substance was obtained daily.

Kinetic Procedures——All kinetic studies were carried out at 35±0.1°C. Ampicillin was dissolved in an appropriate buffer solution (with or without benzaldehyde) which had been preheated to the desired temperature. The initial concentrations of ampicillin and benzaldehyde were 2.5×10^{-4} M and 2.5×10^{-3}—2.5×10^{-2} M, respectively; the two substances were completely soluble in the buffer solution. At suitable intervals, a sample was withdrawn, cooled and assayed for intact ampicillin by I_2-colorimetry as described in the previous paper.

Results and Discussion

Effect of Benzaldehyde on the Degradation of Ampicillin in Aqueous Solution

Time courses of the degradation of total substances biologically active as ampicillin in 0.07 M phosphate buffer (pH 8.00, μ=0.5) at 35°C which initially contained ampicillin and various concentrations of benzaldehyde are shown in Fig. 1. In each case, a plot of the logarithm of residual percent *versus* time gave a straight line with a slope which decreased when benzaldehyde was present in the solution. Thus, increasing concentrations of benzaldehyde tended to decrease the degradation of ampicillin.

Pseudo-first-order rate constants calculated from these slopes were plotted against the concentration of benzaldehyde (Fig. 2). As can be seen in Fig. 2, a linear relationship was not observed in these plots, so this reaction was assumed to occur according to the scheme in Chart 1.[2] Consequently, since the total concentration, $[A]_T$, of intact ampicillin present in the solution was determined by the I_2-colorimetry, the overall reaction rate is given by Eq. (1):

$$-\frac{d}{dt}([A]+[AB]) = -\frac{d}{dt}[A]_T = k_a[A] + k_c[AB] \quad (1)$$

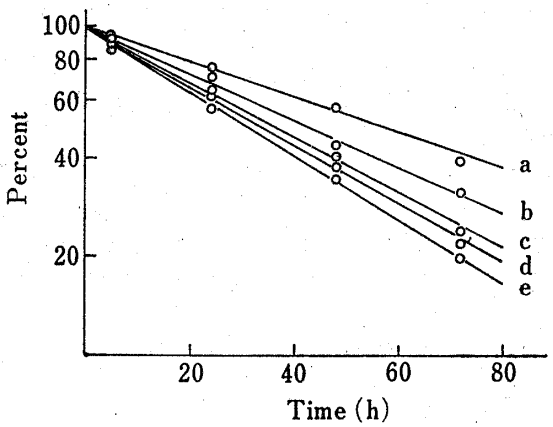

Fig. 1. First-Order Plots for the Degradation of Ampicillin in the Presence and Absence of Benzaldehyde in 0.07 M Phosphate Buffer (pH 8.00) at 35°C and μ=0.5

Ampicillin conc. 2.5×10^{-4} M; benzaldehyde conc. a=2.5×10^{-2}, b=1.0×10^{-2}, c=5.0×10^{-3}, d=2.5×10^{-3} and e=0 M.

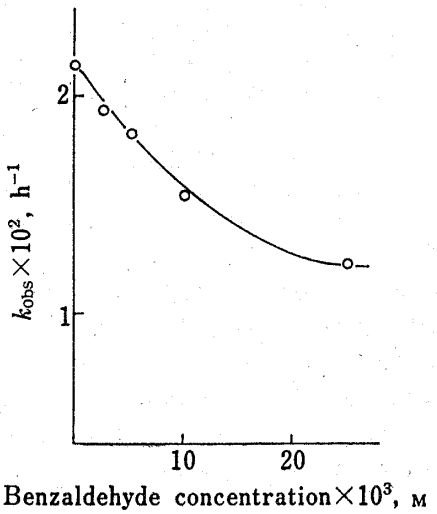

Fig. 2. Effect of Benzaldehyde on the Apparent First-Order Rate Constant (k_{obs}) for Ampicillin Degradation in 0.07 M Phosphate Buffer (pH 8.00) at 35°C and μ=0.5

The solid line is calculated from Eq. (4) and the parameters listed in Table I.

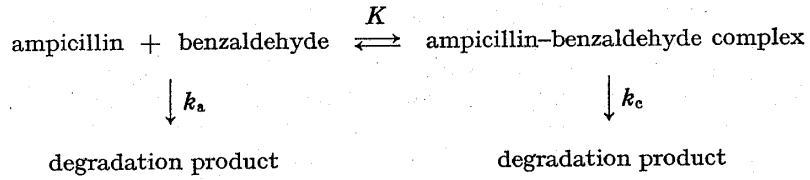

Chart 1

where k_a=first-order rate constant of β-lactam cleavage of ampicillin; k_c=first-order rate constant of β-lactam cleavage of complex; $[A]$=concentration of free ampicillin; $[AB]$=concentration of complex.

If 1 : 1 complex formation is assumed, the stability constant, K, can be expressed as follows:

$$K = \frac{[AB]}{[A][B]} \tag{2}$$

where $[B]$ is the concentration of benzaldehyde.

The reversible rates of complexation in the solution of ampicillin with benzaldehyde seem to be very fast compared with the degradation rates of free ampicillin and the complex, because the overall reaction was observed to follow first-order kinetics.

From Eqs. (1) and (2), Eq. (3) can be derived.

$$-\frac{d}{dt}[A]_T = \left\{k_a\frac{1}{1+K[B]} + k_c\frac{K[B]}{1+K[B]}\right\}[A]_T = k_{obs}[A]_T \tag{3}$$

In order for Eq. (3) to give an apparent first-order reaction, however, it is necessary to use a large excess of benzaldehyde to ampicillin, so that the concentration of benzaldehyde can be regarded as constant even if complex is formed and/or benzaldehyde is decomposed in the reaction process. Under the reaction conditions described in the experimental section, very little loss of benzaldehyde occurred at 35°C within 48 h at pH 8.00 or 5 h at pH 9.00, and the concentration of benzaldehyde used was at least 10 times that of ampicillin. Thus, Eq. (3) should yield a pseudo-first-order reaction.

The pseudo-first-order rate constant, k_{obs}, is given by Eq. (4) from Eq. (3).

$$k_{obs} = \frac{k_a + k_c \cdot K[B]}{1+K[B]} \tag{4}$$

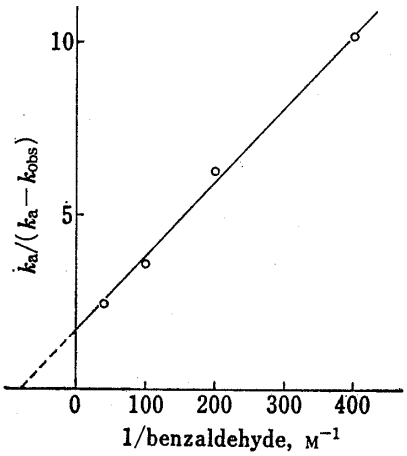

Fig. 3. Double Reciprocal Plot for the Complexation between Ampicillin and Benzaldehyde according to Eq. (5)

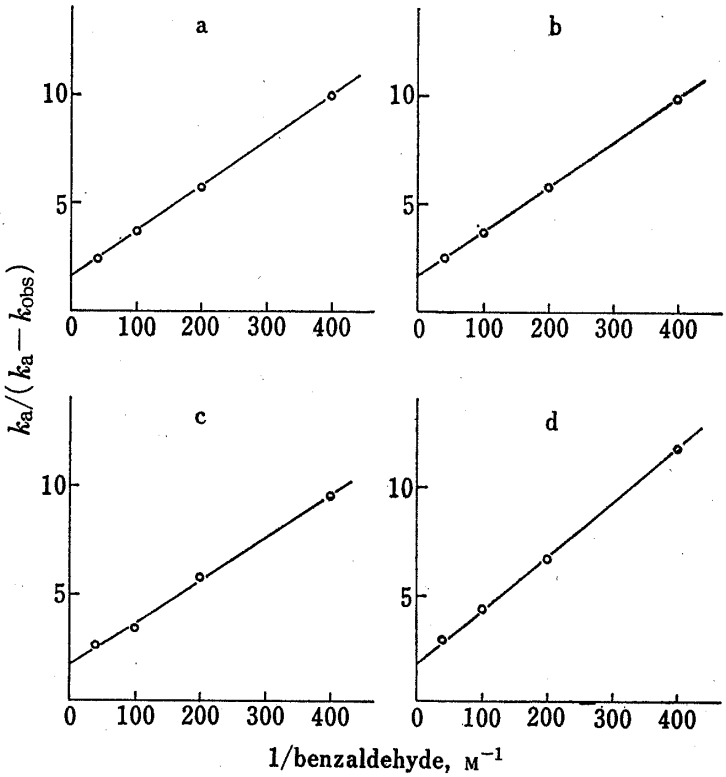

Fig. 4. Double Reciprocal Plots for Various Phosphate Buffer Concentrations (pH 8.00)

a: 0.11 M; b: 0.10 M; c: 0.06 M; d: 0.05 M.

Next, substituting q for $(1-k_c/k_a)$, Eq. (4) is converted to Eq. (5).

$$\frac{k_a}{k_a-k_{obs}} = \frac{1}{qK}\frac{1}{[B]} + \frac{1}{q} \quad (5)$$

By means of a double reciprocal plot[2-4] based on Eq. (5), if the relation between $k_a/(k_a-k_{obs})$ and $1/[B]$ is linear, the stability constant, K, and the degradation rate constant, k_c, can be obtained for the reaction mechanism shown in Chart 1. By the use of various concentrations of benzaldehyde, $[B]$, with the k_{obs} and k_a values from Fig. 2, linear plots similar to that in Fig. 3 were obtained. The values of the intercept and slope were 1.69 and 2.20×10^{-2} M by the least-squares method, and k_c and K were calculated to be 0.88×10^{-2} h^{-1} and 76.9 M^{-1}, respectively.

Catalytic Effect of Buffer on the Degradation of Ampicillin-Benzaldehyde Complex in Aqueous Solution

The degradation of ampicillin is well known to be influenced by general acid–base catalysis, and thus the observed first-order rate constants in phosphate buffer solution increase linearly with the buffer concentration.[5] Since it was thought that the degradation of complex might be similarly affected by buffer, hydrolysis at various buffer concentrations was examined.

Double reciprocal plots were obtained by substituting into Eq. (5) the pseudo-first-order degradation rate constants which were obtained when ampicillin was reacted with various concentrations of benzaldehyde in various phosphate buffer concentrations (pH 8.00, $\mu=0.5$) at 35°C (Fig. 4). A linear relationship was obtained in each plot, and k_c and K, calculated by the least-squares method at each concentration, are summarized in Table I.

Table I. Stability Constant and First-Order Rate Constants of Ampicillin (k_a) and Its Complex (k_c) in Various Phosphate Buffer Concentrations at pH 8.00, 35°C, and $\mu=0.5$

Constant	Buffer concentration (M)				
	0.05	0.06	0.07	0.10	0.11
K M^{-1}	74.6	87.4	76.9	79.0	76.7
$k_c\times10^2$ h^{-1}	0.71	0.83	0.88	1.03	1.11
$k_a\times10^2$ h^{-1}	1.54	1.95	2.15	2.68	2.90

The degradation rate constants of ampicillin, k_a, in the same buffers as described above are also shown in Table I and are in fair agreement with Hou's data. The plots of k_c and k_a *versus* buffer concentration gave straight lines as shown in Fig. 5. This suggests that not only ampicillin but also the complex is hydrolyzed by general acid–base catalysis.

The relations between k_a, k_c and buffer concentration can thus be expressed in terms of Eqs. (6) and (7), respectively.

$$k_a = k_{ap}[P] + k_a^0 \quad (6)$$

$$k_c = k_{cp}[P] + k_c^0 \quad (7)$$

Here, $[P]$ is total phosphate buffer concentration, k_{ap} and k_{cp} are the catalytic rate constants of ampicillin and complex, and k_a^0 and k_c^0 are the buffer-free rate constants of ampicillin and complex, respectively. Therefore, from Eqs. (4), (6) and (7), the pseudo-first-order rate constant, k_{obs}, in phosphate buffer of pH 8.00 and $\mu=0.5$ can be derived as follows:

$$k_{obs} = \frac{1}{1+K[B]}(k_a^0+K[B]k_c^0) + \frac{1}{1+K[B]}(k_{ap}+K[B]k_{cp})[P] \quad (8)$$

By least-squares calculation from the results of Fig. 5, k_{ap}, k_{cp}, k_a^c and k_c^0 were obtained as 0.210 M^{-1} h^{-1}, 0.0609 M^{-1} h^{-1}, 0.00602 h^{-1} and 0.0044 h^{-1}, respectively. Further, as can be

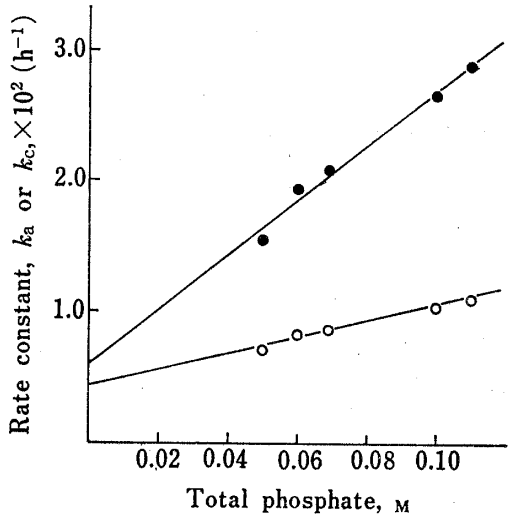

Fig. 5. Plots of Rate Constant, k_a or k_c *versus* Buffer Concentrations at pH 8.00

●, k_a; ○, k_c.

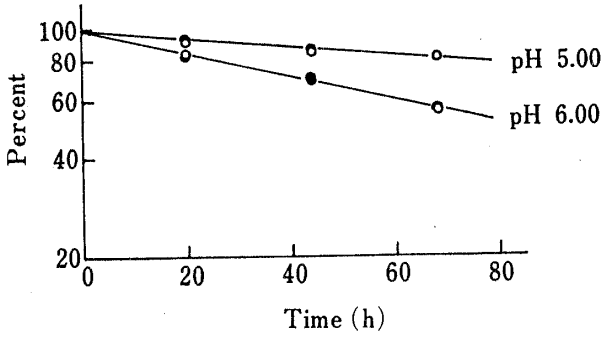

Fig. 6. First-Order Plots for the Degradation of Ampicillin in the Presence (●) and Absence (○) of Benzaldehyde at Various pH Values, 35°C, and $\mu=0.5$

The buffers used were 0.1 M acetate (pH 5.00) and 0.1 M phosphate (pH 6.00). Initial concentrations of ampicillin and benzaldehyde were 2.5×10^{-4} and 1.0×10^{-2} M, respectively.

seen in Table I, K was almost constant regardless of the buffer concentration and on average was 78.9 M^{-1}.

From the above results, it was found that the smaller catalytic effect of phosphate on the complex (k_{cp}) and the slower degradation rate of the complex (k_c^0) compared to that of ampicillin alone contributed to the stabilization of complexed ampicillin as compared with uncomplexed ampicillin in the buffer of pH 8.00.

Effect of pH on the Kinetic Parameters of Ampicillin–Benzaldehyde Complex in Aqueous Solution

As described previously, the complex was not formed and no change of the degradation rate was recognized on addition of benzaldehyde to the buffer of pH 4.00. Further, no effects of benzaldehyde addition were found at pH 5.00 and 6.00, as shown in Fig. 6. The pseudo-first-order rate constant of ampicillin with benzaldehyde, however, became smaller with increase of benzaldehyde concentration above pH 7.00.

At pH's 7—8, di- and monohydrogen phosphate ions catalyze the degradation of ampicillin. Because the degradation of ampicillin–benzaldehyde complex in aqueous solution is also thought to occur similarly, k_{ap} and k_{cp} in Eqs. (6) and (7) can be expressed as follows:

$$k_{ap} = \frac{k_{ap1}aH + k_{ap2}K_2}{aH + K_2} \tag{9}$$

$$k_{cp} = \frac{k_{cp1}aH + k_{cp2}K_2}{aH + K_2} \tag{10}$$

where k_{ap1} and k_{ap2} are the catalytic rate constants of ampicillin degradation due to di- and monohydrogen phosphate ion, k_{cp1} and k_{cp2} are the catalytic rate constants of degradation of the complex due to di- and monohydrogen phosphate ion, respectively, K_2 is the apparent dissociation constant of dihydrogen phosphate ion ($pK_2=7.21$[5]), and aH is the activity of hydrogen ion as measured with the glass electrode. Double reciprocal plots of the pseudo-first-order rate constant of ampicillin degradation *versus* the amount of benzaldehyde at pH 7.50 are shown in Fig. 7. A linear relation was obtained between $1/[B]$ and $k_a/(k_a-k_{obs})$ as at pH 8.00. Values of K and k_c of 64 M^{-1} and 0.00654 h^{-1}, respectively, were obtained from the intercept and slope of the plot by the least-squares method.

Plots of pseudo-first-order rate constants with and without benzaldehyde *versus* buffer concentrations are shown in Fig. 8. By applying Eq. (6) to the results in Fig. 8, k_a^0 and k_{ap} were calculated to be 0.0019 h^{-1} and 0.174 M^{-1} h^{-1}, respectively. Furthermore, since the stabil-

ity constant, K, of ampicillin with benzaldehyde was almost independent of buffer concentration at pH 8.00, this parameter was expressed to be constant at pH 7.50. The changes of first-order rate constants in the presence of benzaldehyde shown in Fig. 8 satisfy Eq. (8), and thus the buffer-free rate constant, k_c^0, can be calculated from the pseudo-first-order rate constants in the presence and absence of benzaldehyde in the phosphate buffer of 0.05 and 0.15 M. The second-order catalytic rate constant, k_{cp}, at pH 7.50 can be determined from k_c^0 at each concentration of the buffer at pH 7.50 and values of 0.00132 h^{-1} for k_c^0 and 0.0522 M^{-1} h^{-1} for k_{cp} were obtained.

Substituting k_{ap} and k_{cp} at pH 7.50 and 8.00 into Eqs. (9) and (10) yielded values of 0.0771, 0.1661, 0.018 and 0.071 M^{-1} h^{-1} for k_{ap1},[5] k_{ap2},[2] k_{cp1} and k_{cp2}, respectively.

The catalytic rate constant of each ionic species of buffer for the degradation of the complex was smaller than that for ampicillin degradation. The stabilization of complexed ampicillin in aqueous solution was found to result from a decrease of the anionic catalytic effect of the buffer.

Borate buffer was used for the runs at pH 8.50 and 9.00, but no apparent catalytic effects of borate buffer were observed on the degradation rate of ampicillin irrespective of the addition of benzaldehyde, while the rate constants became smaller with increase of benzaldehyde concentration. Hou *et al.* demonstrated that borate ion had a negligible effect in comparison to the hydroxide ion catalysis of the degradation of ampicillin.

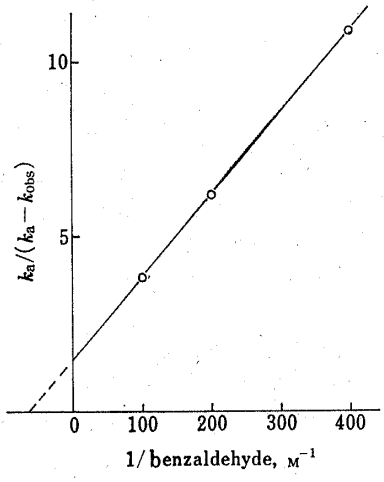

Fig. 7. Double Reciprocal Plot for the Complexation in 0.1 M Phosphate Buffer (pH 7.50) at 35°C and μ= 0.5

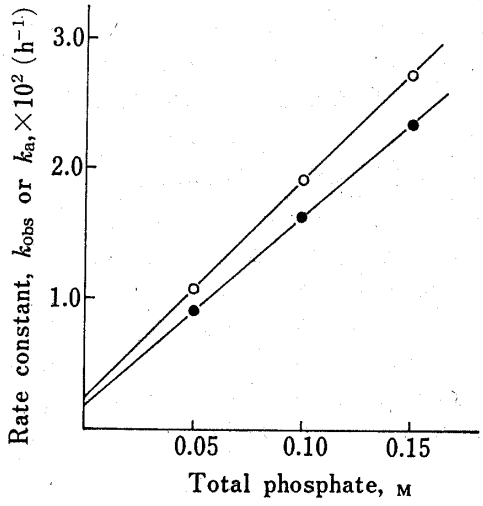

Fig. 8. Plots of First-Order Rate Constant, k_{obs} (●) or k_a (○) *versus* Buffer Concentration at pH 7.50, 35°C, and μ=0.5

Initial concentrations of ampicillin and benzaldehyde were 2.5×10^{-4} and 5.0×10^{-3} M, respectively.

TABLE II. Stability Constant and Buffer-Free Rate Constants of Ampicillin (k_a^0) and Its Complex (k_c^0) at Various pH Values and at 35°C and μ=0.5

Constant	pH							
	4	5	6	7	7.5	8	8.5	9
$k_a^0\times10^2$ h^{-1}				0.09	0.191	0.59	1.70	5.37
$k_c^0\times10^2$ h^{-1}	0	0	0	0.038	0.132	0.44	1.38	4.17
K M^{-1}	0	0	0	48	64	78.9	85	86.8

K, k_a^0 and k_c^0 values obtained from double reciprocal plots at pH 4—9 are summarized in Table II. As can be seen in Table II, the degradation rate constant of the complex is smaller than that of ampicillin, but increases with increase of pH, as in the case of ampicillin. In addition, the stability constant of complexation increased with increase of pH. Plots of the logarithm of the buffer-free degradation rate constant, k_c^0, of the complex against pH gave a slope of unity as shown in Fig. 9.

Thus, the buffer-free degradation rate constant of the complex, k_c^0, can be expressed as follows:

$$k_c^0 = k_{c\,H_2O} + k_{c\,OH} \cdot a\mathrm{OH} \tag{11}$$

where $k_{c\,H_2O}$ and $k_{c\,OH}$ are specific rate constants due to water and hydroxy ion, respectively.

Since only hydroxy ion shows a catalytic effect at pH$>$7.00 and the relation of $k_{c\,H_2O} \ll k_{c\,OH} \cdot a\mathrm{OH}$ is suggested, Eq. (11) reduces to Eq. (12).

$$k_c^0 = k_{c\,OH} \cdot a\mathrm{OH} \tag{12}$$

Eq. (12) can be converted to Eq. (13).

$$\begin{aligned} \log k_c^0 &= \log k_{c\,OH} - \mathrm{pOH} \\ &= \log k_{c\,OH} - \mathrm{p}K_w + \mathrm{pH} \end{aligned} \tag{13}$$

Here, as pK_w is reported to be 13.68[4] at 35°C, a value of 1995.3 $M^{-1}\,h^{-1}$ is obtained for $k_{c\,OH}$ by substituting the results of Fig. 9 into Eq. (13). On the other hand, $k_{a\,OH}$ of ampicillin was 2973.0 $M^{-1}\,h^{-1}$, in accord with the result reported by Hou *et al.* It was thus clear that the nucleophilic attack of hydroxy ion on the β-lactam carbonyl carbon atom of the complex is minor compared with that in the case of ampicillin. Hydrolysis in this pH region is well known to occur by a nucleophilic or general base catalytic reaction.[6,7] Thus, it seems that the fact that the complex is more stable than ampicillin is due to the difficulty of hydroxy ion attack on the β-lactam carbonyl group resulting from an enhancement of the usual amino resonance[8] and/or steric effects in the ampicillin–benzaldehyde complex.

The k_a^0–pH profile for the degradation of ampicillin was not linear at pH$<$7.50 as can be seen in Fig. 9. The slope of the k_c^0 –pH profile of the complex (equal to unity), unlike that of ampicillin, at pH$>$7.00 supports the assumption that there is a hydroxy ion catalytic effect for the complex at pH 7.00 and that the velocity of spontaneous (or H_2O) reaction for free ampicillin[5] is smaller than that of hydroxy ion reaction for the complex.

Relationship between Stability Constant and pH

The results in Table II show that the apparent stability constant, K, obtained from double reciprocal plots changed with pH and increased with increase of pH in the alkaline region. Thus, only specific ionic species of ampicillin were assumed to be involved in the complexation equilibrium with benzaldehyde.

In the pH range where the complex is formed, ampicillin exists as a zwitterion and so the dissociation constant, K_a, of ampicillin is as follows:

$$K_a = \frac{a\mathrm{H}[A^-]}{[A\mathrm{H}^\pm]} \tag{14}$$

where $[A\mathrm{H}^\pm]$ and $[A^-]$ are the zwitterionic species and anionic species of ampicillin, respectively.

As described above, since no complexation occur at pH $<$6.00, and K_a is 8.91×10^{-8} M,[5] the complexation seems to occur between ampicillin anion and benzaldehyde. Thus, the absolute stability constant, K_s, is given by the following equation:

$$K_s = \frac{[AB]}{[A^-][B]} \tag{15}$$

where $[AB]$, $[A^-]$ and $[B]$ are the complex concentration, ampicillin concentration and benzal-

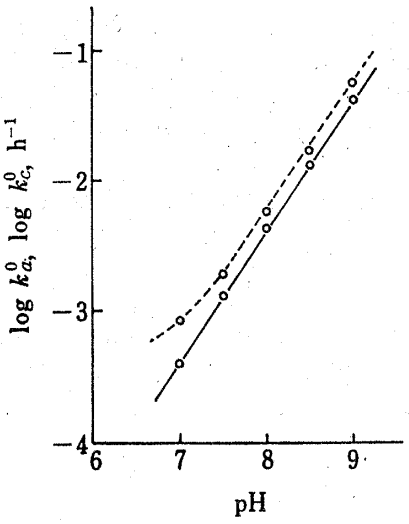

Fig. 9. log k–pH Profile for the Degradation of Ampicillin and the Complex at 35°C and $\mu=0.5$

The solid line was calculated from Eq. (13) using $k_{c\cdot OH}=1995.3$ M^{-1} h^{-1} and the dashed line is the log k_a–pH profile for ampicillin taken from ref. 5. The points are experimental values.

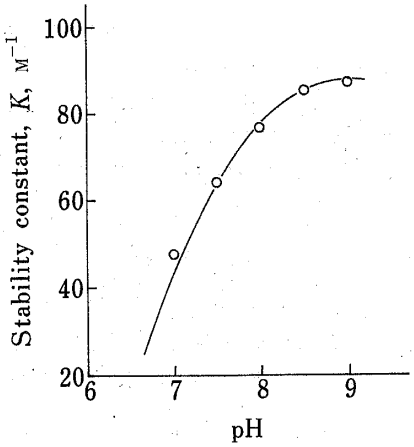

Fig. 10. Plot of the Stability Constant, K, of Ampicillin–Benzaldehyde Complex as a Function of pH in Aqueous Solution at 35°C and $\mu=0.5$

The solid line was calculated by means of Eq. (16) from K_s, K_a and aH values by the least-squares method, while the points represent experimental values.

dehyde concentration, respectively.

The observed stability constant (K) at various pH's therefore, is given by Eq. (16), obtained from Eqs. (14) and (15):

$$K=\frac{[AB]}{[A]_T^f[B]}=K_s\frac{K_a}{K_a+a\mathrm{H}} \tag{16}$$

where $[A]_T^f$ is total ampicillin concentration. A value of K_s of 88.1 M^{-1} was obtained from the K data listed in Table II by means of the least-squares method. Further, a plot of K *versus* pH is shown in Fig. 10; the solid line represents a theoretical value of K calculated from K_s.

The good agreement of the theoretical and experimental relationships of K and pH shown in Fig. 10 supports the validity of the scheme proposed in Chart 1.

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