Acceleration and Inhibition of the Hydrolysis of Penicillin G by Dimerization and Cyclodextrin Inclusion[1)]

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Quantitative treatments for the equilibrium and kinetics of dimerizing, cyclodextrin (CD)-binding, and hydrolyzing systems in unbuffered and buffered solutions have been developed and applied to hydrolysis and pH data on the penicillin G (PCG)-CD system. By dimerization of PCG, the acidic hydrolysis of PCG is accelerated, whereas the basic hydrolysis is inhibited. This result is explicable in terms of electrostatic interactions of the PCG dimer with hydrogen ion and hydroxide ion, similar to the micellar effect on chemical reactions. In buffered solutions, the acidic hydrolysis of PCG is inhibited by all of α-, β-, and γ-CDs. This is explained in terms of the reaction mechanism and the enolation of the secondary hydroxyl group of CD. γ-CD inhibits the acidic hydrolysis most effectively of the three CDs, since its binding constant is the greatest among them. The dimer of PCG can be incorporated into γ-CD, but not incorporated into α- and β-CDs. In a 154 mmol dm^{-3} potassium chloride solution, the acidic hydrolysis of 5 mmol dm^{-3} PCG is enhanced by α- and β-CDs. This striking result can be explained by the catalysis of hydrogen carbonate ion. A commercial sample of α-CD catalyzes the acidic hydrolysis of PCG linearly with the concentration of α-CD, whereas a purified sample catalyzes the same reaction, following Michaelis–Menten-like kinetics. CDs, particularly γ-CD, may be used as an additive for the stabilization of PCG.

Key words penicillin G; hydrolysis; cyclodextrin; dimerization

Cyclodextrins (CDs) can entrap organic hydrophobic compunds into their interior cavities. Generally, for aqueous solutions with no extreme concentrations, the 1 : 1 stoichiometry of CD–guest complexes is predominant.[1-4)] Two guest molecules like methyl orange can be included in one or two γ-CD molecules.[5)] One guest molecule, such as a surfactant, may be entrapped in two β-CD molecules. Thus, depending on the size and shape of guests and CDs, ternary and quaternary complexes of the surfactant can be formed.[6)] As an extreme case, Harada *et al.* "synthesized" a molecular necklace (a rotaxane) which is composed of a polyethylene oxide molecule and many CD molecules.[7)] Several CD dimers, which contain two CD molecules bridged by chemical bonds, can bind the second guest molecule cooperatively[8)] and can speed up chemical reactions more than the corresponding CD monomer.[9)]

A number of organic molecules, including surfactants, dyes, and drugs, self-associate in aqueous media by hydrophobic interactions.[10,11)] For instance, it was shown by chromatography that penicillin G (PCG) can form the dimer without changes in chemical structure.[12)] Following the discovery of penicillin by Fleming, their mass production had been hampered by their chemical instability.[13)] Since penicillins are slowly hydrolyzed in aqueous solutions,[14-17)] their powder is dissolved into a physiological saline solution immediately before injection.[14,15)] The stabilization of penicillins is required for their use in hospitals; for medical use, their stability in unbuffered solutions needs to be investigated. The effects of the self-association of penicillins on CD inclusion and stability are also of interest from an academic viewpoint. Little is known of the binding of self-associable guests to CDs. Since β-CD strikingly catalyzes the hydrolysis of penicillins in alkaline solutions, it was regarded as a model of β-lactamase.[18)] On the other hand, penicillins are stabilized in acidic solutions by α- and β-CDs.[19)] These investigations have been carried out in dilute buffered solutions, where the self-association of penicillins is negligible. Although no investigation on the effect of γ-CD has been made, it could include the dimer of PCG. Ampicillin (α-aminobenzylpenicillin) polymerizes in aqueous media by chemical reactions and this polymerization is inhibited by β-CD.[20)] The 2 : 1 complex of ampicillin with β-CD forms in aqueous solution.[21)]

In this work we develop a quantitative treatment for the equilibria of acid dissociation, dimerization, and CD complexation in buffered and unbuffered solutions and for the kinetics of the hydrolysis of PCG in these systems and analyze the complicated data.

Theoretical

Equilibria of Acid Dissociation, Dimerization, and CD Complexation As Fig. 1 shows, PCG is in molecular form (HP) in acid solutions and is in ionic form (P^-) in alkaline solutions. Furthermore, PCG dimerizes in a weakly acidic solution as follows:

$$\begin{array}{ccc} P^- + HP & \xrightarrow{K_p} & H^+ + 2P^- \\ \Big\uparrow\Big\downarrow K_2 & & \Big\uparrow\Big\downarrow K_2' \\ HP_2^- & \xrightarrow{K_p'} & H^+ + P_2^{2-} \end{array} \qquad (1)$$

where K_p and K_p' denote the acid dissociation constants of PCG and acidic dimer of PCG and K_2 and K_2' denote the equilibrium constants for forming univalent and bivalent dimers of PCG. These equilibrium constants are defined as:

$$K_p = \frac{[H^+][P^-]}{[HP]} \qquad (2)$$

$$K_p' = \frac{[H^+][P_2^{2-}]}{[HP_2^-]} \qquad (3)$$

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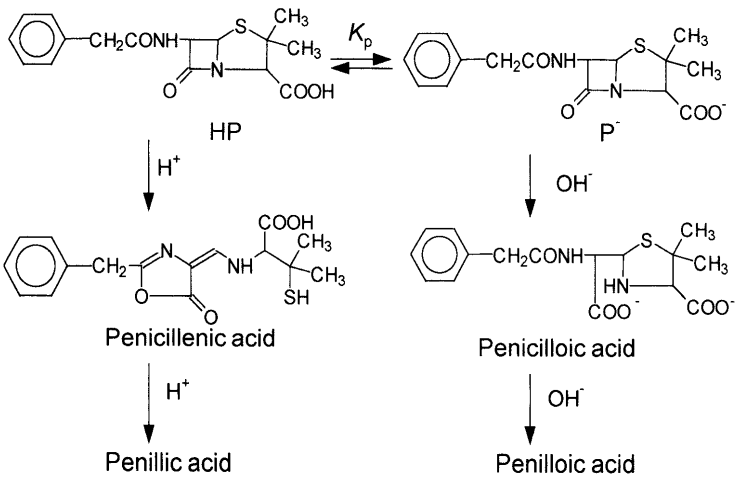

Fig. 1. Mechanisms of the Hydrolysis of PCG

$$K_2=\frac{[HP_2^-]}{[HP][P^-]} \tag{4}$$

$$K_2'=\frac{[P_2^{2-}]}{[P^-]^2} \tag{5}$$

These constants are connected with an equation of $K_p'K_2=K_pK_2'$. The dimer of PCG in molecular form was neglected, since we are concerned with weakly acidic or alkaline solution only.

The complexations of CD (D) with basic PCG monomer (P^-), basic PCG complex (PD^-), and acidic PCG monomer (HP) may be written as:

$$P^- + D \overset{\beta_1'}{\longleftrightarrow} PD^- \tag{6}$$

$$DP^- + P^- \overset{\beta_2'}{\longleftrightarrow} P_2D^{2-} \tag{7}$$

$$HP + D \overset{\beta_1}{\longleftrightarrow} HPD \tag{8}$$

Here, β_1', β_2', and β_1 stand for equilibrium constants of these complexations, respectively:

$$\beta_1'=\frac{[PD^-]}{[P^-][D]} \tag{9}$$

$$\beta_2'=\frac{[P_2D^{2-}]}{[PD^-][P^-]} \tag{10}$$

$$\beta_1=\frac{[HPD]}{[HP][D]} \tag{11}$$

We neglected the complex HP_2D^- and the 2:1 complex of CD and PCG, since these complexes are almost lacking under the present conditions.

Under the above conditions the total concentration, C_p, of PCG can be written as

$$\begin{aligned}C_p&=[P^-]+[HP]+2[P_2^{2-}]+2[HP_2^-]+[PD^-]+[HPD]+2[P_2D^{2-}]\\&=[P^-]+\frac{[H^+][P^-]}{K_p}+2K_2'[P^-]^2+\frac{2K_2'[H^+][P^-]^2}{K_p'}+\beta_1'[P^-][D]\\&\quad+\frac{\beta_1[H^+][P^-][D]}{K_p}+2\beta_1'\beta_2'[P^-]^2[D]\end{aligned} \tag{12}$$

The total concentration, C_D, of CD is calculated from

$$\begin{aligned}C_D&=[D]+[PD^-]+[HPD]+[P_2D^{2-}]=[D]+\beta_1'[P^-][D]\\&\quad+\frac{\beta_1[H^+][P^-][D]}{K_p}+\beta_1'\beta_2'[P^-]^2[D]\end{aligned} \tag{13}$$

From Eq. 13 the concentration of free species of CD is written as

$$[D]=\frac{K_pC_D}{K_p+K_p\beta_1'[P^-]+\beta_1[H^+][P^-]+K_p\beta_1'\beta_2'[P^-]^2} \tag{14}$$

Equilibria in Unbuffered Solution Saturated with Carbon Dioxide The pH of an aqueous solution saturated with carbon dioxide may be written as

$$CO_2+H_2O \rightarrow H_2CO_3 \overset{K_c}{\longleftrightarrow} H^+ + HCO_3^- \tag{15}$$

$$K_c[H_2CO_3]=[H^+][HCO_3^-]=(10^{-pH})^2=\alpha \tag{16}$$

In the present work, the constant α can be determined from the observed pH value for a 154 mmol dm^{-3} KCl solution. From the condition of electric neutrality of the solution, the pH value of an aqueous solution containing CD and PCG may be written as

$$\begin{aligned}C_p+[H^+]&=[P^-]+2[P_2^{2-}]+[HP_2^-]+[PD^-]+[OH^-]+[HCO_3^-]\\&=[P^-]+2K_2'[P^-]^2+\frac{K_2'[H^+][P^-]^2}{K_p'}+\beta_1'[P^-][D]+\frac{K_w+\alpha}{[H^+]}\end{aligned} \tag{17}$$

Here, K_w denotes the ionic product of water.

In the absence of CD, from Eqs. 12 and 17 we can obtain the binomial equation with respect to the concentration of the free PCG ion:

$$K_2[H^+]^2[P^-]^2+[H^+]^2[P^-]+K_p\{[H^+]^2-(K_w+\alpha)\}=0 \tag{18}$$

Solving Eq. 18, we can calculate the concentration of the free PCG ion from

$$[P^-]=\frac{-[H^+]+\sqrt{[H^+]^2-4K_2K_p\{[H^+]^2-(K_w+\alpha)\}}}{2K_2[H^+]} \tag{19}$$

In the presence of CD, from Eqs. 12 and 17 we may obtain

$$[HP]+[HPD]+[HP_2^-]+[H^+]-\frac{\alpha+K_w}{[H^+]}$$

$$= \frac{[H^+][P^-]}{K_p} + \frac{\beta_1[H^+][P^-][D]}{K_p} + \frac{K_2[H^+][P^-]^2}{K_p} + [H^+] - \frac{\alpha + K_w}{[H^+]} = 0 \quad (20)$$

Furthermore, we can obtain the binomial equation for the concentration, C_D, of free species of CD:

$$(AC' - A'C)^2 + (A'B - AB')(BC' - B'C) = 0 \quad (21)$$

From Eqs. 14, 17, and 20 we can obtain the concentration of free PCG ion:

$$[P^-] = \frac{A'C - AC'}{A'B - AB'} \quad (22)$$

Here

$$A = ba' - b'a: \quad B = ca' - c'a: \quad C = da' - d'a$$

$$A' = ba'' - b''a: \quad B' = ca'' - c''a: \quad C' = da'' - d''a$$

$$a = K_2[H^+]^2(K_p\beta_1' + \beta_1[H^+])$$

$$b = (K_p\beta_1' + \beta_1[H^+])[H^+]^2 + K_pK_2[H^+]^2$$

$$c = \{K_p[H^+]^2 - (\alpha + K_w)K_p\}(K_p\beta_1' + \beta_1[H^+]) + K_p[H^+]^2 + K_p\beta_1[H^+]^2C_D$$

$$d = \{K_p[H^+]^2 - (\alpha + K_w)K_p\}K_p$$

$$a' = (2K_pK_2'[H^+] + K_2[H^+]^2)(K_p\beta_1' + \beta_1[H^+])$$

$$b' = (2K_pK_2'[H^+] + K_2[H^+]^2)K_p + K_p[H^+](K_p\beta_1' + \beta_1[H^+])$$

$$c' = K_p^2[H^+] + (K_p\beta_1' + \beta_1[H^+])\{(\alpha + K_w)K_p - K_pC_p[H^+] - K_p[H^+]^2\} + K_p^2\beta_1'C_D[H^+]$$

$$d' = K_p\{(\alpha + K_w)K_p - K_pC_p[H^+] - K_p[H^+]^2\}$$

$$a'' = 2\beta_1'K_pK_2'[H^+] + 2\beta_1'K_2[H^+]^2$$

$$b'' = \beta_1'K_p[H^+] + \beta_1'[H^+]^2 + 2K_2'K_p[H^+] + K_2[H^+]^2$$

$$c'' = -(\beta_1'K_pC_p[H^+] - K_p[H^+]) + \beta_1'C_pC_DK_p[H^+]$$

$$d'' = (\alpha + K_w)K_p - K_pC_p[H^+] - K_p[H^+]^2$$

From Eqs. 17 and 20 we can obtain the equations for the concentrations of complexes HPD and PD^-:

$$[HPD] = \frac{\alpha + K_w}{[H^+]} - \frac{[H^+][P^-]}{K_p} - \frac{K_2[H^+][P^-]^2}{K_p} - [H^+] \quad (23)$$

$$[PD^-] = C_p + [H^+] - [P^-] - 2K_2'[P^-]^2 - \frac{K_2[H^+][P^-]^2}{K_p} - \frac{\alpha + K_w}{[H^+]} \quad (24)$$

From Eq. 9 we can develop the equation for the concentration of free species of CD:

$$[D] = \frac{[PD^-]}{\beta_1'[P^-]} = \frac{1}{\beta_1'[P^-]}\left(C_p + [H^+] - [P^-] - 2K_2'[P^-]^2 - \frac{K_2[H^+][P^-]^2}{K_p} - \frac{\alpha + K_w}{[H^+]}\right) \quad (25)$$

Equilibria in Buffered Solution with and without CD In the absence of CD we can obtain the equation for the concentration of free PCG ion from Eq. 12:

$$[P^-] = \frac{-1 + \sqrt{1 + 8K_2'C_p}}{4K_2'} \quad (26)$$

In the presence of CD, from Eqs. 12 and 14 we can obtain the concentration of ionic species of PCG by solving the following equation:

$$2K'\beta_1'\beta_2'[P^-]^4 + (2K_2'\beta_1' + \beta_1'\beta_2')[P^-]^3 + (\beta_1' + 2K_2' + 2\beta_1'\beta_2'C_D - \beta_1'\beta_2'C_p)[P^-]^2 + (1 + \beta_1'C_D - \beta_1'C_p)[P^-] - C_p = 0 \quad (27)$$

Effects of Dimerization and CD Inclusion on the Hydrolysis of PCG In the absence of CD, the monomer and dimer of PCG are hydrolyzed as follows:

$$\begin{array}{ccc} 2[P^-] & \overset{K_2'}{\longleftrightarrow} & [P_2^{2-}] \\ \downarrow k_1 & & \downarrow k_2 \\ \text{product} & & \text{product} \end{array} \quad (28)$$

where k_1 and k_2 denote the first order rate constants for the monomer and the dimer, respectively.

$$k = \frac{k_1[P^-] + 2k_2[P_2^{2-}]}{C_p} = \frac{k_1[P^-] + 2k_2K_2'[P^-]^2}{C_p} \quad (29)$$

Substitution of Eq. 26 into Eq. 29 yields

$$k = \frac{k_1K_2'(-1 + \sqrt{1 + 8K_2'C_p}) + k_2(1 + 4K_2'C_p - \sqrt{1 + 8K_2'C_p})}{4(K_2')^2C_p} \quad (30)$$

The binary complexes of CD with the ionic monomer, ionic dimer, and molecular species of PCG are hydrolyzed with the first order rate constants, k_{1D}', k_{2D}' and k_D.

$$\begin{array}{ccc} P^- + D & \overset{\beta_1'}{\longleftrightarrow} & PD^- \\ \downarrow k_1 & & \downarrow k_{1D}' \\ \text{product} & & \text{product} \end{array}$$

$$\begin{array}{ccc} P^- + PD^- & \overset{\beta_2'}{\longleftrightarrow} & P_2D^{2-} \\ \downarrow k_1 & & \downarrow k_{2D}' \\ \text{product} & & \text{product} \end{array} \quad (31)$$

$$\begin{array}{ccc} HP + D & \overset{\beta_1}{\longleftrightarrow} & HPD \\ \downarrow k_1' & & \downarrow k_D \\ \text{product} & & \text{product} \end{array}$$

When the equimolar complex of PCG and CD is taken into consideration, we can write the observed rate constant as:

$$k = \frac{k_1[P^-] + 2k_2K_2'[P^-]^2 + k_{1D}'\beta_1'[P^-][D] + 2k_{2D}'\beta_1'\beta_2'[P^-]^2[D]}{C_p} \quad (32)$$

Experimental

Materials PCG potassium salt from Nacalai Tesque Co. was used as received. α-, β-, and γ-CDs were purchased from Nacalai Tesque Co. α-CD was purified by several recrystallizations from propanol,[22] followed by freeze-drying from water. As α-CD was purified, absorbances around 220—300 nm were decreased.[23] β-CD was purified by the method of Sophianopolos and Warner,[23] and finally freeze-dried from water. The ion-exchanged water was doubly distilled before use. Inorganic salts and α-methylglucoside (Sigma Chemical Co.) of analytical grade were used without purification.

Kinetic Procedures Used were four media: a 154 mmol dm^{-3} KCl solution and 20 mmol dm^{-3} phosphate buffer (pH 5.70) for α- and β-CDs,

20 mmol dm^{-3} phosphate buffer (pH 5.65) for γ-CD, and 20 mmol dm^{-3} borate buffer (pH 9.35) without CD. The ionic strength of all the buffers was adjusted at 150 mmol dm^{-3} with KCl. In weakly acidic and neutral solutions the hydrolysis of PCG yields penicillenic acid, which was continuously monitored by observing the absorbance, A, at 322 nm:

$$\ln(A_\infty - A) = -kt + \ln(A_\infty - A_0)$$

The A_∞ value was calculated from the molar absorption coefficient of penicillenic acid[24] and the initial concentration of PCG, and this value was in excellent agreement with the A_∞ value evaluated by best fitting to the above equation. Thus we determined the first-order rate constant for the formation of penicillenic acid from PCG. In the alkaline solution aliquots of a reaction mixture were removed at various time intervals and the remaining amount of PCG was assayed spectrophotometrically by conversion of the penicillin to penicillenic acid in hydrochloric acid containing 1 mmol dm^{-3} mercuric chloride.[25] Thus, we determined the first-order rate constant for the disappearance of PCG in the alkaline solution. The absorbance was measured with a Shimadzu MPS-2000 spectrophotometer. All measurements were carried out at 25 °C.

Results

Effect of the PCG Concentration on the Hydrolysis of PCG without CD As Fig. 2 shows, the effect of the PCG concentration on the hydrolysis of PCG remarkably depends on the pH. In the acidic solutions the hydrolysis is accelerated with increasing PCG concentration, whereas in the alkaline solution it is inhibited. This can be ascribed to the dimerization of PCG.[12] The micellar effect on chemical reactions has been well documented and understood quantitatively,[2a,26-28] although the effect of dimerization has been little investigated. In acidic solutions the hydrolysis of PCG is catalyzed principally by hydrogen ion. Since the negative surface charge density of the dimer of PCG is higher than that of its monomer, the hydrogen ion concentration on the dimer surface exceeds that on the monomer. In alkaline solutions the hydrolysis of PCG is catalyzed mainly by hydroxide ion. The hydroxide ion is expelled from the dimer surface more than from the monomer surface, as the result of the electrostatic repulsion. The solid lines for buffered solutions were calculated from Eq. 30, where a dimerization constant of 2.11 dm^3 mmol^{-1} for PCG, determined in a 154 mmol dm^{-3} KCl solution,[12] was used. The best-fit rate constants for monomer and dimer are shown in Table 1. These constants include the contributions of phosphate ion and borate ion, since these ions catalyze the hydrolysis of PCG as general bases.[15,17] In a neutral or alkaline solution we can practically neglect the molecular form of PCG.

As Fig. 3 shows, the pH of PCG in a 154 mmol dm^{-3} KCl solution increased with the addition of PCG. This is due mainly to the basicity of PCG ion. This effect is not enough to explain the result of Fig. 3 quantitatively: we must take into account the effect of carbon dioxide. Substitution of Eq. 19 into Eq. 17 yields a relation between hydrogen ion and total PCG concentration (C_p). This relation is shown by the solid line, where K_p and K'_p were adjusted to best fit the observed data of Fig. 3. That is, values of pK_p = 1.79 and pK'_p = 2.14 were evaluated.

As Fig. 2 shows, PCG in a 154 mmol dm^{-3} KCl solution is destabilized with increasing PCG concentration. This result cannot be explained by the pH change shown in Fig. 3. The main reason for this will be that the dimer of PCG is less stable than its monomer. Secondly, hydrogen carbonate ion catalyzes this reaction.[15] The hydrogen carbonate ion concentration increases with increasing pH (Fig. 3) and can be calculated from Eq. 16 with the observed pH value. The concentration, [P$^-$], of PCG ion

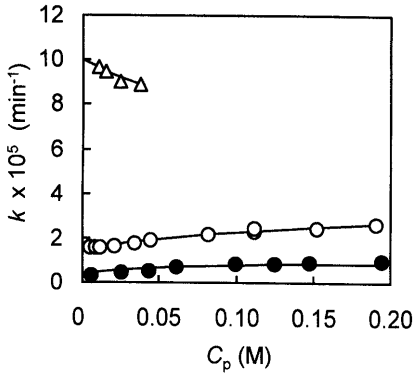

Fig. 2. Hydrolysis Rate Constants of PCG as a Function of PCG Concentration in a 154 mmol dm^{-3} KCl Solution (●), in pH 5.70 Phosphate Buffer (○), and in pH 9.35 Borate Buffer (△)

The solid line for a 154 mmol dm^{-3} KCl solution was calculated from Eq. 33 and those lines for pH 5.70 and pH 9.35 from Eq. 30.

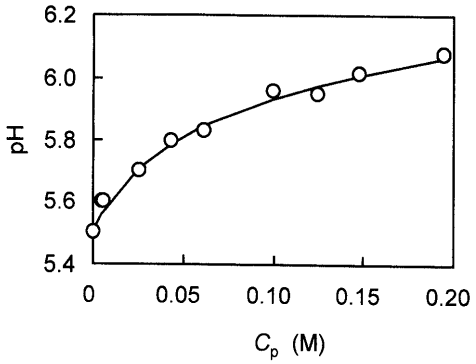

Fig. 3. Change in pH with PCG Concentration in a 154 mmol dm^{-3} KCl Solution

The solid line was calculated using Eqs. 17 and 19.

Table 1. Kinetic and Equilibrium Parameters for PCG and PCG–CD Complexes in Buffer Solutions

CD	pH	C_P (mol dm^{-3})	C_D (mol dm^{-3})	β'_1 (dm^3 mol^{-1})	β'_2 (dm^3 mol^{-1})	k_1 $\times 10^5$ (min^{-1})	k_2 $\times 10^5$ (min^{-1})	k'_{1D} $\times 10^5$ (min^{-1})	k'_{2D} $\times 10^5$ (min^{-1})
—	5.70	−0.19	0	—	—	1.45	4.98	—	—
—	9.35	−0.19	0	—	—	10.4	4.80	—	—
α-CD	5.70	0.005	−0.072	2.6	—	1.45	4.98	0.40	—
β-CD	5.70	0.005	−0.017	30	—	1.45	4.98	0.12	—
β-CD	5.70	0.111	−0.017	30	—	1.45	4.98	0.12	—
γ-CD	5.77	0.005	−0.025	179	0.5	1.14	4.45	0.1	0.1
γ-CD	5.77	0.111	−0.08	179	0.5	1.14	4.45	0.1	0.1

Table 2. Kinetic and Equilibrium Parameters for PCG and PCG–CD Complexes in a 154 mmol dm^{-3} KCl Solution

CD	C_P (mol dm^{-3})	C_D (M) (mol dm^{-3})	β_1' (dm^3 mol^{-1})	β_1 (dm^3 mol^{-1})	k_{1H^+}	$k_{1HCO_3^-}$	k_{2H^+}	$k_{2HCO_3^-}$	k_{1D}'
					$\times 10^6$ (dm^3 mol^{-1} min^{-1})				$\times 10^6$ (min^{-1})
—	−0.19	0	—	—	0.7	0.43	12	0.4	—
α-CD	0.005	−0.072	2.6	110	0.4	0.6	12	0	2
β-CD	0.005	−0.017	40	3200	0.4	0.46	12	0	1
β-CD	0.111	−0.017	40	3200	0.55	0.24	23	0	0.5

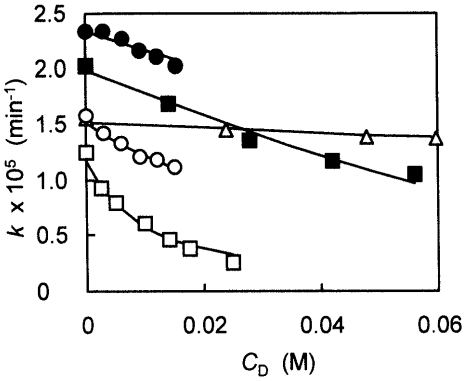

Fig. 4. Effects of pure CDs on the Hydrolysis Rate Constants of PCG at 5 mmol dm^{-3} (Open Symbols) and 111 mmol dm^{-3} (Closed Symbols) in Phosphate Buffers Near pH 5.70

△, α-CD; ○, β-CD; ●, β-CD; □, γ-CD; ■, γ-CD. The solid lines were calculated from Eq. 32.

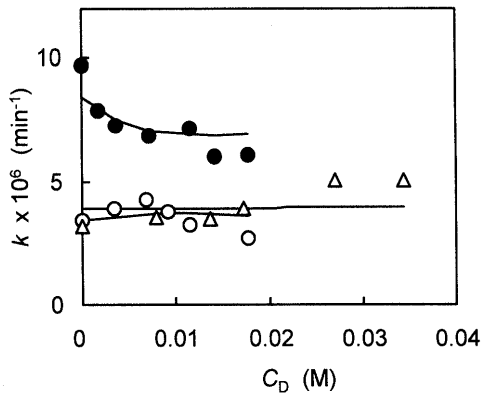

Fig. 5. Effects of CD Concentration on the Hydrolysis Rate Constants of PCG at 5 mmol dm^{-3} (Open Symbols) and 111 mmol dm^{-3} (Closed Symbols) in a 154 mmol dm^{-3} KCl Solution

△, α-CD; ○, β-CD; ●, β-CD. The solid lines were calculated from Eq. 34.

can be calculated from Eq. 19. Using the concentrations of H^+, HCO_3^-, and P^-, we can calculate the rate constant in the 154 mmol dm^{-3} KCl solution from

$$k=\{(k_{1H^+}[H^+]+k_{1HCO_3^-}[HCO_3^-])[P^-]+2K_2'(k_{2H^+}[H^+]+k_{2HCO_3^-}[HCO_3^-])[P^-]^2\}/C_P \quad (33)$$

Here k_{1H^+} and k_{2H^+} denote the catalytic constants of H^+ for monomer and dimer of PCG and $k_{1HCO_3^-}$ and $k_{2HCO_3^-}$ the catalytic constants of HCO_3^- for the monomer and dimer. The catalytic constants best fit to the observed data of Fig. 2 are shown in Table 2. The hydrogen ion catalytic constant for monomer is smaller than that for dimer, whereas the hydrogen carbonate ion catalytic constant for monomer is larger than that of dimer. These results are explicable by the difference in surface charge density between the dimer and monomer: the positive ion on the dimer is concentrated more than that on the monomer, whereas negative ion on the dimer is less than on the monomer.

Effect of Purified Samples of CDs in Phosphate Buffers As Fig. 4 shows, a purified sample of α-CD inhibits the acidic hydrolysis of 5 mmol dm^{-3} PCG. The extent of inhibition increases with increasing size of the CD cavity, which leads to the increased binding constant. The solid lines in Fig. 4 were calculated from Eq. 32, where the concentrations of PCG ion and free CD are calculated from Eqs. 27 and 14 and the rate constants, k_1 and k_2, for monomer and dimer without CD (Table 1) were used. The values of β_1' and k_{1D}' were adjusted to fit the observed data of Fig. 4 and are shown in Table 1. As is evident from comparison of k_1 and k_{1D}', PCG is stabilized by CD inclusion.

Effect of the Purified CD on Hydrolysis and pH in a 154 mmol dm^{-3} KCl Solution Under these conditions CDs vary the hydrolysis rate of PCG, as shown in Fig. 5 and increase the pH, as shown in Fig. 6. Addition of α-CD increases both the rate and the pH. On the other hand, α-CD inhibits the hydrolysis in phosphate buffer (Fig. 4). Since the increase in pH must decelerate the hydrolysis, the enhancement with α-CD would be ascribed to the increase in hydrogen carbonate ion concentration. Therefore, we take into consideration the effects of hydrogen ion, hydrogen carbonate ion, and CD on the hydrolysis of monomer and dimer of PCG:

$$k=\{(k_{1H^+}[H^+]+k_{1HCO_3^-}[HCO_3^-])[P^-]+2K_2'(k_{2H^+}[H^+]+k_{2HCO_3^-}[HCO_3^-])[P^-]^2\}/C_P+k_{1D}'\beta_1'[P^-][D]/C_P \quad (34)$$

Here the hydrogen carbonate ion concentration was calculated from the observed pH value (Fig. 6), the

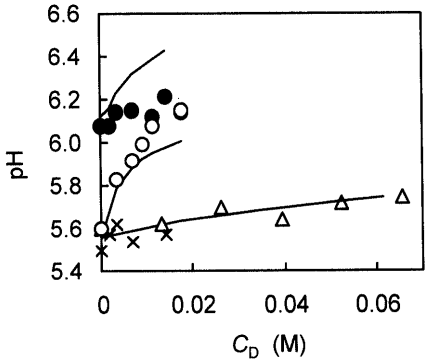

Fig. 6. Changes in pH with CD Concentration in a 154 mmol dm^{-3} KCl Solution at 5 mmol dm^{-3} (Open Symbols) and 194 mmol dm^{-3} (Closed Symbols) PCG

△, α-CD; ○, β-CD; ●, β-CD. The solid lines were calculated from Eqs. 17 and 22. The pH changes of solutions of purified α-CD without PCG (×) are also shown.

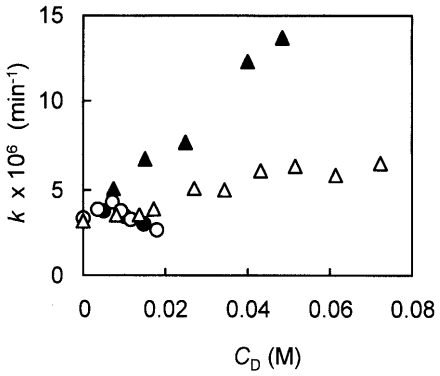

Fig. 7. Effects of Purity of CDs on Hydrolysis Rate Constants of 5 mmol dm^{-3} PCG in a 154 mmol dm^{-3} KCl Solution

○, purified β-CD; ●, commercial β-CD; △, purified α-CD; ▲, commercial α-CD.

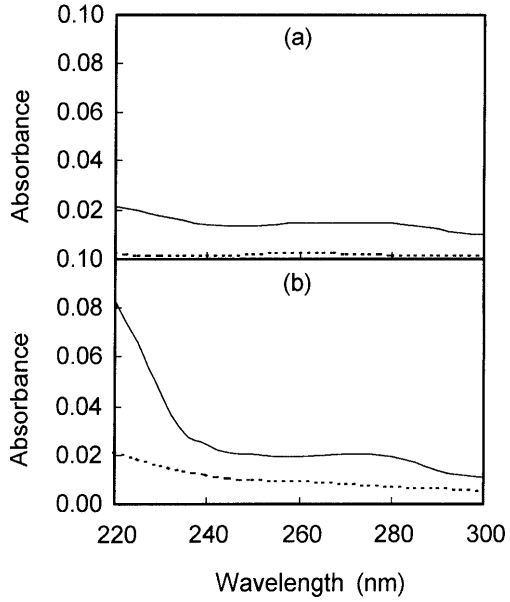

Fig. 8. Absorption Spectra of Commercial (Solid Line) and Purified (Dashed Line) Samples of (a) 20 mmol dm^{-3} α-CD Solutions and (b) 13 mmol dm^{-3} β-CD Solutions

concentration of free PCG ion from Eq. 22, and the concentration of free CD from Eq. 14. The catalytic constants and the binding constants, β_1 and β_1', in Eq. 34 were regarded as parameters fitting to the observed data of Fig. 5. The evaluated values for these catalytic constants and binding constants are shown in Table 2 and were used to calculate the solid lines of Fig. 6.

Effect of Impurities in CDs on the Hydrolysis of 5 mmol dm^{-3} PCG As Fig. 7 shows, the observed rate constant of PCG in a 154 mmol dm^{-3} KCl solution remarkably depends on the kind and purity of CDs. The commercial sample of α-CD catalyzes the hydrolysis linearly with its concentration. This result seems interesting, since it suggests that the CD catalyzes the reaction without inclusion. However, 1 mol dm^{-3} α-methylglucoside, which has the unit structure of CDs and no capability of inclusion, did not affect this hydrolysis (data not shown). Therefore, we suspected that some catalytic impurities were contained in the sample of CD. The hydrolysis catalyzed by a purified sample of α-CD obeys Michaelis–Menten kinetics, indicating that impurities in the original sample of α-CD catalyze the hydrolysis of PCG without complexation. β-CD catalyzes the hydrolysis at low CD concentrations, but inhibits it at high concentrations. The purification of β-CD does not change the rate markedly.

Figure 8 shows the UV spectra of α-CD and β-CD in aqueous solutions. Impure samples of these CDs contain some compounds (probably organic unsaturated compounds) absorbing light around 250 nm.

Discussion

The rate constants for the acid-catalyzed hydrolysis of sodium dodecyl sulfate (SDS) are relatively constant at low concentrations, but increase dramatically with the micelle formation of SDS. Conversely, the hydroxide-ion catalyzed hydrolysis of SDS is inhibited by the micelle formation of SDS.[29] These results can be explained in terms of electrostatic attraction of hydrogen ion and repulsion of hydroxide ion against negative charges of the SDS micelle. The results of Fig. 2 show that the PCG dimer behaves similarly to the SDS micelle, although the dimer is much smaller than the micelle. Little was heretofore known on the reactivity of dimers, although the micellar effects on chemical reactions have been investigated extensively and even quantitatively.[2a,26-28] The dimerization of PCG causes a reduction of the concentration of PCG monomer, and consequently results in a decrease of CD binding of PCG.

As Fig. 7 shows, a commercial sample of α-CD catalyzes the hydrolysis of PCG more effectively than a purified sample. At least two impurities are contained in the commercial sample. One is a light-absorbing and inactive compound, which may have organic unsaturated bonds. This compound will be contained in β-CD more than in α-CD, as is evident from comparison of the UV spectra of α-CD and β-CD (Fig. 8). The second impurity in α-CD would be an inorganic acid or base, so that this component enhances the rate linearly with the CD concentration.

The mechanism of hydrolysis of amides has been established in detail. The carbonyl carbon atom of the amide included in the CD cavity is attacked nucleophilically by the secondary ionized hydroxyl group of the CD, to form a tetrahedral intermediate. The acylcyclodextrin formed is cleaved by the secondary unionized hydroxyl group to form an amine, followed by fission of the acylcyclodextrin.[2b] In the case of PCG, penicilloyl-β-CD is formed as an intermediate of hydrolysis of PCG by β-CD,[2b] with the result that the hydrolysis is accelerated 77 times.[18] In contrast, the hydrolysis of PCG by CDs in acidic buffered solution is inhibited. The structure of the PCG-CD complex will be independent of the pH. Since the secondary hydroxyl group of CD is unionized in acidic solution, penicilloylcyclodextrin would not be formed as a tetrahedral intermediate. The hydrogen ion concentration in the cavity of CD will be smaller than that in bulk solution, owing to the hydrophobic nature of the cavity. This will result in the depression of hydrolysis of PCG by CD in acidic buffered solutions.

As Tables 1 and 2 show, the binding constants are almost independent of the experimental conditions, and are close

to the literature values, regardless of the difference in experimental conditions between the literature and ours. The literature values of the binding constant, β_1', for β-CD are 23 and 21 (at 35 °C) $dm^3 mol^{-1}$. The literature β_1 value for β-CD is *ca.* 700 (at 35 °C) $dm^3 mol^{-1}$ [19]; the literature value of pK_p is 2.74 $dm^3 mol^{-1}$.[19] To our knowledge, no binding constant has been reported for the complex formation of PCG and γ-CD. Since γ-CD can incorporate PCG more strongly than α-CD and β-CD and can include the dimer of PCG, it is the best stabilizer of PCG in acidic solutions among the three cyclodextrins.

The rate constants, k_1 and k_2, shown in Table 1 include the catalytic contributions of hydrogen ion as well as buffer components, although the estimation of each contribution is hard. Such an estimation has been made for micellar systems.[26] As Tables 1 and 2 show, the catalytic coefficients for each of α-CD and β-CD are similar, regardless of the difference in the experimental conditions. The catalytic coefficient of α-CD is greater than that of β-CD; this will be explicable by the cavity size. The hydrolysis rate constants in KCl solutions are influenced by so many factors that they require rigorous analysis. In particular, the presence of CD greatly complicates the reaction system, so that the values of catalytic coefficients shown in Table 2 will include some uncertainties.

Since penicillins are hydrolyzed in aqueous solutions to form harmful products, they are dissolved in a physiological saline solution immediately before injection.[14,15] CDs, especially γ-CD, can stabilize PCG in acidic buffered solution (Fig. 4). For unbuffered systems, it is important to exclude carbon dioxide and β-CD can stabilize PCG in its concentrated solution (Fig. 5). A purified specimen of γ-CD is the best stabilizer of PCG in the CDs investigated.

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References and Notes

1) **Nomenclature**: CD, cyclodextrin; D, free species of CD; HP, acidic PCG monomer; HP_2^-, acidic PCG dimer; HPD, inclusion complex for acidic PCG monomer; PCG, potassium benzyl penicillin; P^-, basic PCG monomer; P_2^{2-}, basic PCG dimer; PD^-, inclusion complex for basic PCG monomer; P_2D^{-2}, inclusion complex for basic PCG dimer; K_p, acid dissociation constant for PCG monomer; K_p', acid dissociation constant for PCG dimer; K_2', dimerization constant for basic form; K_2, dimerization constant for acidic form; k_1, hydrolysis rate constant for basic PCG monomer; k_1', hydrolysis rate constant for acidic PCG monomer; k_2, hydrolysis rate constant for basic PCG dimer; k_D, hydrolysis rate constant for acidic PCG–CD complex; k_{1D}', hydrolysis rate constant for basic PCG–CD complex; k_{2D}', hydrolysis rate constant for basic PCG_2–CD complex; k_{1H^+}, hydrogen ion catalytic constant for PCG monomer; k_{2H^+}, hydrogen ion catalytic constant for PCG dimer; $k_{1HCO^{3-}}$, hydrogen carbonate ion catalytic constant for PCG monomer; $k_{2HCO^{3-}}$, hydrogen carbonate ion catalytic constant for PCG dimer; α, ionic product of carbonic acid saturated with carbon dioxide; β_1, 1 : 1 binding constant for acidic PCG monomer; β_1', 1 : 1 binding constant for basic PCG monomer; β_2', 1 : 2 binding constant for basic PCG dimer.

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