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The Deacylation of Acyl-Cycloamyloses

The Catalytic Effects of Benzimidazole Derivatives on the Rate

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Cycloheptaamylose cinnamate, an intermediate in the hydrolysis of *m*-nitrophenyl cinnamate by cycloheptaamylose, was isolated in pure form. The deacylation of acyl-cycloamyloses (cinnamate and acetate) catalyzed by noncovalently complexed 6-nitrobenzimidazole (**1**) was studied. The reaction was enzyme-like. Saturation of acyl-cycloamylose by **1** was observed; the rate and dissociation constants were determined from Lineweaver–Burk plots. The catalyzed reaction rates at neutral pH were two to three times larger than those of the spontaneous reactions for cycloheptaamylose or cyclohexaamylose cinnamate, respectively. The catalytic effect of **1** on the deacylation rate of cyclohexaamylose cinnamate became smaller as the pH of the solution was raised. The deacylation of cyclohexaamylose acetate was followed by nmr spectroscopy, whereas the deacylation of cycloamylose cinnamates was followed by uv spectroscopy and extraction of *trans*-cinnamic acid with ether. Thermodynamic parameters for the rates of deacylation of cycloamylose cinnamates and dissociation constants of cycloamylose cinnamate–**1** complexes were obtained and discussed.

INTRODUCTION

Soon after it became known that cycloamyloses formed stable inclusion complexes in solution (*1–5*), it was realized that this process might affect the reactivity of organic substances. Particularly, because of the binding of these substances within a cavity of specific dimensions, it was suggested that the cycloamyloses might be useful and relatively simple models for enzymatic action.

VanEtten *et al.* (*6*) found that cyclohexaamylose (α-CA) added in excess to basic aqueous solutions of phenyl acetates increased the rate of the appearance of phenolic products. They found that accelerations were independent of electronic effects and that the cycloamylose system exhibited many characteristics of enzyme-catalyzed reactions, such as saturation, competitive inhibition, and nonproductive binding. They also found that *meta*-substituted phenyl esters were cleaved more rapidly than corresponding *para*-analogs.

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Brass and Bender (*7*) found that rates of substituted phenol release from diphenyl and bis-(*p*-nitrophenyl) carbonates and from diphenyl and bis-(*m*-nitrophenyl)methylphosphonate were accelerated in the presence of cycloamyloses. Griffiths and Bender (*8*) published a detailed review of cycloamyloses as catalysts. Recently, Manor and Saenger (*9*), from X-ray crystallographic studies on the inclusion complexes of α-CA, reported that a conformational change must necessarily be associated with the inclusion process, and they suggested that α-CA is an even better enzyme model than previously assumed.

However, the utility of cycloamyloses as enzyme models is limited by two important factors, namely, (i) at neutral pH's the rate acceleration is very small and (ii) the results are based on phenol release which reflects the acylation process, but the deacylation (the release of acetate or other carboxylate) of the covalent intermediate is very slow. In hydrolytic enzymes both acylation and deacylation are fast. To achieve effective catalysis it is essential either to introduce an internal neucleophile by covalent attachment in the right position of the molecule to accelerate deacylation or to use a nucleophile which will be able both to bind noncovalently in the cavity of the cycloamyloses and to accelerate the hydrolysis of the intermediate.

Two relatively successful attempts have been made toward the former approach. One by Gruhn and Bender (*10*) involved the introduction of a relatively small *N*-methylacetohydroxamate function to a secondary hydroxyl group of α-CA. The other by Iwakura *et al.* (*11*), involved the covalent introduction of histamine by a double nucleophilic displacement of the *p*-tosyl ester and then the iodide of a secondary hydroxyl of α-CA. They observed an acceleration of acylation at two pH's (8.02 and 8.37) and reported a larger effect at lower pH.

We attempted to find a good nucleophile, preferably a benzimidazole derivative, that will bind noncovalently to the cavity of cycloamyloses and thus accelerate the deacylation step. Cinnamoyl and acetyl groups were chosen as the acyl groups of the intermediate acyl-cycloamylose.

EXPERIMENTAL

Materials

Cycloheptaamylose (β-CA) was a gift of the Teijin Co. Ltd. (Tokyo, Japan); α-CA was obtained from the Aldrich Chemical Co. The cycloamyloses were recrystallized from distilled water.

The syntheses of *m*-nitrophenyl cinnamate (**2**) and *m*-nitrophenyl acetate (**3**) were performed by the method of Spasov (*12*). The melting points were 115°C (literature, 114–115°C (*13*)) and 55–56°C (literature, 55–56°C (*14*)) for **2** and **3**, respectively.

The benzimidazole derivatives, 6-nitrobenzimidazole (**1**) 5-nitrobenzimidazole (**4**), and benzimidazole (**5**), were purchased from the Aldrich Chemical Co. All other chemicals used were spectro-grade.

Eastman chromagram sheets were used as tlc plates.

Preparation of Cycloheptaamylose Cinnamate

β-CA (10 g, 8.53×10^{-3} mole) was dissolved in 1000 ml of 5×10^{-4} *M* sodium hydroxide solution containing 500 ml of acetonitrile. Compound **2** (2.5 g, 9.28×10^{-3} mole)

in 100 ml of acetonitrile was gradually added to the above β-CA solution with stirring. The pH of the reaction medium was adjusted to 10.0 by the addition of 0.1 *M* NaOH solution. The reaction temperature was 22°C. After 1 hr, the reaction solution was adjusted to pH 2.5 with a few drops of about 3 *M* HCl solution. Then the reaction mixtute was lyophilized. The lyophilized powder was dissolved in 1000 ml of distilled water and the insoluble compounds were removed by filtration. The contaminants in the filtrate were extracted three times with 1000-ml portions of ether and the aqueous layer was lyophilized again. The lyophilized powder (about 6 g) was recrystallized from about 30 ml of distilled water (yield: ~1.2 g). The product (7) was dried under vacuum at 80°C for 12 hr.

Anal. Calcd for $C_{51}H_{76}O_{36}$ (MW = 1265): C, 48.41; H, 6.01; calcd for $C_{51}H_{76}O_{36}\cdot 2H_2O$ (MW = 1301): C, 47.08; H, 6.21. Found: C, 46.95; H, 6.01.

Anal. Calcd for $C_{42}H_{70}O_{35}$ (MW = 1135): C, 44.44; H, 6.21; calcd for $C_{42}H_{70}O_{35}\cdot 2H_2O$ (MW = 1171): C, 43.08; H, 6.38. Found: C, 43.19; H, 6.56.

A spot corresponding to unreacted and/or regenerated β-CA was not observed on the tlc plates (*15*).

The ir spectrum of **7** was very similar to that of β-CA with the exception of the band at 5.82 nm (string, C=O).

The uv spectral peak of **7** was shifted to about 10-nm-longer wavelength than that of *trans*-cinnamic acid (**6**) and the intensity was increased. The uv spectra of the reaction solutions of **7** (a mixture of **6** and **7**) have two isobestic points at 273 and 232 nm. The purity of **7** was determined by the following equation.

$$\text{Purity of } \mathbf{7}\ (\%) = (Ab_7/Ab_6) \times 100, \tag{1}$$

where Ab_7 is the absorbance of the reaction solution of **7** at the isosbestic point, 273 nm, and Ab_6 is that of a standard solution of **6** which is the same molar concentration as the **7** solution.

The purity of **7** (cycloheptaamylose cinnamate) calculated from Eq. (1) was 102%.

Preparation of Cyclohexaamylose Cinnamate

Five grams (4.78×10^{-3} mole) of α-CA was dissolved in 60% acetonitrile, 4×10^{-4} *M* NaOH solution. To the solution of α-CA was added **2** (2.0 g, 7.45×10^{-3} mole) in 100 ml of acetonitrile. The other reaction conditions and procedures were the same as those of the synthesis of **7** except for the reaction time, volumes of distilled water, and ether used for extraction. The reaction time was 100 min. Distilled water, 300 ml, was used for the solubilization of the first lyophilized powder. The volume of ether used for extraction was 300 ml. As unreacted cycloamylose did not affect the deacylation rate of acyl-cycloamylose (see the results for **7** in Fig. 3), the final recrystallization from distilled water was not made. The purity of cyclohexaamylose cinnamate (**8**) was 39.2%.

Preparation of Cyclohexaamylose Acetate

α-CA was dissolved, 3.88 g (3.71×10^{-3} mole), in 250 ml of pH 9.80 carbonate buffer, I = 0.2 *M*, and 18.6 ml (0.67 g, 3.71×10^{-3} mole) of 0.2 *M* **3** in acetonitrile was added to the solution. The reaction was followed spectrophotometrically (Cary 14 spectrophotometer) at 390 nm. The reaction mixture was magnetically stirred for 13 min to assure more than 98% reaction at room temperature. Then the pH of the reaction

mixture was adjusted to 4.9 with 3 ml of 4 *N* HCl and cooled to 10–12°C. The phenol in the solution was extracted twice with 300 ml of ether. The aqueous layer was immediately lyophilized. The lyophilized powder amounted to 5.5 g. The deacylation of cyclohexaamylose acetate (**9**) might be negligible under these conditions, because it was reported that acylation of α-CA by *m*-nitrophenyl benzoate was much faster than deacylation of α-CA benzoate (~300 times) (*6b*). Since the amount of salts (NaCl and buffer constituents) contained could be calculated, and since the amount of total powder was known, we determined that 63% of this powder was **9**.

Kinetic Procedure for Determination of the Deacylation Rates of Cycloamylose Cinnamates

Buffer systems used were as follows: 1/5 *M* acetate (pH 5.46), 1/15 *M* phosphate (pH 7.35), and 1/20 *M* borate–1/10 M NaOH (pH 9.34 and 10.94).

The uv absorption difference spectrum between **6** and **7** or **8** was used for the determination of the rate constant. Reaction solutions of 4.0×10^{-5} *M* **7** or **8** was placed in a water bath at constant temperature. At appropriate intervals the absorbance of the solution was measured by a Cary 14 spectrophotometer. Pseudo-first-order rate constants were then calculated using the usual first-order equation.

For the nitrobenzimidazole-catalyzed reaction, the **6** in the reaction product was extracted with ether. Two milliliters of the **7** or **8** solution (1.0×10^{-3} *M*) containing nitrobenzimidazole was diluted to 25 ml with 0.1 *M* HCl solution. To 10 ml of the acidic solution an equal volume of ether was added and the mixture was shaken vigorously for 2 min. Five milliliters of the ether layer was added to 10 ml of pH 7.35 phosphate buffer, and **6** was again extracted into the aqueous layer. The absorbance of the aqueous solution was measured at 270 nm (λ_{max} of cinnamate anion). The pseudo-first-order rate constant was determined from the first-order plot. The rate constant determined from this extraction procedure was in good agreement with that from direct uv spectrophotometry.

The initial rate of appearance of **6** was also measured for the very slow reaction of **8** at pH 5.46.

Determination of the Kinetics of the Deacylation of Cyclohexaamylose Acetate

1. Nuclear magnetic resonance spectra of α-CA and 9. Two-tenths gram of α-CA was dissolved in 10 ml of deuterium oxide and the solution was lyophilized. Lyophilized powder, 40–50 mg, was dissolved in 0.5 ml of deuterium oxide–carbonate buffer and the nmr of the solution was taken. The nmr spectrometer used was a Varian T-60. The nmr spectrum shows the following peaks based on the standard HDO peak whose chemical shift was assigned δ 4.70 with respect to TMS (*10*): a doublet at 5.14 (6H) and two superimposed peaks at 3.97 and 3.82 (36H).

The nmr spectrum of **9** shows the following peaks: a doublet at δ 5.14 (5.7H), two superimposed broad peaks at δ 3.97 and 3.82 (36H), a relatively sharp singlet at δ 2.27 (2.6–2.9H), and a small sharp singlet at δ 2.01 (0.1–0.3H).

2. Kinetics by the use of nmr spectra. A preweighed amount of lyophilized **9** powder was dissolved in 0.8 to 1.0 ml of carbonate buffer in D_2O in a 5-ml beaker; an excess of **1** was added directly into the solution. After adjustment of the pH with either dilute HCl

or NaOH in D_2O, the solution was placed in an nmr tube and the nmr spectrum was taken immediately.

The fact that the small peak at δ 2.01 in the spectrum of **9** increased with time the expense of the intensity of the large peak at δ 2.27 made this method feasible to follow the reaction. Actually, the peak at δ 2.27 is attributed to the protons of the methyl group of the acetyl moiety on α-CA. The peak at δ 2.01 was shown to be due to the protons of the acetate ion. The actual kinetics were carried out by taking spectra at different times. The peaks obtained at 100-Hz sweep width were cut and weighed. A spontaneous hydrolysis and a hydrolysis in the presence of **1** were run for each pH.

RESULTS AND DISCUSSION

Deacylation of Cycloheptaamylose Cinnamate.

The effect of benzimidazole derivatives (*10*) on the deacylation of **7** was studied at neutral pH. Figure 1 shows the results at pH 7.35 and 50°C. Nitrobenzimidazoles show a larger catalytic effect than **5** itself, which may be attributable to the hydrophobicity

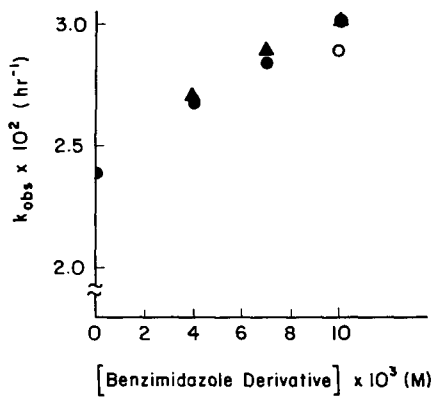

FIG. 1. The effect of benzimidazole derivatives on the rate of deacylation of **7** at pH 7.35 and 50°C. ●, **1**; ▲, **4**; ○, **5**.

of the nitrobenzimidazoles (*6a*). Therefore, the effect of **1** on the rate of deacylation was studied in detail.

Figure 2 shows the catalytic effect of **1** on the deacylation rate of **7** at pH 7.35 and at various temperatures. The observed rate constants (k_{obs}) did not increase linearly with the concentration of **1**. The curves were treated by Lineweaver–Burk plots (*16*) which are also used for enzyme reactions to indicate saturation phenomena. The reaction scheme was generally shown as follows:

$$\begin{array}{l} \mathbf{7} + \mathbf{10} \underset{}{\overset{K_{diss}}{\rightleftharpoons}} \mathbf{7 \cdot 10} \xrightarrow{k_{cat}} \beta\text{-CA} + \mathbf{6} + \mathbf{10} \\ \quad \Big\downarrow k_{un} \\ \beta\text{-CA} + \mathbf{6}, \end{array}$$

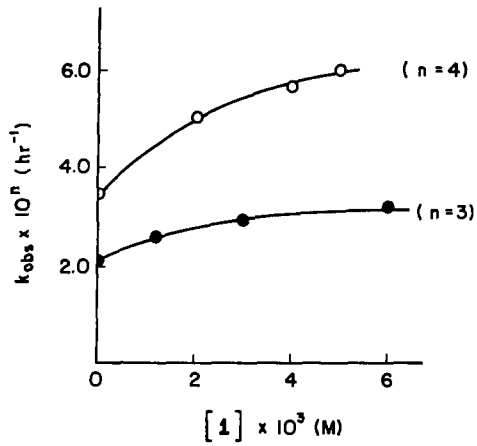

FIG. 2. The effect of concentration of **1** on the rate of deacylation of **7** at pH 7.35 and various temperatures. o, 25°C; ●, 37°C.

where k_{un} and k_{cat} are the first-order rate constants of the spontaneous (uncatalyzed) reaction and the **7·10** complex, respectively, and K_{diss} is the dissociation constant of the complex. The constants were determined using the following equation (*16*).

$$\frac{1}{k_{obs} - k_{un}} = \frac{K_{diss}}{k_{cat} - k_{un}} \cdot \frac{1}{[\mathbf{10}]} + \frac{1}{k_{cat} - k_{un}}, \tag{2}$$

where k_{obs} is the observed rate constant in the presence of **10**.

The rate and dissociation constants calculated from Eq. (2) are summarized in Table 1. For the reaction of **1**, k_{cat} was about two times larger than k_{un}. The following explana-

TABLE 1

RATE AND DISSOCIATION CONSTANTS OF THE DEACYLATION OF **7** IN THE PRESENCE AND ABSENCE OF BENZIMIDAZOLE DERIVATIVES[a]

Catalysts	Temperature (°C)	k_{un} (hr^{-1})	k_{cat} (hr^{-1})	k_{cat}/k_{un}	K_{diss} (M)
1	25	3.47×10^{-4}	7.75×10^{-4}	2.23	3.71×10^{-3}
	37	2.22×10^{-3}	4.72×10^{-3}	2.12	8.00×10^{-3}
	50	2.39×10^{-2}	4.62×10^{-2}	1.93	2.64×10^{-2}
4	50	2.39×10^{-2}	4.01×10^{-2}	1.68	1.63×10^{-2}

[a] $[7] = 1.0 \times 10^{-3}$ M, pH 7.35, I = 0.15 M, 1/15 M phosphate buffer.

tion may be given for this catalytic effect of **1**. The binding of the nitrophenyl group of **1** within the cavity of **7** brings the imidazole portion of the molecule close to the carbonyl group of **7**, leading to nucleophilic catalysis of hydrolysis. The rate ratio, k_{cat}/k_{un}, decreased with increasing temperature. This is attributed to the difference of activation energy between k_{un} and k_{cat}. As temperature increased, K_{diss} increased, which resulted from the endothermic reaction of the complex. In the reaction of **4** with **7**, k_{cat}/k_{un} was somewhat smaller than the reaction of **1** with **7**.

Figure 3 shows the effect of β-CA concentration on the deacylation rate of **7** at pH 7.35 and 50°C. For both k_{un} and k_{obs} there was no effect of β-CA. These results enabled the use of samples of **8** and **9** containing appreciable amounts of α-CA for the kinetics of the deacylation of acyl-cyclohexaamyloses.

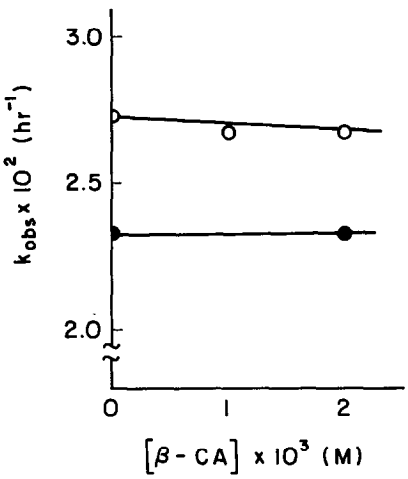

FIG. 3. The effect of β-CA concentration on the rate of deacylation of **7** at pH 7.35 and 50°C. ○, k_{obs} ([**1**] = 6.0×10^{-3} *M*); ●, k_{un} ([**7**] = 1.0×10^{-3} *M*).

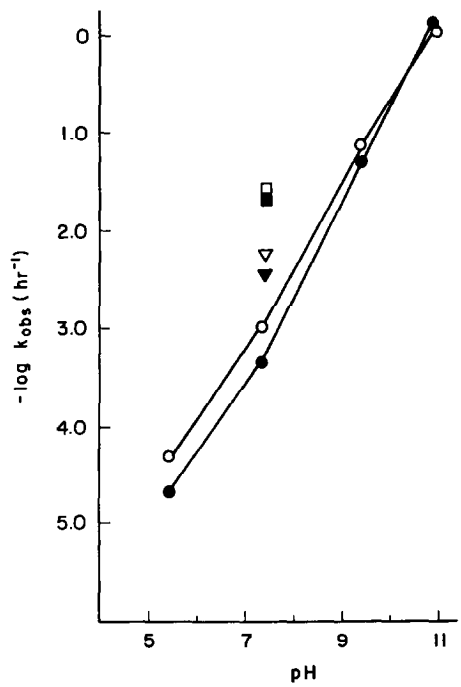

FIG. 4. The pH–rate constant profile for the deacylation of **8**. ●, k_{un} at 25°C ([**8**] = 1.0×10^{-3} *M*); ○, k_{obs} at 25°C ([**1**] = 5.0×10^{-3} *M*); C, k_{un} at 37°C ([**8**] = 1.0×10^{-3} *M*); ▽, k_{obs} at 37°C ([**1**] = 5.0×10^{-3} *M*); ■, k_{un} at 50°C ([**8**] = 1.0×10^{-3} *M*); □, k_{obs} at 50°C ([**1**] = 5.0×10^{-3} *M*).

Deacylation of Cyclohexaamylose Cinnamate

Figure 4 shows the pH–rate profiles for the deacylation of **8** in the presence and absence of **1**. Table 2 gives the values of the rate and dissociation constants calculated

TABLE 2

RATE AND DISSOCIATION CONSTANTS OF THE DEACYLATION OF **8** IN THE PRESENCE AND ABSENCE OF **1**[a]

pH	Temperature (°C)	k_{un} (hr^{-1})	k_{cat} (hr^{-1})	k_{cat}/k_{un}	K_{diss} (M)
5.46	25	2.12×10^{-5}	6.66×10^{-5}	3.14	3.30×10^{-3}
7.35	25	4.62×10^{-4}	1.46×10^{-3}	3.17	5.50×10^{-3}
	37	3.54×10^{-3}	9.09×10^{-3}	2.57	1.08×10^{-2}
	50	2.05×10^{-2}	4.14×10^{-2}	2.02	2.09×10^{-2}
9.34	25	5.42×10^{-2}	1.54×10^{-1}	2.85	1.49×10^{-2}
10.94	25	1.28	—	—	—

[a] $[\mathbf{8}] = 1.0 \times 10^{-3}$ M.

by Eq. [2] for the **8**–**1** system. The slope of the pH–rate constant profile for the spontaneous reaction was +1 in the range of pH 7.3 to 11.0, which was the same result as that obtained previously (*6b*). The catalytic effect of **1** became smaller as the pH was raised. This may be attributed to the incursion of external hydroxide ion catalysis. The dissociation constant between **1** and **8** at pH 9.34 was larger than that at pH 7.35 and 25°C (see Table 2), which may show that anionic **1** (pK_a 10.3) was included less into the cavity of **8** than the free form of **1** (pK_a 3.6 (*17*).

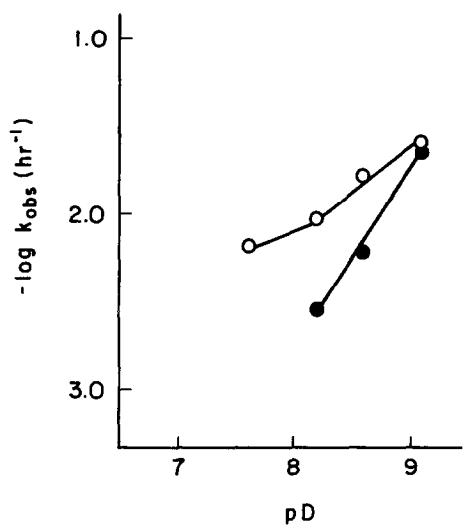

FIG. 5. The pH–rate constant profile for the deacylation of **9** at 22°C. ●, k_{un} ($[\mathbf{9}] = 4.0 \times 10^{-2}$ M); ○, k_{obs} ($[\mathbf{1}] = 1.5 \times 10^{-2}$ M).

At pH 7.35 and 25°C, k_{cat}/k_{un} for **8** was 3.17, whereas the ratio was 2.23 for **7**. This difference might result from the size of the cavity of the cycloamyloses. As α-CA has a smaller cavity than β-CA, the imidazole group of **1** in the hole of **8** may be closer to the carbonyl group of the ester than that in **7**. The effect of temperature on the rate and dissociation constant for **8** at neutral pH was similar to that for **7**.

Deacylation of Cyclohexaamylose Acetate

The pH–rate constant profiles for the deacylation of **9** in the presence and absence of **1** are shown in Fig. 5. The slopes of the profiles were similar in form to the deacylation of **8** and the same explanations as those made for the deacylation of **8** probably hold. Table 3 shows the values of k_{un} and k_{obs} in the presence of **1** and k_{obs}/k_{un} at various pD's in D_2O. There was a somewhat larger catalytic effect of **1** than that for **8**.

TABLE 3

PSEUDO-FIRST-ORDER RATE CONSTANTS FOR THE DEACYLATION OF **9** IN THE PRESENCE AND ABSENCE OF **1**[a]

pD	k_{un} (hr^{-1})	k_{obs} (hr^{-1})	k_{obs}/k_{un}
7.60	—	6.40×10^{-2}	—
8.20	2.56×10^{-3}	8.70×10^{-3}	3.40
8.60	5.33×10^{-3}	1.50×10^{-2}	2.81
9.10	2.26×10^{-2}	2.39×10^{-2}	1.06

[a] $[\mathbf{9}] = 4.0 \times 10^{-2}$ *M*, $[\mathbf{1}] = 1.5 \times 10^{-2}$ *M* in D_2O, room temperature.

The Effect of Temperature on the Deacylation Rates of 7 and 8 and the Gissociation Constants of 7- or 8-1 Complexes

Thermodynamic parameters in the deacylation rates of **7** and **8** and for the dissociation constants of **7** or **8** with **1** are listed in Table 4. Activation energies for the rates of deacylation of **7** or **8** at pH 7.35 were calculated from the Arrhenius equation using the rate constants in Tables 1 and 2. The activation energy for the reaction catalyzed by **1** was lower than that for the spontaneous reactions with both **7** and **8**. Using Van't

TABLE 4

THERMODYNAMIC PARAMETERS FOR THE RATES OF DEACYLATION OF **7** AND **8** AND THE DISSOCIATION CONSTANTS OF THE CORRESPONDING COMPLEXES

	k_{un}	k_{cat}	K_{diss}		
	ΔE_a (kcal/mole)	ΔE_a (kcal/mole)	ΔF^0 (kcal/mole) at 298°K	ΔH^0 (kcal/mole)	$T\Delta S^0$ (kcal/mole) at 298°K
7	31.0	28.8	3.3	13.1	9.9
8	28.1	24.0	3.1	9.5	6.4

Hoff plots the enthalpy change and entropy change for the dissociation constants were determined from the terms $-2.303\ R$ (slope) and $2.303\ R$ (intercept), respectively. The bindings of **1** to **7** or **8** are probably due to a favorable enthalpy change.

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