Imidazole Catalyses in Aqueous Systems. VIII. Spontaneous Hydrolysis and Michaelis-Menten-type Catalytic Hydrolysis of Phenyl Esters in the Presence of Cholic Acid and Its Histamine Derivative[1]

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Spontaneous hydrolyses of cationic and anionic phenyl esters in the presence of cholic acid (VI) and their catalytic hydrolyses with a histamine amide of cholic acid (VII) were investigated using a pH-stat at 30°C in 1.0M aqueous KCl. The rates of spontaneous hydrolysis decreased by 30—70% upon binding of substrates with cholic acid. The variation of the apparent binding constant (7-3650M^{-1}) was considered to reflect the magnitude of hydrophobic forces between cholic acid and substrates, although the values may have to be corrected for the possible involvement of self-association of cholic acid molecules. Imidazole derivative VII catalyzed the hydrolysis of phenyl esters according to Michaelis-Menten kinetics. The long methylene chain in the substrate molecule contributed appreciably to the binding of substrate with cholic acid and its derivative. The magnitude of substrate binding with VII appears to be comparable to the true binding constant with cholic acid. The intra-complex rate constants did not differ much from those expected with other enzyme-like catalysts. Thus, the steroid ring seems to provide a non-specific hydrophobic binding site for the substrates used.

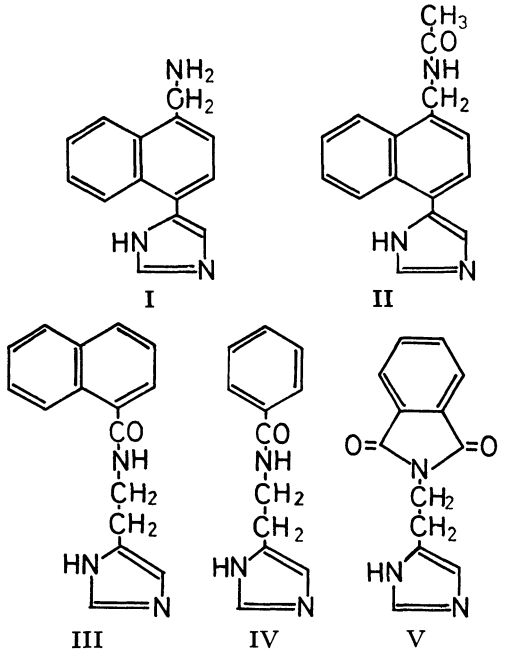
We showed that some imidazole derivatives catalyzed the hydrolysis of *p*-acetoxybenzoic acid (ABA) by second-order kinetics (Eq. (1)) or by the Michaelis-Menten kinetics (Eq. (2)):[2,3]

$$C + S \xrightarrow{k'} C + P \quad (1)$$

$$C + S \underset{K_m}{\rightleftharpoons} CS \xrightarrow{k_{cat}} C + P \quad (2)$$

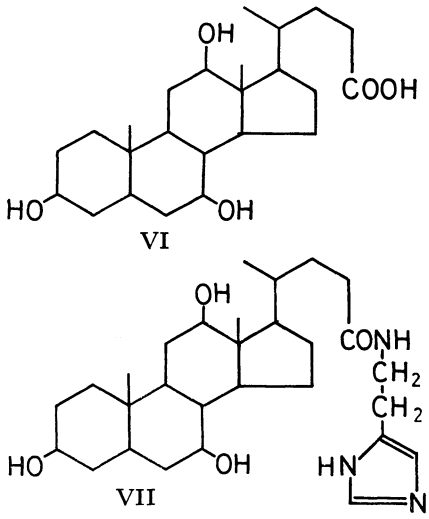
where C, S, and P denote catalyst, substrate, and product, respectively.

The Michaelis-Menten kinetics were observed when imidazole catalysts contained naphthalene ring (I, II, III). The catalysis by less hydrophobic imidazole compounds (IV, V) followed second-order kinetics. Substrate binding in the enzyme-like catalysis was concluded to be ascribable to the hydrophobic interaction. This was also supported by the thermodynamic data obtained for formation of the catalyst-substrate complex.



The intra-complex product formation in the Michaelis-Menten pathway was not very efficient because of extraordinarily large negative $\Delta S^{\ddagger}$ values (*ca.* −50 eu). The unfavorable $\Delta S^{\ddagger}$ term was conceivably related to destruction of the hydrophobic interaction between catalyst and substrate in the transition state of the intra-complex reaction.[3]

Since the intra-complex process would be affected by the structure of the binding site it is interesting to study the catalytic behavior of imidazole compounds with different hydrophobic groups. The steroid ring might provide an interesting binding site as considered from its physiological role. In particular, desoxycholic acid and related compounds are known to form stable inclusion compounds in aqueous solutions.[4] Thus,



1) Contribution No. 234 from this department. Presented at the 24th annual meeting of the Chemical Society of Japan, April, 1971, Osaka.

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2) a) C. Aso, T. Kunitake, and S. Shinkai, *Chem. Commun.*, **1968**, 1483. b) T. Kunitake and S. Shinkai, This Bulletin, **43**, 1109 (1970).

3) T. Kunitake and S. Shinkai, *ibid.*, **43**, 2581 (1970).

4) See for a general reference, K. Takemoto, "Hosetsu Kagobutsu no Kagaku," Tokyo Kagaku Dojin, Tokyo (1969).

imidazole derivatives of these steroids would give rise to interesting catalytic systems. In this paper we report the influence of complexation of cholic aicd (VI) on the spontaneous hydrolysis of several phenyl esters and the catalytic hydrolysis of these phenyl esters by a histamine derivative of cholic acid (VII). Attempts to synthesize a similar catalyst have been reported by French workers.[5]

Experimental

Materials. Commercial cholic acid (Nakarai Chemicals Ltd., Guaranteed Reagent) was used without further purification. Imidazole catalyst VII was prepared from cholic acid (VI) and histamine. Cholic acid (5.1 g, 12.5 mmol) was dissolved in a mixture of tetrahydrofuran (150 m*l*) and acetonitrile (50 m*l*) containing 10 m*l* of triethylamine. The reaction mixture was cooled in an ice bath and 1.5 g (16 mmol) of methyl chloroformate was added with stirring. After stirring for 36 hr, precipitates (triethylamine hydrochloride) were filtered and the filtrate was evaporated to dryness *in vacuo*. The solid residue was dissolved in 60 m*l* of ethanol containing 2 m*l* of 4N aqueous NaOH and was allowed to stand at room temperature for 2 hr. The solvent was again removed *in vacuo* and the residue was dissolved in absolute alcohol in order to separate inorganic materials. Alcohol was evaporated, and the residue which was dissolved in 1N hydrochloric acid was reprecipitated by pouring into a saturated solution of aqueous sodium carbonate. Reprecipitation was repeated once more. Yield 66%. The infrared spectrum showed an amide peak at 1640 cm^{-1} and characteristic peaks of imidazole at 1440 and 1530 cm^{-1}. Another reprecipitation gave a product of mp 117—120°C in 48% overall yield: $pK_a=7.14$ (1.0M KCl, 30°C).

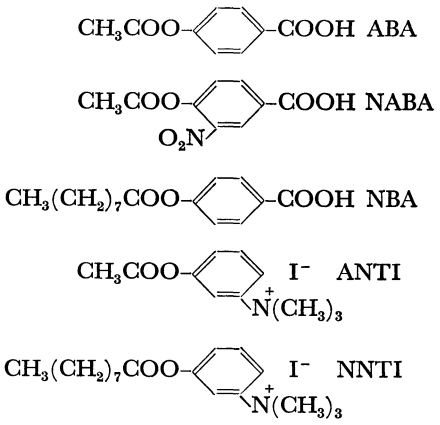
Found: C, 72.12; H, 9.43; N, 7.19%. Calcd for $C_{29}H_{47}N_3O_4$: C, 69.43; H, 9.44; N, 8.38%.

Two spots were observed at $R_f=0.07$ and 0.52 on a silica-gel thin layer chromatogram with acetic acid as developing solvent. The weak spot at $R_f=0.52$ was due to cholic acid. Purity based on the nitrogen content was 85.8%. This preparation containing cholic acid as probably the sole organic contaminant was used as catalyst. When the reprecipitation was repeated five more times, the product showed mp 122—124°C.

Found: C, 70.69; H, 9.47; N, 7.99%; Calcd for $C_{29}H_{47}N_3O_4$: C, 69.43; H, 9.44; N, 8.38%.

Precipitation and characterization of *p*-acetoxybenzoic acid (ABA, mp 192—194°C), 3-nitro-4-acetoxybenzoic acid (NABA, mp 156—158°C), *m*-acetoxy-*N*-trimethylanilinium iodide (ANTI, mp 223—224°C) and *m*-nonanoyloxy-*N*-trimethylanilinium iodide (NNTI, mp 134—135°C) were described previously.[3,6] *p*-Nonanoyloxybenzoic acid (NBA) was prepared from *p*-hydroxybenzoic acid and nonanoyl chloride: 8.4 g (30 mmol) of *p*-hydroxybenzoic acid and 2.8 g (65 mmol) of NaOH were dissolved in water, and 5.3 g (30 mmol) of nonanoyl chloride (bp 126—130°C/30 mmHg. lit,[7] bp 93—96°C/11 mmHg) was added dropwise with stirring. The reaction flask was immersed in an ice bath. White precipitates were formed at the end of the addition. Stirring was continued for 1 hr and pH of the reaction mixture was adjusted to 2—3 by concentrated hydrochloric acid. The precipitates were filtered, washed with water, dried, and recrystallized two times from benzene, yield 76%, mp 113—115°C, IR(KBr): 1750 cm^{-1}(ester), 1675 cm^{-1}(carboxyl).

Found: C, 68.95; H, 7.90%. Calcd for $C_{16}H_{22}O_4$: C, 69.04; H, 7.97%.



Titration and Hydrolysis Procedures. Titration and hydrolysis were carried out at 30.0±0.05°C using a pH-stat connected with a recorder (TOA Electronics Ltd., Models HS-1B and EPR-2T, respectively). The reaction rate was determined from the amount of alkali automatically added to the reaction mixture in order to neutralize the acid formed. The degree of dissociation of the product phenols under the reaction conditions was taken into account in determining the rate of reaction. Details of the procedure have been described.[8]

Results and Discussion

Spontaneous Hydrolyses in the Presence of Cholic Acid. Phenyl esters are hydrolyzed spontaneously in an alkaline medium by the pseudo-first-order kinetics.[3,6,8]

$$v_{spont} = k_s[S] \quad (3)$$

The rate of spontaneous hydrolysis decreased upon addition of cholic acid. Figures 1 and 2 show the effect of cholic acid on the relative rate of spontaneous hydrolysis of five substrates: ABA, NBA, NABA, ANTI, and NNTI. Hydrolysis of NBA was carried out at pH 9.4, because v_{spont} was too small at pH 8.0 to be determined accurately. The relative rate v/v_0 decreased with increasing concentration of cholic acid, and in the case of NBA it decreased to a saturation value with a small amount of cholic acid. The results can be explained by assuming that phenyl esters form complexes with cholic acid and that the resulting complexes show decreased reactivity.

Retardation (or inhibition) of spontaneous hydrolyses of esters in the presence of additives has been studied by some groups of researchers. In particular, Connors and his coworkers made a detailed study on the effect of theophylline and related heterocyclic compounds in the alkaline hydrolysis of several esters.[9-11]

5) G. Defaye and M. Fétizon, *Bull. Soc. Chim. Fr.*, **1969** 1632.
6) T. Kunitake and S. Shinkai, *Makromol. Chem.*, in press.
7) H. E. Fierz-David and W. Kuster, *Helv. Chim. Acta*, **22**, 86 (1939).
8) T. Kunitake, F. Shimada, and C. Aso, *J. Amer. Chem. Soc.*, **91**, 2716 (1969).
9) J. A. Mollica, Jr., and K. A. Connors, *ibid.*, **89**, 308 (1967).
10) P. A. Kramer and K. A. Connors, *ibid.*, **91**, 2600 (1969).
11) K. A. Connors, M. H. Infeld, and B. J. Kline, *ibid.*, **91**, 3597 (1969).

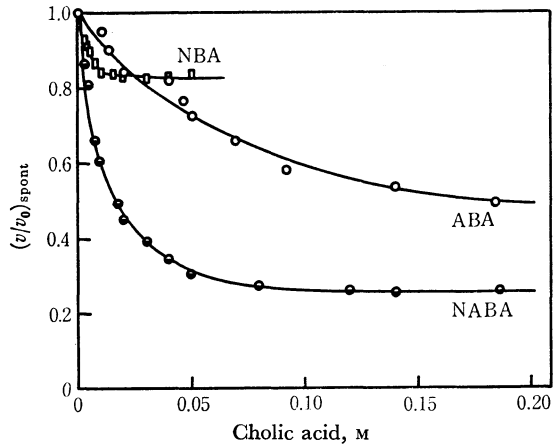


Fig. 1. Spontaneous hydrolysis of phenyl esters in the presence of cholic acid.
ABA: 0.030M, $v_0=1.27\times10^{-5}$M min^{-1} (30°C, 1.0M KCl, pH 8.0)
NABA: 0.015M, $v_0=3.75\times10^{-5}$M min^{-1} (30°C, 1.0M KCl, pH 8.0)
NBA: 0.020M, $v_0=3.22\times10^{-6}$M min^{-1} (30°C, 1.0M KCl, pH 9.4)

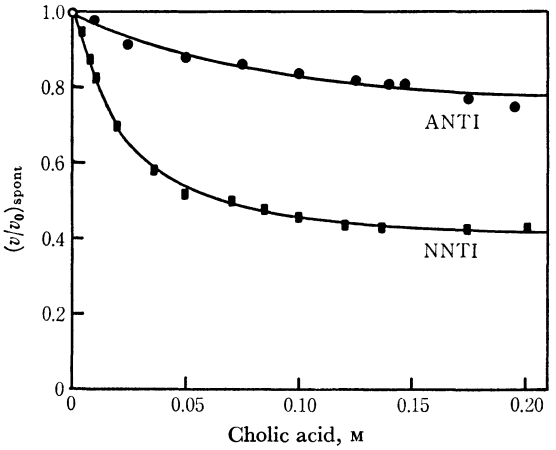


Fig. 2. Spontaneous hydrolysis of phenyl esters in the presence of cholic acid.
ANTI: 0.020M, $v_0=3.18\times10^{-5}$M min^{-1} (30°C, 1.0M KCl, pH 8.0)
NNTI: 0.020M, $v_0=2.34\times10^{-5}$M min^{-1} (30°C, 1.0M KCl, pH 8.0)

The spontaneous hydrolysis of phenyl esters in the presence of cholic acid may be described by the following equations, if substrate S and additive A (cholic acid) interact to form a 1:1 complex SA.

$$S + A \overset{K_c}{\rightleftharpoons} SA \quad (4)$$

$$S \xrightarrow{k_s} \text{products} \quad (5)$$

$$SA \xrightarrow{k_{SA}} \text{products} + A \quad (6)$$

where K_c is the binding constant of phenyl esters and cholic acid and k_{SA} is the pseudo-first-order rate constant for spontaneous hydrolysis of phenyl esters in the complexed state.

The following equation has been obtained according to Connors *et al.* for [A]≫[S]:

$$\frac{k_s}{k_s-k'} = \frac{1}{qK_c[A]} + \frac{1}{q} \quad (7)$$

where k' is the apparent pseudo-first-order rate constant and $q=1-k_{SA}/k_S$. K_c and q are determined by plotting $k_s/(k_s-k')$ against 1/[A].

On the other hand, when the simplifying assumption ([A]≫[S]) cannot be made, the overall rate is expressed by

$$v = k_s[S] + k_{SA}[SA]$$
$$= k_s[S] + k_{SA}\{[S]_0-[S]\} \quad (8)$$

where $[S]_0$ is the total concentration of substrate and [S] is the concentration of free substrate. The following equation holds simultaneously.

$$K_c = \frac{[SA]}{[S][A]} = \frac{[S]_0-[S]}{[S]\{[A]_0-([S]_0-[S])\}} \quad (9)$$

where $[A]_0$ is the total concentration of cholic acid.

We see from Figs. 1 and 2 that the additive concentration is not small compared to the substrate concentration. Therefore, Eqs. (8) and (9) were employed and the values of K_c and k_{SA} were determined by computational trial-and-error as follows. Approximate K_c and k_{SA} values were first estimated from Eq. (7). The corresponding rate v_{calcd} was calculated for respective additive concentrations from the known k_s, $[S]_0$ and $[A]_0$ values and compared with the observed rate v_{obsd}. Then a set of K_c and k_{SA} values was sought which minimized the relative error.

$$\text{relative error} = \sum\left\{\frac{(v_{calcd}/v_{obsd}-1)^2}{n}\right\}^{1/2} \quad (10)$$

where n is the number of experimental runs.

Kinetic constants for the spontaneous hydrolysis of phenyl esters and their complexes with cholic acid are summarized in Table 1. As can be expected from Fig. 1, NBA showed a much greater tendency of binding ($K_c=3650$M^{-1}) than other substrates. This might be attributed to hydrophobic forces between the steroid skeleton of cholic acid and the long methylene chain of the substrate. The increased binding due to the long alkyl chain is also evident in the different K_c values between ANTI and NNTI. The carboxyl group in cholic acid is dissociated completely at pH 8 or 9.4. Therefore, it is possible that both hydrophobic and electrostatic forces contribute to binding of cationic substrates ANTI and NNTI. However, their K_c values were lower than those of related anionic sub-

Table 1. Spontaneous hydrolyses with and without cholic acid[a)]

Substrate	M	K_c[b)] M^{-1}	k_s min$^{-1}\times10^3$	k_{SA}[b)] min$^{-1}\times10^3$	k_{SA}/k_s	relative error[c)] %
ABA	0.030	16	0.423	0.13	0.31	2.9
NBA[d)]	0.020	3650	0.161	0.12	0.73	2.1
NABA	0.015	270	2.50	0.60	0.24	3.7
ANTI	0.020	6.8	1.59	0.88	0.55	1.4
NNTI	0.020	106	1.17	0.47	0.40	1.6

a) Hydrolysis conditions, 30°C, pH 8.0, 1.0M KCl, unless otherwise stated.
b) Obtained by assuming that there is no self-aggregation of chloric acid.
c) *Cf.* Eq. (10).
d) Hydrolysis conditions, 30°C, pH 9.4, 1.0M KCl.

strates (ABA and NBA, respectively). This suggests that the carboxylate group of cholic acid did not contribute noticeably to the binding of the cationic substrates.

In the above experiments, high concentration of cholic acid was employed relative to the substrate concentration except for NBA. Cholic acid molecules might be associated at high concentrations as considered from its tendency to form molecular compounds.[4] If, this is the case, the kinetic analysis based on Eqs. (4) (5), and (6) becomes unreliable for high concentrations of cholic acid. Therefore, it is possible that K_c values are underestimated except for NBA because association of cholic acid is assumed to be absent. Nevertheless, the relative tendency of substrates to form molecular complexes is probably not altered by the occurrence of association.

Underestimation of K_c necessarily leads to that of k_{SA} of the 1 : 1 complex according to Eqs. (8) and (9). As shown in Table 1, retardation of the spontaneous hydrolysis due to complexation was the smallest for NBA substrate. If k_{SA} is underestimated because of the neglect of association of cholic acid, the true k_{SA} value will be greater except for NBA substrate, and the extent of retardation might be closer with each other than that given in Table 1.

In any way, the retardation was not very efficient, k_{SA}/k_s being 0.24 to 0.73. On the other hand, in the alkaline hydrolysis of methyl cinnamate in the presence of theophylline, the corresponding relative rate was close to zero, indicating almost complete inhibition of hydrolysis due to complexation.[9] This contrast is interesting in connection with the nature of the binding site. The chemical environments of the ester group of substrates complexed with cholic acid do not appear to differ much from those of the ester group of free substrates. In the case of inhibition by theophylline, Connors *et al.* suggested that the molecular complexes of theophylline with cinnamate esters were formed in such a way as to permit extensive local dipole and induced dipole interactions in the face-to-face orientation.[11] The electronic interaction of the ester group may be much greater with a dipolar, flat theophylline molecule than with a hydrocarbon-like skeleton of cholic acid.

Catalytic Hydrolysis. Catalyst VII was not completely free from cholic acid as shown by elemental analysis and thin layer chromatography. However, cholic acid present as contaminant (*ca.* 10^{-5}M) would show negligible influence. Catalyst concentration was corrected for the purity of the catalyst. The results of catalytic hydrolysis are shown in Figs. 3 and 4. The catalytic rate v_{cat} clearly tends to level off at high substrate concentrations. The results are similar to those previously observed with imidazole catalysts containing naphthalene rings,[2,3] and can be described by the Michaelis-Menten kinetics. In the case of the enzyme-like catalytic pathway (Eq. (2)), the catalytic rate is given by

$$v_{cat} = \frac{k_{cat}[C][S]}{K_m + [S]} \qquad (11)$$

The kinetic constants K_m and k_{cat} can be determined from Lineweaver-Burk plotting between $1/v_{cat}$ and $1/[S]$.[12]

$$\frac{1}{v_{cat}} = \frac{1}{k_{cat}[C]} + \frac{K_m}{k_{cat}[C]} \cdot \frac{1}{[S]} \qquad (12)$$

The Lineweaver-Burk plots of the data of Figs. 3 and 4 were linear, and K_m and k_{cat} values for respective substrates were obtained from the slope and the intercept as given in Table 2. The binding tendency varied with substrates in approximately the same manner as with K_c values. The catalyst concentrations were much smaller than the cholic acid concentration of the spontaneous hydrolysis. In addition, substrates were used in excess of the catalyst in all the catalytic hydrolyses. Therefore, association of the catalyst molecules can be neglected in catalytic hydrolysis in contrast to spontaneous hydrolysis, and $1/K_m$ will reflect the true binding capacity of the catalyst.

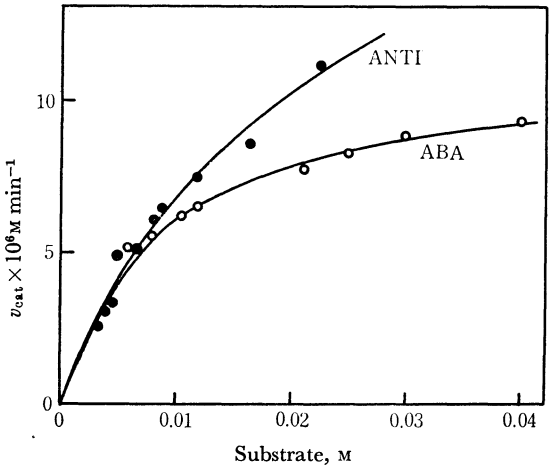


Fig. 3. Catalytic hydrolysis of phenyl esters.
○: substrate ABA, catalyst 4.54×10^{-4}M, 30°C, 1.0M KCl, pH 8.0
●: substrate ANTI, catalyst 2.27×10^{-4}M, 30°C, 1.0M KCl, pH 8.0

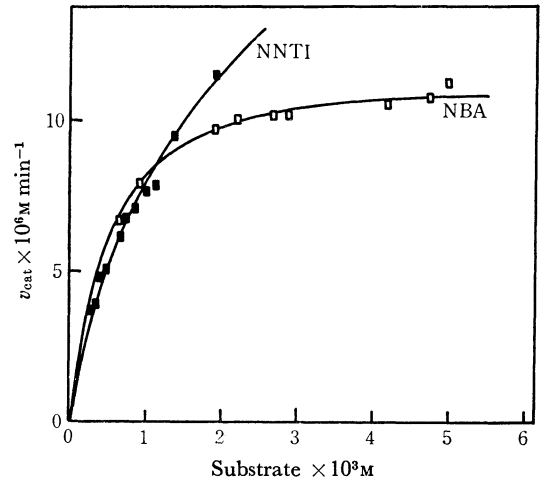


Fig. 4. Catalytic hydrolysis of phenyl esters.
□: substrate NBA, catalyst 5.04×10^{-4}M, 30°C, 1.0M KCl, pH 9.4
■: substrate NNTI, catalyst 2.27×10^{-4}M, 30°C, 1.0M KCl, pH 8.0

12) H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).

TABLE 2. CATALYTIC HYDROLYSES[a)]

Substrate	Catalyst M×10⁴	K_m mM	k_{cat} min⁻¹	k_{cat}/K_m min⁻¹M⁻¹	$1/K_c$ mM
ABA	4.05	8.0	0.028	3.0	63
NBA[b)]	4.50	0.53	0.033	62	0.27
ANTI	2.02	20	0.099	5.0	150
NNTI	2.02	0.94	0.075	80	9.4
ABA	8.2[c)]	16.4	0.021	1.3	—

a) Hydrolysis conditions: 30°C, pH 8.0, 1.0M KCl, unless otherwise stated.
b) Hydrolysis condtions: 30°C, pH 9.4, 1.0M KCl.
c) Catalyst, III. From Ref. 3.

It is obvious that the nonanoyl group enhanced substrate binding due to hydrophobic interaction. The binding tendency of cationic substrates was smaller relative to that of the anionic substrates (ABA *vs.* ANTI, NBA *vs.* NNTI). Since the catalytic species VII does not carry a negative charge, it is not necessary to take into account the electrostatic interaction between the opposite charges in the catalytic hydrolysis. Table 2 includes the rate data of ABA with naphthoylhistamine III. From a comparison of K_m values it is indicated that the steroid skeleton is twice as effective as the naphthalene ring as a hydrophobic binding site. As for the k_{cat} term, these catalysts showed very similar efficiencies, suggesting that the reactivity of the histamine moiety is not affected by this extent of difference in the binding site.

The binding capacities of cholic acid and its histamine derivative can be compared by using $1/K_c$ and K_m given for each substrate in Table 2. As mentioned above, K_c may be underestimated except for NBA. If this is taken into consideration, the $1/K_c$ values for ABA, ANTI, and NNTI will be smaller than those given in Table 2, and K_m and $1/K_c$ values may become close in the respective substrate. It is probable that the nature of the binding site is not greatly altered by incorporation of the histamine moiety.

TABLE 3. FREE ENERGIES OF SUBSTRATE BINDING

Substrate	ΔG_u kcal/mol	$\Delta\Delta G_u$ kcal/mol	Free energy of binding per methylene group, $\Delta\Delta G_u/7$, kcal/mol.
ABA	−5.33	−1.63	−0.23
NBA	−6.96		
ANTI	−4.78	−1.84	−0.26
NNTI	−6.62		

The unitary free energy change ΔG_u (kcal/mol) for substrate binding is defined as follows.[13)]

$$\Delta G = -RT\ln(1/K_m) \quad (13)$$

$$\Delta G_u = \Delta H - T\Delta S_u = \Delta H - T(\Delta S + 7.98) \quad (14)$$

ΔG_u values in the catalytic hydrolysis are summarized in Table 3. The $\Delta\Delta G_u$ value reflects the contribution of the nonanoyl group to substrate binding, and is similar in the anionic and cationic substrates. The contribution of the methylene unit to binding, *ca.* −0.25 kcal/mol, was in the same range as observed with some polymer catalysts.[6)] The hydrophobic contribution of the methylene group has been estimated to be *ca.* −0.75 kcal/mol. The smaller contribution of the methylene unit in the present case suggests that the alkyl chain is not fully available for the hydrophobic interaction with the catalyst.

In conclusion, the steroid ring in cholic acid and its derivative was shown to constitute a substrate binding site based on hydrophobic forces. This hydrophobic region did not show specific interactions at least with the substrates used, contrary to our expectation.

The authors are deeply grateful to Professor Chuji Aso, head of our research group, for his unfailing encouragement and advice.

13) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).