Rationalization of the Rate of the Acylation Step in Chymotrypsin-Catalyzed Hydrolysis of Amides

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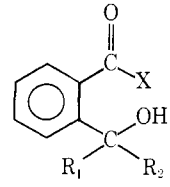
Abstract: The effect on lactonization rates of substituting a phenyl and a methyl group for the methylene hydrogen atoms of 2-hydroxymethylbenzoic acid (**1c**) and *N*-methyl-2-hydroxymethylbenzamide (**1a**) was studied at 25° in 3.2% ethanol–water. The 2-hydroxymethylphenylmethylbenzoic acid (**2c**) exhibited rate constants for hydrogen ion and hydroxide ion catalyzed cyclization which were 2700 and 610 times greater than that exhibited by the unsubstituted compound. The equilibrium constant for formation of the phthalide from the un-ionized acid was 1.1×10^3 times greater for the substituted derivative. The ionization constant for the substituted acid **2c** (4.64×10^{-4}) was within a factor of 3 of the ionization constant of the unsubstituted derivative **1c** (1.79×10^{-4}). The substituted phthalide differed by less than a factor of 3 from the unsubstituted derivative with respect to the rate constants for hydrogen ion and hydroxide ion catalyzed hydrolysis of the phthalide to the acid. Substitution of the two methylene hydrogen atoms for phenyl and methyl groups increased the rate constants of the hydrogen ion, hydroxide ion, and imidazole-catalyzed lactonization of the *N*-methylamides of acids **1c** and **2c** by factors of 200, 250, and 140, respectively. From the rate constant of 4.0×10^{-2} min^{-1} M^{-1} for the imidazole-catalyzed lactonization of the *N*-methylamide of **2c** and from the 15.5-fold enhancement observed for the imidazole-catalyzed cyclization on going from the *N*-methylamide of **1c** to the amide of **1c**, a rate constant of 0.62 min^{-1} M^{-1} was estimated for the imidazole-catalyzed elimination of ammonia from the amide of **2c**. It was shown that the rate constant for this reaction is high enough to account for the rate of the acylation step in the chymotrypsin-catalyzed hydrolysis of its best known amide substrates.

Previous work from this laboratory demonstrated that lactonization of 2-hydroxymethylbenzamides serves as a good model for the acylation step in the catalytic cycle of serine proteinases acting on amide substrates.[1] Both the enzymic and model reactions are catalyzed by imidazole.[1,2] Both reactions appear to involve two steps, formation and decomposition of a tetrahedral intermediate.[1-5] Interestingly, the rate-determining step for both the enzymic and model reaction changes with changing pH.[1,3-5] The imidazole-catalyzed lactonization of 2-hydroxymethylbenzamide **1b** comes within a factor of 23–280 of accounting for the rate of formation of the acyl enzyme intermediate in the catalytic cycle of chymotrypsin acting on its best amide substrates.[1] The inability of the rate of the imidazole-catalyzed lactonization of model compound **1b** to completely account for the acylation step in the catalytic cycle of chymotrypsin was attributed to differences in the relative orientation of the hydroxyl and carboxamido groups in the model and enzyme–substrate complex.[1] Bender[6] has postulated that a perpendicular approach of the nucleophile to the plane determined by the carbonyl carbon atom is the preferred geometry for the transition state for nucleophilic attack on a carbonyl carbon atom. Bunnett and Hauser[7] have shown that the acid-catalyzed lactonization of 2-hydroxymethylbenzoic acids is facilitated by bulky substituents positioned ortho to either the carboxyl or hydroxymethyl group. Bunnett and Hauser[7] rationalized this steric acceleration of lactonization by pointing out how ortho substituents would favor a conformation in which the attacking hydroxyl group was aligned perpendicular to the plane determined by the carboxyl group.[8]

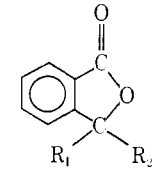
In this work, the lactonization of compounds **1** and **2** was studied in order to assess the sensitivity of the rate of displacement to the orientation of the attacking hydroxyl group, the leaving group X, and the catalyst.

Equilibrium constants for formation of phthalides **3** and **4** from acids **1c** and **2c** were also evaluated along with rate constants for the acid- and base-catalyzed hydrolysis of phthalides **3** and **4** in order to gain insight into the effect of substitution on the relative free energies of the acids, transition states, and phthalides.

As shown in Figure 1, the phenyl and methyl groups in **2** like Bunnett and Hauser's ortho substituents should also



	R_1	R_2	X
1a	H	H	$NHCH_3$
1b	H	H	NH_2
1c	H	H	OH
2a	Ph	Me	$NHCH_3$
2b	Ph	Me	NH_2
2c	Ph	Me	OH



3, $R_1 = R_2 = H$
4, $R_1 = Ph; R_2 = Me$

stabilize a conformation which favors lactonization. The bulky substituents should tend to be oriented above and below the plane of the benzene ring and away from the carbonyl carbon atom. Thus, the hydroxyl group is forced into the plane of the benzene ring and toward the carbonyl carbon atom. Interactions between the carbonyl group and the hydroxyl group should tend to force the amide bond and the benzene ring out of coplanarity. When the plane determined by the amide bond is oriented approximately perpendicular to the plane determined by its pendant benzene ring, the attacking hydroxyl group should be in van der Waals contact with the carbonyl carbon atom and aligned for formation of the tetrahedral intermediate. This work demonstrates that large rate enhancements for the acid- and base-catalyzed lactonization of the hydroxy acids and amides are associated with the steric stabilization of a conformer favoring lactonization. It is also shown in this work that the rate of imidazole-catalyzed lactonization of a substituted 2-hy-

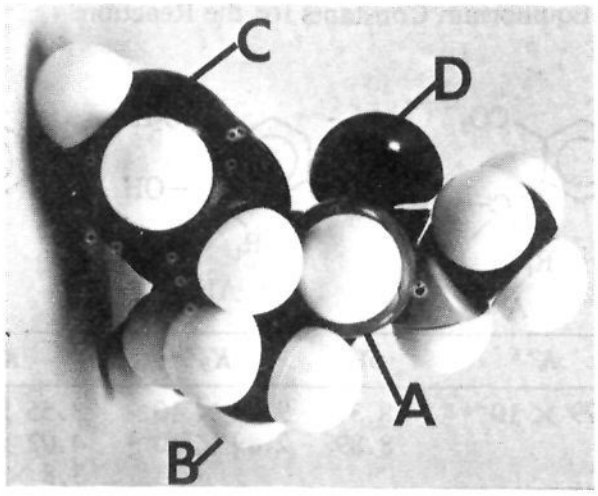


Figure 1. Space filling model of a reactive conformation of *N*-methylamide **2a.** A, B, and C are the hydroxy, methyl, and phenyl substituents of the 2-hydroxymethylphenylmethyl group. D is the carbonyl oxygen of the amide group.

droxymethylbenzamide is high enough to account for the highest known rates of formation of the acyl enzyme intermediate in the chymotrypsin-catalyzed hydrolysis of amide substrates.

Experimental Section

Materials. Phthalide **3** was obtained from Matheson Coleman and Bell and recrystallized from water: mp 73–74° cor (lit.[9] mp 73°).

N-Methyl-2-hydroxymethylbenzamide (**1a**) was prepared by stirring 5 g of phthalide (37 mmol) in 30 ml of 3.4 *N* methylamine (102 mmol) in methanol for 3 hr at room temperature. After cooling the reaction mixture in an ice bath, the product crystallized. The crystals were triturated with carbon tetrachloride to extract any remaining trace of phthalide and dried under reduced pressure at room temperature: mp 123–124° cor (lit.[10] 122–123°).

2-Hydroxymethylbenzamide (**1b**) was prepared by reacting **3** with ammonia by the method of Belke, *et al.*:[1] mp 148–149° (lit.[1] 148–149°).

N-Methylbenzamide was prepared by adding 48 g (0.34 mol) of redistilled benzoyl chloride (from Matheson Coleman and Bell) dropwise over 2 hr to a stirred solution of 15 *M* methylamine in water (from Matheson Coleman and Bell), which was cooled in an ice–salt bath. After completion of the reaction, excess methylamine was evaporated under reduced pressure at room temperature and the solution brought to pH 8 with 1 *M* HCl. The mixture was extracted with 300 ml of ether and the ether layer washed twice with water. The ether extract was dried over anhydrous sodium sulfate and the ether removed on a rotary evaporator. The resulting oil solidified on cooling. Recrystallization of the solid from dry ether gave 27 g (60% yield) of *N*-methylbenzamide: mp 77–78° (*lit.*[11] *mp* 77–78°).

N-Methyl-2-hydroxymethylphenylmethylbenzamide (**2a**) was synthesized by ortho metalation of *N*-methylbenzamide with *n*-butyllithium and subsequent condensation of the dilithioamide with acetophenone according to a previously described method.[12] The crude product was recrystallized from acetonitrile: mp 157.5–159.5° cor (lit.[12] 157–159°).

α-Phenylmethylphthalide (**4**) was prepared by stirring 2.2 g (8.6 mmol) of amide **2a** in hot methanol until liberation of methylamine was complete. The methanol was then evaporated under reduced pressure and the remaining white solid crystallized from ether to give **4** in 90% yield: mp 76.5–78° cor (lit.[13] mp 76.8–78°).

Imidazole, obtained from the Aldrich Chemical Co., was recrystallized three times from benzene and sublimed under reduced pressure: mp 88–89° cor (lit.[14] mp 90.2–90.6°).

Methylamine hydrochloride was obtained from Eastman Organic Chemicals.

Fluorescamine (Fluram) was a gift from Hoffman-La Roche Inc.

The distilled water supplied to the laboratory was run through a demineralizer and redistilled in an all-glass still. All other chemicals used were Mallinckrodt, Fisher, or Baker-Adamson analytical reagents.

Methods. Measurements of pH were made using a Radiometer Model 4B pH meter which was standardized with a 1:1 phosphate–NBS primary standard solution.[15] The response of the glass electrode was determined with NBS primary standard solutions (borax and phthalate). The glass electrode response (pH meter reading) was essentially a linear function of the pH between 4 and 9. Measurements of pH were made before and after each kinetic run, and the average value of pH was used. The total change in pH during a kinetic run in a buffered solution rarely exceeded 0.02 unit.

Hydrogen ion concentrations, $[H^+]$, were related to pH by $-\log([H^+]\gamma_H) = pH$. A value of 0.72 was used for γ_H at $\Gamma/2$ 0.87, 25°. This value was interpolated from mean activity coefficients of HCl in KCl listed by Harned and Owen.[16] The hydroxide ion concentration was related to the hydrogen ion concentration by $[H^+][OH^-] = K_w\alpha_{H_2O}/\gamma_H\gamma_{OH} = K_w'$. The formal dissociation constant of water, K_w', was interpolated from data listed in ref 16 ($K_w' = 1.75 \times 10^{-14}$ at $\Gamma/2$ 0.87, 25°).[17]

Rate Measurements. All reactions were carried out at 25 ± 0.2° in 3.2% ethanol, $\Gamma/2$ 0.87. Replacing ethanol with acetonitrile did not alter any rate constants. The hydrolysis of phthalides **3** and **4** and the cyclization of acids **1c** and **2c** were followed spectrophotometrically at 275 nm, the absorption maximum for phthalide. At pH values less than 7, the phthalide produced was either stable or underwent hydrolysis so slowly that the rate constants for the cyclization of amide **2a** could also be determined from the first-order approach of the absorbance at 275 nm to its final value. At pH values greater than 7, the cyclization of **1a** and **1b** was followed at 258.6 nm and the cyclization of **2a** was followed at 262 nm. These wavelengths are the isobestic points for **1c** and **3,** and **2c** and **4,** respectively. Thus, subsequent hydrolysis of the phthalide does not alter the time dependence of the absorbance at these wavelengths. For all the studies discussed above, the initial concentrations of reactants were in the range 0.16–0.32 m*M*. Pseudo-first-order rate constants were calculated from the slopes of linear plots of $-\ln|D_t - D_f|$ *vs.* time where D_t and D_f represent the absorbance reading at time t and the final absorbance reading, respectively. In some cases, rate constants were evaluated by the method of Guggenheim[18] from the slopes of plots of $-\ln|D_t - D_{t+\Delta t}|$ *vs.* time, where $D_{t+\Delta t}$ is the absorbance at a constant time after time t. When the absorbance of the reaction mixture was monitored continuously, it was kept at 25° in the thermostated cell compartment of a Gilford Model 240 multiple sample absorbance recorder. Slower reactions and reactions occurring in unbuffered or weakly buffered solutions were kept at 25° in a constant temperature bath. Reactions occurring in unbuffered or weakly buffered solutions were maintained at constant pH with a Radiometer TTT1 pH-Stat.

Reactions having pseudo-first-order rate constants greater than 1 min^{-1} (some of the reactions occurring in HCl and NaOH) were followed on an Aminco-Morrow stopped-flow apparatus (from the American Instrument Co.) coupled to a Beckman DU spectrophotometer equipped with a logarithmic photometer. Reactions in the stopped flow apparatus were initiated by adding an aliquot of a freshly prepared solution of the reactant in ethanol to distilled water, just prior to reaction. After thermal equilibration, an equal volume of the reactant in distilled water was mixed with a HCl or NaOH solution made to $\Gamma/2$ 1.74 with KCl. All other reactions were initiated by adding an aliquot of a freshly prepared solution of the reactant in acetonitrile or ethanol to the aqueous reaction mixture, thermally equilibrated at 25°. At pH values greater than 12, the lactonization of amides **1a, 1b,** and **2a** was also followed from the buildup and decay of absorbance at 275 nm. Values of the rate constants for phthalide formation and hydrolysis were evaluated using a computer program to fit the time dependence of the absorbance to the rate equation.[1] The initial concentrations were in the range 0.6–1.0 m*M* for **1a** and **1b** and 0.16–0.33 m*M* for **2a.** The rate constants for phthalide formation agreed with those measured at the isosbestic wavelengths. The rate constants for phthalide hydrolysis determined from the buildup and decay of the absorbance at 275 nm were in good agreement with those determined from separate hydrolysis of phthalides **3** and **4.** In imidazole buffer, at pH 7.9, rate constants for the cyclization of amides **1a** and **2a** were evaluated from the initial rates of release of methylamine. The initial concentration of **1a** and **2a** was 1.3 and 0.32 m*M,* respectively. Rate constants determined for **2a** from the first-order approach of absorbancies to their final values and the rate constants determined from the initial rate of release of methylamine were within 5% of each other. Rate constants for the cycli-

zation of amide **1a** were determined only from the initial rate of release of methylamine. In initial rate measurements, the release of methylamine was followed to less than 6% reaction and the rate constant calculated from the initial rate of methylamine release divided by the initial concentration of amide. Methylamine was determined by adding 400 μl of the reaction mixture to 2.0 ml of borate buffer (pH 8, 0.2 M in boric acid + borate) immediately followed by 1 ml of fluorescamine in dioxane (0.23 mg/ml). The fluorescamine was added while the solution was mixed on a vortex mixer. After 5–10 min, the fluorescence of the solution was measured at 475 nm (excitation at 390 nm). Since the excess fluorescamine is rapidly destroyed in the aqueous buffer, continuing reaction of excess fluorescamine with cyclizing amide was not a complicating factor. A standard curve relating fluorescence to methylamine concentration was prepared by assaying reaction solutions which contained known concentrations of methylamine instead of the amide. Calibration curves were linear up to 85 μM methylamine in the 400-μl aliquot being assayed. However, these standard curves varied with the imidazole concentration. Fluorescence measurements were made using an electronic ratio fluorimeter built by Dr. David Ballou and Mr. Gordon Ford.

The acid dissociation constants of acids **1c** and **2c** were determined from the pH values of partially neutralized solutions. The solutions were at 25°, $\Gamma/2$ 0.87, and 3.2% ethanol. The salt of the acid was prepared by diluting 1 part of the phthalide in ethanol with 30 parts of 0.02 M NaOH, 0.88 M KCl. The mixture was stirred over a N_2 atmosphere for several hours and any precipitated material removed by centrifugation. An aliquot (V_1, ml) of this solution was titrated with 0.1 M HCl, 3.2% ethanol, 0.77 M KCl. The amount of hydroxy acid in the solution, Y, was determined from the difference between the amount of sodium hydroxide originally added, X, and the amount of HCl used to neutralize the excess sodium hydroxide; 0.0645 mequiv in 6 ml and 0.021 mequiv in 5 ml of the salts of **1c** and **2c** were titrated. All pH measurements were made prior to precipitation of the acid and before any significant formation of phthalide could have occurred. With phthalide **3**, K' was evaluated from pH measurements between 3.1 and 4.1, and with phthalide **4**, K' was evaluated from measurements between 3.3 and 3.8. K' was evaluated from $K' = \alpha_H[A]/[AH]$. The concentration of salt, [A], and acid, [AH], was obtained from eq 1 and 2 where V_1 = volume of titrant (0.1 M in HCl) required to

$$[A] = [X(1 - f_1f_2) - 0.1(V_t - V_{bt}f_1)]/V \quad (1)$$

$$[HA] = (Y/V) - [A] \quad (2)$$

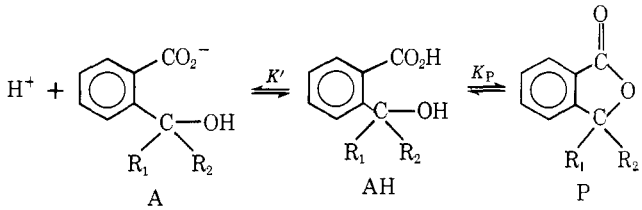
bring V_1 (ml) of the original sample solution to a given pH, V_{bt} = volume of titrant required to bring V_{b1} (ml) of a blank (containing NaOH but no hydroxy acid) to the same pH as the sample, $V = V_t + V_1$, $V_b = V_{bt} + V_{b1}$, $f_1 = V/V_b$, and $f_2 = V_{b1}/V_1$.

Equilibrium constants (K_p) for lactonization of the hydroxy acids were calculated from the relationship $K_P = K_P'(\alpha_H + K')/E_H$, where $K_P = [P]_e/[AH]_e$, $K_P' = [P]_e/([AH]_e + [A]_e)$. The equilibrium concentrations of phthalide, hydroxy acid, and its conjugate base are represented by $[P]_e$, $[AH]_e$, and $[A]_e$, respectively. The fraction (f_P) of material present as phthalide at any time was evaluated from the absorbance (a) of the solution at 275 nm, using the relationship

$$f_P = [P]/([AH] + [A] + [P]) = (a - a_A)/(a_P - a_A) \quad (3)$$

The absorbance of the solution when all the material is present as P and when all the material is present as an equilibrium mixture of AH and A is given by a_P and a_A, respectively. Equilibrium was approached from the phthalide and acid side. Initial values of absorbance gave accurate values of a_P and a_A. The reaction from the phthalide side was initiated by adding 0.2 ml of an ethanolic solution of phthalide (32 mM **3** or 8 mM **4**) to 3 ml of 0.1 M borate buffer ($\Gamma/2$ = 0.9) and 3 ml of 0.9 M KCl. The reaction from the acid side was initiated by first mixing 0.4 ml of the ethanolic solution of phthalide with 3 ml of 0.1 N KOH, 0.85 M KCl, and waiting until all the phthalide was converted to the acid salt. While mixing this solution on a vortex mixer, 6 ml of 0.1 N borate buffer ($\Gamma/2$ = 0.9) and 3 ml of 0.1 M HCl, 0.85 M KCl, were added. It may be shown that at any given time after mixing, K_P' can be calculated from the relationship $K_P' = f_{PA}/(1 - f_{PP})$, where f_{PA} and f_{PP} represent the fraction of phthalide present at a given time after mixing in the solution originally containing acid and phthalide, respectively. In some cases, the value of K_P' was evaluated from the ratio of the initial rates of the forward and reverse direction and from the equilibrium ratio of $f_P/(1 - f_P)$. In all cases, these values of K_P' were in reasonable agreement with each other.

Table I. Equilibrium Constants for the Reaction[a]



A AH P

Acid	K'[b]	pH	K_P'[c]	K_P[d]
1c[e]	1.79×10^{-4} [f]	8.31	9.71×10^{-2}	3.55×10^3
		8.89	2.89×10^{-2}	4.02×10^3
				3.8×10^3 (av)
2c[e]	4.64×10^{-4}	9.30	5.00	4.63×10^6
		10.35	0.377	3.92×10^6
				4.3×10^6 (av)

[a] All measurements made at 25°, $\Gamma/2$ 0.87, 3.2% ethanol, pH 8.3–9.3 borate buffer, pH 10.35 carbonate buffer. [b] K', acid dissociation constant, $pK' = pH + \log [AH]/[A]$. [c] $K_P' = [P]_e/([A]_e + [AH]_e)$. [d] $K_P = [P]_e/[AH]_e$. [e] **1c**, $R_1 = R_2 = H$. **2c**, $R_1 = Ph$; $R_2 = CH_3$. [f] A value of K' of 1.45×10^{-4} at low ionic strength has been reported in ref 7.

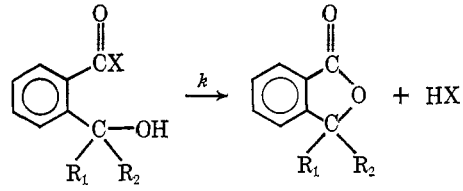
Results and Discussion

The equilibrium constants listed in Table I reveal a 1.1×10^3 fold increase in the equilibrium constant for lactonization of hydroxymethylbenzoic acid when the two hydrogen atoms of the methylene carbon atom are substituted with a phenyl and a methyl group. The rate constants for cyclization listed in Table II show that substitution at the hydroxymethyl group has a pronounced effect on the rate of cyclization. It is unlikely that these changes in cyclization rate constants are primarily the result of differences in electronic or solvation effects at the carbonyl carbon atom of **1** and **2.** Less than a three-fold difference between the acid dissociation constants, K', of **1c** and **2c** was observed (Table I), whereas 140–2700-fold increases in cyclization rate constants were observed on substitution at the methylene carbon atom. It is also unlikely that electronic effects accompanying substitution caused a change in reactivity of the hydroxyl group which could account for the differences in rate constants listed in Table II. The change in the Taft σ^* value (0.105)[19] on going from a methyl group to a $PhCH_3CH$– group should characterize the change in electronic effects on the hydroxyl group on going from **1** to **2.** Thus, a value of about 22 (*i.e.*, 2.3/0.105) for ρ^* would be required to explain, in terms of electronic effects, a 200-fold increase in the acid- and base-catalyzed cyclization rate constants on going from **1a** to **2a.**

Rate constants for the hydrolysis of phthalides **3** and **4** are listed in Table III. Comparison of the data in Tables II and III reveals that the more than 1000-fold increase in the stability of the substituted phthalide **4** over the unsubstituted compound **3** is reflected mainly in increases in the rate constants for the acid- and base-catalyzed lactonization rather than by decreases in the rate constants for acid- and base-catalyzed ring opening. This result leads to some interesting conclusions concerning how substitution facilitates lactonization.

Assuming that the base-catalyzed interconversion of acid and phthalide proceeds through pathway 1, the rate con-

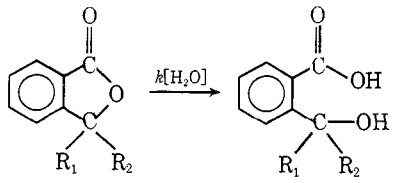
Table II. Rate Constants for the Reaction[a]



Compd	X = NHCH$_3$			X = NH$_2$			X = OH	
	k_H	k_{OH}[b]	k_{IM}	k_H	k_{OH}[b]	k_{IM}[c]	k_H	k_{OH}[d]
1[e]	1.3×10^{-2}	1.1	2.9×10^{-4}	0.170	2.4	4.5×10^{-3}	0.293[f]	7.1×10^{4}
2[e]	2.6	2.8×10^{2}	4.0×10^{-2}				8.0×10^{2}	4.3×10^{7}
2/1	2.0×10^{2}	2.5×10^{2}	1.4×10^{2}				2.7×10^{3}	6.1×10^{2}

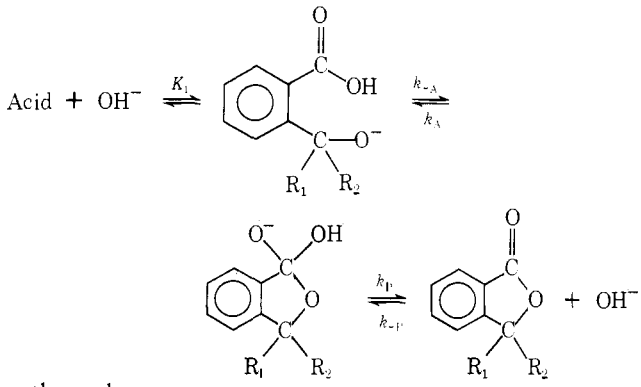
[a] At 25°, Γ/2 0.87, 3.2% ethanol. k values in min^{-1} M^{-1}. [b] At 12.3 > pH > 10. Above pH 12.3 ionization of the amide inhibits cyclization and also gives rise to another reaction, which probably involves displacement of the hydroxyl group. [c] From ref 1, 25°, Γ/2 0.822, 3.2% ethanol. [d] Calculated from K_P and the second-order rate constant for hydroxide ion catalyzed hydrolysis of the phthalide. [e] **1**, $R_1 = R_2 = H$. **2**, R_1 = Ph; $R_2 = CH_3$. [f] A value of 0.43 min^{-1} M^{-1} (at 30°, low ionic strength, 9% ethanol) has been reported in ref 7.

Table III. Rate Constants for the Reaction[a]



Compd	$k_H[H_2O]$,[b] min^{-1} M^{-1}	$k_{OH}[H_2O]$, min^{-1} M^{-1}
3[c]	7.7×10^{-5}	18.7
4[c]	1.9×10^{-4}	9.9
Ratio **4/3**	2.5	0.53

[a] T = 25°, Γ/2 0.87, 3.2% ethanol. The concentration of water was taken as unity. [b] Calculated from K_P and the second-order rate constant for hydronium ion catalyzed cyclization of the acid. [c] **3**, $R_1 = R_2 = H$. **4**, R_1 = Ph; $R_2 = CH_3$.



pathway 1

stants for phthalide formation and hydrolysis are given by $K_1 k_P k_{-A}/(k_P + k_A)$ and $k_A k_{-P}/(k_P + k_A)$. The second-order rate constants for the hydroxide ion catalyzed hydrolysis of phthalides **3** and **4**, ethyl acetate (6.8 min^{-1} M^{-1}),[20] and ethyl benzoate (1.8 min^{-1} M^{-1})[21] are all within an order of magnitude of each other. This result, coupled with the fact that $k_P \sim k_A$ for the alkaline hydrolysis of normal alkyl esters,[22] strongly suggests that substitution does not markedly alter the individual rate constants which comprise the expression $k_A k_{-P}/(k_P + k_A)$. Thus, the substituent mediated increase in the hydroxide ion catalyzed lactonization on going from **1c** to **2c** is primarily due to an increase in the rate constant (k_{-A}) for the attack of the neighboring alkoxide ion on the carbonyl carbon atom. It seems reasonable that for the other reactions in Table II, the primary effect of substitution is also to increase the rate constant for attack by the neighboring hydroxyl group, without markedly altering the rate constants for the decomposition of the tetrahedral intermediate to either phthalide or starting material. A transition state diagram for formation and hydrolysis of esters is shown in Figure 2. The increased reactivity of the substituted hydroxymethylbenzoic acids can be explained by assuming that the average orientation of the starting material more closely resembles that of the transition state. This explanation is consistent with the idea that the hydroxy acid reacts through a well-defined conformation which it assumes in a rapid, prior equilibrium step. If internal vibrations and rotations are restricted in this conformer so that all molecules in this conformation meet the geometric requirements of the transition state for formation of the tetrahedral intermediate, the free energy of activation would be zero, and the structure of the conformer would be identical with the transition state. If, on the other hand, all geometric requirements are met except for the distance between the carbonyl carbon atom and the hydroxyl group, the free energy of activation would correspond to the difference in bond energies between a C=O bond and two partial C—O bonds.

Assuming that the hydroxyl groups in **1c** and **2c** are similar in acidity to benzyl alcohol, a value of 3.2/55 can be calculated for K_1 from the relative acidities of water and benzyl alcohol reported by Hine and Hine.[23] Using this value along with a value of 4.3×10^7 min^{-1} determined for the second-order rate constant for hydroxide ion catalyzed cyclization of the acid, one obtains a value of 1.5×10^9 min^{-1} for k_{-A} (assuming $k_A \sim k_P$ and that OH$^-$ is not acting on **2c** as a general base catalyst). This value corresponds to a free energy of activation of 7.4 kcal/mol. The uncertainty in the differences in bond energy between a C=O bond and two partial C—O bonds makes it difficult to comment on how closely the ground state of **2c** meets the geometric requirements of the transition state for formation of the tetrahedral intermediate.

The pH-rate profile for the cyclization of **2a** is depicted in Figure 3. A break in the pH-rate profile similar to that observed for other hydroxamides[1,3,24] is seen in Figure 3. This break undoubtedly reflects a change in rate-controlling step, from formation to decomposition of a tetrahedral intermediate. Figures 4 and 5 illustrate the effect of phosphate, imidazole, and carbonate buffers on the rate of cyclization. In certain buffers, there is a nonlinear relationship between the observed pseudo-first-order rate constant and the buffer concentration. Such an effect is expected when the buffer catalyzes the step which is rate controlling at zero buffer concentration more efficiently than it catalyzes the step which is not rate controlling at zero buffer concen-

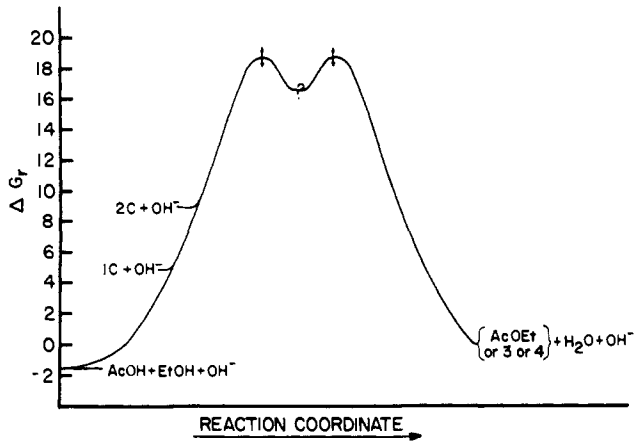


Figure 2. Transition-state diagram for hydroxide ion catalyzed ester formation and hydrolysis at 25°. ΔG_r represents the free energy difference between a given state and the parent ester plus water and the catalyst. The activity of water in the reaction mixture was set to unity. Variation in free energy of activation is indicated by ‡. Uncertainty in the position of the minimum corresponding to the tetrahedral intermediate is indicated by "?." Equilibrium and rate constants from W. P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, **86**, 4651 (1964), and J. F. Kirsch and W. P. Jencks, *ibid.*, **86**, 837 (1964), were used for calculating free energy differences for ethyl acetate.

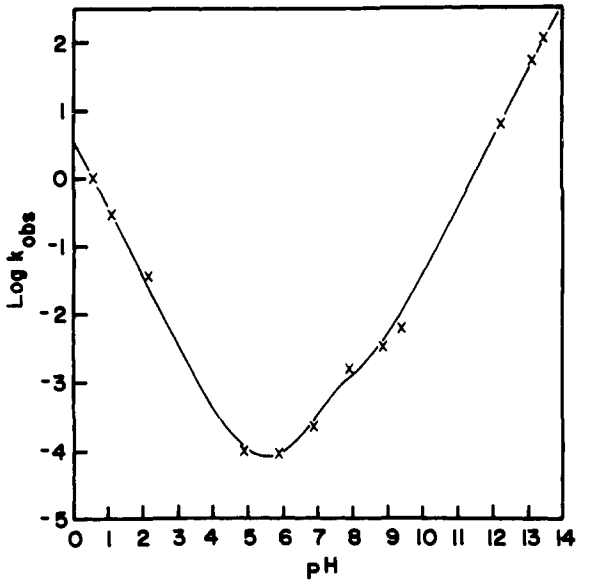


Figure 3. pH-rate profile for the hydrolysis of *N*-methylamide **2a** at zero buffer concentration, 25°, Γ/2 0.87. The solid line was calculated from the following relationships. At pH <5.5, $k_{obsd} = k_a = 2.6[H^+] + 2.8 \times 10^3[OH^-] + 6.0 \times 10^{-5}$ min^{-1}. At pH ≥5.5, $k_{obsd} = k_a k_b/(k_a + k_b)$, where $k_b = 2.76 \times 10^2[OH^-] + 1.8 \times 10^{-3}$ min^{-1}.

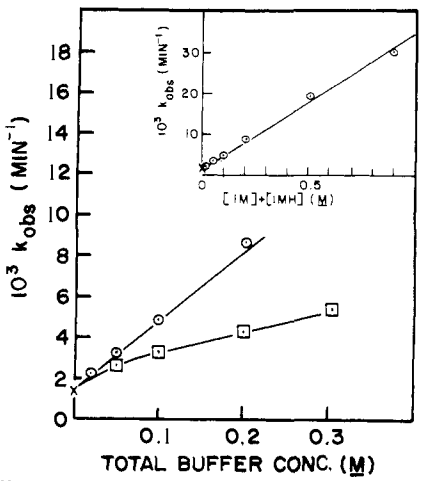


Figure 4. Effect of phosphate and imidazole buffer (pH 7.90 ± 0.02, 25°, Γ/2 0.87) on the pseudo-first-order rate constant (k_{obsd}) for the lactonization of *N*-methylamide **2a**: ×, no buffer, pH controlled with a pH-stat; ⊡, $H_2PO_4^-$ + HPO_4^{2-}; ⊙, imidazole + imidazole hydrochloride.

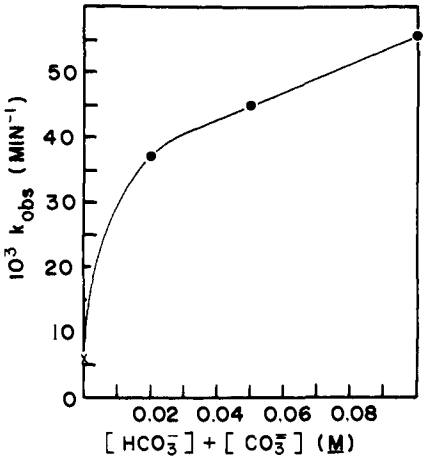


Figure 5. Effect of carbonate buffer (pH 9.44 ± 0.04, 25°, Γ/2 0.87) on the pseudo-first-order rate constant for the lactonization of *N*-methylamide **2a**: ×, no buffer, pH controlled with a pH-stat; ●, $[HCO_3^-]$ + $[CO_3^{2-}]$.

tration. The linear dependence of the rate constant on the imidazole concentration depicted in Figure 4 for the lactonization of **2a**, at pH 7.9, in the region of the change in the rate-determining step shows that imidazole can catalyze both formation and decomposition of the tetrahedral intermediate. This observation contrasts with the observations of Cunningham and Schmir[24] who found that only acid and bases that could act as both proton donors and acceptors could catalyze the cyclization of hydroxybutyramides. Furthermore, these acids and bases appear to catalyze only the decomposition of the tetrahedral intermediate.[24] The increased stability of the tetrahedral intermediate in the hydrolysis of 2-hydroxymethylbenzamides may be responsible for simple bases, such as imidazole, being able to efficiently catalyze lactonization. A plot (Figure 6) of the apparent second-order rate constant based on total imidazole concentration *vs.* the fraction of unprotonated imidazole yielded a value of 4.0×10^{-2} min^{-1} M^{-1} for the imidazole-catalyzed cyclization of **2a**. Figure 7 depicts the linear dependence of the pseudo-first-order rate constant for the imidazole-catalyzed lactonization of **1a** on the imidazole concentration in 80.4% neutralized imidazole at pH 7.9. The rate constant for imidazole-catalyzed lactonization increases by a factor of 15.5 on going from the *N*-methylamide **1a** (2.9×10^{-4} min^{-1} M^{-1}) to amide **1b** (4.5×10^{-3} min^{-1} M^{-1}).[1] Assuming a similar increase for the imidazole-catalyzed lactonization on going from *N*-methylamide **2a** (4.0×10^{-2} min^{-1} M^{-1}) to amide **2b**, a value of 0.62 min^{-1} M^{-1} ($4.0 \times 10^{-2} \times 15.5$ min^{-1} M^{-1}) was obtained for the imidazole-catalyzed elimination of ammonia from **2b**.[25]

It is interesting to consider the imidazole-catalyzed elimination of ammonia from **2b** as a model for the acylation step in the chymotrypsin-catalyzed hydrolysis of amide substrates. The rate constant for formation of an acyl enzyme from an enzyme–substrate complex in which the relative orientation of the enzyme–hydroxyl and substrate–carboxamido group was similar to that existing in model compound **2b** would be 0.62 min^{-1} times the effective local concentration of imidazole on the enzyme. Since proton transfer has no steric requirements, it is reasonable to assign a

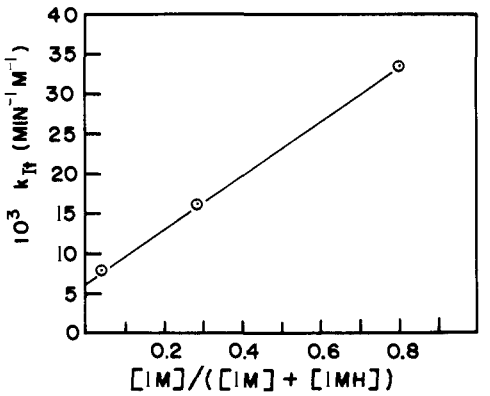


Figure 6. Dependence of the apparent second-order rate constant for the lactonization of *N*-methylamide **2a** catalyzed by imidazole buffer on the fraction of imidazole present as the free base (25°, Γ/2 0.87).

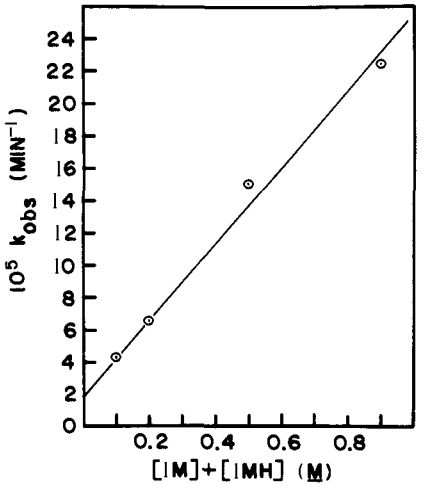


Figure 7. Effect of imidazole buffer (pH 7.90 ± 0.02, 25°, Γ/2 0.87) on the lactonization of *N*-methylamide **1a.**

value of 20 *M* to the local effective concentration of an imidazole residue around the hydroxyl group of the "active serine" residue of a serine proteinase.[26] We can therefore now account for a rate constant as high as 12 min^{-1} for the rate-determining acylation step in the chymotrypsin-catalyzed hydrolysis of amide substrates. This number is close to the rate constant for the chymotrypsin-catalyzed hydrolysis of its best known low molecular weight amide substrates. At 25°, pH 7.9, the rate constants for the chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-tyrosinamide, and *N*-isonicotinyl-L-tyrosinamide are 2.1, 9.5, and 25 min^{-1}, respectively.[27] Thus the rate constants for the acylation step in the chymotrypsin-catalyzed hydrolysis of its best substrates can be accounted for by assuming that in the enzyme-substrate complex the relative orientation of the serine hydroxyl group and the carbonyl carbon atom is similar to that existing in derivatives **2a-c.** A less favorable orientation between the α-carbonyl carbon atom of the substrate and the hydroxyl group of the "active serine" residue in the chymotrypsin-substrate complex could easily account for the decreased rate constants observed with other amide substrates.

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