Electrostatic Facilitation of General Acid Catalyzed α-Oxonium Ion Formation in a Lysozyme-Like Environment: Kinetic Investigations

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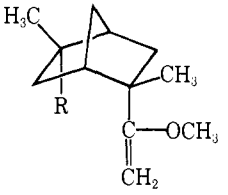
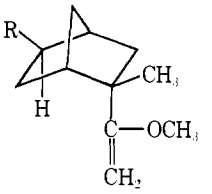
Abstract: The hydrolyses of *exo*-2,5-dimethyl-*endo*-2-(1-methoxyvinyl)bicyclo[2.2.1]heptyl-*endo*-5-carboxylic acid (**1**) and a "control" compound, *exo*-2-methyl-*endo*-2-(1-methoxyvinyl)bicyclo[2.2.1]heptyl-*exo*-5-carboxylic acid (**2**), have been studied as a function of buffer concentration and pH in order to assess the role of proximal carboxylate ions in assisting general acid catalyzed α-oxocarbonium ion formation. Buffer catalysis (acetic acid) in **1** is accelerated only 2.3-fold by carboxyl ionization, and it is argued that the un-ionized carboxyl compound does not hydrolyze unusually rapidly. Buffer catalysis (acetic acid) of the hydrolysis of **2** is accelerated 2.1-fold by a similar ionization. Hydronium ion catalysis is accelerated twofold in **2** by carboxyl ionization, but in **1** the apparent acceleration is 37-fold. The acceleration in the latter case is attributed to inefficient intramolecular general acid catalysis, with the "effective concentration" of the proximal acid group only 1.3 M. The pH–rate profile for **2** shows two breaks; the break at higher pH seems to require a change in the hydrolytic mechanism. On the basis of the modest accelerative effect of carboxyl ionization in this and other studies, it is concluded that significant electrostatic facilitation of general acid-catalyzed α-oxonium ion formation has received no experimental support and is probably not important in lysozyme.

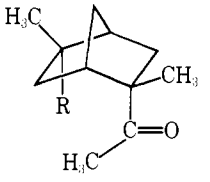
In the previous paper,[1] we outlined both the rationale and the synthesis of a model compound **1** and its control **2** designed for the investigation of electrostatic facilitation of α-oxocarbonium ion formation in an environment of limited solvent accessibility. We herein report our kinetic studies on the hydrolysis of **1** and **2** to **3** and **4**, respectively, the results



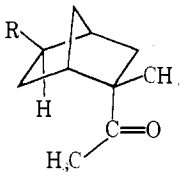
1a, R = CO_2H
b, R = CO_2^-



2a, R = CO_2H
b, R = CO_2^-



3a, R = CO_2H
b, R = CO_2^-



4a, R = CO_2H
b, R = CO_2^-

of which more strongly underscore the argument that carboxylate electrostatic stabilization of α-oxocarbonium ion formation is very weak.

Results

Kinetics. The hydrolysis of **1** (to **3**) and **2** (to **4**) at 25 °C was carried out in 5% ethanol at 1 M total ionic strength (maintained with KCl) and showed clean first-order kinetics over the entire pH range utilized in these studies. At a given pH, variation of buffer concentration at constant pH and ionic strength showed that the pseudo-first-order rate constant, k_ψ, conformed to the following equation

$$k_\psi = k_0 + \bar{k}_{cat}[A_T] \quad (1)$$

in which $[A_T]$ is the total stoichiometric buffer concentration [HA + A], where HA is the buffer conjugate acid and A is the buffer conjugate base. This behavior is illustrated for **1** in Figure 1, and Table I contains a summary of the kinetic data for both compounds **1** and **2**.[2] The division of $\bar{k}_{cat}$ into contributions from HA and A was readily assessed from the equation[3]

$$\bar{k}_{cat} = (k_{HA} - k_A)f_{HA} + k_A \quad (2)$$

in which k_{HA} is the catalytic constant for catalysis by HA, k_A is that for catalysis by A, and f_{HA} is the fraction of the stoichiometric buffer concentration in the HA form. The constant k_A was always found to be zero within experimental error,[4] so that eq 1 may be rewritten

$$k_\psi = k_0 + k_{HA}[HA] \quad (3)$$

The pH dependence of k_0 for **1**, $k_{0,1}$, is shown in Figure 2 and was observed to be of the functional form

$$k_{0,1} = \frac{k_{011}[H^+]^2 + k_{012}[H^+]}{K_{SH1} + [H^+]} \quad (4)$$

in which $pK_{SH1} = 5.17 \pm 0.08$, and k_{01i} are empirical constants whose mechanistic interpretation will be dealt with below. Compound **1** was too reactive to allow direct pK_a measurement, but measurement of the pK_a of keto acid **3** (by half-neutralization) gave a value 5.10 ± 0.02 in this solvent system. Thus, it appears reasonable to assign the kinetic pK_a to that of the carboxyl group in **1**. The derived constants from a fit of the data to eq 4 are presented in Table III. The kinetically equivalent interpretations of eq 4 will be dealt with in the discussion section below.

The pH dependence of k_0 for **2**, $k_{0,2}$, was expected to be similar to that of **1** with a considerably less pronounced break, since the carboxyl group is farther away. The observed pH dependence of k_0 for **2** is shown in Figure 3; the inset of that figure shows the superposition of the data for the pH dependence of the hydrolysis rates of **1** and **2**. The data for **2** are not well fit by an equation of the form of eq 4 and even more poorly fit by a linear log k_0 vs. pH dependence. A careful inspection of the data of Figure 3 revealed the presence of two breaks, one around pH 4 and the other, more pronounced, around pH 6. In fact, the data were adequately fit by eq 5, in which two apparent ionization con-

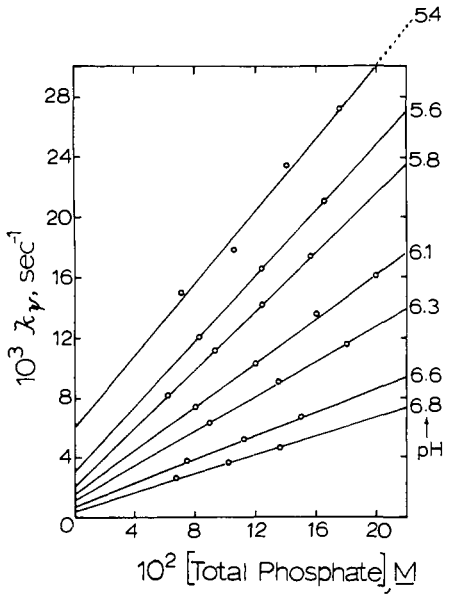


Figure 1. Catalysis of the hydrolysis of **1** by phosphate buffers at various pH values. The points are experimental, and the lines are calculated from the linear least-squares fit to eq 1.

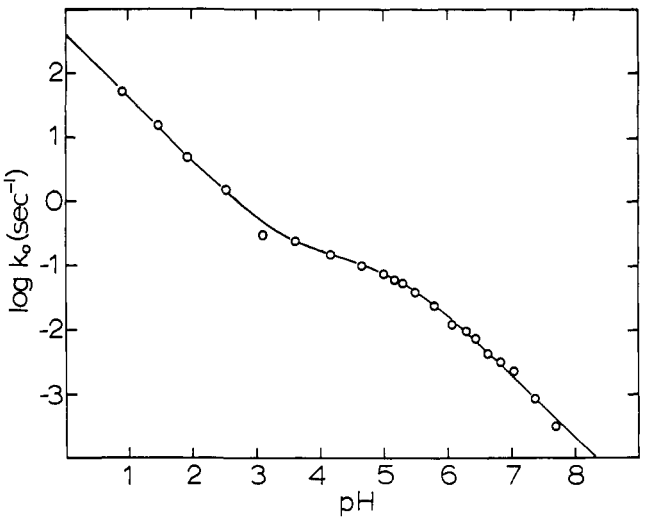


Figure 2. The pH dependence of the buffer-independent hydrolytic rate constant, k_0 (eq 1 or 3) for **1**. The points are experimental, and the line is calculated from eq 4 using the parameters given in Table III.

stants (K_{SH2}, K'_{SH2}) are indicated, and k_{02i} are again empirical constants.

$$k_{0,2} = \frac{k_{021}[H^+]^3 + k_{022}K_{SH2}[H^+]^2 + k_{023}K_{SH2}K'_{SH2}[H^+]}{[H^+]^2 + K_{SH2}[H^+] + K_{SH2}K'_{SH2}} \quad (5)$$

The derived constants from the fit of the data to this equation are presented in Table IV. The less pronounced

Table I. Summary Parameters for Hydrolysis of **1** and **2**

pH, f_{HA}[a]	$\tilde{k}_{cat}$,[b] M^{-1} s^{-1}	k_0,[b] s^{-1}	pH, f_{HA}[a]	$\tilde{k}_{cat}$,[b] M^{-1} s^{-1}	k_0,[b] s^{-1}
	Hydrolysis of **1**			Hydrolysis of **2**	
Formate buffer			Formate buffers		
3.62, 0.500	1.52 ± 0.16	(2.54 ± 0.12) × 10^{-1}	3.64, 0.500	(8.80 ± 0.76) × 10^{-1}	(1.42 ± 0.06) × 10^{-1}
Acetate buffer			Acetate buffers		
4.18, 0.742	(4.74 ± 0.93) × 10^{-1}	(1.53 ± 0.07) × 10^{-1}	3.88, 0.852	(4.15 ± 0.13) × 10^{-1}	(7.67 ± 0.10) × 10^{-2}
4.66, 0.488	(3.77 ± 0.22) × 10^{-1}	(1.07 ± 0.02) × 10^{-1}	4.17, 0.747	(4.03 ± 0.01) × 10^{-1}	(4.09 ± 0.01) × 10^{-2}
5.00, 0.304	(2.30 ± 0.31) × 10^{-1}	(8.13 ± 0.29) × 10^{-2}	4.40, 0.635	(3.36 ± 0.23) × 10^{-1}	(3.17 ± 0.16) × 10^{-2}
5.17, 0.228	(1.98 ± 0.21) × 10^{-1}	(6.46 ± 0.16) × 10^{-2}	4.70, 0.466	(2.62 ± 0.22) × 10^{-1}	(1.92 ± 0.20) × 10^{-2}
5.31, 0.176	(1.78 ± 0.12) × 10^{-1}	(5.31 ± 0.12) × 10^{-2}	5.02, 0.294	(2.17 ± 0.09) × 10^{-1}	(9.42 ± 0.78) × 10^{-3}
5.48, 0.126	(1.58 ± 0.20) × 10^{-1}	(3.84 ± 0.19) × 10^{-2}	5.33, 0.170	(1.38 ± 0.02) × 10^{-1}	(5.04 ± 0.16) × 10^{-3}
Phosphate buffer			5.50, 0.121	(9.56 ± 0.49) × 10^{-2}	(3.87 ± 0.43) × 10^{-3}
5.49, 0.932	(1.70 ± 0.02) × 10^{-1}	(4.13 ± 0.02) × 10^{-2}	Phosphate buffers		
5.78, 0.876	(1.67 ± 0.16) × 10^{-1}	(2.43 ± 0.17) × 10^{-2}	5.37, 0.948	(1.20 ± 0.10) × 10^{-1}	(6.08 ± 1.33) × 10^{-3}
6.08, 0.780	(1.36 ± 0.07) × 10^{-1}	(1.25 ± 0.11) × 10^{-2}	5.43, 0.941	(1.25 ± 0.01) × 10^{-1}	(4.02 ± 0.09) × 10^{-3}
6.30, 0.681	(1.04 ± 0.03) × 10^{-1}	(9.93 ± 0.36) × 10^{-3}	5.59, 0.916	(1.08 ± 0.01) × 10^{-1}	(3.18 ± 0.09) × 10^{-3}
6.44, 0.608	(8.89 ± 1.02) × 10^{-2}	(7.82 ± 1.30) × 10^{-3}	5.76, 0.881	(9.71 ± 0.07) × 10^{-2}	(2.16 ± 0.08) × 10^{-3}
6.63, 0.500	(7.68 ± 0.31) × 10^{-2}	(4.45 ± 0.32) × 10^{-3}	6.06, 0.788	(7.31 ± 0.27) × 10^{-2}	(1.62 ± 0.40) × 10^{-3}
6.83, 0.387	(5.72 ± 0.44) × 10^{-2}	(3.29 ± 0.47) × 10^{-3}	6.32, 0.671	(5.81 ± 0.21) × 10^{-2}	(1.20 ± 0.40) × 10^{-3}
7.05, 0.275	(3.52 ± 0.24) × 10^{-2}	(2.38 ± 0.22) × 10^{-3}	6.46, 0.597	(4.93 ± 0.16) × 10^{-2}	(1.19 ± 0.19) × 10^{-3}
7.37, 0.154	(2.29 ± 0.10) × 10^{-2}	(9.07 ± 0.82) × 10^{-4}	6.47, 0.591	(4.77 ± 0.11) × 10^{-2}	(1.06 ± 0.14) × 10^{-3}
7.70, 0.078	(1.35 ± 0.08) × 10^{-2}	(3.35 ± 0.56) × 10^{-4}	6.65, 0.489	(3.96 ± 0.04) × 10^{-2}	(7.68 ± 0.45) × 10^{-4}
HCl solutions[c]			6.83, 0.387	(3.14 ± 0.10) × 10^{-2}	(4.50 ± 1.09) × 10^{-4}
0.90, *d*		55.5 ± 3.4	7.01, 0.294	(2.36 ± 0.01) × 10^{-2}	(3.11 ± 0.03) × 10^{-4}
1.48, *d*		15.8 ± 0.3	7.32, 0.170	(1.48 ± 0.01) × 10^{-2}	(9.54 ± 1.66) × 10^{-5}
1.94, *d*		5.40 ± 0.18	7.51, 0.119	(1.03 ± 0.01) × 10^{-2}	(3.99 ± 0.02) × 10^{-5}
2.53, *d*		1.62 ± 0.02	HCl solutions[c]		
3.12, *d*		(3.17 ± 0.22) × 10^{-1}	1.45, *d*		12.1 ± 1.0
3.14, *d*		(3.14 ± 0.33) × 10^{-1}	1.94, *d*		4.78 ± 0.22
Deuterioacetate buffers[e]			2.55, *d*		1.23 ± 0.02
4.98, 0.500	(5.00 ± 0.19) × 10^{-2}	(1.45 ± 0.02) × 10^{-2}	3.35, *d*		(2.22 ± 0.06) × 10^{-1}
5.45, 0.333	(5.20 ± 1.00) × 10^{-2}	(8.52 ± 1.17) × 10^{-3}	Deuterioacetate buffers[e]		
Deuteriophosphate buffers[e]			4.98, 0.500	(4.70 ± 0.13) × 10^{-2}	(2.08 ± 0.13) × 10^{-3}
6.56, 0.750	(2.32 ± 0.24) × 10^{-2}	(1.52 ± 0.20) × 10^{-3}	5.45, 0.333	(3.85 ± 0.12) × 10^{-2}	(5.15 ± 1.35) × 10^{-4}
7.48, 0.278	(8.09 ± 0.42) × 10^{-3}	(2.24 ± 0.43) × 10^{-4}	Deuteriophosphate buffers[e]		
DCl solutions[e]			6.56, 0.750	(1.28 ± 0.02) × 10^{-2}	(1.64 ± 0.13) × 10^{-4}
1.32, *d*		5.09 ± 0.04	7.50, 0.278	(4.25 ± 0.26) × 10^{-3}	(5.04 ± 2.66) × 10^{-5}
2.60, *d*		4.39 ± 0.02	DCl solutions[e]		
			1.32, *d*		4.42 ± 0.08
			2.60, *d*		(3.70 ± 0.08) × 10^{-1}

[a] Defined in eq 2. [b] Equation. [c] H_3O^+ catalysis only. [d] Stopped-flow. [e] Values in pH column are pD values.

Table III. Constants of Eq 4 Derived for the H_3O^+-Catalyzed Hydrolysis of **1**

k_{011}, M^{-1} s^{-1}	$(4.22 \pm 0.58) \times 10^2$
k_{012}, s^{-1}	$(1.05 \pm 0.49) \times 10^{-1}$
K_{SH1}, M	$(6.8 \pm 1.2) \times 10^{-6}$
pK_{SH1}	5.17 ± 0.08
Standard deviation[a]	0.02

[a] Standard deviation of fit of log k_0 vs. pH to eq 4.

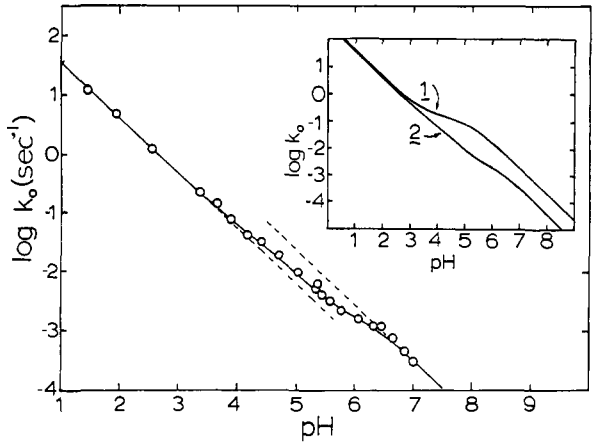


Figure 3. The pH dependence of the buffer-independent hydrolytic rate constant, k_0 (eq 1 or 3) for **2**. The points are experimental, and the line is calculated from eq 5 using the parameters given in Table IV. The inset is a superposition of the pH dependence of k_0 for compounds **1** and **2**.

Table IV. Constants of Eq 5 Derived for H_3O^+-Catalyzed Hydrolysis of **2**

k_{021}, M^{-1} s^{-1}	$(3.71 \pm 0.26) \times 10^2$
k_{022}, M^{-1} s^{-1}	$(6.26 \pm 0.94) \times 10^2$
k_{023}, M^{-1} s^{-1}	$(2.89 \pm 0.29) \times 10^3$
K_{SH2}, M	$(1.3 \pm 2.2) \times 10^{-4}$
pK_{SH2}	3.9 ± 0.8
K'_{SH2}, M	$(5 \pm 2) \times 10^{-7}$
pK'_{SH2}	6.30 ± 0.17
Standard deviation[a]	0.03

[a] Standard deviation of fit of log k_0 vs. pH to eq 5.

break in the pH-rate profile at pH $\simeq$4 (pK_{SH2} = 3.9 ± 0.8) is poorly defined by the data and, indeed, is expected to be ill-defined because of the weak effect on the rate anticipated for the carboxyl group, as noted above. The pK_a of **4** was found to be 4.81 ± 0.03, so that pK_{SH2} might reasonably be ascribed to that of the substrate carboxyl group, if the large error in this parameter is considered. On the other hand, the break at pH $\approx$6 (pK'_{SH2} = 6.30 ± 0.17) is somewhat more pronounced and better defined by the data; yet, it is the more difficult break for which to account, since there is only one ionizable group in the molecule. We shall consider the possible significance of this latter break below.

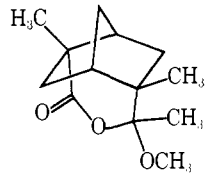
Solvent Isotope Effects. Solvent deuterium isotope effects, as well as buffer isotope effects, were measured for both **1** and **2** at both acidic and alkaline extremes of pH, well away from the breaks in the pH-rate profiles, in the linear regions of the log k_0 vs. pH plots. These isotope effects are tabulated in Table V and are all consistent with a rate-determining proton-transfer mechanism, as expected for this reaction.

Products and Possible Intermediates. Production determinations were made at both low and high pH for both **1** and **2**, and the products of hydrolysis of these compounds were the expected keto acids **3** and **4**, respectively, isolated in excellent yield. An effort was made to investigate the possible intermediacy of "lactal" **5** in the reaction of **1**. The observation of strict first-order kinetics at the wavelengths of both vinyl ether and ketone chromophores rules out **5** as an intermediate present in high concentration. In addition, an effort was made to trap **5** by running the hydrolysis of **1** in the presence of hydroxylamine at pH 7.8. The reaction gave a negative $FeCl_3$ test relative to a control solution containing no **1**.

Table V. Isotope Effects for Hydrolysis of 1 and 2[a]

Compound	$k_{H_3O^+}/k_{D_3O^+}$	k_{HA}/k_{DA}
1a	3.3	
1b	3.4	5.3[b]
2a	3.4	
2b	4.1	4.7[c]

[a] These isotope effects were determined well away from breaks in the pH–rate profile. [b] Acetate buffers. [c] Phosphate buffers.



5

Deuterium Incorporation Studies. Deuterium incorporation studies were carried out to ascertain whether the double bond protonation step is reversible. Experiments of two types were carried out. In the acid pH region (pH 1.45), reactions were much too rapid to monitor incorporation of deuterium into starting material, so that incorporation into products were measured. Products **3** and **4** were isolated and converted with CH_2N_2 to their respective methyl esters, **6** and **7**, and first analyzed by NMR. This analysis revealed that the products had NMR spectra identical with authentic materials, with the exception of the acetyl methyl signal, which appeared as a multiplet characteristic of deuterium substitution. Integration of this signal was inexact because of overlapping resonances, but this analysis served to establish the position of deuteration. Duplicate samples were subjected to a direct determination of atom excess deuterium. Compound **6** (derived from **1**) contained 0.88 atom of D/molecule, and **7** (derived from **2**) contained 0.84 atom of D/molecule. The NMR and analytical results together establish the position and amount of deuterium. In the basic region of pH, deuterium incorporation into unreacted starting material could be monitored for **1** and **2** in the NMR spectrometer, provided that the alcohol was omitted from the kinetic solution. Relative integrations of vinyl to vinyl ether methoxy proton signals through about 70% reaction gave time-invariant values of 0.64 ± 0.01 for **1** (pH 9.1) and 0.68 ± 0.04 for **2** (pH 7.7). These values are in accord with the theoretical value of 0.67 expected for no deuterium incorporation. It should be noted that all deuterium incorporation results were obtained well below or well above all breaks in the pH-rate profiles for both compounds and in the absence of buffers.

Nucleophilic Buffer Contributions. In selected cases, we wished to test the rate of the reaction as a function of the nucleophilicity of the medium. Thus, the solvent was made more nucleophilic by replacement of half of the KCl with KBr and less nucleophilic by replacement of half of the KCl by KNO_3; neither change significantly affected the pH of the solution. Full buffer plots were determined in the former case, and single reaction rates were measured in the

Table VI. Tests of Nucleophilic Component to Hydrolysis of **1**

Compound	Conditions[a]	k_{cat},[b] M^{-1} s^{-1}	k_o,[b] s^{-1}
1b	pH 6.66,[c] 1:1 KBr:KCl	$(7.20 \pm 0.77) \times 10^{-2}$	$(4.93 \pm 0.09) \times 10^{-3}$
1b	pH 6.66,[c] KCl only	$(7.68 \pm 0.31) \times 10^{-2}$	$(4.45 \pm 0.32) \times 10^{-3}$
2b	pH 5.77,[c] 1:1 KBr:KCl	$(7.94 \pm 0.89) \times 10^{-2}$	$(4.20 \pm 1.08) \times 10^{-3}$
2b	pH 5.77,[c] KCl only	$(7.71 \pm 0.07) \times 10^{-2}$	$(2.16 \pm 0.6) \times 10^{-3}$
1b	pH 6.66,[c,d] 1:1 KNO_3:KCl		1.69×10^{-2}[e]
1b	pH 6.66,[c,d] KCl only		1.58×10^{-2}[e]
2b	pH 6.66,[c,d] 1:1 KNO_3:KCl		7.56×10^{-3}[e]
2b	pH 6.66,[c,d] KCl only		6.70×10^{-3}[e]

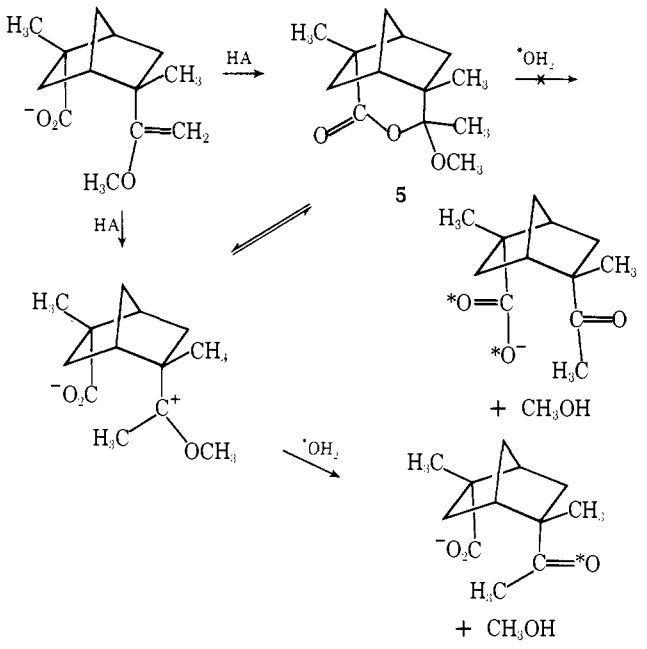
[a] Ionic strength maintained with a neutral salt mixture of the indicated composition. [b] Equation 1. [c] Phosphate buffers. [d] 0.150 M total phosphate; single kinetic determination. [e] Value of observed k, k_ψ; estimated error ≤5%.

latter. The results of these experiments are presented in Table VI.

Discussion

Reaction Products and Deuterium Exchange. The products isolated from the kinetic solutions were identified as the keto acids **3** and **4**. We failed to trap the hypothetical intermediate **5** with hydroxylamine, although the interpretation of this experiment is not straightforward. Hydroxylamine would be reasonably expected to react with **5** only at the carbonyl group, which we have previously shown[1] to be in a rather restricted environment. Thus, **5** may be formed, but might conceivably break down by alkyl-oxygen cleavage, a mode of cleavage known to exist even for unstrained acylals.[6] Models of **5** suggest that it is rather strained, so that relief of strain would accompany such a mode of cleavage. Further, in our synthesis of **1**,[1] there were ample opportunities for 2,5-lactonization to occur, yet none was found; this observation additionally underscores the strain inherent in a 2,5-bridge of the type found in **5**. Thus, the possible remaining roles of **5** are indicated in Scheme I. The

Scheme I

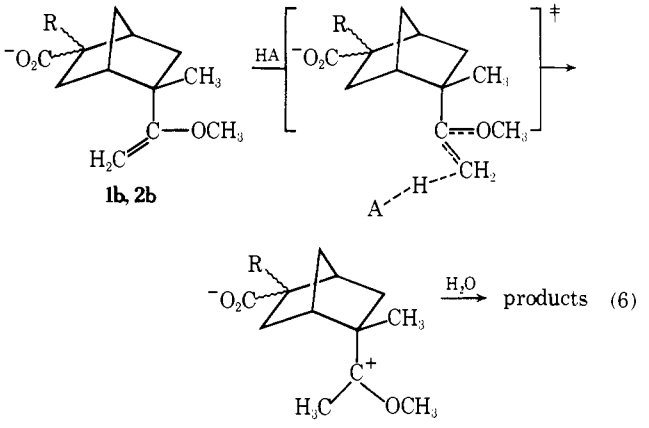


lack of a large rate enhancement in the buffer-catalyzed reaction for the ionized compound **1b** (see below) leads us to conclude that **5** is certainly of no kinetic consequence in the hydrolysis of **1b**.

In the acid region of pH, deuterium incorporation into product was assessed by NMR (to locate its position) and direct deuterium analysis (to determine the amount). Any mechanism in which proton transfer is rate determining predicts 1.0 deuterium/molecule in the product. The observed ratios of 0.84 and 0.88 for **1a** → **3a** and **2a** → **4a**, respectively, localized in the acetyl methyl group, are consistent with this prediction. The presence of a few percent of the protic keto acids **3** and **4** in the kinetic samples of **1** and **2** is impossible to rule out with certainty, although the NMR spectra of these compounds do not show obvious impurities; such impurities would account for the less than theoretical amount of deuterium incorporated. Likewise, the effect of any HDO in the D_2O solvent will be "amplified" in the deuterium incorporation work by the solvent isotope effect. Similar kinds of results were obtained by us previously.[7,8]

In the basic region of pH, deuterium incorporation into unreacted starting material in unbuffered solution gave the result that, for both **1b** and **2b**, no detectable deuterium appeared in these compounds during the course of hydrolysis. This result is also predicted by the rate-determining proton-transfer mechanism. The effect of the presence of buffer was not assessed because of the lack of availability of an oxygen acid which simultaneously met the criteria of a reasonable pK_a (so that the reaction had a sufficiently slow rate to be monitored by NMR) and freedom from interfering signals in the NMR.

Buffer Catalysis. Buffer catalysis was found to be general acid catalysis, with no apparent contribution from the buffer conjugate base. A two-point Bronsted correlation for phosphate ($H_2PO_4^-$) and acetate buffers gave a Bronsted slope, α, of 0.60 for **1b** and 0.65 for **2b**.[9,10] Deuterium isotope effects for buffer catalysis were found to be considerably greater than unity; this fact, together with the incorporation results previously noted, suggests the mechanism shown in eq 6 for buffer catalysis in **1** and **2**, which encompasses the feature of rate-limiting proton transfer in the transition state. In this mechanism, any effect of the carboxyl group is attributed to a primarily through-space in-



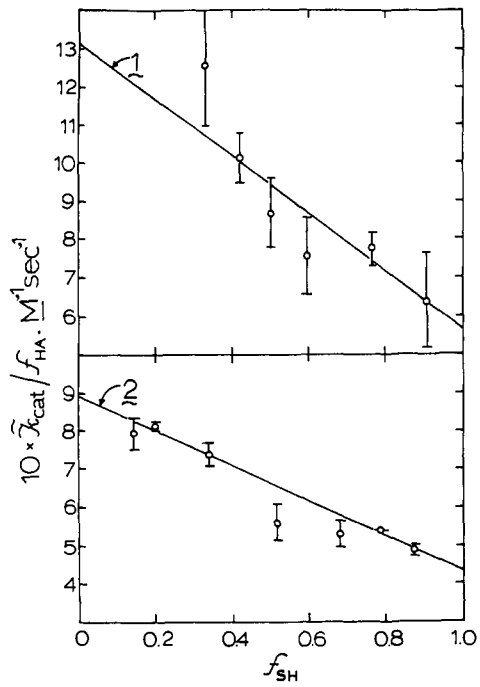


Figure 4. The effect of ionization of substrate carboxyl group on the specific rate of acetic acid catalyzed hydrolysis of **1** (top) and **2** (bottom). The points are experimental, using 5.17 as the pK_a of **1** and 4.81 as the pK_a of **2**, and the lines are calculated from eq 7 using the parameters of Table VII.

teraction[12] between the group and the developing charge at the reaction center. To assess the effect of ionization of the carboxyl group on buffer catalysis, we recognize that only apparent general acid catalysis by the buffer is observed (cf. eq 2 and 3), so that

$$k_{HA} = \bar{k}_{cat}/f_{HA} = k_{HA,SH}f_{SH} + k_{HA,S}f_S = (k_{HA,SH} - k_{HA,S})f_{SH} + k_{HA,S} \quad (7)$$

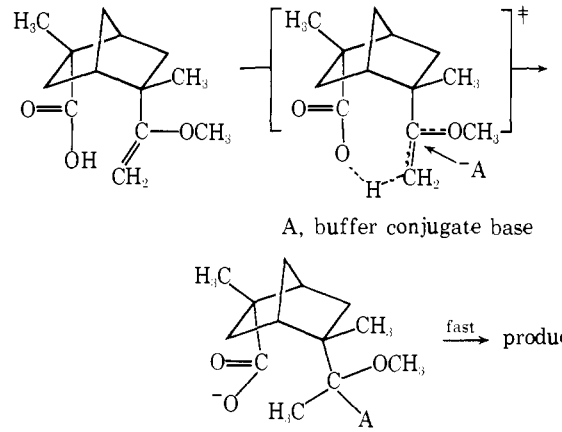
where $\bar{k}_{cat}$ and k_{HA} were previously defined (eq 2 and 3), $k_{HA,SH}$ is the second-order rate constant for buffer acid-(HA) catalyzed hydrolysis of the un-ionized substrate (SH) **1a** or **2a,** and $k_{HA,S}$ is the second-order rate constant for buffer acid-catalyzed hydrolysis of **1b** or **2b**. From a correlation based on eq 6 (Figure 4), the values of the rate constants given in Table VII are derived. Two features are evident: first, the ionization of the carboxyl group has only a slight effect on the rate; and second, the effect of the carboxyl ionization on the rate is not very different for the two compounds. In **2**, the source of the effect of the ionization of the carboxyl group is unambiguous; it could be due only to the through-space interaction of the carboxyl group and the developing positive charge at the site of hydrolysis. In **1**, the apparent modest effect of the carboxyl ionization may be due to unusually fast reactions of both **1a** and **1b,** such that the difference in the two rates is not large (i.e., participation by *both* ionized and un-ionized carboxyl groups), or to a truly feeble effect of carboxyl ionization on the reaction rate, over and above a normal hydrolysis rate for the un-ionized **1a**. Kresge et al.[13] found that ethyl cyclopentenyl ether (whose hydrolysis has essentially the same Bronsted α as those studied here) was hydrolyzed by acetic acid at 25 °C which a k_{HOAc} of 7.58×10^{-1} M^{-1} s^{-1}; compound **2a** hydrolyzes with a rate of 4.34×10^{-1} M^{-1} s^{-1} for acetic acid catalysis; and the rate for **1a** is 5.67×10^{-1} M^{-1} s^{-1}. Evidently, the rate for **1a** is not abnormally high, so that the ionization of the carboxyl group in **1** has only a weak accelerative effect on the rate of the reaction.

Table VII. Catalytic Constants for Buffer-Catalyzed Hydrolysis (Acetic Acid) of Ionized and Un-Ionized Formes of 1 and 2[a]

Compd	$k_{HOAc,SH}$, M^{-1} s^{-1}	$k_{HOAc,S}$, M^{-1} s^{-1}	$k_{HOAc,S}/k_{HOAc,SH}$
1	$(5.67 \pm 2.23) \times 10^{-1}$	1.31 ± 0.21	2.31
2	$(4.34 \pm 0.39) \times 10^{-1}$	$(8.93 \pm 0.22) \times 10^{-1}$	2.06

[a] Errors are standard deviations.

Another mechanism kinetically indistinguishable from that shown in eq 6 is the following (eq 8), which is sterically



(8)

reasonable for **1b** but not for **2b**. This mechanism would display kinetic behavior equivalent to general acid catalysis of the ionized substrate **1b,** which is observed. However, this mechanism implies a nucleophilic contribution of buffer, and there should be such a contribution from each nucleophile in solution. The data in Table VI show that when the medium is made more nucleophilic, the effect on the hydrolysis of **1** (k_0 term) is essentially nil within experimental error. A Swain-Scott[14] correlation shows that the calculated effect under these conditions should be about a 2.5-fold increase in k_0 (the k_0 term isolates the effect of nucleophiles Cl^- and Br^- from that of buffer anion). Compound **2b,** for which this mechanism is impossible, shows a salt effect which, within error, is not much different from that shown by **1b**. Similarly, making the medium less nucleophilic, if anything, increases the rate, although the one-point determination represents an observed effect on both k_0 and k_{HA}. Finally, one would expect for a different mechanism a considerable rate enhancement over the effect shown for the carboxyl group ionization in **2**, for which this mechanism is impossible; such a rate enhancement is not observed. Granting the assumptions inherent in these arguments, there seems to be no evidence to support the mechanism of eq 8 as the mechanism of buffer catalysis.

There are other mechanisms which, although formally consistent with the kinetic data, have been ruled out for other vinyl ether hydrolyses[7,8] and would be ruled out here on similar grounds.

Compound **1** was specifically designed as a situation in which solvation of the carbonium ion intermediate is poor, and in the previous paper[1] we have recorded observations which support the realization of this intention (the highly hindered attack of nucleophiles at the proacyl carbon). In view of this point, one might reasonably raise several questions about the hydrolysis rates of **1** and **2**. (1) Why is the hydrolysis of **1a** not unusually slow relative to that of **2a,** where such steric hindrance to solvation is presumably less severe? (2) Why should not nucleophilic attack of solvent on the carbonium ion become rate determining in hindered **1**? (3) Why do the effects of carboxyl ionization differ so little in **1** and **2**? To the first question, one may respond that

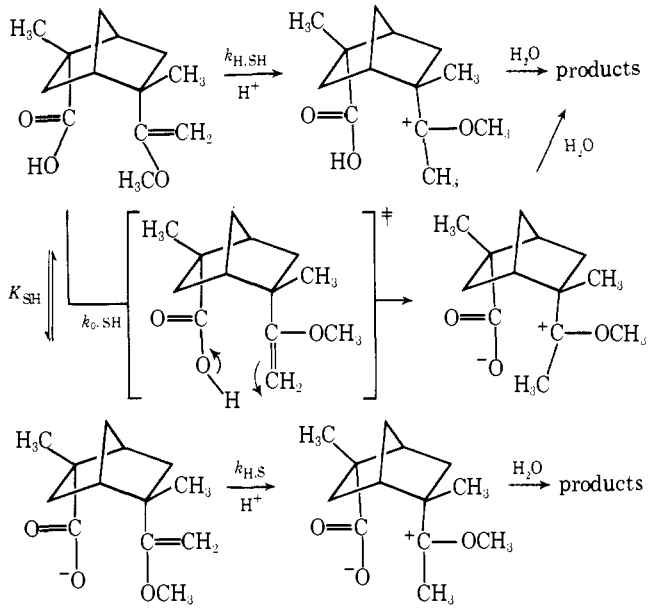
"solvation" may have a steric requirement less severe than that for overt nucleophilic attack. On the other hand, the un-ionized carboxyl group of **1a** with a strong, favorably oriented dipole may offset the lack of solvent interaction; i.e., the neutral carboxyl group may not be a bad "solvating group". To the second question, we note that if direct attack of solvent on the proacyl carbon of the α-oxocarbonium ion is hindered, conversion of the carbonium ion to product may yet occur by attack of solvent on the methoxyl methyl group. In addition, it is perhaps trivial to point out that, although attack of water on the proacyl carbon of the α-oxocarbonium ion might be retarded, because of steric hindrance, several orders of magnitude, such a retardation may not be sufficient to render this process rate determining. To the last question, there is the speculation that the carboxylate-carbonium ion interaction of **2b**, although occurring over a longer distance than the corresponding interaction in **1b**, might occur through a region of lower effective dielectric constant[16] than that in **1b**. Uncertainties in cavity shape make quantitation of this effect difficult, but a similar explanation may be advanced for the larger substituent effect of para relative to meta substituents on pK_a values of benzoic acids for substituents which cannot exert a resonance effect.[17] Finally, we of course recognize that the evidence that our model incorporates the "solventless" region we designed it to have rests only on a number of self-consistent observations during its synthesis and a consideration of models; there are, of course, limitations to such reasoning. Nevertheless, the fact remains: there is little rate enhancement of buffer catalysis by a proximal carboxyl group in **1**.

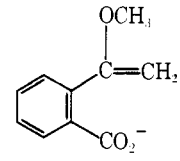
Hydronium Ion Catalysis. The catalysis of the hydrolysis of **1** and **2** by hydronium ion yielded the pH-rate curves shown in Figures 2 and 3. The pH dependence of the buffer-independent hydrolysis rate of **1** is rationalized by the mechanisms of Scheme II. Equation 9 follows from

$$k_{0,1} = \frac{k_{H,SH}[H^+]^2 + (k_{0,SH} + k_{H,S}K_{SH})[H^+]}{K_{SH} + [H^+]} \qquad (9)$$

Scheme II, and the constants relate to those in empirical eq 4 as follows: $k_{011} = k_{H,SH}$; $k_{012} = (k_{0,SH} + k_{H,S}K_{SH})$; and $K_{SH1} = K_{SH}$. Separation of the two terms of k_{012} cannot of course be made by kinetics; one term corresponds to internal general acid catalysis of **1a** hydrolysis, whereas the other corresponds to external general acid catalysis of **1b** hydrolysis. If k_{012} is dominated by the second term, then $k_{H,S}/k_{H,SH}$ may be calculated from Table III to be 36.7. The corresponding number for acetic acid catalysis (from Table VII), $k_{HOAc,S}/k_{HOAc,SH}$, is 2.3. The analogous ratios for the hydrolysis of **8** were found to be[7] 11.6 and 7, respectively, and those for **2** where internal general acid catalysis is impossible are 1.96 and 2.1, respectively (see below); thus, the value of 36.7 seems rather high. There could be two reasons for this observation. (1) Electrostatic facilitation of hydrolysis by carboxyl ionization could be more significant for positively charged acids than for neutral ones by a greater amount in the norbornyl compound **1** than in **8** or **2** because of anomalous electrostatic effects,[18] or (2) k_{012} is not dominated by $k_{H,S}K_{SH}$, but rather by the $k_{0,SH}$ term (internal acid catalysis). If explanation 1 is correct, then amine buffers, also positively charged, should show an anomalously large value of $k_{RNH_3^+,S}/k_{RNH_3^+,SH}$. Thus, we chose an amine buffer with a pK_a near that of **4** (methoxyamine, pK_a = 4.80 in our solvent system) with the intention of constructing a plot like that in Figure 4 for methoxyamine catalysis. Buffer catalysis by methoxyamine was absent, however, in the hydrolysis of **1** over the entire pH range investigated, so that the desired ratio is indeterminate. Slight buffer catalysis may be present in **2**. The failure to observe buffer catalysis of at least the ionized form of **1** suggests, however, that reason 1 does not explain the large $k_{H,S}/k_{H,SH}$ ratio. Therefore, internal "general acid catalysis" appears to be the major contributor to the high pH branch of the pH-rate profile of **1**. To estimate the rate acceleration due to this mode of catalysis, we may first estimate the contribution expected of hydronium ion catalysis via the second term of k_{012} by extrapolating our Bronsted correlation to the pK_a of the hydronium ion. Straightforward application of the Bronsted relationship yields a predicted ratio $k_{H,S}/k_{H,SH}$ = 5.3. Since $k_{H_3O^+}$ rates often deviate from Bronsted correlations (and actually do so in the case of **2**), a correction to the Bronsted pK_a of **1** was applied using the ΔpK_a observed for **2**. A predicted rate enhancement $k_{H,S}/k_{H,SH}$ = 2.3 was obtained.[20] Thus, the contribution of the $k_{H,S}$ term of k_{012} may be estimated to be about 6-14% of this term. The remainder may be readily ascribed to "general acid catalysis" by the internal carboxyl group ($k_{0,SH}$). This analysis gives for $k_{0,SH}$ a range of values of (9.0-9.8) × 10^{-2} s^{-1}. A carboxylate buffer of pK_a = 5.17 (=pK_{SH}) would be expected to have a catalytic rate constant of about 0.074 M^{-1} s^{-1} for hydrolysis of **1a**; applying the reasonable and perhaps excessively small[21] proximity correction of 10 M, the calculated value of $k_{0,SH}$ is about 0.74 s^{-1}. The observed value of 0.090 to 0.098 s^{-1} is only 12-13% of this value, so that this internal acid catalysis is extremely weak. Put another way, the "effective concentration" of the internal acid catalyst is only 1.3 M. Presumably, a more favorably disposed carboxyl group would show rate enhancements of the order of 600 M, as observed by Fife for ketal hydrolysis,[22,23] although this contention has not been tested for vinyl ether hydrolysis. Thus, our initial assessment that internal acid catalysis would not be favorable in **1** has been nicely borne out, although it is present in sufficient magnitude that it is barely observable.[24]

Scheme II





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The buffer-independent hydrolysis of **2** below ca. pH 5.5 may be interpreted in a manner similar to that of **1**, except that the effect of carboxyl ionization must be electrostatic in origin. The slight break observed near pH 4 is consistent with such an effect, so that eq 9 without the $k_{0,SH}$ term and Scheme II without the internal acid catalysis pathway, which is sterically unreasonable for **2**, appears to adequately describe the hydrolysis of **2** in this pH region. The rate acceleration due to ionization of the carboxyl group is 1.96.

The break at pH 6.3 is harder to rationalize. Before postulating reasons for its existence, we were concerned whether it did indeed exist. We considered it possible that there was only one break, but that random data error coincidentally produced two apparent breaks. Nevertheless, a data fit using only one apparent ionization was severely deteriorated, even when the ionization was constrained to values clustered around the expected pK_a of **2**. A large number of data points were gathered in the region of this break, and they served only to verify its existence. Our observation that ammonium ions do not catalyze the hydrolysis of **2** may provide us serendipitously with a method of subsequently determining this profile with even greater certainty. However, if this break is real, its apparent pK_a appears to be ca. 6.3. This is nowhere near the pK_a of any reasonable ionizable group in the molecule. Such a break can be evidence for a change of mechanism (bend away from the pH axis), along with a change of rate-determining step (bend toward the pH axis). Deuterium-exchange results in unbuffered solution at both ends of the profile require that the proton transferred remain nonequivalent to the olefinic protons prior to the rate-determining step; this requirement limits candidate mechanisms to those involving more than one step prior to formation of the α-oxocarbonium ion or its kinetic equivalent. Because the deuterium-exchange work was carried out under slightly different conditions, because the break is so small, and because the precision of the buffer-independent rates is poor (a result due to strong buffer catalysis), we shall defer a temptation toward mechanistic speculation until the pH-rate behavior can be more thoroughly investigated.

Electrostatic Catalysis by Asp^{52} in Lysozyme. This work reinforces the conclusion that stabilization of a developing α-oxocarbonium ion by electrostatic or nucleophilic interaction with a carboxylate group is not significant. This conclusion contrasts with the well-established role of carboxyl functions acting as general base or nucleophilic catalysts in other reactions,[26] not to mention the previously cited role of intramolecular carboxylic acid groups as proton donors in ketal hydrolysis and, presumably, in lysozyme itself. Rogers and Bruice[27] recently reported their aptly constructed model system designed to assess the "charge-relay" contribution of carboxyl groups in the serine proteases; the small effect they found might be essentially electrostatic in origin. To paraphrase their statement made for those enzymes, if Asp^{52} does, in fact, accelerate significantly glycoside hydrolysis via electrostatic or nucleophilic participation, such an acceleration must be ascribed to some "little understood factor not duplicated in these model studies". We have even attempted to provide an unusual "microenvironment" for this reaction, and the evidence in the preceding paper bears on our success in this objective. We are becoming increasingly convinced that Asp^{52}, if indeed an essential residue in lysozyme, must be assigned a role other than the one investigated here.

Recently, a new class of lysozymes, much different from hen lysozyme ("black swan type") have been studied,[28] and it will be of extreme interest to learn whether the active site of these proteins retains a carboxyl duo analogous to Glu^{35}-Asp^{52}. The x-ray crystal structure of bacteriophage T4 lysozyme was recently presented.[29] Although not homologous to hen lysozyme, this enzyme does have a molecular weight in the range of that of the "black swan" enzyme. Within the active site are two carboxyl groups, Glu^{11} and Asp^{20}; the latter has been identified as essential based on studies with phage mutations.[30] The former, however, whose essentiality is yet to be established, is involved in a salt bridge with Arg^{145}, and a direct catalytic (as opposed to structural) role for this residue would be difficult to envision. It is interesting to note that Asp^{52} of hen lysozyme also seems to be heavily hydrogen bonded to proximal residues in the crystal structure of the enzyme.[31]

Experimental Section

Kinetic Measurements. The solvent system for kinetic measurements was 5% ethanol/1 M ionic strength (KCl). Typically, 5 vol % absolute ethanol was added to a volumetric flask containing the required amounts of buffers and KCl dissolved in a little distilled water, and the flask was diluted to volume with doubly distilled, deionized water. We chose ethanol rather than dioxane as our organic solvent component because of the high absorbance of even carefully purified dioxane in the low-wavelength uv region. We found in dilute acid solutions that the pH meter reading very closely matched known $p[H^+]$ values, which were assumed to equal pa_H in the pH region under standard conditions of 1 M ionic strength. Likewise, the observed pH of buffer solutions was very close to that calculated on the basis of mass action using the pK_a of the buffer in this solvent system, measured by half-neutralization. Buffer pK_a values (25 °C, ±0.02) were found to be formic acid, 3.64; acetic acid, 4.64; methoxyamine hydrochloride, 4.80; and $H_2PO_4^{2-}$, 6.63. These values are very close to those found in pure water and in the 5% dioxane/1 M KCl system used previously. We also established that pD = pH + 0.31 by the usual method.[33] Deuterated solvents utilized C_2H_5OD in place of C_2H_5OH, and other active hydrogen compounds were either purchased in deuterated form or extensively exchanged prior to use.

Our general kinetic procedures have been previously described. The hydrolysis of vinyl ethers **1** and **2** were followed spectrophotometrically on a Cary Model 1605 spectrophotometer thermostated at 25 °C, at two different wavelengths. The hydrolysis reaction resulted in a large decrease of optical density at λ <230 nm, due to the disappearance of the vinyl ether chromophore, and this spectral region was generally used. The appearance of the ketone $n \rightarrow \pi^*$ transition, at 280 nm for both compounds, could also be utilized at higher substrate concentrations; thus, one region was essentially characteristic of vinyl ether and the other of ketone product. Kinetic determinations based on either transition were, for a given set of conditions, characterized by clean pseudo-first-order kinetics and identical rate constants. Scanning of the reaction at various times revealed tight isosbestic points for both compounds.

In the more acidic regions of pH, reactions became too fast to measure on the Cary, and we utilized an Aminco-Morrow stopped-flow device interfaced to Beckman DU optics and thermostated at 25 °C. The device was modified by replacing all air hoses with copper tubing, a change which gave highly reproducible drive syringe operation. Typically, a stock solution ca. 8×10^{-4} M in vinyl ether sodium salt, 5% ethanol, and 1 N in KCl was mixed with an acid or buffer solution of twice the desired final molarity, also 1 M in ionic strength and 5% in ethanol. Triplicate determinations were recorded on a Tektronix Type 549 storage oscilloscope and photographed with a Polaroid camera. Exchange of the two solutions between drive syringes had no effect on the traces obtained. A few runs were duplicated on both stopped-flow and Cary spectrometers, and these gave concordant results.

Our methods of calculation are on record in previous papers,[7] except that the Wentworth procedure[34] was adapted for nonlinear least-squares analysis of first-order rate curves on a Hewlett-Packard 9810A Calculator.

Product Studies. Sodium salts of **1** or **2** were hydrolyzed to completion in HCl or buffer solutions of the appropriate pH (or in DCl, in concurrent deuterium incorporation work) containing 5% ethanol, ionic strength 1 M (KCl). The solutions were extracted with ether, and the extracts were dried and concentrated. The white solids remaining in both cases had NMR and ir spectra iden-

tical with those of authentic ketones. This residue was esterified with CH_2N_2 and the product identified by both gas chromatography and GC–mass spectrometry as the methyl ester of the keto acid. The column used for the GC work was a 6 ft × ⅛ in. 20% Carbowax 20M column. Both high- and low-pH product determinations were carried out, the former requiring acidification of the medium prior to extraction.

Deuterium Incorporation Studies. At low pH, deuterium incorporation into product keto acid was studied, since the reaction was too fast to monitor incorporation into starting material. The isolation procedure described above in the product studies was used to obtain keto acids from DCl solutions; these were esterified (CH_2N_2) and preparatively gas chromatographed, and the resulting methyl esters of **1** and **2** were submitted to Mr. Josef Nemeth, University of Illinois, for direct analysis of deuterium incorporation. The NMR spectra of these compounds established the position of deuterium incorporation.

At high pH, sodium salts of **1** or **2** were hydrolyzed to completion in argon-blanketed, unbuffered deuterated kinetic solutions at pD 7.7–10.5 (1 M KCl). C_2H_5OD was not added because of interfering signals in the NMR spectrometer; thus, interpretations of this experiment are subject to the reasonable assumption that elimination of this small amount of ethanol would not result in a change of mechanism. For a similar reason, buffered media were not used, and thus, in the discussion, we have assumed that the presence of buffer would have no effect on the exchange results. That this is a reasonable assumption is supported by the linear buffer plots and the large k_{HA}/k_{DA} values. The pH in these solutions was found to be stable to within 0.1 unit. Deuterium incorporation in these cases was monitored by observing directly the vinyl ether methylene and methoxy signals. The analysis was carried out to 70–80% reaction, at which point the resonances became indiscernible.

Detection of Intermediates. An effort to trap a possible intermediate **5** was adapted from a procedure of Jencks and Blackburn[35] in a reaction mixture containing phosphate buffer, pH 7.8, 3.75×10^{-3} M 4, 3.75×10^{-2} M NH_2OH, and the other usual components of the kinetic medium. A ferric chloride test at several points during the reaction was compared to a blank and was found to be negative. Lactal **5** is not a known compound, and its synthesis was not anticipated to be trivial, so that a control to set limits of detectability was not available.

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Supplementary Material Available. A complete listing of the kinetic results (Table II) for hydrolysis of **1** and **2** (4 pp). Ordering information is given on any current masthead page.

References and Notes

(1) D. E. Ryono and G. M. Loudon, *J. Am. Chem. Soc.*, preceding paper in this issue.
(2) Table II in the microfilm edition is a detailed presentation of our kinetic data.
(3) Cf. Figure 3 and eq 2 in W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, N.Y., 1969, p 163. Our eq 2 readily follows from this equation by multiplication and division of all buffer terms by [total buffer] and recognizing that $f_{HA} + f_A = 1$.
(4) A plot of eq 2 in the form k_{cat} vs. f_{HA} for $H_2PO_4^-/HPO_4^{2-}$ shows severe upward deviation from linearity for both compounds **1** and **2** at f_{HA} values >0.6. This was shown quantitatively to be consistent with very efficient catalysis of the reaction by the very small amount of H_3PO_4 present.[5] This feature has also been observed for structurally unrelated vinyl ethers and only for phosphate buffers.[5] Monoprotic acids give linear plots as expected from this equation.
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(8) G. M. Loudon and C. Berke, *J. Am. Chem. Soc.*, **96**, 4508 (1974).
(9) Kresge[11] has shown that phosphate is expected to lie above a Bronsted plot for carboxylic acid catalysis of vinyl ether hydrolysis, so that these α values are probably lower limits.
(10) The k_{HOAc} was obtained by extrapolation of plots of $\bar{k}_{cat}/f_{HA}$ vs. f_{SH} to $f_{SH} = 0$; f_{HA} is the fraction buffer in the un-ionized form, and f_{SH} is the fraction substrate in the un-ionized form; cf. eq 7.
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