Metal Ion Effects on Intramolecular Nucleophilic Carboxyl Group Participation in Amide and Ester Hydrolysis. Hydrolysis of *N*-(8-Quinolyl)phthalamic Acid and 8-Quinolyl Hydrogen Glutarate

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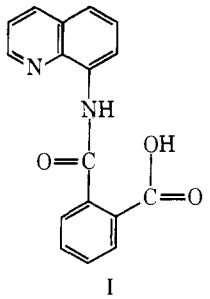
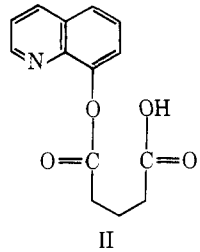
Abstract: The hydrolysis of the carboxyl substituted amide *N*-(8-quinolyl)phthalamic acid at 50 °C is characterized by a bell-shaped plot of log k_{obsd} vs. pH, indicating maximum reactivity of the neutral or zwitterionic species. The relatively fast rate of hydrolysis, the shape of the pH–rate constant profile, and the lack of a significant D_2O solvent isotope effect indicate that the carboxyl group is participating as a nucleophile. The pH–log rate constant profile for hydrolysis of 8-quinolyl hydrogen glutarate at 30 °C has, in addition to a bell-shaped region from pH 2 to 7, a plateau from pH 7 to 9 and, at high pH, a slope of 1.0. These regions correspond to nucleophilic participation by the carboxylate anion in reactions of the zwitterionic and anionic species and hydroxide ion catalyzed hydrolysis of the anion. Divalent metal ions (Cu^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}) have little effect on the intramolecular nucleophilic reaction of either the amide or ester. Metal ions in these systems would be expected to bind more strongly to the products, and consequently to the transition state, than to the reactant, but transition state effects were not detected. At pH values above 6 a metal ion promoted ^-OH-catalyzed reaction is observed in hydrolysis of the ester but not the amide. The order of reactivity of the metal ion complexes is Zn(II) > Co(II) > Ni(II). Rate enhancements range from 10^2 to 2×10^4 at a 100-fold excess of metal ion over substrate (nonsaturating). However, metal ion facilitated ^-OH catalysis, although capable of very large rate enhancements, cannot compete with the carboxylate ion nucleophilic reaction below pH 6, even though the latter reaction is not of maximum efficiency owing to poor steric fit of the carboxyl group and the carbonyl. Thus, in reactions of carboxypeptidase A with ester substrates, both types of mechanisms could be operative at appropriate pH values. The pH–rate constant profile for hydrolysis of 8-quinolyl hydrogen glutarate in the presence of Zn^{2+} is strikingly similar in shape to the pH–k_{cat} plots for the carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate and its chloro derivative.

Carboxypeptidase A has Zn(II) chelated at the active site.[1-3] The metal ion presumably complexes the carbonyl of ester and amide substrates. Glutamic acid-270 has also been implicated as a participant, and both general base and nucleophilic mechanisms involving Glu-270 have been proposed. There is strong evidence that the mechanisms are different with esters and amides.[4] It has been suggested that Zn(II) might polarize the carbonyl group of the substrate thereby increasing its susceptibility to nucleophilic attack.[1-3] The metal ion could also exert an effect by stabilization of the leaving group.[5] Such an effect might be especially important in the hydrolysis of peptides. There is, however, little chemical information available which would allow critical assessment of such proposals. Studies of metal ion effects on intramolecular carboxyl group participation in ester and amide hydrolysis are, therefore, of great relevance to an understanding of the mechanism of action of the enzyme.

Metal ion chelation of *N*-(2-phenanthrolyl)phthalamic acid[6] strongly inhibits carboxyl-catalyzed hydrolysis. This effect is most likely due to inhibition of breakdown of a tetrahedral intermediate to products. It is sterically not possible in that system for the metal ion to chelate the phenanthroline nitrogens and the amide nitrogen leaving group. Consequently, it is important to determine the effects of metal ions on intramolecular nucleophilic reactions of amides and esters in cases where the metal ion can complex the leaving group. In the present work, we have studied the hyrolysis of the carboxyl substituted amide, *N*-(8-quinolyl)phthalamic acid (I), and ester, 8-quinolyl hydrogen glutarate (II), in the presence of divalent metal ions. With both I and II, metal ion binding to the products,[7] and presumably to the transition state, will be much stronger than to the reactant so that possible transition state effects may be ascertained.



I



II

A further mechanistic possibility for carboxypeptidase A is nucleophilic attack by Zn(II) coordinated hydroxide ion.[8] Divalent metal ion promoted attack of ^-OH at the ester carbonyl[9,10] will give rise to rate enhancements in ester hydrolysis of 10^3–10^5, which are sufficient to overcome any contribution to the observed rate from general base catalysis by a neighboring carboxyl group.[9] Stable Co(III) complexes of peptides hydrolyze rapidly via internal nucleophilic attack by coordinated hydroxide ion,[11] but in cases where the complexes can dissociate, the effects of metal ion promoted ^-OH catalysis are generally smaller in amide hydrolysis than in ester hydrolysis.[12] An important chemical question in regard to the mechanism of carboxypeptidase A is whether a neighboring carboxyl group functioning as a nucleophile will be competitive with metal ion facilitated ^-OH catalysis.

Experimental Section

Materials. *N*-(8-Quinolyl)phthalamic acid (I) was prepared by refluxing equivalent amounts of phthalic anhydride (Mallinckrodt) and 8-aminoquinoline (Eastman) for 2 h in dry benzene. The resulting suspension was suction filtered. The light green solid was recrystallized from absolute ethanol and vacuum dried over calcium sulfate. The white crystals had mp 225–226 °C. Anal. Calcd for $C_{17}H_{12}N_2O_3$: C, 69.86; H, 4.16; N, 9.58. Found: C, 69.60; H, 4.19; N, 9.28.

8-Quinolyl Hydrogen Glutarate (II). Glutaric anhydride (Matheson) was recrystallized once from dry ether. Equivalent amounts of the purified anhydride and 8-hydroxyquinoline (Matheson) were dissolved in dry benzene and refluxed overnight. The resulting solution was allowed to cool to room temperature, and the white solid which pre-

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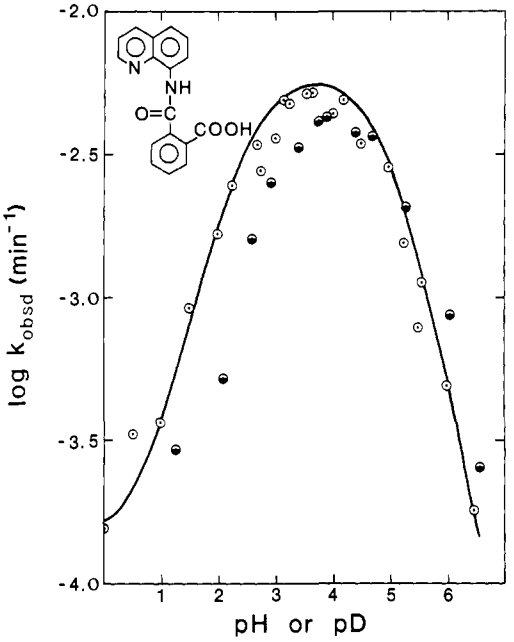


Figure 1. Plot of log k_{obsd} vs. pH or pD for hydrolysis of *N*-(8-quinolyl)-phthalamic acid (I) at 50 °C and μ = 0.5 with $LiClO_4$ in H_2O (⊙) and D_2O (◒). The line was calculated from eq 1 using the appropriate values of the constants.

cipitated was subsequently recovered by suction filtration, recrystallized from absolute ethanol, and vacuum dried over calcium sulfate in an Abderhalden apparatus. The compound had mp 115–117 °C (lit.[13] 115–117 °C).

Kinetic Methods. Stock solutions of substrate (10^{-2} M) were made up in methanol. In studies employing a Beckman Model 25 spectrophotometer, 20 μL of the substrate stock solution was injected into the reaction cuvette containing 2 mL of buffer, and the reaction was monitored at the appropriate wavelength after stirring (290 or 265 nm (I), 260 or 315 nm (II)). The spectrum of the solution upon completion of the reaction was invariably both qualitatively and quantitatively that of equivalent concentrations of the appropriate products, amine or alcohol plus acid. Temperature was controlled at 30 ± 0.1 °C or 50 ± 0.1 °C, and the ionic strength of the buffers was kept constant at 0.5 M with either KCl or $LiClO_4$. Each reaction was measured in duplicate or triplicate. Pseudo-first-order rate constants were calculated with an IBM 360 computer or an Olivetti-Underwood Programma 101.

Reaction solution pH values were measured with a Radiometer Model 22 pH meter or a Beckman Model 3500 digital pH meter standardized with Mallinckrodt standard buffer solutions.

To avoid trace metal ion contamination in buffers the following precautions were taken. Deionized water was used throughout. Plastic labware was used whenever possible. Buffer solutions were extracted with a 0.001 M solution of dithizone in carbon tetrachloride[14] to remove metal ion from the buffer itself and/or salts (KCl or $LiClO_4$) used to maintain ionic strength. Spot checks of k_{obsd} values were made using the same buffer plus 10^{-5} M EDTA, and values of the rate constants were identical in the presence and absence of EDTA.

Spectrophotometric Determination of pK_a Values. *N*-(8-Quinolyl)phthalamic acid is not sufficiently soluble in H_2O for accurate titrimetric determination of the pK_a values. However, it was found that large changes in absorbance at appropriate wavelengths occurred with changes in pH so that a pK_a value could be determined spectrophotometrically. A series of buffer solutions were prepared to cover a pH range of 0–12, and the ionic strength was kept constant at 0.5 M with KCl. A 10^{-2} M stock solution (20 μL) was injected into a cuvette containing 2 mL of buffer which had been thermally equilibrated at 50 ± 0.1 °C. After mixing, the absorption spectrum was taken using a Beckman Model 25 spectrophotometer. A plot of absorbance at the appropriate wavelength vs. pH was made. Amide I has one discernible pK_a of 4.85 at 50 °C measured at 260 nm.

Results

Figure 1 presents a plot of log k_{obsd} vs. pH or pD[15] for hydrolysis of *N*-(8-quinolyl)phthalamic acid. The plot shows an apparent dependence of k_{obsd} on the ionization of two groups. Hydroxide ion catalysis was not observed. At pH 8.23 in Tris buffer and pH 11.15 (KOH) significant changes in absorbance were not detected over a period of 48 h. The data give a good fit to the equation

$$k_{obsd} = \frac{k_2 K_a a_H}{a_H^2 + K_a a_H + K_a K_a'} \quad (1)$$

where K_a and K_a' are the apparent dissociation constants and k_2 is the rate constant for carboxyl participation when the compound is neutral or in the equivalent zwitterionic form. Employing the measured pK_a' of 4.85, and assuming a pK_a of 2.45 to give the best fit to the experimental data, the rate constant k_2 for maximum participation by the carboxyl group is 6.2×10^{-3} min^{-1} in H_2O and 5.2×10^{-3} min^{-1} in D_2O ($k_2^{H_2O}/k_2^{D_2O}$ = 1.19). Significant buffer catalysis was not observed in the hydrolysis of I at pH 3.00 (formate) or pH 4.45 (acetate) over a total buffer concentration range of 0.05–0.4 M.

Table I lists the rate constants calculated for the hydrolysis of I in the presence of 10^{-2} M metal ion (Co^{2+}, Ni^{2+}, and Zn^{2+})[16] at 50 °C at various pH values. The presence of a 200-fold excess concentration of metal ion over amide does not affect the values of k_{obsd} significantly.

Figure 2 presents a plot of log k_{obsd} vs. pH for hydrolysis of 8-quinolyl hydrogen glutarate (II). The plot indicates an apparent dependence of k_{obsd} on the ionization of two groups, and at pH values greater than 8.5, hydroxide ion catalysis is observed. The data give a satisfactory fit to the equation

$$k_{obsd} = \frac{k_H a_H^2}{K_a + a_H} + \frac{[k_1 a_H + k_2 K_a' + k_{OH}(OH^-)K_a']K_a}{K_a K_a' + K_a a_H + a_H^2} \quad (2)$$

where K_a and K_a' are apparent dissociation constants, k_H is the rate constant for acid-catalyzed hydrolysis of the nondissociated ester, k_1 is the rate constant for participation of the carboxyl group when the quinoline nitrogen is protonated, k_2 is the rate constant for participation when the quinoline nitrogen is unprotonated, and k_{OH} is the rate constant for hydroxide ion catalyzed hydrolysis of the ionized ester. From the data, k_{OH} was determined to be 112 M^{-1} min^{-1}. The theoretical line in Figure 2 was calculated using this k_{OH} value, plus the values of Maugh and Bruice[13] for k_H (1.45×10^{-3} M^{-1} min^{-1}), k_1 (1.16 min^{-1}), k_2 (5.8×10^{-3} min^{-1}), K_a (4.79×10^{-4}), and K_a' (2.69×10^{-5}).

Figure 3 is a plot of log k_{obsd} vs. pH for the hydrolysis of II in the presence of 10^{-2} M Ni^{2+}, Co^{2+}, and Zn^{2+} (100-fold excess over ester).[17] The plot is identical with that of II in the absence of metal ion except that the inception of hydroxide ion catalysis is observed at lower pH values. The second-order rate constants k_{OH} are given in Table II. The enhancements in k_{OH} produced by the metal ions range from 4.38×10^2 with Ni^{2+} to 1.92×10^4 with Zn^{2+}. Figure 4 shows the dependence of the rate of hydrolysis on Ni^{2+} concentration at 30 °C at pH 8.95. The plot is linear and no saturating effects are observed at Ni^{2+} concentrations as high as 2×10^{-2} M (200-fold excess).

Discussion

Hydrolysis of I and II. The pH–rate constant profile for hydrolysis of I is bell shaped, and the observed rate constants at 50 °C are 10^4 greater than those observed for the analogous amide *N*-(2-pyridyl)benzamide[6] at 90 °C or calculated for hydrolysis of benzamide at 109 °C.[18,19] Thus, the carboxyl group substituent must be participating in the reaction. As with the other phthalamic acid derivatives that have been investi-

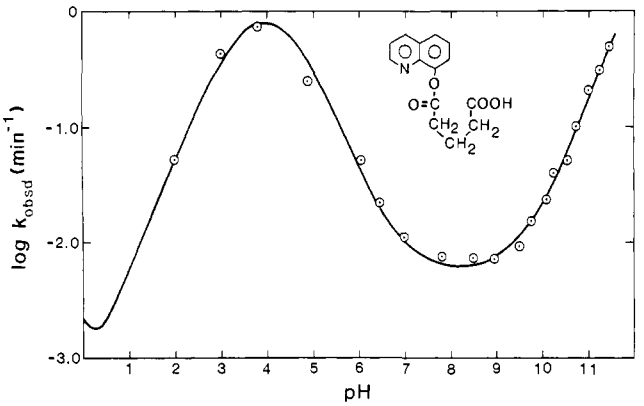


Figure 2. Plot of log k_{obsd} vs. pH for hydrolysis of 8-quinolyl hydrogen glutarate (II) in H_2O at 30 °C and μ = 0.5 with $LiClO_4$. The line was calculated from eq 2 using the appropriate values of the constants.

Table I. Rate Constants for the Hydrolysis of *N*-(8-Quinolyl)-phthalamic Acid[a] (I) in the Presence of Metal Ions at 50 °C (μ = 0.5 M with $LiClO_4$)

pH	Metal ion	Concn, M × 10^3	k_{obsd} × 10^3, min^{-1}
2.75[b]	None	0	3.60
	Co^{2+}	10	3.27
	Ni^{2+}	10	2.85
	Zn^{2+}	10	3.43
4.00[c]	None	0	5.17
	Co^{2+}	10	4.69
	Ni^{2+}	10	4.32
	Zn^{2+}	10	4.06
5.00[c]	None	0	3.06
	Co^{2+}	10	3.01
	Ni^{2+}	10	2.92
	Zn^{2+}	10	2.98
6.45[d]	None	0	0.180
	Co^{2+}	1	0.094
	Ni^{2+}	1	0.099
	Zn^{2+}	1	0.109
8.23[e]	None	0	0[f]
	Co^{2+}	1	0[f]
	Ni^{2+}	1	0[f]
	Zn^{2+}	1	0[f]

[a] Amide concentration was 10^{-4} M. [b] $HClO_4$. [c] Acetate (0.1 M). [d] Cacodylate (0.1 M). [e] Tris (0.1 M). [f] No significant absorbance change was observed over a 48-h period.

gated,[6,20,21] this participation is most likely by a nucleophilic pathway through an anhydride intermediate. The D_2O solvent isotope effect close to unity indicates that the carboxyl group is functioning as a nucleophile. A general base or general acid mechanism would be expected to proceed much more slowly in D_2O than in H_2O. The intermediate anhydride would hydrolyze rapidly to phthalic acid in a fast step. Phthalic anhydride has been indirectly demonstrated to be an intermediate in the hydrolysis of phthalamic acid[20] and phthalate monoesters.[22] It hydrolyzes in the pH range 1.6–5.7 in a pH-independent reaction with a rate constant of 0.739 min^{-1} at 30 °C,[22b] which is several orders of magnitude greater than the k_{obsd} values for I in that pH range at higher temperature. *N*-Methylphthalimide is formed in addition to hydrolysis products in reactions of *N*-methylphthalamic acid,[23] but in the present study of I there was no evidence for imide formation. Likewise, no evidence was found for imide formation during hydrolysis of phthalanilic acids in aqueous solution.[21]

The bell-shaped profile for hydrolysis of I indicates that the neutral species or the kinetically equivalent zwitterion is the most reactive species (eq 3). Breakdown of a tetrahedral in-

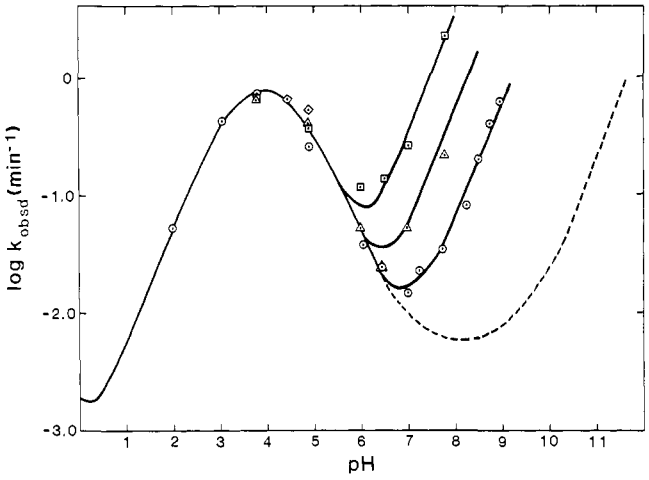
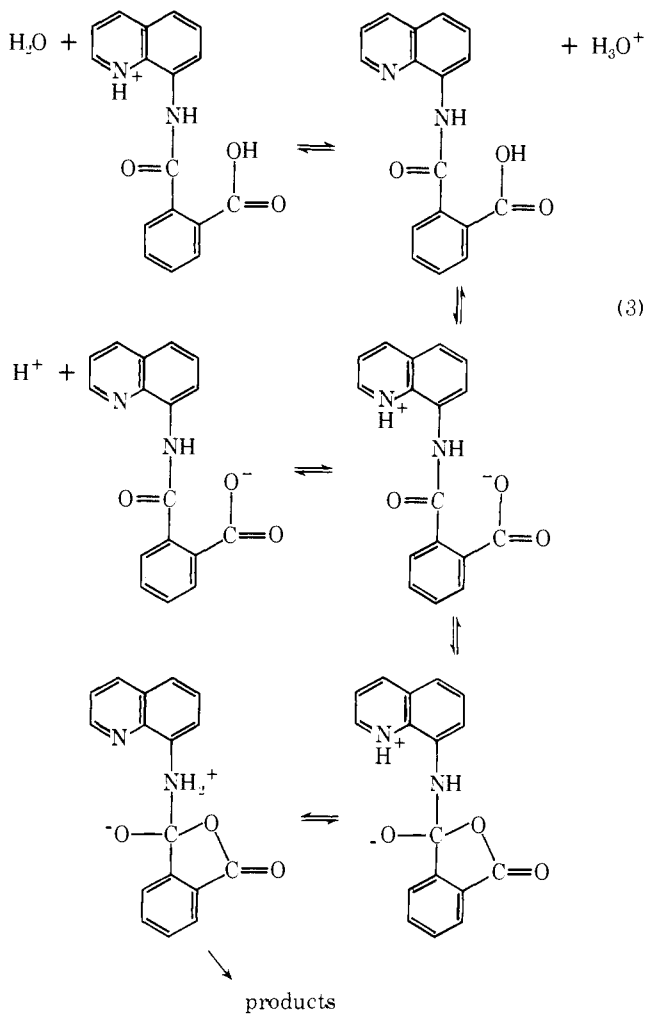


Figure 3. Plot of log k_{obsd} vs. pH for hydrolysis of 8-quinolyl hydrogen glutarate (II) at 30 °C and μ = 0.5 with $LiClO_4$ in the presence of 10^{-2} M metal ion (100-fold excess). The dotted line (- - - - -) shows the pH dependence of k_{obsd} in the absence of metal ion and the solid line (——) indicates the pH dependence of k_{obsd} in the presence of metal ion: Co^{2+} (△); Cu^{2+} (◇); Ni^{2+} (○); and Zn^{2+} (□). Copper ion effects were studied only up to pH 4.90 because of precipitation problems.



(3)

termediate must be rate limiting in view of the low basicity of the carboxylate anion. Nucleophilic attack could only be rate determining if departure of the amine leaving group from the tetrahedral intermediate were more favorable than breakdown to reactant. Hawkins[21] has presented evidence supporting rate-limiting tetrahedral intermediate breakdown in hydrolysis of phthalanilic acid in 0.1 M HCl. A characteristic of nu-

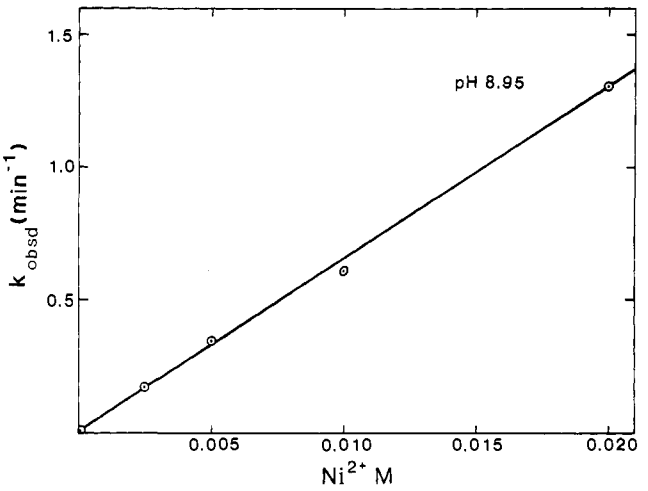
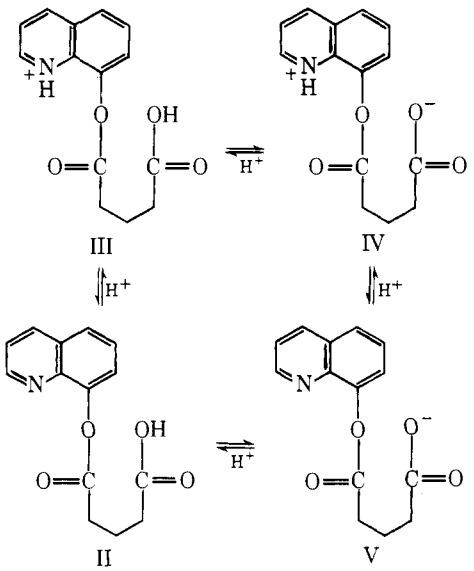


Figure 4. Plot of k_{obsd} vs. Ni^{2+} concentration for hydrolysis of 8-quinolyl hydrogen glutarate (II) at 30 °C and pH 8.95 with μ = 0.5 with $LiClO_4$.

cleophilic reactions of amides is a requirement for protonation of the leaving group, as in the scheme of eq 3, to avoid expulsion of a highly unstable amine anion from the tetrahedral intermediate.[24-26] Proton transfer to the leaving group may be a preequilibrium process as depicted, or may be concerted with bond breaking or wholly rate limiting.[27] The latter possibilities are less likely than the former in view of the lack of significant D_2O solvent isotope effects. The pH-rate constant profile for hydrolysis of *N*-(2-pyridyl)phthalamic acid is also bell shaped, and a scheme analogous to eq 3 was suggested.[6]

The hydrolysis of 8-quinolyl hydrogen glutarate has been studied previously[13] in the pH range 0-9. The pH-rate constant profile is bell shaped at pH values less than 7, whereas the reaction is pH independent from pH 7 to 9. In comparison with the hydrolysis of 8-acetoxyquinoline, the glutarate ester hydrolyzes 10^3-fold faster. The bell-shaped pH-rate profile was interpreted as arising from nucleophilic participation by the carboxylate anion and a changing electronic effect due to dissociation of a proton from the quinoline nitrogen, i.e., maximum reactivity is displayed by the zwitterionic species IV in eq 4. The plateau at higher pH is due to carboxylate anion



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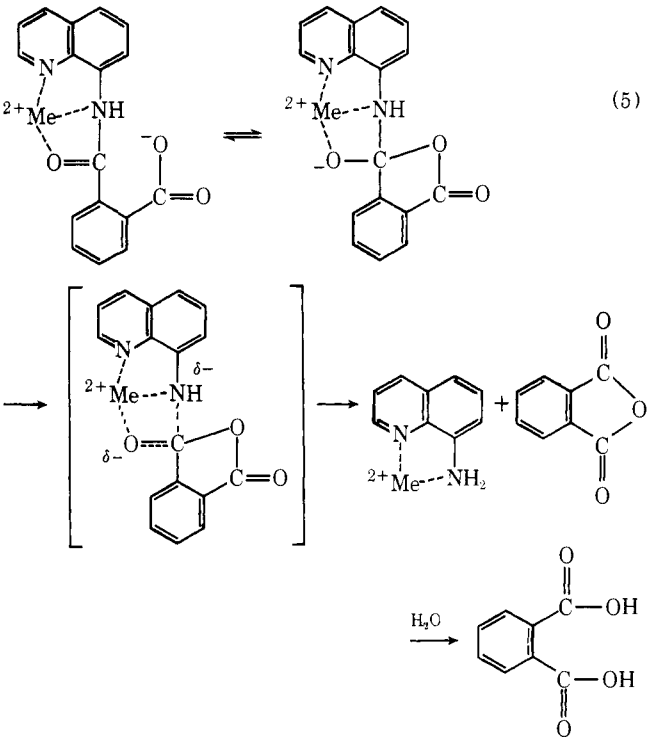
attack when the nitrogen is unprotonated. The hydrolysis of glutarate aryl monoesters has been examined in detail,[28,29] and sizable rate enhancements have been obtained from nucleophilic attack by the carboxylate anion at the ester carbonyl. However, the carboxylate nucleophile is not restricted in close proximity to the carbonyl. Degrees of freedom exist, and, in fact, the preferred conformation in aqueous solution may have the carboxylate anion extended into the solvent away from the carbonyl.[28] Thus, nucleophilic attack does not occur with maximum efficiency. This is advantageous in studying possible competing mechanisms in the same system (metal ion promoted attack of hydroxide ion), since the rate enhancement due to carboxyl participation will not necessarily overwhelm other pathways.

Metal Ion Effects. Divalent metal ions must bind poorly to I and II. Pronounced spectroscopic absorbance changes could not be detected upon the addition of relatively high concentrations of Co^{2+}, Ni^{2+}, or Zn^{2+}, in contrast with the marked absorbance changes observed in binding of these metal ions to salicyl phenanthroline-2-carboxylate[9] or *N*-(2-phenanthrolyl)phthalamic acid.[6] It is unlikely that the carboxyl groups of I and II are chelated because of the unfavorable ring size that would be required in a 1:1 complex. Chelation of a metal ion with the hydrolysis products 8-hydroxyquinoline and 8-aminoquinoline must be considerably stronger than binding to the reactant.

Divalent metal ions do not affect the shape of the pH-rate constant profile for hydrolysis of I. There is only a small inhibitory effect (<twofold) of a 100-fold excess concentration of Ni^{2+}, Co^{2+}, and Zn^{2+}. This small effect is similar to that observed in hydrolysis of *N*-(2-pyridyl)phthalamic acid[6] and in contrast with the great hydrolytic stability of the metal ion complexes of *N*-(2-phenanthrolyl)phthalamic acid where coordination to the reactant is very strong. The present work shows that complexation of a metal ion in the transition state for breakdown of a tetrahedral intermediate (eq 5) has no



(5)

catalytic effect. Thus, in these reactions, chelation of a metal ion to the nitrogen of the leaving group offers no kinetic advantage in comparison with a proton. Basicity of an amide nitrogen is quite low. Also, coordination of a metal ion with the amide nitrogen in the reactant would result in loss of resonance stabilization. As a consequence, strong binding of metal ions to the leaving group cannot occur until C-N bond breaking is appreciable. However, in view of the very poor leaving group (amine anion), the bond cannot break without protonation or comparable complexing with a positively charged species. A

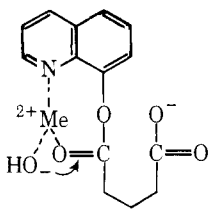
Table II. Second-Order Rate Constants for Hydroxide Ion Catalyzed Hydrolysis of 8-Quinolyl Hydrogen Glutarate (II) in the Presence of Metal Ions at 30 °C (μ = 0.5 M with $LiClO_4$)[a]

Metal ion	k_{OH}, M^{-1} min^{-1} [b]	k_{rel} [c]
None	1.12×10^2	1.00
Ni^{2+}	4.91×10^4	4.38×10^2
Co^{2+}	3.34×10^5	2.98×10^3
Zn^{2+}	2.15×10^6	1.92×10^4

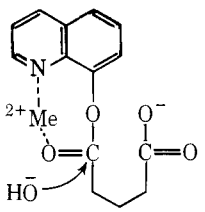
[a] Ester concentration was 10^{-4} M. Metal ion concentration was 10^{-2} M. [b] In calculating second-order rate constants at 30 °C, the ion product of water K_w was taken to be 1.47×10^{-14}: R. C. Weast, Ed., "Handbook of Chemistry and Physics", 54th ed, Chemical Rubber Publishing Co., Cleveland, Ohio, 1973, p D131. [c] The rate constants k_{OH} were obtained at nonsaturating concentrations of metal ions. Therefore, relative rate ratios represent minimum values.

proton that is completely transferred to nitrogen must be more efficient in facilitating bond breaking than a weakly chelated divalent metal ion. It should be noted in Figure 1 that metal ion promoted attack of hydroxide ion does not influence the pH–rate constant profile at pH 6, and at pH 8.23 a hydrolytic reaction could not be detected.[30]

In contrast with the amide I, divalent metal ions catalyze the hydrolysis of the ester II but only at pH values greater than 6. The linear pH–log rate constant profiles with slope of 1.0 indicate that the catalyzed reaction must proceed through intramolecular attack of metal bound hydroxide ion (VI) or by metal ion promoted attack of external hydroxide ion (VII).



VI

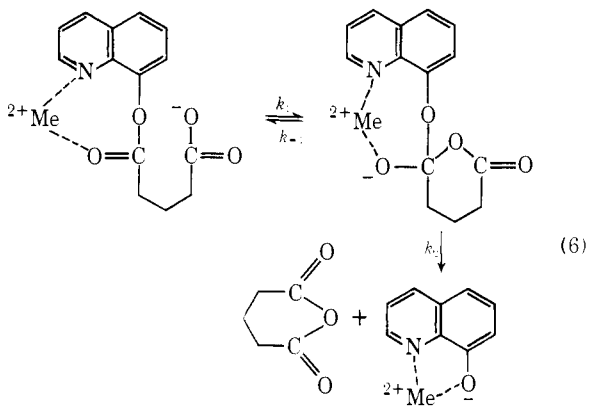


VII

The order of reactivity of the various metal ions is Zn(II) > Co(II) > Ni(II). The Zn(II) facilitated hydroxide ion catalyzed reaction at high pH is catalyzed by a factor of 2×10^4 at a metal ion concentration of 0.01 M (100-fold excess).[31] The rate enhancements are comparable to those for saturating concentrations of metal ions in hydrolysis of salicyl phenanthroline-2-carboxylate where metal ion binding is strong.[9] However, the order of reactivity of the complexes in that case is Cu(II) > Ni(II) > Co(II) > Zn(II), which is the order of increasing stability constants for the complexes of 1,10-phenanthroline.[32] The inverse order for the complexes of II might be the result of different geometrical requirements; a seven-membered ring would be required for chelation of the quinoline nitrogen and the carbonyl oxygen. The reactions in the presence of metal ions could not be studied at high pH values owing to precipitation problems and, as a consequence, the pK_a of metal-bound water could not be determined. Therefore, the order of reactivity of the metal ion complexes could be altered at high pH. It should be noted that even a 0.02 M concentration of metal ion is not saturating in this system. Even larger enhancements in rate could in principle be observed if larger concentrations of metal ions were experimentally feasible.

One of the striking features of the pH–log rate constant profile of Figure 3 is that it shows a complete lack of effect of metal ions at pH values less than 6, where the zwitterion of II is the reactive species. The rate constants in the presence of a 100-fold excess concentration of Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} are identical with the rate constants in the absence of metal ion. Clearly, complexes form at high pH in view of the large catalysis of the rate of hydrolysis, but at low pH there will be a competition with protons for binding. Significant rate enhancements have been observed in the hydrolysis of 8-quinolyl phosphate[33] and 8-acetoxyquinoline[34] in the presence of divalent metal ions at pH values as low as 3. Therefore, it is likely that complexes of II form at pH <6. From the profile of Figure 3 it can be calculated that the limits for metal ion catalysis of the carboxyl nucleophilic reaction of the anionic species (pH 7–9) are 20-fold in the presence of Zn(II) and 3 with Ni(II). These maximum enhancements give values of k_{obsd} that are considerably less than those for reaction of the zwitterionic species. Therefore, k_{obsd} in the pH region where the nucleophilic reaction of the zwitterionic species is of importance (pH 2–6) *cannot* be enhanced by metal ions.

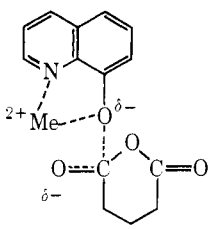
Either the metal ion complexes have no kinetic effect on carboxyl group participation or there are compensating effects on the rate constants in eq 6. The expression for k_{obsd} is given in eq 7.



(6)

$$k_{obsd} = \frac{k_1 k_2}{k_{-1} + k_2} \qquad (7)$$

There is abundant evidence that in carboxylate anion nucleophilic reactions of aryl esters the critical transition state must resemble products with expulsion of phenolate ion.[28,29,35] Thus the reactions may be concerted or, in terms of the scheme of eq 6, $k_{-1} > k_2$. A concerted reaction should be significantly catalyzed by metal ions since both nucleophilic attack and departure of the leaving group would be enhanced. The stability constants for complexing of metal ions to 8-hydroxyquinoline are very large.[7] A metal ion might be expected to chelate strongly to the leaving group, thereby producing a large transition state effect (VIII) similar to that observed in metal



VIII

ion catalyzed hydrolysis of 8-quinolyl phosphate,[33] although, when the leaving group is good, as in the case of II, the critical transition state may be reached before metal ion binding becomes effective. The near absence of metal ion effects on nucleophilic carboxyl group participation may then imply compensating effects on the three rate constants for the formation and breakdown of a tetrahedral intermediate. Metal ion binding to the carbonyl oxygen should enhance nucleophilic attack (k_1), but the attack step cannot be rate limiting. Rate-determining breakdown of the intermediate ($k_{-1} > k_2$) should be the case because of the large difference in basicity of the carboxyl group nucleophile and the leaving group.

It is probable that the lack of significant metal ion effects on carboxyl group participation with I and II reflect the fact that nucleophilic attack is a preequilibrium process. Metal ion catalysis is observed in the hydroxide ion catalyzed reactions of esters since the attack step is then rate limiting. Catalytic effects have been observed in hydroxide ion catalyzed amide hydrolysis,[12] but these effects are small in comparison with esters, reflecting the differences in the ratio of k_{-1}/k_2. It appears improbable that large metal ion catalysis will be observed in nucleophilic reactions in which the nucleophile is much less basic than the leaving group. Consequently, if Glu-270 is acting as a nucleophile in reactions of carboxypeptidase A, it is unlikely that Zn(II) is involved catalytically in the formation of an anhydride intermediate.

8-Quinolyl hydrogen glutarate represents a system in which both nucleophilic carboxyl participation and metal ion promoted $^-$OH-catalyzed hydrolysis can be observed at appropriate pH values. The effect of metal ions on the pH–log rate constant profile of II is to shift the line of slope 1.0 (hydroxide ion catalysis) three to four pH units to the left. Thus the nucleophilic reaction of the anionic species (pH 7–9) is overcome by the metal ion facilitated $^-$OH reaction, but the nucleophilic reaction of the zwitterionic species (pH 2–6) is unaffected. This is a consequence of the fact that the rate enhancement from the carboxyl group nucleophilic reaction at low pH is sufficiently large that it can effectively compete. Nucleophilic mechanisms usually give rate enhancements[36,37] of 10^4–10^7 so that nucleophilic attack will be the preferred pathway at pH values below neutrality even though it is uncatalyzed by metal ion and steric fit of the nucleophile and the carbonyl is less than maximal. Removal of degrees of freedom for rotation of the nucleophile away from the carbonyl, as, for example, with succinate or phthalate monoesters, would enlarge the bell-shaped portion of the profile and shift the intersection of the plots for the carboxyl nucleophilic mechanism and metal ion promoted $^-$OH reaction to the right. It is therefore clear that highly efficient carboxyl group participation would restrict the metal ion facilitated $^-$OH reaction to only high pH values.

Carboxypeptidase A. The plot of log k_{obsd} vs. pH for hydrolysis of II in the presence of divalent metal ions is remarkably similar to the published pH–k_{cat} profile for carboxypeptidase A catalyzed hydrolysis of the specific ester substrates, *O*-(*trans*-cinnamoyl)-L-β-phenyllactate and its chloro derivative.[38,39] In fact, the k_{cat} profile can be considered to represent hydroxide ion catalysis superimposed on a bell-shaped region. Suh and Kaiser[38] suggested that the pH–k_{cat} plots for the cinnamoyl phenyllactate esters might reflect nucleophilic participation by Glu-270 and rate-determining attack of hydroxide ion on an intermediate anhydride at high pH.[42] It is possible, however, that the high pH region of the profile might represent metal ion promoted attack of $^-$OH on the substrate, i.e., at pH <8 an anhydride intermediate might be formed, but at pH >8 such an intermediate is not formed.

Proposals for the mechanism of carboxypeptidase A mediated hydrolysis of peptide substrates have been based on the supposition that the amide carbonyl could coordinate with the zinc ion in the active site thereby becoming more susceptible to nucleophilic attack.[2,3,43] Support for these proposals was considered to reside in x-ray crystallographic studies which demonstrated that the carbonyl oxygen of the poor substrates Gly-L-Tyr and Phe-Gly-Phe-Gly was coordinated with Zn(II).[43,44] However, the rate-determining step should not be attack by Glu-270. Catalytic effects in a nucleophilic mechanism due to complexation of the substrate to the zinc ion would therefore not be expected unless the metal ion can duplicate the role of a proton in facilitating breakdown of a tetrahedral intermediate to products. Such effects are absent in the hydrolysis of *N*-(8-quinolyl)phthalamic acid where chelation of the leaving group nitrogen is sterically possible.

Intramolecular attack of metal ion bound hydroxide ion is capable of giving rise to large rate enhancements in the hydrolysis of stable Co(III) complexes of amides.[11] The pH–k_{cat} profile for carboxypeptidase A catalyzed hydrolysis of the peptide substrate carbobenzoxy-Gly-Gly-L-Phe shows dependence on the ionization of a group with a pK_a of 6.[45] Thereafter, the rate remains constant until at least pH 10.5. Thus, there is no evidence for a Zn(II)-promoted attack of hydroxide ion on the peptide substrate, nor does such a reaction occur at accessible pH values in the hydrolysis of *N*-(2-phenanthrolyl)phthalamic acid[6] or *N*-(8-quinolyl)phthalamic acid. Thus, in the enzyme-catalyzed hydrolysis of amides it is likely that either the metal ion is not involved mechanistically or the reaction proceeds with general base catalysis by Glu-270.[46] There is, however, no evidence that the latter mechanism could provide sufficient rate enhancements to be effective.

The key question in regard to the enzyme mechanism is whether the carboxyl group of Glu-270 is sterically capable of participating efficiently in a nucleophilic reaction. Evidence has been obtained at very low temperatures for an anhydride intermediate in ester hydrolysis.[47] If the steric fit of Glu-270 and the substrate carbonyl is reasonably good, then nucleophilic attack would be the preferred pathway at pH values less than 7–8 with ester substrates even if Zn(II) does not catalyze the reaction.

Conclusions

The following conclusions can be drawn from the present work.

1. In hydrolytic reactions of amides where nucleophilic attack by carboxyl occurs, chelation of divalent metal ions results in inhibition of the rate of hydrolysis regardless of whether coordination to the leaving group nitrogen is sterically possible, i.e., transition state effects are not observed.

2. In the hydrolysis of an ester which has a neighboring carboxyl group that can function as a nucleophile, this reaction is not catalyzed by metal ions, although a 10^4-fold increase in the rate of hydroxide ion catalysis is observed. Metal ion promoted attack of $^-$OH will not successfully compete with carboxylate anion participation at pH <6 when steric fit of the carboxyl and carbonyl is sufficiently good that the nucleophilic reaction proceeds with efficiency.

3. Intracomplex nucleophilic attack by Glu-270 is likely in the carboxypeptidase A catalyzed hydrolysis of esters below pH 7, if in fact the carboxyl group is in position to do so efficiently. Metal ion promoted attack of $^-$OH at pH >7 must also be considered a likely mechanism, and both mechanisms might occur in the enzyme reaction at the appropriate pH values.

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Lack of Concertedness in the Catalysis of the Enolization of Oxaloacetic Acid by General Acids and Bases. Formation of a Carbinolamine Intermediate in the Tertiary Amine Catalyzed Enolization Reaction

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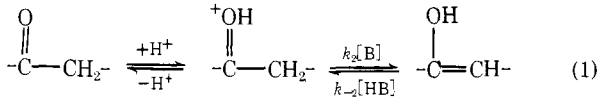
Abstract: The interconversion of the keto-enol tautomers of oxaloacetic acid exhibits general-acid catalysis with acetate and pyridine buffers, is subject to general-base catalysis in the presence of carbonate and phosphate (HPO_4^{2-}/PO_4^{3-}) buffers, and exhibits both general-acid- and general-base-catalyzed pathways with imidazole and phosphate ($H_2PO_4^-/HPO_4^{2-}$) buffers. A Brønsted $-\alpha$ value of 0.43 and a β value of 0.35 were obtained for the general-acid and general-base catalytic rate constants. None of the buffer systems employed gave any evidence of the concerted general acid-general base catalyzed mechanism that had previously been reported to occur in the enolization of oxaloacetic acid. In the presence of tertiary amines of $pK_a > 8$, enolization occurs via the formation of a zwitterionic carbinolamine intermediate followed by amine-catalyzed elimination of a proton and tertiary amine from the protonated carbinolamine. Apparently, the quaternary ammonium group of the carbinolamine serves as an electron sink to enhance the rate of proton removal from the α-carbon of the ketone by the second molecule of tertiary amine. The elimination reaction apparently occurs only through protonated carbinolamine. The β value for the reaction of tertiary amines with oxaloacetic acid is 0.77.

Introduction

The interconversion of keto-enol tautomers may take place by two possible stepwise mechanisms, one subject to general-acid catalysis (eq 1) and the other to general-base catalysis (eq 2). In both of these reaction pathways, two consecutive steps are involved and the rate-limiting step in each is proton abstraction from the α-carbon atom. Alternatively, the reaction may take place through a concerted mechanism (eq 3) in which



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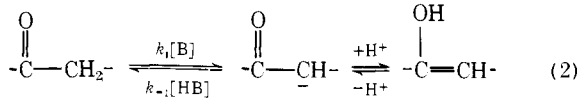

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