

Metal Ion Catalysis of Anhydride Hydrolysis. Metal Ion Promoted Water and Hydroxide Ion Catalyzed Reactions of Mixed Cinnamic Acid Anhydrides

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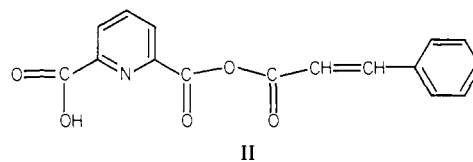
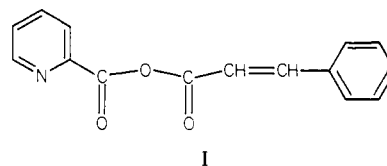
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Abstract: Rate constants have been determined for hydrolysis of cinnamic picolinic anhydride and cinnamic 6-carboxypicolinic monoanhydride in 50% dioxane-H₂O at 30 °C. At pH values less than 7, both water and hydronium ion catalyzed reactions are observed in hydrolysis of cinnamic picolinic anhydride. The latter reaction is also seen with cinnamic isonicotinic anhydride and is very likely associated with protonation of the pyridine nitrogen. Divalent metal ions (Cu²⁺, Ni²⁺, Co²⁺, and Zn²⁺) exert a significant catalytic effect in the hydrolysis of cinnamic picolinic anhydride even though binding is weak (saturation effects were not observed). Rate enhancements ranging from 200-fold with 0.0005 M Cu²⁺ to 10-fold with 0.005 M Zn²⁺ were obtained. The reactions at constant metal ion concentration are pH independent. Metal ion catalysis is not observed in the hydrolysis of cinnamic isonicotinic anhydride. Therefore, metal ion catalysis in the hydrolysis of cinnamic picolinic anhydride must be specifically associated with a chelation effect. The metal ion binding to cinnamic 6-carboxypicolinic monoanhydride is quite strong, and saturation occurs at low metal ion concentrations. Only a metal ion promoted OH⁻-catalyzed reaction is then observed in the presence of Cu²⁺. However, at saturating concentrations of Ni²⁺, Co²⁺, and Zn²⁺, both OH⁻- and water-catalyzed reactions occur. Rate enhancements are >10² in the water reactions catalyzed by saturating concentrations of the metal ions. These results show that metal ion promoted water-catalyzed reactions will occur in the hydrolysis of anhydrides, and with Ni²⁺, Co²⁺, and Zn²⁺, such reactions can be competitive with metal ion promoted OH⁻ catalysis even when the metal ion is strongly chelated to the anhydride.

Carboxypeptidase A is a Zn (II) metalloenzyme that catalyzes the hydrolysis of peptides and *O*-acyl derivatives of α -hydroxy carboxylic acids.² X-ray crystallographic analysis at 2-Å resolution has revealed the presence of the carboxyl group of glutamic acid-270 in the active site.²⁻⁴ Both nucleophilic and general-base mechanisms have been suggested for the enzyme involving Glu-270.^{3,4} Cryogenic experiments (low temperature and a mixed solvent system) have provided evidence for a nucleophilic reaction under these conditions with ester substrates,⁵ and it was suggested that breakdown of an anhydride intermediate is rate determining in reactions of *p*-chlorocinnamoyl-*L*- β -phenyllactic acid.⁶ This had also been previously suggested on the basis of the D₂O solvent isotope effect.⁷ An understanding of the chemistry of metal ion effects in anhydride hydrolysis is therefore of fundamental importance in regard to the mechanism of action of the enzyme.

The k_{cat} -pH profile for carboxypeptidase A catalyzed hydrolysis of *O*-(*trans*-cinnamoyl)-*L*- β -phenyllactic acid is sigmoidal ($\text{p}K_{\text{app}} = 6.2$) at pH < 9 and has a rapidly ascending arm with increasing pH at pH > 9.⁸ In view of the magnitude of the rate constants ($k_{\text{cat}} \sim 100 \text{ s}^{-1}$ at pH > 6 and 25 °C), anhydride hydrolysis is very likely catalyzed by the metal ion. Both metal ion promoted water- (pH 6-9) and OH⁻ (pH > 9) catalyzed reactions would be required if the $\text{p}K_{\text{a}}$ of Zn(II)-bound water is greater than 9.^{9,10}

There have been few studies of metal ion catalyzed anhydride solvolysis.^{11,12} In a system where the metal ion was strongly chelated, only metal ion promoted OH⁻ catalysis was observed.¹¹ Since the metal ion promoted OH⁻-catalyzed reaction in ester hydrolysis is enhanced by strong chelation,^{13,14} it is of particular importance to establish whether water catalysis is indeed a feasible mechanism in cases where the metal ion is tightly bound to the anhydride. In view of the multistep enzyme reaction, it is also important to determine the magnitude of the rate constants and the rate enhancements that can be obtained in the metal ion catalyzed reactions of anhydrides so that comparisons may be made with those in ester hydrolysis reactions. Cinnamate esters have been extensively studied as substrates for carboxypeptidase A.⁵⁻⁸ The intermediate in the enzymatic reaction would then be a mixed cinnamic acid anhydride. Consequently, we have studied the hydrolysis reactions of the mixed anhydrides I and II in the presence of Cu²⁺, Co²⁺, Ni²⁺, and Zn²⁺.



Experimental Section

Materials. With the exception of II, the mixed anhydrides were prepared by stirring equivalent amounts (0.005 mol) of freshly distilled cinnamoyl chloride, triethylamine, and benzoic or picolinic acid in dry ether for about 3 h. After filtration to remove the precipitated tri-

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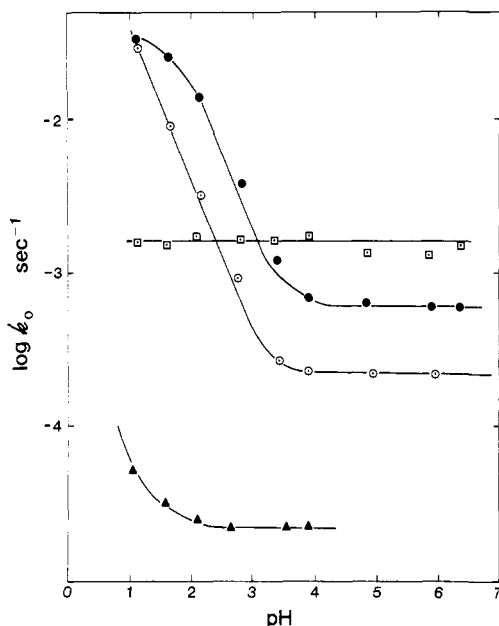


Figure 1. Plots of $\log k_0$ vs. pH for hydrolysis of I (O), II (□), and III (●) at 30 °C and IV (▲) at 50 °C in 50% dioxane-H₂O (v/v) ($\mu = 0.1$ M with KCl). Rate constants were obtained in HCl solutions or by extrapolation to zero buffer concentration.

ethylamine hydrochloride, the ether was removed by rotary evaporation. The remaining solid was washed with a small amount of petroleum ether and recrystallized from hexane or chloroform-hexane.

Cinnamic picolinic anhydride (I); mp 58–59 °C. Anal. Calcd for C₁₅H₁₁NO₃: C, 71.15; H, 4.35. Found: C, 71.16; H, 4.44.

Cinnamic isonicotinic anhydride (III); mp 75–76 °C. Anal. Calcd for C₁₅H₁₁NO₃: C, 71.15; H, 4.35; N, 5.53. Found: C, 71.01; H, 4.46; N, 5.45.

Benzoic cinnamic anhydride (IV); mp 52–53 °C. Anal. Calcd for C₁₆H₁₂O₃: C, 76.19; H, 4.76. Found: C, 76.27; H, 4.95.

Cinnamic 6-carboxypicolinic monoanhydride (II) was prepared by adding a solution of 1 g (0.006 mol) of cinnamoyl chloride in 20 mL of dry THF dropwise to a hot stirring suspension of 10 g (0.06 mol) of 2,6-pyridinedicarboxylic acid in 100 mL of THF. After the hot suspension was filtered, the solvent was removed by rotary evaporation. The remaining solid was extracted and recrystallized from benzene. The compound decomposed at 145 °C. Anal. Calcd for C₁₆H₁₁NO₅: C, 64.65; H, 3.70; N, 4.71. Found: C, 64.60; H, 3.84; N, 4.81.

The dioxane used for kinetic studies was spectral grade (Mallinckrodt) and was refluxed over sodium borohydride for at least 3 h and freshly distilled prior to use.

Kinetic Measurements. The rates of reaction of the mixed anhydrides were measured spectrophotometrically at 30 or 50 °C ($\mu = 0.1$ M with KCl) in water or 50% dioxane-water (v/v) by employing a Beckman Model 25, Pye-Unicam SP8-100, or Durrum D110 stopped-flow spectrophotometer. In all cases, disappearance of reactant at 320 nm was monitored. Stock solutions of the anhydrides were 10⁻² M in THF. In a typical experiment, 15–20 μ L of stock solution was injected into 3 mL of buffered reactant solution. In the studies of metal ion catalysis carried out with the Durrum stopped-flow apparatus, one drive syringe contained 100 μ L of anhydride stock solution in 15 mL of pH 3 (with HCl) solution while the other syringe contained the metal ion and appropriate buffer. The drive syringes were suspended in a water trough whose temperature was maintained at 30 °C. Optical density changes after mixing were recorded on a Hewlett-Packard storage oscilloscope (Model 1270B).

Buffer solutions used in the hydrolysis of the anhydrides in the absence of metal ion contained 2 \times 10⁻⁵ M EDTA as a precaution against trace metal in the buffer or salt. Buffer concentrations were 0.01–0.02 M in the metal ion studies, and no correction was made for buffer-metal ion complexation. In all cases, the rate constant for hydrolysis in the buffer alone was subtracted from k_{obsd} obtained in the buffer plus metal ion. The buffers used were HCl (pH 1–3), chloroacetate (pH 4–5), 2,6-lutidine (pH 5–6), and *N*-ethylmorpholine (pH 6–8). Reaction pH values were measured with a Radiometer Model 22 pH meter. The glass electrode gives the correct pH reading in concentrated dioxane-water mixtures.¹⁵

Table I. Rate Constants for Anhydride Hydrolysis at 30 or 50 °C in 50% Dioxane-H₂O (v/v) ($\mu = 0.1$ M)

compound	T, °C	k_1/K_a , M ⁻¹ s ⁻¹	$k_0' \times 10^4$ s ⁻¹
I	30	0.40	2.20
	50	1.0	
II	30		16.1 167 ^a
	50		45.0
III	30	1.9	6.0
	50	3.0	
IV	50	4 \times 10 ⁻⁴	0.22

^a In H₂O.

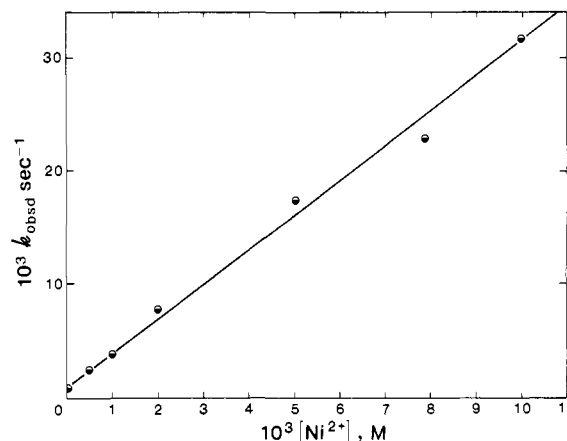


Figure 2. Plot of k_{obsd} vs. Ni²⁺ concentration for hydrolysis of cinnamic picolinic anhydride at 30 °C in 50% dioxane-H₂O (v/v) ($\mu = 0.1$ M with KCl) at pH 4.98 (0.02 M 2,6-lutidine buffer).

Results

A plot is presented in Figure 1 of $\log k_0$ vs. pH for hydrolysis of the series of mixed cinnamic acid anhydrides at 30 °C in 50% dioxane-H₂O (v/v) with $\mu = 0.1$ M, where k_0 is the rate constant obtained by extrapolation to zero buffer concentration or in HCl solutions. There is a pH-independent reaction in all cases, but with the anhydrides I and III, there is also a hydronium ion catalyzed reaction at low pH (slope of -1.0 in the $\log k_0$ vs. pH profile), so that eq 1 is followed, where K_a is the dissociation

$$k_0 = \frac{k_1 a_{\text{H}^+} + k_0' K_a}{a_{\text{H}^+} + K_a} \quad (1)$$

constant of the anhydride. The value of k_1 for hydrolysis of cinnamic isonicotinic anhydride is 4.2×10^{-2} s⁻¹ and K_a is 2.2×10^{-2} M⁻¹. A hydronium ion catalyzed reaction was not observed with cinnamic 6-carboxypicolinic monoanhydride (II). In that case, the values of k_0 are independent of pH in the range 1.12–6.36. The reactions could not be followed at higher pH values because the pronounced buffer catalysis made accurate determination of the intercepts of plots of k_{obsd} vs. buffer concentration difficult. In H₂O as the solvent, the value of k_0' for II is 10-fold larger than in 50% dioxane-H₂O. Rate constants for these reactions are given in Table I.

Divalent metal ions (Cu²⁺, Ni²⁺, Co²⁺, or Zn²⁺) exert a significant catalytic effect on the hydrolysis of cinnamic picolinic anhydride. Figure 2 presents the linear plot of k_{obsd} vs. the concentration of Ni²⁺ at pH 5.0. Saturation effects are not observed even at a metal ion concentration of 0.01 M. The metal ion catalyzed reaction is pH independent at pH < 5 at constant metal ion concentration (0.005 or 0.0005 M in the case of Cu²⁺), as shown in Figure 3. With Co²⁺ and Zn²⁺, the rate constants increase slightly with increasing pH at pH > 5. Rate constants are given in Table II. Metal ion catalysis was not observed in the hydrolysis of cinnamic isonicotinic anhydride.

Saturation effects are observed in hydrolysis of cinnamic 6-carboxypicolinic monoanhydride (II) at low metal ion concen-

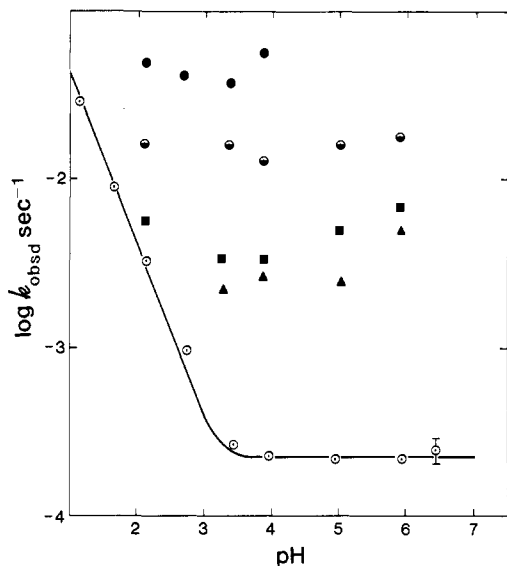


Figure 3. Plots of $\log k_{\text{obsd}}$ vs. pH for hydrolysis of cinnamic picolinic anhydride at 30 °C in 50% dioxane-H₂O (v/v) ($\mu = 0.1$ M with KCl) in the presence of 0.005 M Co²⁺ (■), Zn²⁺ (▲), or Ni²⁺ (●) or 0.0005 M Cu²⁺ (●) and in the absence of metal ion (○).

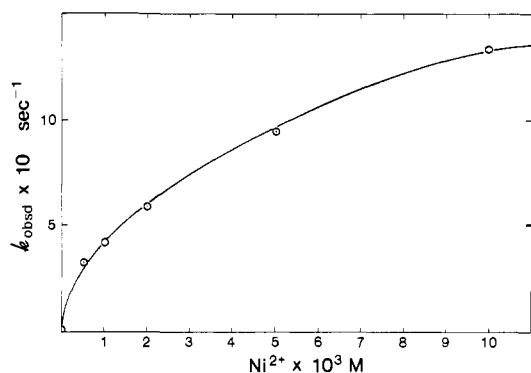


Figure 4. Plot of k_{obsd} vs. Ni²⁺ concentration for hydrolysis of cinnamic 6-carboxypicolinic monoanhydride at 30 °C in 50% dioxane-H₂O (v/v) ($\mu = 0.1$ M with KCl) at pH 5.0 (0.02 M 2,6-lutidine buffer).

Table II. Rate Constants for Metal Ion Catalyzed Hydrolysis of Cinnamic Picolinic Anhydride at 30 °C in 50% Dioxane-H₂O (v/v) ($\mu = 0.1$ M)

metal ion ^a	$k_o' \times 10^4 \text{ s}^{-1}$	$k_{\text{Me}}',^b \text{ M}^{-1} \text{ s}^{-1}$
none	2.20	
Ni ²⁺	160	3.2
Co ²⁺	34	0.7
Zn ²⁺	25	0.5
Cu ²⁺	450	90.0

^a 0.005 M except with Cu²⁺, which had a concentration of 0.0005 M. ^b $k_{\text{Me}}' = k_o' / (\text{Me}^{2+})$.

trations as seen in Figure 4. The equation for k_{obsd} is given in eq 2, where K_a' is the second dissociation constant of the anhydride.

$$k_{\text{obsd}} = \frac{k_{\text{Me}}(\text{lim})K_a'K_{\text{Me}}[\text{Me}^{2+}]}{a_{\text{H}} + K_a' + K_a'K_{\text{Me}}[\text{Me}^{2+}]} \quad (2)$$

This equation reduces to eq 3 when $K_a' > a_{\text{H}}$. The data give a

$$k_{\text{obsd}} = \frac{k_{\text{Me}}(\text{lim})K_{\text{Me}}[\text{Me}^{2+}]}{(1 + K_{\text{Me}}[\text{Me}^{2+}])} \quad (3)$$

good fit to eq 3, where k_{Me} is the limiting rate constant for metal ion catalysis at saturation and K_{Me} is the association constant of the metal ion complex. At pH < 5 with Ni²⁺, Co²⁺, and Zn²⁺, the metal ion concentration was not sufficiently high at 0.01 M that the reaction became completely independent of metal ion concentration. However, at pH > 5, 0.01 M metal ion did produce

Table III. Values of k_{Me} and K_{Me} app for Metal Ion Catalyzed Hydrolysis of Cinnamic 6-Carboxypicolinic Monoanhydride (II) at 30 °C in 50% Dioxane-H₂O (v/v) ($\mu = 0.1$ M)

metal ion	pH	$k_{\text{Me}}, \text{ s}^{-1}$	$K_{\text{Me}} \times 10^{-2} \text{ M}^{-1}$
Ni ²⁺	3.23	0.378	0.959
	3.99	0.723	2.35
	5.00	1.39	4.71
	5.83	6.02	3.59
	6.42	23.4	3.75
Co ²⁺	3.23	0.0692	3.59
	4.07	0.165	5.66
	4.81	0.541	2.62
	5.41	1.14	4.97
	6.19	8.61	3.09
	7.14	54.2	
Zn ²⁺	3.23	0.263	1.23
	3.99	0.207	7.39
	5.14	0.216	
	6.00	0.812	9.75
	6.76	3.56	
Cu ²⁺	2.09	0.355	<i>a</i>
	3.11	2.39	
	3.97	15.6	
	4.74	85.3	
	5.16	200	

^a Saturation occurred at such low values of Cu²⁺ that only a lower limit of 10³ M⁻¹ could be obtained.

Table IV. Rate Constants for Metal Ion Catalysis of the Hydrolysis of Cinnamic 6-Carboxypicolinic Monoanhydride at 30 °C in 50% dioxane-H₂O (v/v) ($\mu = 0.1$ M)

metal ion	$k_o', \text{ s}^{-1}$	$k_{\text{OH}},^a \text{ M}^{-1} \text{ s}^{-1}$
none	0.00161	
Ni ²⁺	0.398	4.8×10^8
Co ²⁺	0.16	2.5×10^8
Zn ²⁺	0.178	3.4×10^7
Cu ²⁺		1.2×10^{11}

^a Values of k_{OH} were calculated by using $K_w = 1.47 \times 10^{-14}$, i.e., the value in H₂O. Therefore, these rate constants are consistently calculated but can be employed only for internal comparison.

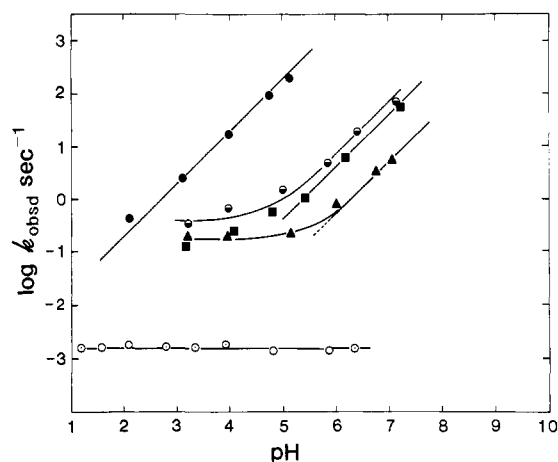


Figure 5. Plots of $\log k_{\text{obsd}}$ vs. pH for hydrolysis of cinnamic 6-carboxypicolinic monoanhydride at 30 °C in 50% dioxane-H₂O (v/v) ($\mu = 0.1$ M with KCl) in the presence of saturating concentrations of Cu²⁺ (●), Ni²⁺ (○), Co²⁺ (■), or Zn²⁺ (▲) and in the absence of metal ion (○).

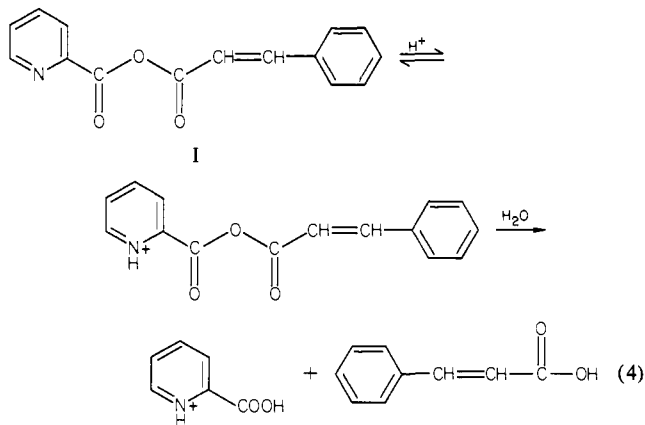
complete saturation, and with Cu²⁺ as the catalyst, the reactions were independent of metal ion concentration below 0.005 M at all pH values studied. Values of $k_{\text{Me}}(\text{lim})$ and K_{Me} are given in Table III. The reported K_{Me} constants are apparent values since K_a' is unknown. However, the data suggest that $K_a' \approx 10^{-4}$ M. The true K_{Me} values would then be Ni²⁺, $3 \times 10^2 \text{ M}^{-1}$; Co²⁺, $5 \times 10^2 \text{ M}^{-1}$; Zn²⁺, 10^3 M^{-1} ; and Cu²⁺, $> 10^3 \text{ M}^{-1}$. A plot of log

k_{obsd} vs. pH for the hydrolysis reaction at saturating metal ion concentration is shown in Figure 5. Only OH^- catalysis is observed in the presence of Cu^{2+} . However, both metal ion promoted OH^- and H_2O -catalyzed reactions are detected in the reactions catalyzed by Ni^{2+} , Co^{2+} , and Zn^{2+} . Rate constants for these reactions are given in Table IV.

Discussion

The log k_0 vs. pH profiles for hydrolysis of I and III indicate that both water and hydronium ion catalysis occur as a function of pH. Reactions that are pH independent have been observed previously in the hydrolysis of anhydrides.¹⁶⁻²⁰ These reactions are characterized by a large D_2O solvent isotope effect, indicating proton transfer in the transition state.²⁰ The pH-independent reactions of I and II occur more than 100-fold faster than in the case of benzoic cinnamic anhydride. The neighboring pyridyl nitrogen of I could participate in the reactions as a nucleophile or a general base; however, the rate of hydrolysis of cinnamic isonicotinic anhydride, with which the pyridyl nitrogen is in the para position, is in fact slightly larger than that of I. Furthermore, protonation of nitrogen increases the rate of hydrolysis, which is not compatible with significant neighboring-group participation. Intramolecular nucleophilic attack would necessarily occur at the cinnamoyl carbonyl, which would result only in a reversible rearrangement reaction; this might not compete favorably with facile water catalysis. The water reaction of II is approximately 7-fold faster than that of I at pH 4 and only 2-fold faster than that of III. The 6-carboxyl of II is therefore influencing the reaction only slightly.

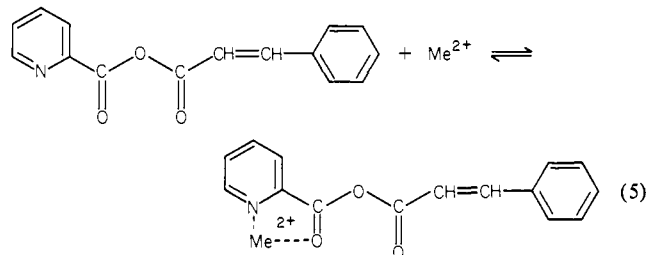
The apparent hydronium ion catalyzed reactions observed at low pH in hydrolysis of I and III are quite favorable in comparison with those in hydrolysis of other types of anhydrides,^{19,21} e.g., IV. Hydronium ion catalysis of the reactions of I and III may reflect protonation of the pyridine nitrogen (eq 4), which will increase



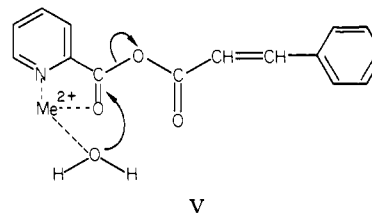
electron withdrawal from the carbonyl carbon and thereby make it more susceptible to nucleophilic attack by water. Consequently, the lack of observable hydronium ion catalysis in hydrolysis of II at pH 1.0 indicates a lower $\text{p}K_a$ for the pyridine nitrogen than in the case of I.

Divalent metal ions (Cu^{2+} , Ni^{2+} , Co^{2+} , and Zn^{2+}) exert a significant catalytic effect in hydrolysis of I, even though binding of metal ion to the anhydride is quite weak (saturating effects are not observed). At constant metal ion concentration, the reactions are pH independent as seen in Figure 3. Thus, metal ion catalysis is being exerted in the water reaction with rate

enhancements ranging from 200-fold with Cu^{2+} (0.0005 M) to 10-fold with Zn^{2+} (0.005 M). Larger catalytic effects could, of course, be obtained at higher metal ion concentrations. The observed catalysis does not result from simple binding to the pyridyl nitrogen, analogous to hydronium ion, because metal ion catalysis is not detected in reactions of the 4-pyridyl derivative. Therefore, a chelation effect must be important as in eq 5. The



hydrolysis reaction then involves either metal ion promoted attack of an external water molecule or an internal attack of metal ion bound water V.



Metal ion promoted OH^- catalysis (plots of log k_{obsd} vs. pH with slopes of 1.0) has been observed in ester hydrolysis in cases where there is an additional functional group present that can chelate the metal ion.^{13,14,22-24} This reaction is facilitated by increased strength of metal ion binding to the reactant. Such an effect would, therefore, also be expected in metal ion catalyzed anhydride hydrolysis. Consequently, a question arises as to whether a metal ion catalyzed water reaction would be detectable when metal ion binding to the anhydride is strong since the water reaction would necessarily have to be competitive with the OH^- -catalyzed reaction. The additional metal ion chelating group in cinnamic 6-carboxypicolinic monoanhydride (II) results in very strong metal ion binding as compared with I. Saturation effects are observed at low metal ion concentrations in the hydrolysis of II, and facile metal ion promoted OH^- -catalyzed reactions occur ($\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+}$). The metal ion promoted OH^- reaction is so favorable with Cu^{2+} that a water reaction is not observed. At pH 6.5, the difference in k_{obsd} due to the presence of the metal ion is Cu^{2+} , 3.7×10^6 ;²⁵ Ni^{2+} , 1.5×10^4 ; Co^{2+} , 8.3×10^3 ; and Zn^{2+} , 1.1×10^3 . These enhancements are, of course, minimum values and could be considerably larger if rate measurements at higher pH were experimentally feasible. Nevertheless, at saturating concentrations of Ni^{2+} , Co^{2+} , and Zn^{2+} , pH-independent reactions are clearly detectable even though the length of the plateau regions in the pH-log (rate constant) profiles is restricted by the OH^- -catalyzed reactions. The pH-independent Zn^{2+} -catalyzed reaction extends over at least 2.5 pH units. Therefore, a metal ion catalyzed water reaction occurs even when metal ion binding to the substrate is very strong. The rate enhancements in the water reaction range from 245 with Ni^{2+} to 110 with Zn^{2+} . It is noteworthy that although the rate of the metal ion promoted OH^- -catalyzed reaction depends on the identity of the metal ion, this is not the case in the water reactions. The rate constants are similar for all the metal ions. Even Cu^{2+} cannot

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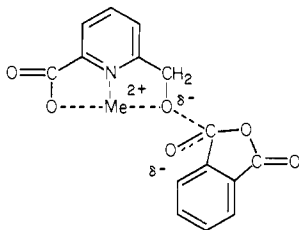
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(25) The calculated rate enhancement for Cu^{2+} is based on a rate constant obtained by extrapolation to pH 6.5. At pH 5, the experimentally determined rate enhancement for Cu^{2+} is 1.2×10^5 .

have a k_0' much larger than that of Ni^{2+} or Zn^{2+} .

These findings are quite important in regard to possible mechanisms for carboxypeptidase A catalyzed hydrolysis of esters. Breakdown of an anhydride intermediate appears to be rate limiting in enzyme-catalyzed hydrolysis of substituted cinnamoyl- β -phenyllactate esters.⁵⁻⁷ The k_{cat} vs. pH profiles suggest both OH^- - and H_2O -catalyzed reactions that are very likely promoted by the zinc ion. The observation of such reactions in hydrolysis of II, with which metal ion binding is strong, shows that a water reaction can be competitive with metal ion promoted OH^- catalysis.²⁶

The rate constants for hydrolysis of II at saturating concentrations of metal ions are approximately 4 orders of magnitude greater than in the intramolecular carboxyl nucleophilic reaction of 2-(6-carboxypyridyl)methyl hydrogen phthalate, with which the leaving group has a $\text{p}K_{\text{a}}$ comparable to that of β -phenyllactic acid.¹⁴ The steric fit of the carboxyl and the carbonyl group of a phthalate monoester is, of course, excellent. Metal ions bind strongly to the ester and catalyze the nucleophilic reaction through a transition-state effect in which the leaving group is stabilized (VI). The rate enhancements in the metal catalyzed reactions

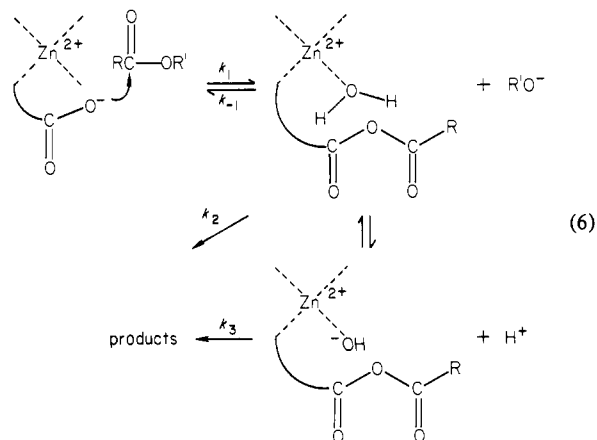


VI

are however comparable in the carboxyl nucleophile reaction and in water-catalyzed hydrolysis of the anhydride II ($\sim 10^2$).

(26) The possibility of a metal ion promoted water reaction in carboxypeptidase A catalyzed reactions is in accord with the proposed structure of the metal ion, anhydride intermediate complex: Kuo, L. C.; Makinen, M. W. *J. Biol. Chem.* **1982**, *257*, 24.

Therefore, in view of the reactivity of mixed cinnamic acid anhydrides, for anhydride hydrolysis to be rate determining in carboxypeptidase A catalyzed hydrolysis of cinnamate esters, either Glu-270 attack (anhydride formation) is facilitated to an extent not duplicated in the chemical reactions of the phthalate monoester (VI) and/or the enzymatic reaction is reversible as in the simplified scheme of eq 6 with $k_{-1} > k_2$. Phenyllactic acid is bound strongly



in the active site ($K_i = 5.8 \times 10^{-5}$ M at pH 7.5)⁸ and should be in excellent position to reverse the reaction, although reversibility has not as yet been detected in reactions catalyzed by carboxypeptidase A.^{7,27}

Acknowledgment. This work was supported by research grants from the National Institutes of Health.

Registry No. I, 83693-14-5; II, 83693-15-6; III, 83693-16-7; IV, 25388-78-7; Ni^{2+} , 14701-22-5; Co^{2+} , 22541-53-3; Zn^{2+} , 23713-49-7; Cu^{2+} , 15158-11-9; cinnamoyl chloride, 102-92-1; 2,6-pyridinedicarboxylic acid, 499-83-2.

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Equilibration of 1-Octanol with Alcohol Dehydrogenase. Evidence for Horse Liver Alcohol Dehydrogenase Responsibility for Exchange of the 1-*pro-S* Hydrogen Atom[‡]

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Abstract: Equilibration of hydrogen atoms of 1-octanol with water, mediated by the system horse liver alcohol dehydrogenase-NAD/NADH-diaphorase, involves a rapid exchange of 1-*pro-R* hydrogen atoms and a slow exchange of 1-*pro-S* hydrogen atoms. Yeast alcohol dehydrogenase has an apparent absolute stereospecificity for the 1-*pro-R* hydrogen atom of 1-octanol; replacement of horse liver alcohol dehydrogenase by yeast alcohol dehydrogenase in the above system results in exchange of only the 1-*pro-R* hydrogen atom of 1-octanol. In the absence of horse liver or yeast alcohol dehydrogenase, no exchange of C-1 hydrogen atoms of 1-octanol occurs. Thus, horse liver alcohol dehydrogenase is directly responsible for promoting exchange of the 1-*pro-S* hydrogen atom of 1-octanol with water hydrogen atoms.

The equilibration of C-1 hydrogen atoms of primary alcohols with water, mediated by horse liver alcohol dehydrogenase-NAD/NADH-diaphorase, has been presumed to involve the

stereospecific exchange of the 1-*pro-R* hydrogen atom of the alcohol.^{1,2} Recently we found that this reaction is *not* stereospecific: the 1-*pro-S* hydrogen atom of 1-octanol is also exchanged,

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[‡]Dedicated to Prof. E. Lederer on his 75th birthday.

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