Foundation is gratefully acknowledged. Computer funds were supplied by the University of Illinois Research Board. M.T.M. thanks the National Science Foundation for support through a predoctoral fellowship, 1970-1973.

Supplementary Material Available. Positions of nuclei, thermal parameters, and a listing of structure factor amplitudes will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JACS-75-192.

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(7) See, for example, M. Bixon, H. Dekker, J. D. Dunitz, H. Eser, S. Lifson, C. Mosselman, J. Sicher, and M. Svoboda, *Chem. Commun.*, 360 (1967).
(8) R. A. Bauer, W. R. Robinson, and D. W. Margerum, *J. Chem. Soc., Chem. Commun.*, 289 (1973).
(9) The synthesis of the 1,4-dimethylcyclam complex was accomplished by a modification of the procedure recently described for Ni(cyclam)$^{2+}$ using 5,8-dimethyl-1,5,8,12-tetraazadodecane as the starting tetramine: see E. K. Barefield, *Inorg. Chem.*, **11**, 2273 (1972). Complete details of the synthesis of this complex will appear in a later publication.

M. J. D'Aniello, Jr., M. T. Mocella, F. Wagner
E. Kent Barefield,* Iain C. Paul
W. A. Noyes Laboratory, School of Chemical Sciences
University of Illinois
Urbana, Illinois 61801
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Very Fast Zinc-Catalyzed Hydrolysis of an Anhydride. A Model for the Rate and Mechanism of Carboxypeptidase A Catalysis

Sir:

One of the most attractive mechanisms[1] for the hydrolysis of peptides or esters by carboxypeptidase A involves two steps and an anhydride (acyl-enzyme) intermediate. In the first step Zn^{2+} of the enzyme electrophilically activates the substrate carbonyl toward nucleophilic attack by a glutamate residue. Departure of an alkoxyl group (with ester substrates) or an amino group (with peptide substrates, assisted by an enzyme tyrosine) results in production of the anhydride between the enzyme glutamate and the scissile carboxyl group. The hydrolysis of this anhydride can be catalyzed only by the Zn^{2+}, the only remaining necessary[2] catalytic group.

There is some direct evidence for a two-step mechanism in the observation[3] that carboxypeptidase catalyzes ^{18}O exchange with the terminal carboxyl group of amino acid derivatives. Such exchange can be interpreted[4] as the catalyzed re-formation of the intermediate anhydride, followed by its hydrolysis with $H_2{}^{18}O$. However, this ^{18}O exchange evidence is not unambiguous evidence for an anhydride intermediate. Furthermore, an intermediate has never been trapped with nucleophiles such as hydroxylamine.[5] An additional question about such an anhydride intermediate is whether indeed simple Zn^{2+} catalysis *could* result in a hydrolysis rate on the enzymatic time scale, the k_{cat}'s[6] for several esters lying in the range of 0.5-230 sec^{-1}.

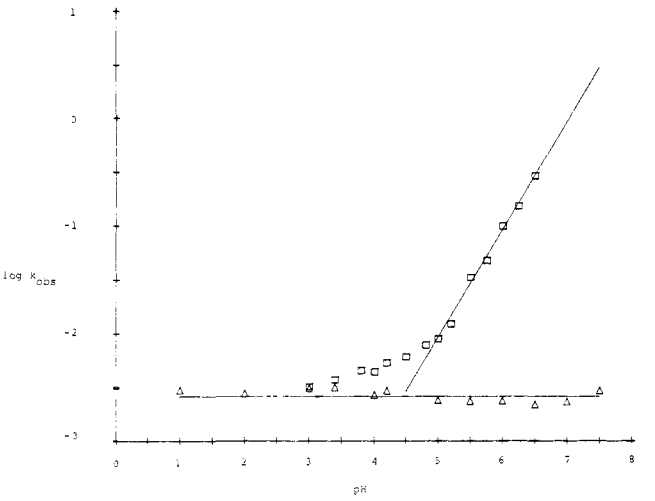


Figure 1. Hydrolysis pseudo-first-order rate constants of anhydride I *vs.* pH: Δ, I; □, I·Zn^{2+}. All runs at 25.0° with 0.3 *M* buffers having μ = 0.5 *M*. See Table I for experimental details.

Table I. Pseudo-First-Order Hydrolysis Rate Constants[a-c] at pH 7.50[d]

Compound	k_{obsd} (sec^{-1})	k_{rel}
I	$(2.7 \pm 0.5) \times 10^{-3}$	1.0
I·Zn^{2+}	3.0 ± 0.5	10^3
II	$(5.5 \pm 0.5) \times 10^{-3}$	2.0
II·Zn^{2+}	1.5 ± 0.5	5×10^2

[a] Hydrolyses in aqueous 0.3 *M* buffered solutions held at 25° were monitored spectrophotometrically at 400 nm using a Cary 17 or Gilford 2400 spectrophotometer. The least-squares computer-processed kinetic data from two or more runs were averaged for each datum point in Figure 1. Titrimetric kinetic studies with II were also conducted at pH 4.00 and identical results were obtained. [b] Buffers used were 0.3 *M* formate from pH 3.0 to 4.2, 0.3 *M* acetate from pH 4.0 to 5.2, and 0.3 *M* 2-(N-Morpholino)ethane sulfonic acid from pH 5.5 to 7.5. The ionic strength was adjusted with KCl or $NaClO_4$ to μ = 0.5 *M* for all buffers. [c] Buffer concentrations were varied, and the rate constants were corrected for small buffer terms when observed. The zinc-catalyzed hydrolysis of I showed no buffer catalysis from pH 3.0 to 6.5 (the total range studied). [d] Optimum esterase and peptidase activity for carboxypeptidase A has been determined to occur at pH 7.5. Values for the zinc-catalyzed hydrolysis rate constants were extrapolated to pH 7.5 through the use of plots like those shown in Figure 1.

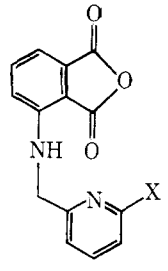
As part of our series of model studies on carboxypeptidase, we have examined the hydrolysis of anhydrides I[7] and II[7] with and without zinc (and other metal ions). We find that rate accelerations of this process do indeed bring it into the rate region mentioned above for the overall enzymatic process itself. Furthermore, we find that the mechanism of this process explains the failure to trap an anhydride intermediate in the enzymatic reaction with nucleophiles other than water, and indeed demonstrates that the large preference of this enzyme for water over other nucleophiles is evidence in favor of the anhydride mechanism.

Since in carboxypeptidase A the natural metal is Zn^{2+} coordinated to two nitrogens and a carboxylate, our principal emphasis has been on the study of the zinc catalyzed reactions of anhydride I, which is fully saturated with Zn^{2+} with metal concentrations greater than 0.005 *M*. The results from the kinetic study are displayed in Figure 1, and the kinetic constants from the studies are listed in Table I. These data show that in the absence of metal the anhydride hydrolysis is independent of pH in the region 1.00-7.50, as with phthalic anhydride.[8] However, with coordinated zinc ion the cleavage is first order in hydroxide above pH 5.00.

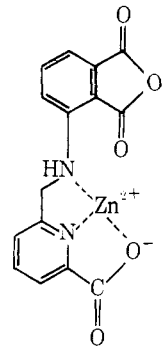
The rate was extrapolated to the enzymatically important pH of 7.50 since it was too fast to measure in this region by simple conventional techniques.

The rate enhancement of this cleavage due to coordinated metal ion is of a magnitude similar to that for other known metal ion accelerations, but with a relatively reactive anhydride function the rate constant at pH 7.50 is 3.0 ± 0.5 sec^{-1}. This falls in the region of values of k_{cat} mentioned for the enzyme, and it does indicate that such a zinc-catalyzed cleavage of the anhydride is a reasonable step to invoke in the course of the enzymatic reaction. The additional acceleration required to make this step fast compared with the hydrolysis of the fastest substrates for the enzyme could presumably be supplied if some of the freedom still present in our model III were restricted in the enzyme-substrate complex.

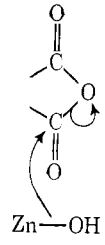
The data in Figure 1 contain an indication of the mechanism of this catalysis. The pH dependence indicates that Zn^{2+} catalyzes the attack of hydroxide on the anhydride but does not detectably catalyze the attack of neutral water. Such an effect would not be expected if zinc were functioning as a Lewis acid to facilitate attack on the coordinated anhydride by an external nucleophile; in a more reactive coordinated anhydride the preference for a better nucleophile should be decreased, not increased. However, if the mechanism of this attack involves coordination of hydroxide to the Zn^{2+}, followed by nucleophilic attack by such a coordinated hydroxide on an uncoordinated anhydride carbonyl (IV), then the effect would be understandable. This mechanism is the one expected for catalyzed cleavage of an anhydride, in which the catalytic problem to be solved is that of supplying a sufficient concentration of nucleophile, not of stabilizing the leaving group.



I, X = CO_2H
II, X = H



III



IV

Studies of the opening of anhydride I with hydroxylamine also support this mechanism. In the absence of Zn^{2+}, hydroxylamine is an effective nucleophile toward I, but its attack is *not catalyzed* by Zn^{2+}. Instead the data fit eq 1

$$-\frac{d[I]}{dt} = k_1[I \cdot Zn^{2+}][OH^-] + k_2[I_{total}][NH_2OH] \quad (1)$$

under a variety of conditions. When [I] is 5×10^{-5} M and $[NH_2OH_{total}]$ is 5×10^{-4} M at pH 5.50 in the absence of Zn^{2+}, for example, the rate for the disappearance of I due to hydroxylamine attack completely dominates that due to spontaneous hydrolysis ($\sim 10^2$ faster). When enough Zn^{2+} is added to completely saturate I with the metal under these conditions, an increase in the overall rate by 30% is observed. This rate increase is exactly that expected from the addition of the now important first term in eq 1.[9] The balance between these two terms will be determined by pH and the effectiveness of Zn^{2+} catalysis. Thus, in the enzyme at pH 7.50, with decreased conformational mobility and good proximity of Zn^{2+}, we would expect that the catalyzed attack by coordinated OH^- would be much faster than the uncatalyzed attack by hydroxylamine.

The results from our model systems indicate that the failure to trap an anhydride intermediate in the enzymatic reaction[5] is expected because of the mechanism by which Zn^{2+} would catalyze its cleavage, and this would explain[10] the observed preference of carboxypeptidase A for water (OH^-) over any other lytic agent. The two-step mechanism thus remains one of the most attractive explanations of all the data on the reactions catalyzed by carboxypeptidase A. In this mechanism the Zn^{2+} catalyzes the first step, anhydride formation, by acting as a Lewis acid. It catalyzes the second step by the delivery of a specific nucleophile to the anhydride intermediate.

Acknowledgments. Helpful discussions with Mr. David Wernick are gratefully acknowledged. This work was supported by the National Institutes of Health.

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(4) D. Wernick, in unpublished calculations, has shown that the ^{18}O exchange rate is as fast as expected for the enzyme-catalyzed endothermic resynthesis of an anhydride. The calculation involves the known equilibrium constant for anhydride hydrolysis and an enzyme-catalyzed rate of hydrolysis fast enough to fit the enzyme time scale for substrate hydrolyses.
(5) D. Wernick, unpublished work. Mr. Wernick has also shown that carboxypeptidase A prefers $H_2O(OH^-)$ over $CH_3OH(CH_3O^-)$ by at least 10^2. Related observations have apparently been made in other laboratories.
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(9) Conversion of I to its Zn^{2+} complex III neither increases nor decreases its reaction rate with NH_2OH (a 10% change would be detectable). The flexibility in III permits the Zn^{2+} to lie away from the anhydride, and electronic effects are apparently small.
(10) Most other nucleophiles could not be delivered by Zn^{2+}. Even CH_3O^- would be selected against, for reasons discussed by R. Breslow, R. Fairweather, and J. Keana, *J. Amer. Chem. Soc.*, **89**, 2135 (1967). An additional likely possibility is that in mechanism IV the hydroxide is not transferred away from zinc, but instead undergoes a further deprotonation after it attacks the carbonyl group. This would generate a carboxylate ion still coordinated to zinc. Such a further deprotonation is not possible for methoxide.
(11) National Research Council of Canada Postdoctoral Fellow, 1972–1974.
(12) Undergraduate Summer Research Participant, 1974.

R. Breslow,* D. E. McClure
R. S. Brown,[11] J. Eisenach[12]
Department of Chemistry, Columbia University
New York, New York 10027

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Trans Addition of Halogens to Tetrakis(triphenylphosphine)platinum(0)

Sir:

The oxidative additions of bromine and iodine to $Pt(PPh_3)_4$ have been reported both explicitly and implicitly to give invariably *cis*-$PtBr_2(PPh_3)_2$ and *cis*-$PtI_2(PPh_3)_2$,