The Mechanism of Inhibition of Carboxypeptidase A by 1,10-Phenanthroline*

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Received December 26, 1961

The chelating agent 1,10-phenanthroline inhibits native zinc carboxypeptidase A by competing with the apoenzyme for its zinc atom and removing it. This time-dependent inhibition is fully reversible on dilution or on addition of Zn^{++} ions. The inhibition is also a function of enzyme and 1,10-phenanthroline concentrations. The peptide substrate, carbobenzoxyglycyl-L-phenylalanine, interferes with the removal of the zinc from the enzyme by 1,10-phenanthroline, and it blocks the instantaneous reconstitution of the metal-free apoenzyme on addition of Zn^{++} ions. It is concluded that the peptide substrate binds to amino acid side-chains of the active center to form the enzyme-substrate complex, thus permitting the metal mercaptide to exercise its hydrolytic function.

The enzymatic activity of native carboxypeptidase A depends crucially on the binding of 1 g atom of zinc to one mole of the metal-free, inactive apoenzyme (Vallee *et al.*, 1958b, 1960). The physical-chemical characteristics of this enzyme have been studied, and the stability constant of the complex between zinc and the apoenzyme has been determined (Coleman and Vallee, 1961).

A chelating agent, 1,10-phenanthroline, inhibits the enzyme and removes zinc from it (Vallee and Neurath, 1955; Vallee *et al.*, 1960). The nature and mechanism of this inhibition have now been examined kinetically and by physical-chemical methods. Further, it is shown that a peptide substrate, carbobenzoxyglycyl-L-phenylalanine, interferes with the binding of zinc to the apoenzyme.

MATERIALS AND METHODS

Beef Pancreas Carboxypeptidase [(CPD) Zn][1].—Four-times-recrystallized zinc carboxypeptidase was prepared from beef pancreas acetone powder[2] by the method of Allan, Keller, and Neurath (in preparation). The solution of the final crystals in 1.0 M NaCl, 0.1 M Tris buffer, *p*H 7.5, was homogeneous in the ultracentrifuge and by moving boundary electrophoresis when examined in 0.3 ionic strength LiCl buffers at *p*H 6.6 to 10.5. The proteolytic coefficient, *C*, of this preparation was 25 to 30 at *p*H 7.5, 25°, and 6 to 7 at *p*H 7.5, 0°. The esterase activity, expressed as a zero order rate constant, *k* (see below), was 1.15×10^3 μM H^+/min./mg N at *p*H 7.5, 25°. The zinc-to-protein ratio was 1950 μg per g of protein or 1.03 g atom per mole, assuming a molecular weight of 34,300 for the protein (Smith and Stockell, 1954; Vallee and Neurath, 1955).

Zinc-Free Apocarboxypeptidase (CPD).—Zinc-free apocarboxypeptidase was prepared by dialyzing the native enzyme against 1,10-phenanthroline at *p*H 7.0 followed by dialysis against buffer; precautions were taken against contamination by metal ions (Coleman and Vallee, 1960; Vallee *et al.*, 1960). The preparations of "zinc-free apocarboxypeptidase" used in these experiments contained between 26 and 39 μg of zinc per g of protein, or 1.3 and 2% of the original zinc content; they exhibited 2% of the original activity.

Zn^{65} Carboxypeptidase [(CPD) Zn^{65}].—Zn^{65} carboxypeptidase was prepared by dialyzing the metal-free apoenzyme against a buffered solution of $Zn^{65}Cl_2$ (Coleman and Vallee, 1960). The final product contained 1850 μg of zinc per g of protein and had a specific radioactivity of 6750 cpm/mg Zn. The proteolytic coefficient (*C*) at 25°, *p*H 7.5, was 30.

Metal Solutions.—Standard solutions of Zn^{++} and Cu^{++} were prepared by dissolving spectrographically pure metals (Johnson Matthey Co., Ltd.) in a minimum of metal-free hydrochloric acid which was then diluted with buffer to the desired *p*H and molarity. A weighed amount of reagent-grade ferrous sulfate, $FeSO_4 \cdot 7H_2O$, was dissolved in metal-free water just before use.

1,10-Phenanthroline.—Reagent-grade crystalline hydrochloride (G.F. Smith Co.) was used without further purification. Solutions in the appropriate buffers were standardized by the absorbance at 265 mμ E_{265}^{1mM}, = 31.5 (Vallee *et al.*, 1960).

Enzymatic Activity.—Peptidase activity was determined with carbobenzoxyglycyl-L-phenylalanine (CGP) as the substrate (Mann Chemical Company). Activity is expressed as an apparent proteolytic coefficient, *C* (Coleman and Vallee, 1960; Neurath and DeMaria, 1950; Snoke and Neurath, 1949). The assays were performed either at 25° or at 0° in 0.1 M Tris buffer containing 1 M

* This work was supported by the Howard Hughes Medical Institute and by a grant-in-aid from the National Institutes of Health of the Department of Health, Education and Welfare, No. H-3117 (C4).

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[1] The abbreviations used are: [(CPD) Zn], zinc carboxypeptidase where (CPD) represents the metal-free apoenzyme and the brackets indicate the firm binding of zinc in the native or reconstituted zinc carboxypeptidase. These abbreviations are employed only when needed in formulations or required for clarity. OP, 1,10-phenanthroline; CGP, carbobenzoxyglycyl-L-phenylalanine.

[2] A gift kindly supplied by the Eli Lilly Research Laboratories.

NaCl, *p*H 7.5, and *C* was calculated from the linear portion of the first-order reaction plots observed when hydrolysis did not exceed 15%. All assays were carried out by sampling the reaction mixture at 5-minute intervals for 20 minutes except in the experiments illustrated in Figures 4 and 5, where the samples were assayed at intervals of 1 minute.

Protein Concentrations were measured either by precipitation with 10% trichloracetic acid followed by drying at 104° (Hoch and Vallee, 1953) or by absorbancy at 278 mμ (Davie and Neurath, 1955). The results of the two procedures were in excellent agreement.

A Beckman DU spectrophotometer with photomultiplier attachment and thermostatically controlled cell housing was employed throughout; *p*H was measured with a Leeds and Northrup *p*H indicator equipped with a general-purpose external glass electrode. Contamination from adventitious metal ions was controlled by prior extraction of substrate and buffer solutions with dithizone (Vallee *et al.*, 1960). Water was purified by passage over a mixed resin. All solutions were stored and the reactions carried out in polyethylene vessels whenever possible (Thiers, 1957).

The Continuous Variations Method was applied to the [(CPD) Zn]–1,10-phenanthroline system to determine the stoichiometry of the enzyme–1,10-phenanthroline complex as described (Vallee and Coombs, 1959; Vallee, 1960b). Mixtures of [(CPD) Zn] and 1,10-phenanthroline were prepared in which the concentration of each component was varied from 100 to 0 mole % [(CPD) Zn] and 0 to 100 mole % 1,10-phenanthroline respectively, while the molar sum was kept constant. The low solubility of the enzyme restricted the total concentrations that could be employed to 5×10^{-5} and 1×10^{-4} M. The degree of complexation occurring in each [(CPD) Zn]–1,10-phenanthroline mixture was calculated from the measured increase in absorbancy at 3275 A, the wave length of the absorption maximum resulting from the interaction of [(CPD) Zn] with 1,10-phenanthroline (Vallee *et al.*, 1958a). A_{3275A} is plotted as a function of the mole % [(CPD) Zn] to give a standard continuous variations curve (Fig. 1).

Results

Time-Dependent Inhibition by 1,10-Phenanthroline.—When 1,10-phenanthroline is preincubated with zinc carboxypeptidase prior to dilution into a reaction mixture containing buffer and substrate, activity is lost as a function of the concentration of the inhibitor and the time of exposure (Fig. 2). Inhibition increases rapidly at first, but then becomes constant after 1 hour of preincubation at 0°: the enzyme is inhibited 50% by 5×10^{-4} M 1,10-phenanthroline after 1 minute, by 2×10^{-4} M after 5 minutes, by 1.5×10^{-4} M after 10 minutes, and by 7×10^{-5} M after 1 and 2 hours of incubation.

Increasing the temperature of preincubation to 25° only increases the *rate* at which the inhibition is produced. The final *degree* of inhibition is not affected, however. As a consequence, at 25° equilibrium is reached after 10 minutes, instead of after the 1 hour required at 0°.

The inhibition of carboxypeptidase with 1,10-phenanthroline is dependent upon the concentration of the enzyme in the reaction mixture. The activities resulting from the preincubation of increasing concentrations of enzyme with 1×10^{-4} M 1,10-phenanthroline are shown in Table I. When the enzyme is 10^{-6} M a 5-fold increase in its concentration results in a 7.5-fold decrease of inhibition.

Table I

Effect of Enzyme Concentration on Inhibition of [(CPD) Zn] by 1,10-Phenanthroline

The enzyme was incubated with OP for 1 hr. at 0° in 1 M NaCl–0.1 M Tris, *p*H 7.5. Peptidase assays as in Fig. 2. Uninhibited activity is *Vc*, inhibited activity, *Vi*.

([(CPD) Zn]) (M × 10^6)	(OP) (M × 10^4)	*Vi*/*Vc*
1.0	1.0	0.04
1.5	1.0	0.06
2.0	1.0	0.09
5.0	1.0	0.30

Reversibility and Mechanism of the Inhibition.—The dialysis of [(CPD) Zn^{65}] against a 5-ml volume of 3.3×10^{-4} M 1,10-phenanthroline for 24 hours results in the removal of 96% of Zn^{65} and the loss of 97% of the activity. When the same enzyme solution, serving as a control, is dialyzed against buffer only, but under conditions which are otherwise identical, essentially no Zn^{65} or activity is lost (Table II). The loss of both zinc and activity is reversed on dilution of the 1,10-phenanthroline to 3.3×10^{-5} M, brought about by a tenfold increase in the dialyzing volume of the buffer to 50 ml. In 24 hours, 46% of the original Zn^{65} and 40% of the activity are restored to the enzyme. The control remains essentially unchanged throughout.

Table II

Effect of 1,10-Phenanthroline and Volume of Dialysate on Activity and Zn^{65} Content of [(CPD) Zn^{65}]

1×10^{-5} M enzyme was employed throughout. Tenfold dilution of the dialyzing volume (line 3) dissociates the $[Zn^{65}\ OP]^{++}$ complexes formed on removal of zinc from the enzyme with subsequent restoration of Zn^{65} and activity to it. 1 M NaCl–0.1 M Tris, *p*H 7.5, 0°. Peptidase assays as in Fig. 2. Uninhibited activity is *Vc*, inhibited activity, *Vi*.

Sample	Dialyzing Vol. (ml)	Zn^{65} Bound (g at/M)	*Vi*/*Vc*
[(CPD) Zn]	0	0.99	1.00
[(CPD) Zn] + 3.3×10^{-4} M OP	5	0.04	0.03
[(CPD) Zn] + 3.3×10^{-4} M OP	50	0.46	0.40
[(CPD) Zn] Control	5	0.96	0.97
[(CPD) Zn] Control	50	0.96	0.95

Prevention and Reversal by Metal Ions.—The incubation of the 1:1 Zn^{++}, Cu^{++}, or Fe^{++} complexes of 1,10-phenanthroline with [(CPD) Zn] for 1 hour does not result in inhibition (Table III). Addition of Zn^{++} ions, equimolar to the 1,10-phenanthroline employed for preincubation with the enzyme, instantaneously restores 90% of the

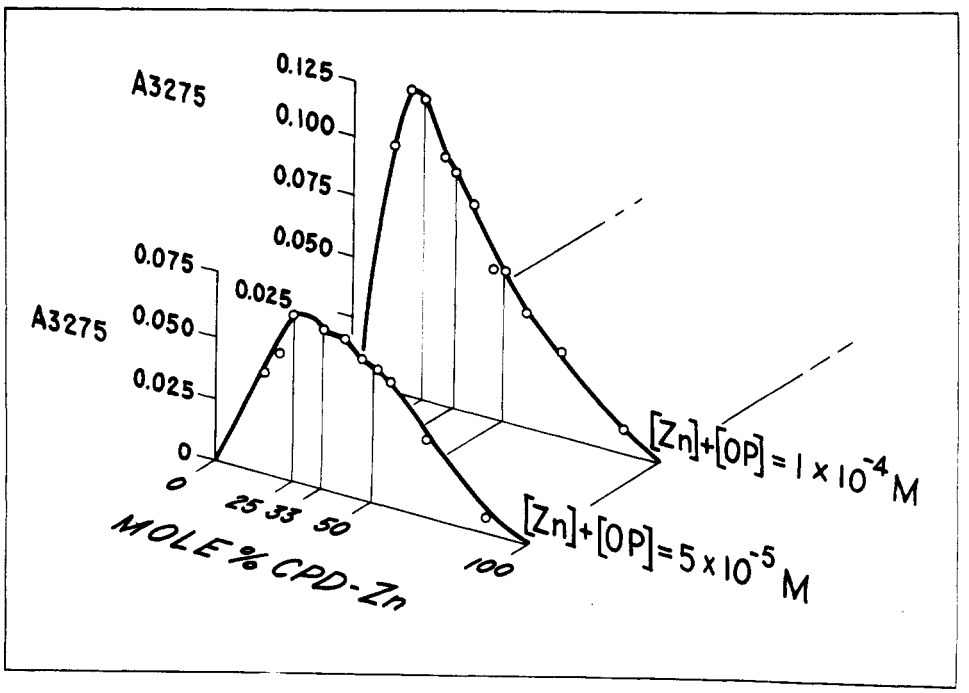

FIG. 1.—Job's method of continuous variations applied to the interaction of the zinc of carboxypeptidase with 1,10-phenanthroline. The absorbancy, A, was measured at 3275 A. Each curve represents measurements of absorption at the indicated total molarity of [(CPD)Zn] + OP; the moles per cent of the two species are varied as indicated on the abscissa. The maximal formation of the 1:1, 1:2, and 1:3 $[Zn(OP)]^{++}$ complexes are indicated by vertical guide lines. Conditions: 1 M NaCl–0.1 M Tris, *p*H 8.0, 0°; Beckman DU spectrophotometer, 5-cm path length cells.

original activity after 3 and 30 or more minutes of preincubation (Fig. 3).

TABLE III

PREVENTION OF 1,10-PHENANTHROLINE INHIBITION OF [(CPD) Zn] BY METAL IONS

1×10^{-5} M Me^{++} ions were added to 1×10^{-5} M OP before preincubation with 3.8×10^{-7} M enzyme for 1 hr. at 0° in 1 M NaCl–0.1 M Tris, *p*H 7.5. Peptidase assays as in Fig. 2. Uninhibited activity is *Vc*, inhibited activity, *Vi*.

Sample	*Vi*/*Vc*
Control	1.00
Control + OP	0.22
Control + [OP + Zn^{++}]	0.95
Control + Zn^{++}	0.94
Control + [OP + Cu^{++}]	0.81
Control + Cu^{++}	0.34
Control + [OP + Fe^{++}]	0.93
Control + Fe^{++}	1.00

Stoichiometry of the [(CPD) Zn]-1,10-Phenanthroline Complex.—At a total concentration of [(CPD) Zn] + OP = 5×10^{-5} M, a plot of the absorbancy due to all Zn-OP complexes, A_{3275A}, as a function of the mole % [(CPD) Zn], results in a broad maximum at 25 mole % of [(CPD) Zn]; this represents the formation of a $[Zn(OP)_3]$ complex (Job, 1928). When the total concentration of [(CPD) Zn] + OP is increased to 1×10^{-4} M, the curve becomes much steeper, and the maximum is demarcated even more sharply at 25 mole % of [(CPD) Zn], representing an increase in the proportion of the total of the $[Zn(OP)_3]$ complex formed (Fig. 1).

Functional Effects of the Substrate.—The presence of the substrate interferes markedly with the rebinding of Zn^{++} ions to the metal-free apoenzyme, and, hence, with the restoration of activity. This phenomenon is readily demonstrated when the order of additions of apoenzyme, substrate, and Zn^{++} ions is varied to start the reaction (Fig. 4). When equimolar concentrations of apoenzyme and Zn^{++} ions are preincubated and carbobenzoxyglycyl-L-phenylalanine is added last, activity equal to that of native [(CPD) Zn] is obtained. However, when apoenzyme and carbobenzoxyglycyl-L-phenylalanine are preincubated and then Zn^{++} ions are added last, activity is not restored; in fact, the activity obtained does not differ materially from the control to which Zn^{++} ions have not been added (Fig. 4).

Similarly, the order of addition of carboxypeptidase, 1,10-phenanthroline, and carbobenzoxyglycyl-L-phenylalanine affects the inhibition of carboxypeptidase by 1,10-phenanthroline. When the enzyme is added both to substrate and 1,10-phenanthroline in the reaction mixture, inhibition is not detected until the reaction has already proceeded for 3 minutes. However, when the 1,10-phenanthroline is first added to the enzyme followed by the substrate, activity is inhibited instantaneously (Fig. 5).

Kinetics of the Inhibition by 1,10-Phenanthroline.—The degree of inhibition of carboxypeptidase by a given concentration of 1,10-phenanthroline depends not only on the time of preincubation but

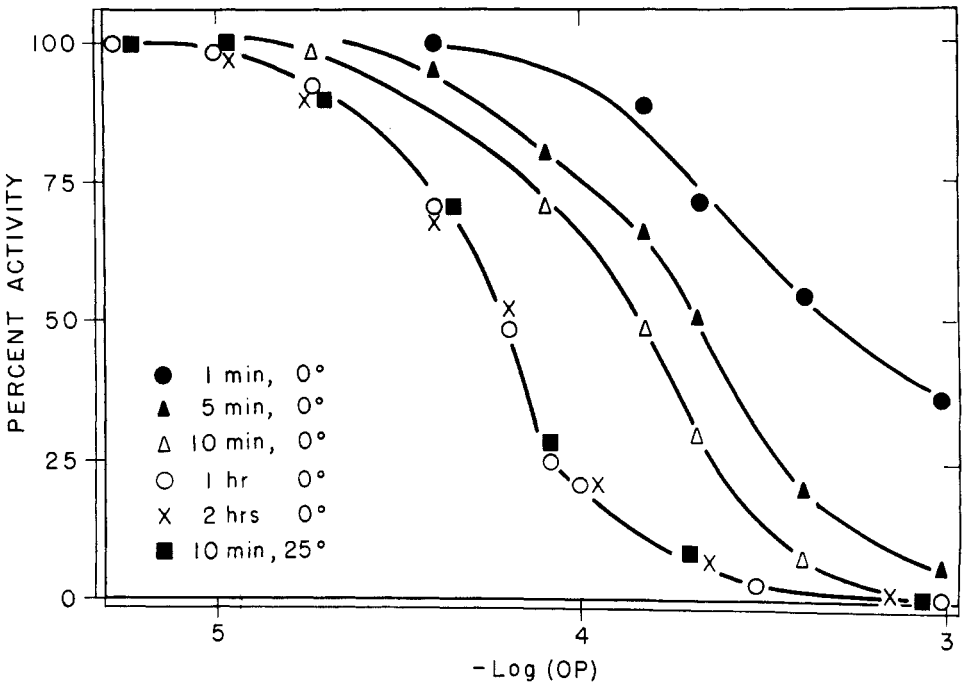

FIG. 2.—Time-dependent inhibition of carboxypeptidase by 1,10-phenanthroline. [(CPD)Zn], 3.8×10^{-7} M, was incubated with increasing concentrations of 1,10-phenanthroline in 1 M NaCl–0.1 M Tris, *p*H 7.5, at 0° or 25°. Aliquots of 0.1 ml were removed for activity measurements after 1 minute at 0° (●); 5 minutes at 0° (▲); 10 minutes at 0° (Δ); 1 hour at 0° (○); 2 hours at 0° (X); and 10 minutes at 25° (■). Peptidase assays were performed with 3.8×10^{-8} M enzyme and 0.02 M carbobenzoxyglycyl-L-phenylalanine in 1 M NaCl–0.1 M Tris, *p*H 7.5, 25°.

also on the concentration of the substrate. The relationships between the substrate concentration and inhibitor concentration were, therefore, determined quantitatively as a function of time. Lineweaver-Burke plots of the reciprocal velocities, v^{-1}, as previously defined (Elkins-Kaufman and Neurath, 1948), versus the reciprocal carbobenzoxyglycly-L-phenylalanine concentrations, after 1, 5, and 60 minutes of exposure to increasing concentrations of 1,10-phenanthroline, demonstrate a series of straight lines with increasing slopes both as a function of 1,10-phenanthroline concentration and of the time of reaction. These lines, however, do not converge to a common point of intersection under any of the experimental conditions examined.[3]

DISCUSSION

The properties of chelating agents pertinent to the study of the mechanism of action of metalloenzymes have been discussed and their mode of action has been studied extensively in a variety of enzymes (Vallee, 1955, 1960a). Carboxypeptidase is inhibited by 1,10-phenanthroline, $\alpha\alpha'$-dipyridyl, and 8-hydroxyquinoline-5 sulfonic acid (Vallee, 1955), and these agents have been shown to act at the metal atom site (Vallee *et al.*, 1958a). The failure of the metal ion complexes of 1,10-phenanthroline to inhibit the enzyme (Table III) indicates further that the metal-coordinating groups of the chelating agent are responsible for this inhibitory action.

While in the active enzyme zinc is firmly bound, the 1:1 complex between the apoenzyme and zinc is dissociable. Therefore

$$[(\text{CPD})\ \text{Zn}] \rightleftharpoons (\text{CPD}) + \text{Zn}^{++} \qquad (1)$$

and

$$K_e\ app = \frac{((\text{CPD}))(\text{Zn}^{++})}{([(\text{CPD})\ \text{Zn}])} \qquad (2)$$

where $K_e\ app$ is the apparent dissociation constant, which has been found to be 4.7×10^{-9} M at *p*H 8.0 (Coleman and Vallee, 1961).

In principle, inhibition of metalloenzymes by 1,10-phenanthroline operating at the metal atom site may be thought to occur by two different mechanisms: The chelating agent may compete successfully with the apoenzyme—a second metal-binding agent—for the metal atom to form the enzymatically inactive, metal-free, apoenzyme plus the metal-chelate as in equation (3),

$$[(E)\ \text{Me}] + n\ \text{OP} \rightleftharpoons (E) + [\text{Me}\ (\text{OP})_n]^{++} \qquad (3)$$

where n is the number of moles of chelating agent combining with each mole of metal. This type of inhibition may or may not be reversible, as indicated by the broken arrow. When such a reaction is

[3] The conditions for showing competitive inhibition involve the assumption of reversibility of the reaction, an assumption which does not here pertain. Hence documentation of its nonexistence did not seem warranted.

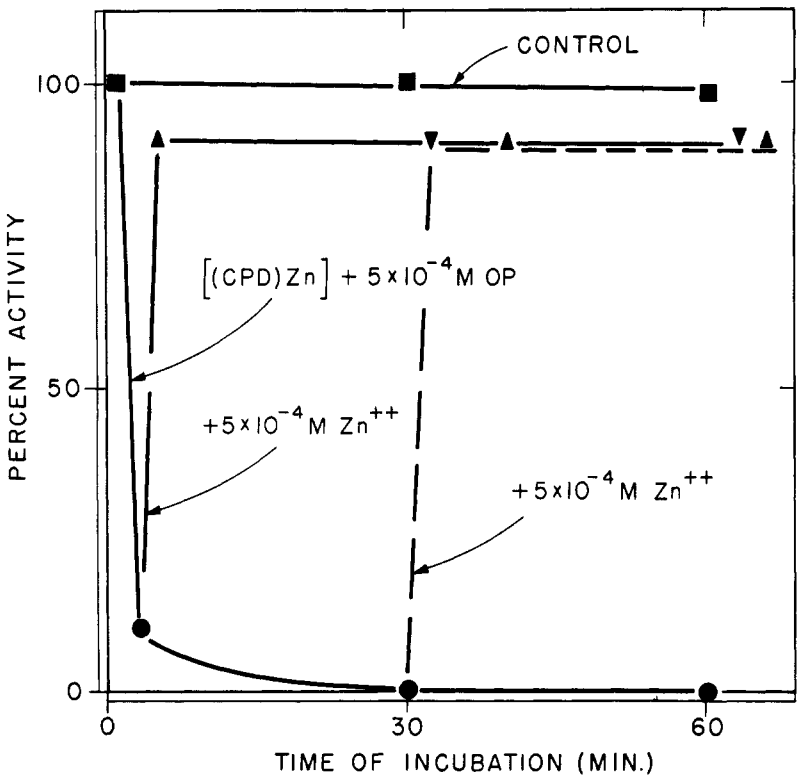

FIG. 3.—Reversal of 1,10-phenanthroline inhibition of carboxypeptidase by Zn^{++} ions. The decrease in the partial activity, expressed as percent of the control, when 5.7×10^{-7} M [(CPD)Zn] is incubated with 5×10^{-4} M 1,10-phenanthroline at 0° is indicated by solid circles (●—●). The triangles, (▲—▲) and (▼—▼), represent the change in activity when Zn^{++} ions in a concentration equimolar to that of 1,10-phenanthroline are added after 3 and 30 minutes of incubation respectively. The squares, (■—■), indicate the activity of the control which contains enzyme only. Conditions for incubation and peptidase assays as in Figure 2.

irreversible, characteristically this type of inhibition has been shown to be time-dependent, irreversible on dilution and irreversible on readdition of metal ions (Vallee and Hoch, 1955; Williams *et al.*, 1958). The irreversibility has been attributed to changes in the binding site or in the conformation of the protein.

Alternatively, the chelating agent might combine with the metal atom while "*in situ*," *i.e.*, bound to the protein, forming an enzymatically inactive, dissociable mixed complex; protein-Me-OP as in equation (4).

$$[(E)\ \mathrm{Me}] + n\ \mathrm{OP} \rightleftarrows [(E)\ \mathrm{Me}]\ \mathrm{OP}_n \qquad (4)$$

Such inhibitions have been shown to be *instantaneous*, reversible on dilution and independent of the time of exposure (Hoch *et al.*, 1958; Vallee *et al.*, 1959). The experiments employing the method of continuous variations provide conclusive, direct evidence in regard to the mechanism of this type of inhibition (Vallee, 1960b; Vallee *et al.*, 1959). Three moles of 1,10-phenanthroline are the maximum number which can combine with zinc, since this element's maximum number of coordination sites is six. When 1,10-phenanthroline is in sufficient excess, ionic zinc will always form the 1:3 complex (Kolthoff *et al.*, 1951). This situation would naturally be altered when any of these six sites are already occupied in some manner, for instance when the metal is bound firmly to a protein.

When in a protein–zinc–1,10-phenanthroline system it can be shown that a 1:1 Zn:OP complex is the major species present at concentrations of 1,10-phenanthroline at which the 1:3 complex predominates in an ionic zinc system, this can only be interpreted to imply that zinc is bound to protein, demonstrating the mechanism of equation (4). The formation of the 1 Zn:3 OP complex, as in Figure 1, in the presence of carboxypeptidase denotes that the inhibition of the enzyme by 1,10-phenanthroline is accompanied by the removal of zinc, *i.e., the mechanism of equation* (3) *applies.*

Since both the interchange of Zn^{++} ions with the apoenzyme and with 1,10-phenanthroline is fully reversible (Kolthoff *et al.*, 1951; Vallee *et al.*, 1960), the inhibition proceeding as in equation (3) should also be reversible if the ligand does not undergo changes subsequent to the removal of the metal ion. This is borne out by the experiments utilizing [(CPD)Zn^{65}] (Table II). The [Zn^{65}OP]$^{++}$

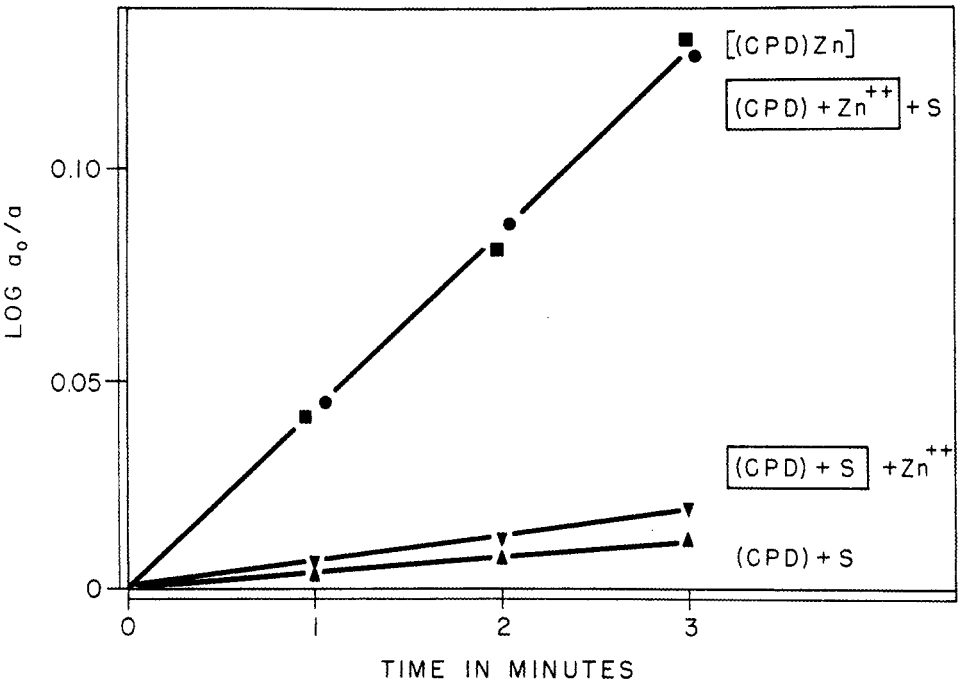

FIG. 4. Prevention of restoration of activity to apocarboxypeptidase (CPD) by variation in the sequence of addition of Zn^{++} ions and of the substrate, carbobenzoxyglycyl-L-phenylalanine (*S*). First-order progression curves; native zinc carboxypeptidase (■); (CPD) + S (▲) is the control; (CPD) was first incubated with 1 g atom of Zn^{++} ions for 1 minute and substrate, *S*, was then added to start the reaction (●); (CPD) was first incubated with substrate, *S*, for 1 minute and 1 g atom of Zn^{++} ions was then added to start the reaction (▼). Enzyme, 1×10^{-6} M, peptidase assay conditions as in Figure 2. Preincubation of (CPD) with the substrate carbobenzoxyglycyl-L-phenylalanine, *S*, prevents restoration of activity with Zn^{++} ions.

complexes formed on removal of Zn^{65} from the apocarboxypeptidase are dissociated by dilution, and Zn^{65} re-enters the enzyme and restores the activity. Further, the addition of Zn^{++} ions to the inhibited enzyme immediately restores full activity, and the added ions are retained after either 3 or 30 minutes of preincubation with the inhibitor (Fig. 3). Equation (3) indicates that under these conditions the degree of inhibition would be expected to depend on the relative concentrations of both enzyme and 1,10-phenanthroline.

The kinetic studies to discern the mechanism of inhibition of carboxypeptidase A with 1,10-phenanthroline have been unsatisfactory compared to those performed at equilibrium by physical-chemical approaches. Such ambiguities in the kinetics can be traced to intrinsic characteristics of the system. Importantly, the method for the measurement of the rate of hydrolysis is too slow for the study of mechanisms of the *instantaneous* inhibition. Further, the examination of the time-dependent inhibition requires preincubation and subsequent dilution into a reaction mixture. When the enzyme and 1,10-phenanthroline are preincubated alone, inhibition occurs through removal of zinc (Fig. 2) and as a function of enzyme concentration (Table I); on dilution partial *reactivation* of the enzyme takes place as the $[ZnOP]^{++}$ complexes are dissociated (Table II). A further and significant complication becomes apparent on addition of the substrate which, on the one hand, protects the enzyme against removal of zinc (Fig. 5), and, on the other, prevents the potentially freely reversible binding of Zn^{++} ions by the apoenzyme (Fig. 4).

Thus, the reversibility of the system varies continuously as a function of the time of contact, of the sequence of addition, and of the concentration of enzyme, substrate, and inhibitor, during the period both of preincubation and of the lengthy assay thereafter.

In our hands this method of assay proved incapable of yielding the accurate data necessary for the measurement of instantaneous kinetics during periods which must be even shorter than those employed in the technically demanding experiments shown in Figures 4 and 5. The activities observed on variation of the inhibitor concentration and on changing concentrations of the substrate fail to meet a common point of intersection when plotted according to Lineweaver and Burke (1934). With increasing time of contact between enzyme and inhibitor the plots progressively diverge until the lines become parallel at 60 minutes; although there is marked convergence, not even the lines plotted on the basis of assays performed over the interval of only 1 minute meet a common point of intersection either on the ordinate or the negative abscissa.

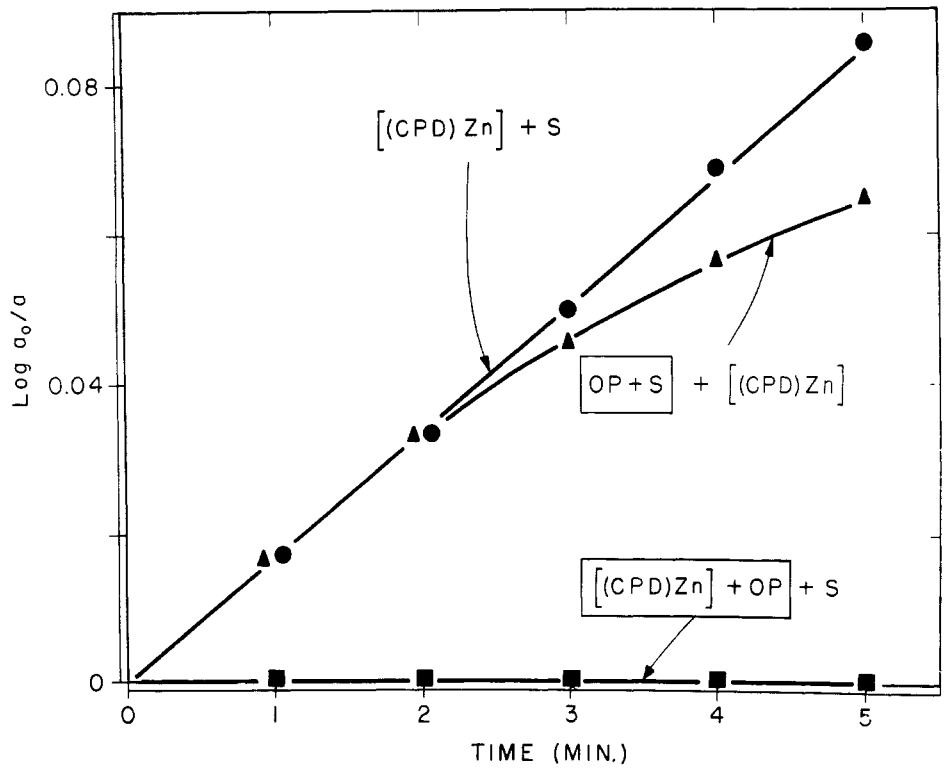

FIG. 5.—Prevention of 1,10-phenanthroline inhibiton of carboxypeptidase by the substrate, carbobenzoxyglycyl-L-phenylalanine (S). First-order progression curves: carboxypeptidase + substrate, S (●) constitutes the control; carboxypeptidase was first preincubated with 1×10^{-2} M 1,10-phenanthroline for 1 minute and then the substrate, S, was added to start the reaction (■). Substrate S and 1×10^{-2} M 1,10-phenanthroline were first preincubated for 1 minute and the enzyme was then added to start the reaction (▲). Enzyme, 1×10^{-6} M, peptidase assay conditions as in Figure 2. Presence of the substrate prevents the otherwise instantaneous inhibition of [(CPD)Zn].

Thus the kinetic criteria for fully reversible reactions cannot be employed in this particular case to reach final conclusions concerning the nature of the competition between the chelating inhibitor, 1,10-phenanthroline, and the peptide substrate, carbobenzoxyglycyl-L-phenylalanine.

The peptide substrate prevents the removal of zinc from the enzyme by 1,10-phenanthroline, but in addition it blocks the otherwise instantaneous complete reconstitution of the active metalloenzyme. It is clear from variations in the sequence of addition of inhibitor and substrate to the enzyme (Fig. 4 and 5) that the substrate interacts with loci on the apoenzyme in addition to zinc while 1,10-phenanthroline does not. Experiments with these two agents would be unlikely to yield conclusive kinetic evidence concerning the mode of substrate attachment. It appears that the metal atom is concerned chiefly with hydrolytic cleavage once the enzyme-substrate complex has been formed (Coombs and Felber, 1961). The interference of the substrate with the binding of metal to the apocarboxypeptidase indicates that both the metal and the substrate binding sites of the protein must be in close proximity. Figure 4 would suggest, *e.g.*, that the substrate might prevent reactivation by covering the metal binding site. Such evidence further delineates the topography of the catalytically active region of the molecule and identifies the active center of this enzyme as a small and chemically unique locus. The present experiments, however, do not identify the specific nucelophilic groups of the substrate to which the metal atom may bind, inescapable as such a hypothesis may seem. The precise physical and chemical nature of the mode of substrate binding to carboxypeptidase is under investigation.

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Use of the *p*H-Stat in Kinetic Studies of Reactions Whose Products Are Capable of Functioning as Buffers*

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Received September 28, 1961

The *p*H-stat has been used to follow the α-chymotrypsin–catalyzed hydrolysis of α-*N*-acylated L-tyrosinamides, hydrazides, and hydroxamides under conditions where one of the reaction products was capable of functioning as a buffer. The limits of usefulness of this application have been defined and α-*N*-acetyl-L-tyrosinhydroxamide has been found to be a particularly useful substrate. Attention has been directed to the nature of the dependence of the kinetic constants of a variety of substrates upon the concentration of added salts.

The kinetics of many enzyme-catalyzed reactions are determinable through use of the *p*H-stat (Jacobsen *et al.*, 1957; Applewhite *et al.*, 1958). These reactions include a number whose reaction products are capable of functioning as buffers (Waley and Watson, 1953; Richards, 1955; Haugaard and Haugaard, 1955; Ottesen, 1956). However, with the latter substrates no attempt has been made to determine the limits of applicability of the *p*H-stat, or to compare reaction kinetics so determined with those evaluated by other procedures. Since kinetic data of the latter kind are available for many systems involving α-chymotrypsin and substrates whose reaction products are capable of buffering the reaction system, we have examined the usefulness of the *p*H-stat for evaluation of their kinetic constants.

We may represent the general case by reactions (1) to (4) inclusive,

$$RCOB + H_2O \longrightarrow RCO_2H + BH \qquad (1)$$

$$RCO_2H \overset{K_1}{\rightleftharpoons} RCO_2^{\ominus} + H^{\oplus} \qquad (2)$$

$$BH + H^{\oplus} \overset{K_2}{\rightleftharpoons} BH_2^{\oplus} \qquad (3)$$

$$BH \overset{K_3}{\rightleftharpoons} B^{\ominus} + H^{\oplus} \qquad (4)$$

where B may be OR′, NHOH, NH_2, $NHNH_2$, or NHR″, when RCOB is an α-*N*-acylated α-amino acid ester, hydroxamide, amide, hydrazide, or a peptide, representing the range of substrates normally encountered in α-chymotrypsin–catalyzed hydrolyses. In all preceding examples, except for phenolic esters, $B^{\ominus}$ is a strong base, hence as a product is present in aqueous reaction systems of $pH < 10$ as the species BH. Since α-chymotrypsin–catalyzed reactions are ordinarily studied in the region from *p*H 6 to 9, reaction (4) may be ignored, except for phenolic esters.

The dissociation constant $K_1 = [RCO_2^{\ominus}][H^{\oplus}]/[RCO_2H]$ of reaction (2) must satisfy the condition, $pK_1 \leq (pH_R - 2)$, where pH_R is the *p*H of the reaction system if more than 99% of the acid is to be titrated. This latter condition is usually satisfied, since for α-*N*-acylated α-amino acids and peptides $pK_1 \leq 3.4$ (Greenstein and Winitz, 1961). Therefore, for most cases we shall be concerned with the consequences of participation of reaction (3).

Kinetic studies require knowledge of the degree to which the stoichiometry, in terms of addition of standard base to the reaction system, is altered by the presence of buffer and in what way, if any, the

* Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service. Contribution no. 2747 from the Gates and Crellin Laboratories of Chemistry.

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