Metallocarboxypeptidases: Mechanism of Inhibition by Chelating Agents, Mercaptans, and Metal Ions*

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The chelating agents α,α'-dipyridyl, 8-hydroxyquinoline-5-sulfonic acid, and 1,10-phenanthroline inhibit zinc, nickel, and manganese carboxypeptidases by removing the metal ions from the apoenzyme. The mercaptans cysteine, thioglycollic acid, and mercaptoethanol inhibit the zinc enzyme by an identical mechanism. Hg^{++}, Cd^{++}, Pb^{++}, Cu^{++}, and H^+ all abolish peptidase activity by displacing zinc from native carboxypeptidase. Buffer anions and Cl^- form stable complexes with some of these ions in solution, lowering the concentration of free ions available for exchange. The observed inhibition constants, K_I, are direct functions of the magnitudes of the apparent stability constants of the metallocarboxypeptidases, K_E, and of the metal ligand complexes, K_L. The data indicate that chelating agents and mercaptans compete with the nitrogen-sulfur binding site of the enzyme for the zinc atom and metal ions displace it.

The enzymatic activity of native carboxypeptidase A of bovine pancreas is reversibly lost and then regained when zinc is removed and added back to the metal-free, inactive apoenzyme (Vallee *et al.*, 1958, 1960b). Activity toward both synthetic peptides and esters is also restored when Co^{++}, Ni^{++}, Fe^{++}, and Mn^{++} are substituted for Zn^{++} (Coleman and Vallee, 1960, 1961b). Binding of Cd^{++}, Hg^{++}, or Pb^{++} to apocarboxypeptidase restores esterase but not peptidase activity (Coleman and Vallee, 1961b).

The magnitude and sequence of the stability constants of these metallocarboxypeptidases has contributed to the identification of the chemical nature of the active enzymatic site of apocarboxypeptidase. Apparently, the sulfhydryl group of the single cysteine and a nitrogen-containing residue, most probably the α-amino group of the amino-terminal asparagine, together with a zinc atom, are essential components of the active site of native carboxypeptidase (Coleman and Vallee, 1961a,b; Vallee *et al.*, 1960a). The data to be presented demonstrate that the mechanisms of inhibition by a variety of chemically diverse agents can be understood and predicted from the chemical details of the active site. A preliminary report has been made (Coombs and Felber, 1961).

MATERIALS AND METHODS

Beef pancreas carboxypeptidase [(CPD)Zn],[1] zinc-free apocarboxypeptidase (CPD), and carboxypeptidase containing Zn^{65} [(CPD)Zn^{65}], cobalt, nickel, and manganese were all prepared as described previously (Felber *et al.*, 1962; Coleman and Vallee, 1960).

Metal Solutions.—Standard solutions of Zn^{++}, Co^{++}, Ni^{++}, Mn^{++}, Cd^{++}, Hg^{++}, Cu^{++}, and Pb^{++} were prepared by dissolving the spectrographically pure metals or salts (Johnson Matthey Co., Ltd.) in dilute metal-free HCl. The solutions were diluted with buffer to the desired *p*H and molarity.

Chelating Agents and Mercaptans.—These were the reagent grade salts and were used without further purification.

Enzymatic Activity.—Peptidase activity was determined with carbobenzoxyglycyl-L-phenylalanine (CGP) (Mann Chemical Company) as the substrate. Activity is expressed as an apparent proteolytic coefficient, *C*, as previously defined and employed (Coleman and Vallee, 1960). The assays were carried out at either 25° or 0° in either 0.04 M sodium veronal or 0.1 M Tris buffer containing 1 M 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%.

Esterase activity was determined with hippuryl-*dl*-β-phenyllactate[2] (HPLA) as the substrate (Coleman and Vallee, 1961b).

Protein Concentrations.—These were measured either by precipitation with 10% trichloracetic acid followed by drying at 104° (Hoch and

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[1] The abbreviations used are: [(CPD)Zn], zinc carboxypeptidase, with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc; [(CPD)Me], metallocarboxypeptidase, where Me = Co, Ni, or Mn; 8 OHQ5SA, 8-hydroxyquinoline-5-sulfonic acid; OP, 1,10-phenanthroline; α,α'-D, α,α'-dipyridyl; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-*dl*-β-phenyllactate. These abbreviations are employed in formulations only and when required for differentiation.

[2] A gift of Dr. H. Neurath.

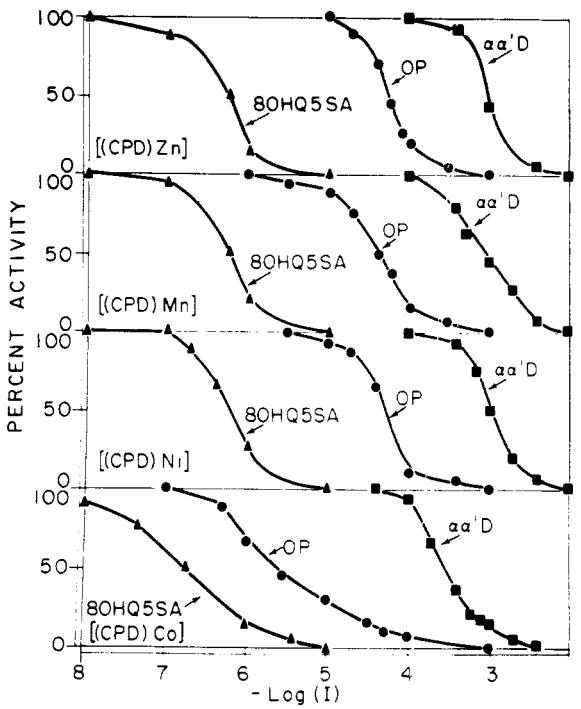

FIG. 1.—Inhibition of zinc, manganese, nickel, and cobalt carboxypeptidases by chelating agents. 6.7×10^{-7} M of each metalloenzyme was incubated with increasing concentrations of α,α'-dipyridyl (α,α'D), ■—■; 1,10-phenanthroline (OP), ●—●; or 8-hydroxyquinoline-5-sulfonic acid (8OHQ5SA), ▲—▲, in 1 M NaCl–0.1 M Tris, *p*H 8.0, for 1 hour at 0°. Peptidase activities were measured with 2×10^{-8} M enzyme and 0.02 M carbobenzoxyglycyl-L-phenylalanine in 1 M NaCl–0.1 M Tris, *p*H 7.5, 25°. The concentration of dissociated inhibitor[3] is plotted as a function of the partial activity, V_i, expressed as per cent of the unihibited enzyme, V_c, acting as a control.

Vallee, 1953) or from the absorbancy at 278 mμ with use of the extinction coefficient as previously reported (Davie and Neurath, 1955). The results of the two procedures were in excellent agreement.

The 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 (Coleman and Vallee, 1960). Water was purified by passage over a mixed resin (IR-120 and IRA-410, Rohm and Haas Co.). All solutions were stored and reactions carried out in polyethylene containers whenever possible (Thiers, 1957).

RESULTS

The chelating agents 8-hydroxyquinoline-5-sulfonic acid (8-OH-Q-5SA), 1,10-phenanthroline (OP), and α,α'-dipyridyl (α,α'-D) inhibit the zinc, cobalt, nickel, and manganese carboxypeptidases (Fig. 1). All four metallocarboxypeptidases are inhibited in the order 8-hydroxyquinoline-5-sulfonic acid > 1,10-phenanthroline > α,α'-dipyridyl. The inhibition concentration curves[3] of the zinc, manganese, and nickel enzymes span about two log units of concentration of inhibitor from full activity to complete inactivation and are quite similar in slope, and the K_I values are similar in magnitude. The inhibition of cobalt carboxypeptidase with 8-hydroxyquinoline-5-sulfonic acid and 1,10-phenanthroline, however, differs from that of the other metallocarboxypeptidases; the slopes of these curves span four rather than two log units of inhibitor concentration and the concentrations required to achieve 50% inhibition are somewhat lower.

Column A of Table I shows both the apparent

TABLE I

INHIBITION OF METALLOCARBOXYPEPTIDASES BY CHELATING AGENTS

Comparison of the apparent stability constants at *p*H 8.0, K_E, of [(CPD) Zn], [(CPD) Ni], and [(CPD) Co] (Coleman and Vallee, 1961b), the stability constants, K_L, of the corresponding 1:1 metal ion chelates (Bjerrum *et al.*, 1957; Irving, 1959), and the concentrations, K_I, at 50% inhibition. $\bar{n}$ is the average number of moles of ligand complexed per mole of enzyme and is calculated from $\phi = K_I \cdot I^{\bar{n}}$ where $\phi = \left(\frac{V_c}{V_i} - 1\right)$ (Kistiakowsky and Shaw, 1953).

Complex	A log K_E or log K_L	B −log K_I	C $\bar{n}$
[(CPD) Zn]	10.5	—	—
[(α,α'-D) Zn]	5.4	2.9	2.6
[(OP) Zn]	6.3	4.2	2.3
[(80HQ5SA) Zn]	8.65	6.4	1.9
[(CPD) Ni]	8.2	—	—
[(α,α'-D) Ni]	[a]	3.0	2.2
[(OP) Ni]	8.0	4.3	2.2
[(80HQ5SA) Ni]	9.75	6.2	1.9
[(CPD) Co]	7.0	—	—
[(α,α'-D) Co]	[a]	3.5	2.1
[(OP) Co]	7.0	5.6	0.7
[(80HQ5SA) Co]	9.2	6.7	1.2
[(CPD) Mn]	5.6	—	—
[(α,α'-D) Mn]	[a]	3.0	1.9
[(OP) Mn]	4.8	4.4	1.8
[(80HQ5SA) Mn]	6.6	6.3	1.9

[a] No constant on record.

[3] The concentration given is that fraction of the species which is fully dissociated at the *p*H of the preincubation medium and, therefore, capable of forming a complex with the metal ion. The acid dissociation constants used were obtained from Näsänen and Uusitalo (1954) for 8-hydroxyquinoline-5-sulfonic acid, from Stricks *et al.* (1954) for thioglycollic acid, and from Stricks and Kolthoff (1951, 1953) and Tanaka *et al.* (1955) for cysteine. The pK_a of the sulfhydryl group of mercaptoethanol was assumed to be the same as that of thioglycollic acid.

stability constant, log K_E, of the metallocarboxypeptidase at *p*H 8.0, 4°, and the stability constants, log K_L, of the 1:1 metal complexes of the chelating agents here employed as inhibitors. Column B indicates the negative log of the concentration of dissociated inhibitor, $-\log K_I$, which is required to produce 50% inhibition at *p*H 8.0, the *p*H at which the inhibitions were studied.

Column C of Table I shows $\bar{n}$, the average number of moles of ligand complexed per mole of enzyme, calculated from the slope of the inhibition curves according to the equation $\phi = K_I \cdot I^{\bar{n}}$, where I is the concentration of inhibitor, K_I is the inhibition constant, and $\phi = \left(\frac{V_c}{V_i} - 1\right)$ where V_c is the activity of the control and V_i, the activity of the inhibited enzyme (Kistiakowsky and Shaw, 1953). It is apparent that the effectiveness of these chelating agents in bringing about inhibition is directly related to the magnitude of the stability constant of the metal-inhibitor complexes.[4]

Since zinc is bound to a sulfur-nitrogen site of carboxypeptidase, ligands containing these donor groups and mercaptans would be expected to compete for the metal atom and inhibit the enzyme. Carboxypeptidase has been shown previously to be inhibited completely by 0.01 M cysteine (Smith and Hanson, 1949). This finding is here confirmed. In addition, it is shown that cysteine, thioglycollic acid, and mercaptoethanol inhibit zinc carboxypeptidase as a function both of the mercaptan concentration and of the time of their preincubation with the enzyme (Fig. 2 and 3). Here too, the high stability constants, K_L, of these mercaptides are reflected in the inhibition constants, K_I (Table II).

Equilibrium dialysis of [(CPD)Zn^{65}] with L-cysteine and thioglycollic acid demonstrates that both of these reagents remove Zn^{65} from the enzyme (Fig. 3). Zinc content and enzymatic activity remaining at the end of dialysis correspond closely. After 16 hours of exposure to L-cysteine both Zn^{65} and activity are lost completely. After the same period of dialysis against thioglycollate, however, 0.8 g atoms of Zn^{65} remain bound to the enzyme, which is 80% active. The stability constants of zinc complexes with cysteine and thioglycollic acid fully account for the differential effectiveness of these inhibitors (Table II).

TABLE II

INHIBITION OF ZINC CARBOXYPEPTIDASE BY MERCAPTANS

Comparison of the apparent stability constant at *p*H 8.0, K_E, of [(CPD) Zn], the stability constant, K_L, of the corresponding metal mercaptides (Bjerrum *et al.*, 1957), and the inhibitor concentration, K_I, at 50% inhibition.

Complex	log K_E or log K_L	− log K_I
[(CPD) Zn]	10.5	—
[(Mercaptoethanol) Zn]	[a]	4.7
[(Thioglycollate) Zn]	7.44	5.7
[(Cysteine) Zn]	9.86	7.9

[a] No constant on record.

[4] The higher the stability constant of the complex compared to that of the metalloenzyme, the less inhibitor is required to bring about 50% inhibition.

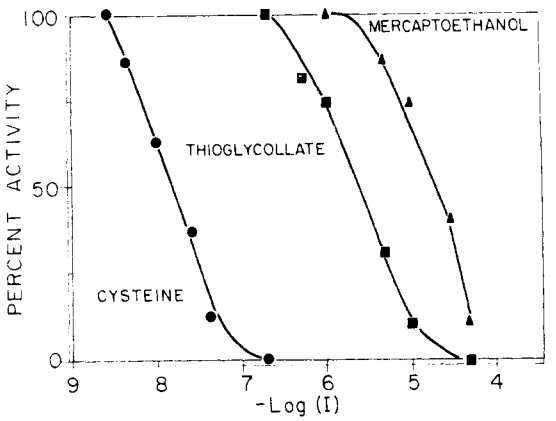

FIG. 2.—Inhibition of zinc carboxypeptidase by mercaptans. 1×10^{-6} M [(CPD) Zn] was incubated with increasing concentrations of mercaptoethanol, ▲, thioglycollic acid, ■, and L-cysteine, ●, in 1 M NaCl–0.1 M Tris, *p*H 7.0, for 72 hours at 0°, under N_2. Peptidase activities were measured as in Figure 1 after incubation with mercaptoethanol and thioglycollic acid. Esterase activities were measured after incubation with L-cysteine using 2×10^{-3} M enzyme and 0.01 M hippuryl-DL-phenyl lactic acid in 1 M NaCl – 0.005 M Tris, *p*H 7.5, 25°. The concentration of dissociated inhibitor[3] is plotted as a function of the partial activity expressed as per cent of the uninhibited enzyme acting as a control.

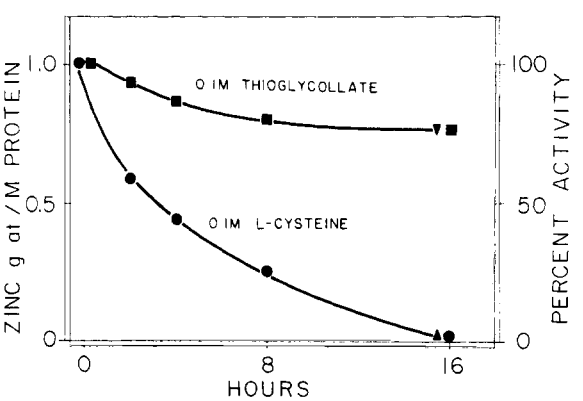

FIG. 3.—Effect of mercaptans on zinc content and activity of carboxypeptidase. 1×10^{-4} M [(CPD) Zn^{65}] was dialyzed for 16 hours against 100-ml volumes of 0.1 M thioglycollic acid, ■, or 0.1 M L-cysteine, ●, in 1 M NaCl–0.1 M Tris, *p*H 7.5, 0°, under N_2. Enzyme-bound metal was measured as a function of time as described (Coleman and Vallee, 1960). Peptidase or esterase activities were measured at the end of the dialysis as in Figure 2 (▼, ▲).

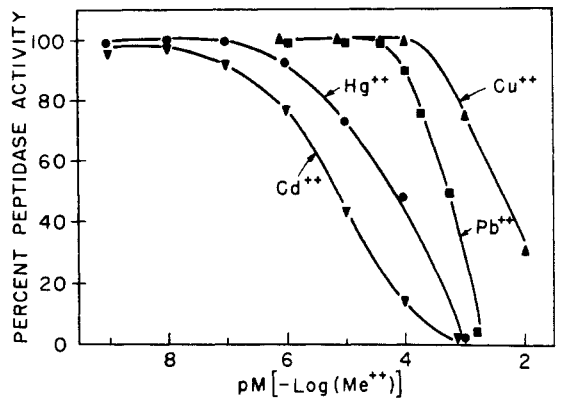

FIG. 4.—Inhibition of zinc carboxypeptidase by metal ions. 2.5×10^{-6} M [(CPD) Zn] was incubated with increasing concentrations of Cu^{++} (▲), Pb^{++} (■), Hg^{++} (●), and Cd^{++} (▼) for 72 hours in 1 M NaCl–0.05 M Tris, pH 8.0, 4°. Remaining peptidase activities, measured as in Figure 1, are plotted as a function of the negative log of the *total* metal ion concentration, *p*M.

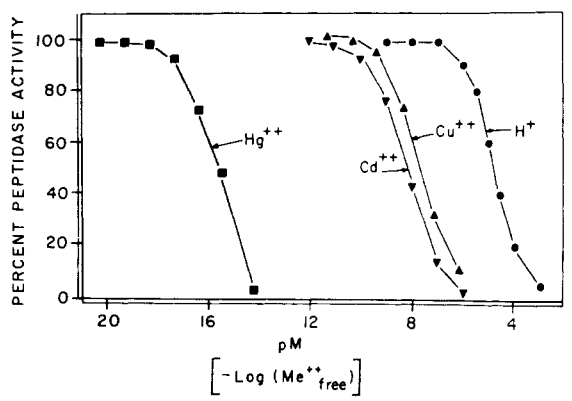

FIG. 5.—Inhibition of zinc carboxypeptidase by metal and hydrogen ions. The total concentrations of Me^{++} are corrected for that fraction which is bound to chloride and Tris (Coleman and Vallee, 1960). Partial peptidase activities are plotted as a function of the negative log of the *free* Me^{++} or H^+ ion concentrations, pM or pH. H^+ (●), Cu^{++} (▲), Cd^{++} (▼), Hg^{++} (■).

The inhibition of carboxypeptidase by lead and cuprous (Smith and Hanson, 1949) and by zinc ions (Vallee *et al.*, 1960b) is well known, but silver and mercuric ions have failed to inhibit the enzyme in previous investigations (Smith and Hanson, 1949; see also Vallee *et al.*, 1960a). Since the replacement of zinc in native carboxypeptidase A by a series of metal ions, which includes mercury, results in alterations or abolition of peptidase and esterase activity (Coleman and Vallee, 1960, 1961b), the mechanism of inhibition of this enzyme by metal ions was investigated further. The anion composition of the incubation mixture was found to account for the apparent differences in inhibition by metal ions. In 0.04 M veronal buffer, Cd^{++}, Pb^{++}, and Cu^{++} inhibit the enzyme both at a faster rate and to a greater degree than in 0.1 M Tris. Moreover, in veronal buffer equilibrium is achieved in 24 hours, whereas 72 hours are required in Tris buffer. Hg^{++} ions, on the other hand, fail to inhibit the enzyme in veronal buffer even after 24 hours, but in Tris buffer the enzyme is inhibited significantly under otherwise identical conditions (Table III).

TABLE III

DEPENDENCE OF PEPTIDASE INHIBITION OF ZINC CARBOXYPEPTIDASE BY Me^{++} IONS ON BUFFER COMPOSITION

5×10^{-7} M [(CPD) Zn] was incubated with 1×10^{-4} M Me^{++} ions in either 1 M NaCl–0.1 M Tris or 1 M NaCl–0.04 M veronal buffer, *p*H 7.5, 0°. Peptidase activity was measured at *p*H 7.5, 25°, in the same buffer with 0.02 M carbobenzoxyglycyl-L-phenylalanine as the substrate. Control activity in the absence of inhibitor is V_c, inhibited activity, V_i.

Metal Ions Added (1×10^{-4} M)	V_i/V_c After Preincubation for 1 Hour		24 Hours	
	Tris	Veronal	Tris	Veronal
Hg^{++}	1.00	1.00	0.40	1.00
Cd^{++}	1.00	0.10	0.15	0.10
Pb^{++}	0.90	0.63	0.65	0.52
Cu^{++}	1.00	0.50	0.86	0

Further studies, at equilibrium, were undertaken in 0.05 M Tris + 1 M NaCl, *p*H 8.0, the buffer system employed for the determination of the stability constants of the metallocarboxypeptidases. Plotted as a function of the *total concentration* of metal ions added, the order of the resultant inhibitions is $Cd^{++} > Hg^{++} > Pb^{++} > Cu^{++}$ (Fig. 4) and the corresponding $-\log K_I$ values are 5.1, 4.3, 3.0, and 2.2 respectively. However, the *total* concentrations of the added metal ions must be corrected for that fraction which is bound to both Tris and chloride ions. When the inhibitions are plotted as a function of *free* metal or hydrogen ions in solution, *p*M or *p*H, the relative order of effectiveness is changed to $Hg^{++} > Cd^{++} > Cu^{++} > H^+$ (Fig. 5). This order is identical with that of the stability constants of the corresponding metal or protonated carboxypeptidases (Table IV). The data for H^+ ion inhibition are given for comparison. The total concentration for Pb^{++} was not corrected, since the appropriate constants for Pb^{++} were not available.

TABLE IV

METALLOCARBOXYPEPTIDASES

Comparison of the concentrations of *free* mercuric, cadmium, or cupric ions (*p*M) or hydrogen ions (*p*H) causing 50% inhibition of [(CPD) Zn] activity with the apparent stability constants at *p*H 8.0, log K_E, of the corresponding metallocarboxypeptidases (see Fig. 4).

	Hg^{++}	Cd^{++}	Cu^{++}	H^+
*p*M or *p*H	16.0	8.3	7.8	4.8
log K_E	21.0	10.8	10.6	8.4

Cupric ions inhibit carboxypeptidase by displacing the zinc atom in a manner similar to that by which cadmium and mercury ions have previously been shown to inhibit peptidase activity. Zn^{65} carboxypeptidase was dialyzed against 1×10^{-2} M Cu^{++} ions and Zn^{65} bound to the apoenzyme, peptidase, and esterase activities were measured simultaneously in the course of dialysis. Figure 6 shows that the loss of both peptidase and esterase activities is a direct function of the displacement of Zn^{65}. Chemical measurements of copper and zinc after 48 hours of dialysis confirm that the replacement of zinc by copper is complete when all activity has disappeared. Conversely, exposure of the newly formed copper carboxypeptidase to 1×10^{-5} M Zn^{65} ions results in rapid exchange of copper for Zn^{65++} until 1.0 g atom of Zn^{65} per mole of enzyme is bound and peptidase and esterase activities are fully restored.

Discussion

We have shown previously that 1,10-phenanthroline inhibits carboxypeptidase by removal of the zinc atom at the active site (Vallee *et al.*, 1960b; Felber *et al.*, 1962). These studies are here extended to the nickel, cobalt, and manganese carboxypeptidases and the chelating agents α,α'-dipyridyl and 8-hydroxyquinoline-5-sulfonic acid.

A comparison of the stability constants of the metallocarboxypeptidases, K_E, and of the metal complexes of these inhibitors, K_L, with the inhibition constants K_I (Table I), indicates the inhibition to be a result of competition between the apoenzyme and these bidentate ligands for the respective metal ions: The K_I values are direct functions of the K_E values relative to the K_L values. The calculations of K_I are based on that concentration of the chelating agents which is fully dissociated and, hence, capable of complexing a metal ion at *p*H 8.0, the *p*H at which incubations are performed.

The concentrations of each chelating agent required to inhibit zinc, nickel, cobalt, or manganese carboxypeptidases do not differ by orders of magnitudes (Fig. 1). This is in accord with expectation since the stability constants of the metal-inhibitor complexes, K_L, decrease in the same relative order as do those of the metallocarboxypeptidases (Table I) (Coleman and Vallee, 1961b). Compared to the other carboxypeptidases here examined, the zinc enzyme is more stable under the conditions employed, a circumstance which is reflected in the slightly higher concentrations of α,α'-dipyridal and 1,10-phenanthroline required for inhibition of zinc carboxypeptidase (Fig. 1). This relatively higher stability may be attributed to the zinc mercaptide bond (Coleman and Vallee, 1961b). The remarkably effective inhibition of zinc carboxypeptidase by mercaptans is a further reflection of the preference of this ion for the mercapto groups of these ligands (Fig. 2).

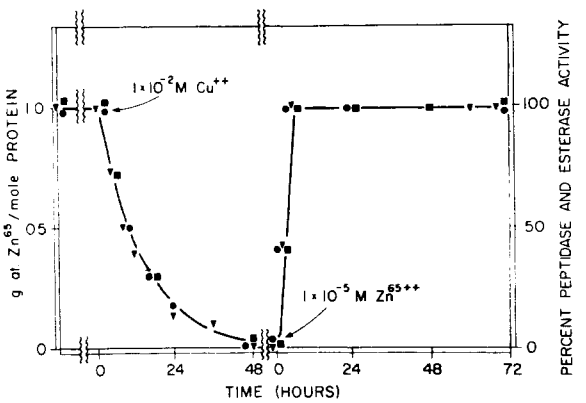

FIG. 6.—Effect of copper replacement on peptidase and esterase activities of zinc carboxypeptidase. 1×10^{-5} M [(CPD) Zn^{65}] was dialyzed for 48 hours against a 200-ml volume of 1×10^{-2} M Cu^{++} in 1 M NaCl–0.05 M Tris, *p*H 8.0, 4°. Zn^{65} bound to the enzyme (▼), the partial peptidase activity (■), and esterase activity (●) were measured as a function of time. After 48 hours, the dialysate was changed to a 200-ml volume of 1×10^{-5} M Zn^{65++} in the same buffer system and the dialysis continued for a further 72 hours under the same conditions. Peptidase assays as in Figure 1. Esterase assays as in Figure 2. Measurements of Zn^{65} as described (Coleman and Vallee, 1960). At 48 hours 1 g atom of copper was present by chemical analysis.

It has been pointed out (Smith *et al.*, 1951; Williams and Vallee, 1955) that $\bar{n}$, the average number of molecules of inhibitor participating in inhibition of an enzyme, can be determined from the slope of concentration/inhibition curves, provided the inhibition is freely reversible and the substrate does not compete. These considerations apply to the fully reversible inhibition of carboxypeptidase by 1,10-phenanthroline (Felber *et al.*, 1962). The molar stoichiometry of the Zn^{++}, Co^{++}, Ni^{++}, and Mn^{++} ion complexes with the chelating agents here employed as inhibitors are known; hence $\bar{n}$, calculated from inhibition experiments, can be compared directly to the molar stoichiometry observed in simple systems (Kolthoff *et al.*, 1951; Bjerrum *et al.*, 1957). This comparison presumes that the chelating agents remove the metal ions from the apoenzyme, an assumption proven to be correct for the [(CPD)Zn]:OP system.

Three moles of 1,10-phenanthroline and α,α'-dipyridyl are the maximum number which can combine with Zn^{++}, Ni^{++}, and Mn^{++} ions in solution, while 8-hydroxyquinoline-5-sulfonic acid maximally forms 2:1 complexes. The concentrations of these ligands must be in considerable excess over those of the respective metal ions to approach the maximum value of $\bar{n}$, since the 3:1 complex, for example, is in equilibrium with the 2:1 and 1:1 complexes which predominate at lower ligand concentrations (Kolthoff *et al.*, 1951). This stoichiometry would be modified and reduced further if any of the coordination sites of the metal

atom were to be occupied by being bound to the protein. Values of $\bar{n}$ calculated from the inhibition of zinc, nickel, and manganese carboxypeptidase by 1,10-phenanthroline and α,α'-dipyridyl vary between 2 and 3, and those for 8-hydroxyquinoline-5-sulfonic acid approximate 2 (Table I). This is completely consistent with the hypothesis that the removal of the metal atoms is the basis of the inhibition in all instances; the values of $\bar{n}$ are, in fact, very close to those which are calculated as a result of the contributions of the 1:1 and 2:1 complexes.

Cysteine, thioglycollic acid, and mercaptoethanol, like 1,10-phenanthroline, inhibit zinc carboxypeptidase as functions of time of incubation and of enzyme concentration, and this fact is also consistent with the mechanism described for the reversible removal of a metal atom (Felber *et al.*, 1962) (Fig. 3). The complete removal of Zn^{65} by cysteine on equilibrium dialysis, in contrast to its partial removal by thioglycollate under comparable conditions (Fig. 3), reflects the difference between the stability constants of the zinc cysteinate and thioglycollate complexes (Table II).

The stability constant of the zinc-mercaptoethanol complex has not been reported; its K_I, however, is lower than that of the other two mercaptans studied. The sequence of the K_I values shown in Table II suggests that the stability constant of this complex will also be found to be lower.

The conclusions which can be drawn from the inhibition of cobalt carboxypeptidase are less certain. The slope of the inhibition curves of cobalt carboxypeptidase indicate that $\bar{n} = 0.7$ with 1,10-phenanthroline and 1.2 with 8-hydroxyquinoline-5-sulfonic acid (Fig. 1 and Table I). These numerical values suggest a different interaction of these agents with cobalt carboxypeptidase. On the one hand these data could indicate the formation of a mixed complex, $[(CPD)Co]\cdot(I)$, where one mole of inhibitor, I, is bound to the cobalt atom, and the metal is not removed. Alternatively, these results might be brought about by alterations in the oxidation state of the cobalt atom resulting in a change of its coordination sphere and, hence, in the number of moles of ligand bound. These propositions are under study, and results so far do not favor the formation of a mixed complex as the explanation.

The magnitude and sequence of K_E, and the apparent stability constants of the metallocarboxypeptidases containing metal atoms of the first transition series and of II B group elements, suggests that, at equimolarity, any member of the series forming a more stable complex with the apoenzyme should be able to displace the less stable neighbors of the sequence $Hg^{++} > Cd^{++} > Cu^{++} > Zn^{++} > Ni^{++} > Co^{++} > Mn^{++}$ (Coleman and Vallee, 1961b). The data presented here constitute a test of this hypothesis with those elements of this series which abolish peptidase activity.

It has been shown previously that Hg^{++} and Cd^{++} displace zinc from carboxypeptidase; mercury and cadmium carboxypeptidases are inactive toward peptides (Coleman and Vallee, 1961b). It is now demonstrated that Cu^{++} inhibits carboxypeptidase by a similar mechanism (Fig. 4 and 5). Pb^{++} was first shown by Smith and Hanson (1949) to inhibit carboxypeptidase. Though the stability constant for lead carboxypeptidase is not known, the avidity of this element for sulfur (Irving, 1959) suggests a mechanism of inhibition by Pb^{++} ions similar to that described for Hg^{++}, Cd^{++} and Cu^{++}.

The media in which the incubations are carried out profoundly affect both the magnitude and rate of inhibition with these metal ions (Table III). NaCl is required in large concentrations in both Tris and veronal buffers in order to render the enzyme soluble. Both Hg^{++} and Ag^{+} form very stable chloride complexes, reducing markedly the concentration of their free ions available for reaction with the enzyme. This circumstance accounts for the apparent failure of these ions to displace zinc instantaneously from the native enzyme and to react with the mercapto group, which is revealed when the metal-free enzyme is titrated with these ions (Vallee *et al.*, 1960a). When the reaction is given sufficient time to reach equilibrium, however, inhibition, and therefore presumably displacement, may eventually be observed, even though the concentration of free ions is low (Table III).

Tris and veronal form metal complexes also, thereby further reducing the concentration of free metal ions available for competition with the zinc ion, accounting for the differences of inhibition that are observed in these buffers.

The observed order of effectiveness of inhibition $Cd^{++} > Hg^{++} > Cu^{++}$ in Table III is at variance with that predicted from the known stability constants for the metallocarboxypeptidases. Correction of the total metal ion concentration for that fraction which is complexed by Tris and chloride changes the order of effectiveness of inhibition to $Hg^{++} > Cd^{++} > Cu^{++} > H^{+}$, the order which is consistent with that expected from the stability constants of the respective metalloenzymes (Table IV).

The present data indicate that chemically diverse agents inhibit carboxypeptidase through their common action on the nitrogen-zinc-sulfur site. Thus both chelating agents and mercaptans successfully compete with the apoenzyme for the metal atom and inhibit enzymatic activity by removing the metal. Certain divalent metal ions inhibit peptidase activity by *displacing* zinc.

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Studies of Soybean Trypsin Inhibitor. II. Conformational Properties*

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Ultraviolet difference spectra and optical rotation measurements were carried out on soybean trypsin inhibitor (STI) at various *p*H's, ionic strengths, and temperatures. At room temperature, the conformation is essentially independent of *p*H in the range 2 to 7, but changes in the alkaline range. The protein undergoes a rather sharp thermal transition (detected by difference spectra) which is reversible at *p*H 6.6 and 9.0 at low ionic strength but irreversible at *p*H 1.3. The transition temperature depends on *p*H and ionic strength. Since the optical rotation data yield essentially temperature-independent b_0-values which are not very different from zero, it is concluded that the native protein may contain mostly randomly coiled regions plus a small percentage of regular structure, the latter being a mixture of left- and right-handed helices, and that the sharp transition observed by difference spectra is due to the conversion of the helices of both senses to random coils. The sign of the changes in optical density implies that tyrosyl and tryptophanyl chromophores pass from water into a region containing non-polar groups and/or negatively charged ones, as the extended helical forms are transformed into the more compact random coil forms. Since spectral changes are produced either by heating or by making the solution alkaline at room temperature, the constancy of the electrostatic factor, w, between *p*H 2 and 7, and its increase at alkaline *p*H (Wu and Scheraga, 1962), are consistent with this conclusion. Optical rotation measurements carried out on the STI-trypsin compound suggest that no significant change in the conformation of either protein accompanies association.

Studies of reversible denaturation can provide information about the nature of side-chain interactions and conformation in protein molecules (Scheraga, 1960, 1961, 1962). Crystalline soybean trypsin inhibitor (STI) was selected for such a study, since its denaturation has been reported to be reversible (Kunitz, 1947). In a previous paper (Wu and Scheraga, 1962) results were reported on several physicochemical properties of

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