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Metallocarboxypeptidase-Inhibitor Complexes*

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Previous gel-filtration and metal-ion-exchange studies have shown that apocarboxypeptidase A forms stable complexes with peptide substrates. Ester substrates, in contrast, form complexes only with the metalloenzyme. The present studies with [^{14}C]-β-phenylpropionate confirm the conclusions drawn from experiments employing gel-filtration and metal-ion exchange that a number of competitive inhibitors similarly form complexes only with the metalloenzyme. One mole of [^{14}C]-β-phenylpropionate binds to zinc carboxypeptidase when the concentration of this inhibitor is 10^{-3} M, while under the same conditions none binds to apocarboxypeptidase. Binding of the inhibitor increases the half-life for the exchange of $^{65}Zn^{2+} \rightleftharpoons Zn^{2+}$ at the active site from 5 to 576 hours. The stability of metalloenzyme-inhibitor complexes can be measured by their effects on metal-ion exchange. The stabilities are functions both of the species of metal ion at the active site and of the chemical structure of the inhibitors. Inhibitors bind progressively firmer in the order $Co^{2+} < Cd^{2+} < Zn^{2+}$. Substitutions of R groups in the basic acetate structure of inhibitors result in progressively greater affinities for the active site in the order $CH_3 <$ Br < I < phenyl < indole < benzyl. The obligatory stereospecificity for the C-terminal amino acid of the substrate is L; that required for binding these inhibitors is D. A model for inhibitor binding is proposed.

The effect of substrates on the association and dissociation of metal ions at the active site[1] of carboxypeptidase A[2] has provided a means of separating the catalytic event from substrate binding and has given insight into the chemical details of the latter process. Peptide substrates form stable apocarboxypeptidase-substrate complexes and thus do not require the metal ion for binding. In contrast, the metal is mandatory for the binding of ester substrates (Coleman and Vallee, 1962a,b).

These studies have now been extended to encompass the interaction of competitive inhibitors of carboxypeptidase including D-phenylalanine, D-tryptophan, D-leucine, iodoacetate, bromoacetate, phenylacetate, indole-3-acetate, propionate, β-iodopropionate, and β-phenylpropionate. [^{14}C]-β-Phenylpropionate has been employed to confirm directly the results obtained by the metal-ion-exchange methods. A preliminary account has been rendered (Coleman, 1963).

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[1] The designation "active site" will refer specifically to the nitrogen-metal-sulfur bond essential for hydrolysis. "Active center" will refer to all those features of primary, secondary, and tertiary structure of the enzyme, including the "active site," which are required for substrate binding, specificity, or hydrolysis of the substrate.

[2] In this paper "carboxypeptidase" refers to carboxypeptidase A only. All the experiments were carried out with δ-carboxypeptidase (for nomenclature see Bargetzi *et al.*, 1963) prepared from acetone powder of beef pancreas (Allan *et al.*, 1964) and containing N-terminal asparagine.

Experimental Procedures

Bovine Pancreatic Carboxypeptidase A [(CPD)Zn].[3]—Four-times-recrystallized δ-carboxypeptidase was prepared by the method of Allan *et al.* (1964). The ratio zinc/protein of the preparation was between 0.98 and 1.03 g-atoms/mole based on a molecular weight of 34,300 (Smith and Stockell, 1954; Vallee and Neurath, 1955; Brown *et al.*, 1961).

Apocarboxypeptidase, cadmium carboxypeptidase and cobalt carboxypeptidase were prepared as described (Coleman and Vallee, 1961; 1962a). *The inhibitors* D-phenylalanine, D-leucine, D-tryptophan, indole-3-acetic acid, *N*-acetylhistidine, *N*-acetyl-D-phenylalanine (Mann Research Laboratories) and β-phenylpropionic acid–[^{14}C] carboxyl (California Corp. for Biochemical Research) were chromatographically pure. The iodoacetic, β-iodopropionic, bromoacetic, propionic, and β-phenylpropionic acids (Eastman Organic Chemicals), iodoacetamide (K and K Laboratories), and phenyl-

[3] The abbreviations used are in formulations only and when required for differentiation: [(CPD)Zn], zinc carboxypeptidase, with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc or other metals substituting for it; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-β-phenyllactic acid; Tris, tris(hydroxymethyl)aminomethane; β-ϕ-P, β-phenylpropionate,

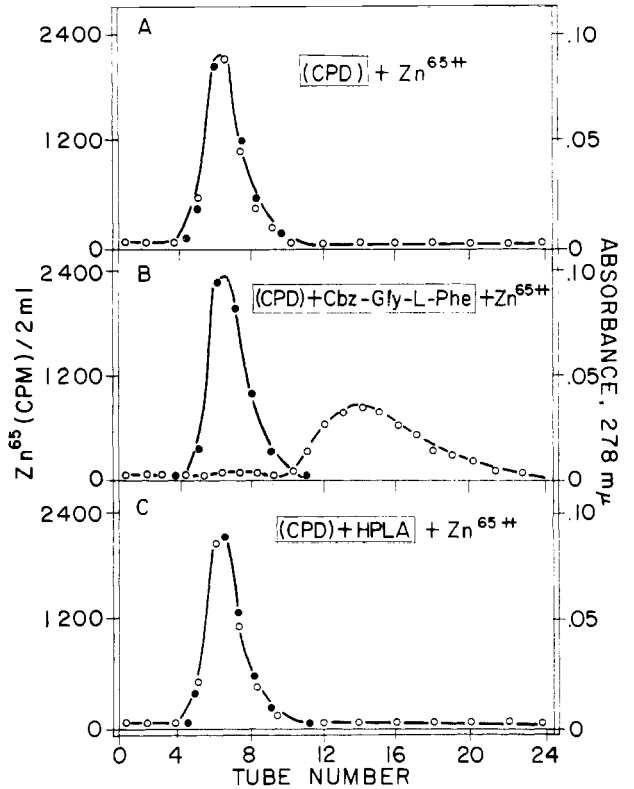


FIG. 1.—Effect of carbobenzoxyglycyl-L-phenylalanine and hippuryl-*dl*-β-phenyllactate on binding of $^{65}Zn^{2+}$ to apocarboxypeptidase. One-ml samples of 5×10^{-6} M apocarboxypeptidase plus 5×10^{-6} M $^{65}Zn^{2+}$ (A), plus 5×10^{-2} M Cbz-Gly-L-Phe plus 5×10^{-6} M $^{65}Zn^{2+}$ (B), plus 4×10^{-2} M hippuryl-*dl*-β-phenyllactate plus 5×10^{-6} M $^{65}Zn^{2+}$ (C) were passed over a 1×30-cm Sephadex G-25 column at a flow rate of 1 ml/min. Absorbance at 278 mμ (●) and $^{65}Zn^{2+}$ counts per minute per 2-ml fraction (O) were determined. Ionic $^{65}Zn^{2+}$ passed over the column is retarded and emerges in fractions 12–24 with a maximum at fraction 14, while enzyme-bound $^{65}Zn^{2+}$ emerges with the protein.

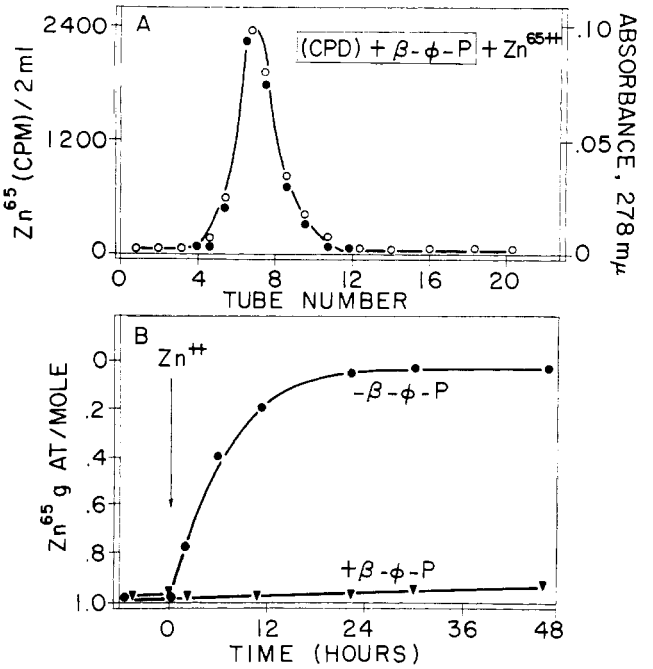


FIG. 2.—(A) Effect of β-phenylpropionate on binding of $^{65}Zn^{2+}$ to apocarboxypeptidase.—A 1-ml sample of 5×10^{-6} M apocarboxypeptidase plus 5×10^{-2} M β-phenylpropionate plus 5×10^{-6} M $^{65}Zn^{2+}$ was passed over a Sephadex G-25 column (1×30 cm) at a flow rate of 1 ml/min. Absorbance at 278 mμ (●) and counts per minute per 2-ml fraction (O) were determined. (B) Prevention of the exchange $^{65}Zn^{2+} \rightleftarrows Zn^{2+}$ at the active site by β-phenylpropionate. [(CPD)^{65}Zn], 1×10^{-5} M, was exposed to 1×10^{-5} M Zn^{2+} as indicated, and the enzyme-bound $^{65}Zn^{2+}$ (●) was measured as a function of time. (▼), same in the presence of 2×10^{-2} M β-phenylpropionate.

acetic acid (Mann Research Laboratories) all were the reagent grade compounds. All of the acids were dissolved in 1 M NaCl–0.05 or 0.005 M Tris, *p*H 7.5 or 8.0, and hence were present in the ionized form.

Peptidase activity was determined with carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) and under conditions previously described (Coleman and Vallee, 1960). The apparent proteolytic coefficients, *C* (Riordan and Vallee, 1963), for the metallocarboxypeptidases here employed were: [(CPD)Zn] = 32.0; [(CPD)Cd] = 0.0; [(CPD)Co] = 51.5.

Esterase activity was determined by *p*H titration (Snoke *et al.*, 1948) with 0.1 N NaOH of the hydrogen ions released on hydrolysis using a *p*H-stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). Assays were performed at 25° with 5 ml of 0.01 M hippuryl-*dl*-β-phenyllactate in 0.2 M NaCl–0.005 M Tris buffer, *p*H 7.5. Activities, expressed as zero-order velocity constants, *k*, with units of mmoles H^+ per minute per μmole of enzyme were as follows: [(CPD)Zn] = 5.0; [(CPD)Cd] = 7.5; and [(CPD)Co] = 4.7.

Protein concentrations were measured either by precipitation with 10% trichloroacetic acid followed by drying at 104° (Hoch and Vallee, 1953) or by absorbance at 278 mμ. The molar absorptivity of native carboxypeptidase A is 6.42×10^4 M^{-1} cm^{-1} (Simpson *et al.*, 1963).

Binding of [^{14}C]-β-*Phenylpropionate to Carboxypeptidase.*—One-ml samples of 2×10^{-4} M metallocarboxypeptidase or apocarboxypeptidase were dialyzed against varying concentrations of β-phenylpropionate labeled with a tracer amount of β-phenylpropionate-[^{14}C] carboxyl. The equilibrium-dialysis technique was identical to that previously described (Coleman and Vallee, 1961). The specific activity of the [^{14}C]-β-phenylpropionate solution in 1 M NaCl–0.05 M Tris, *p*H 8.0, 4°, was approximately 8000 cpm/ml. The protein samples were equilibrated with 50 ml of the [^{14}C]-β-phenylpropionate solution for 24 hours. A 0.5-ml aliquot of the protein was then removed from the dialysis bag with a calibrated syringe, and an identical aliquot was taken from the dialysate. The samples were placed in 10×125-mm test tubes, evaporated to dryness at 95° for 3 hours, and then taken up in 2 ml of hydroxide of hyamine (Packard Instrument Co.) by gentle heating in a water bath for 2 hours at 70°. These samples were then extracted twice with 10-ml volumes of liquid scintillator [2,5-phenyloxazole and *p*-bis-2-(5-phenyloxazolyl)-1-benzene (Pilot Chemicals, Inc.) dissolved in toluene]. The extracts were placed in 20-ml vials and counted in a well-type liquid scintillation counter (Packard Instrument Co.). The ^{14}C-labeled material bound to the protein was determined by subtracting the radioactivity of the dialysate from that of the protein sample. The moles of inhibitor bound to the enzyme were then calculated from the specific activity of the initial solution of [^{14}C]-β-phenylpropionate.

Gel Filtration and Metal-Ion Exchange: Principles and Illustration of Methods.—Gel-filtration and metal-ion-exchange techniques employing $^{65}Zn^{2+}$ detect two types of substrate binding to carboxypeptidase as reported previously in detail (Coleman and Vallee,

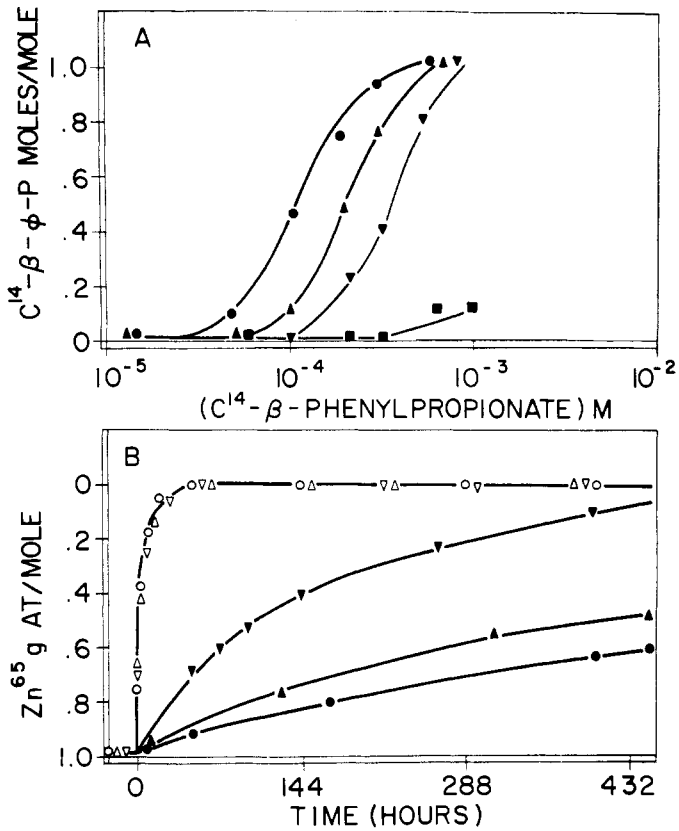


FIG. 3.—(A) Metal-dependent binding of [^{14}C]-β-phenylpropionate to carboxypeptidase. One-ml samples of zinc carboxypeptidase (●), cadmium carboxypeptidase (▲), cobalt carboxypeptidase (▼), and apocarboxypeptidase (■), all 2 × 10^{-4} M, were equilibrated with 50 ml of [^{14}C]-carboxyl-β-phenylpropionate in concentrations varying from 10^{-6} M to 10^{-3} M as indicated on the abscissa of this semilogarithmic plot. Moles of β-phenylpropionate bound per mole of protein were determined as described under Experimental Procedures. (B) Comparative effects of β-phenylpropionate on $^{65}Zn^{2+} \rightleftharpoons Zn^{2+}$ (○, ●), $^{115}Cd^{2+} \rightleftharpoons Cd^{2+}$ (△, ▲), and $^{60}Co^{2+} \rightleftharpoons Co^{2+}$ (▽, ▼): exchanges at the active site of carboxypeptidase. Radioactively labeled metallocarboxypeptidases [(CPD)^{65}Zn], [(CPD)^{115}Cd], or [(CPD)^{60}Co], all 1 × 10^{-4} M, were exposed to the corresponding stable isotopes, 1 × 10^{-5} M, at zero time and the enzyme-bound ^{65}Zn (○), ^{115}Cd (△), and ^{60}Co (▽) were followed as functions of time. (●), (▲), (▼), the same, respectively, in the presence of 0.02 M β-phenylpropionate.

1962a,b). Dipeptide substrates such as carbobenzoxyglycyl-L-phenylalanine form stable complexes with the inactive *apoenzyme* thus preventing the association of the metal ion and, hence, restoration of activity (Fig. 1B). In contrast, ester substrates such as hippuryl-*dl*-β-phenyllactate bind only to the *metalloenzyme* and prevent neither the binding of the metal ion nor the consequent restoration of activity (Fig. 1C).

However, both peptide and ester substrates form metalloenzyme-substrate complexes which prevent the *dissociation* of the metal ion and, therefore, the exchange of one metal ion for another. The details of these procedures have been described (Coleman and Vallee, 1962a,b), but are here recalled for convenience to permit comparison of the results of these approaches with those of direct binding studies with ^{14}C-labeled material.

As a matter of convenient differentiation the firmness of metal-protein binding will be expressed as a *stability constant*, i.e., the reciprocal of the dissociation or equilibrium constant, since this is the terminology in general usage to describe metal complexes (Cotton and Wilkinson, 1962). Hence *dissociation constants* will refer only to protein-substrate or inhibitor complexes.

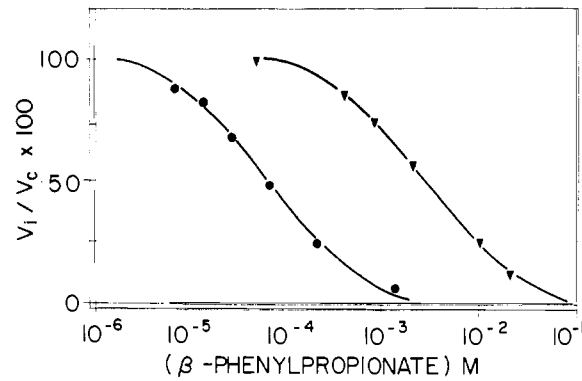


FIG. 4.—Inhibition of peptidase (●) and esterase (▼) activity of [(CPD)Zn] by β-phenylpropionate. Both peptidase and esterase reaction mixtures contained β-phenylpropionate in the concentrations indicated on the abscissa. Assays were performed as described under Experimental Procedures using 0.01 M carbobenzoxyglycyl-L-phenylalanine and 0.01 hippuryl-*dl*-β-phenyllactate and approximately 10^{-3} μmoles of enzyme.

RESULTS

Like the ester substrate, hippuryl-*dl*-β-phenyllactate, the inhibitor, β-phenylpropionate, does not prevent the binding of $^{65}Zn^{2+}$ to apocarboxypeptidase. The sequence of additions of the metal and of the inhibitor do not affect the restoration of the metalloenzyme and, therefore, of activity. The metal atom binds to the apoenzyme regardless of the sequence of addition of the metal ion and inhibitor (Fig. 2A). Further, β-phenylpropionate, like the ester, markedly retards the exchange of $^{65}Zn^{2+} \rightleftharpoons Zn^{2+}$ at the active site and, hence, dissociation (Fig. 2B). At equimolarity of [(CPD)^{65}Zn] and Zn^{2+}, the half-life of the exchange is 5 hours, but increases to 576 hours in the presence of 0.02 M β-phenylpropionate (Fig. 2B, Table I). By this criterion, β-phenylpropionate only forms a complex with the metalloenzyme, not with the apoenzyme.

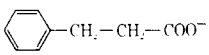
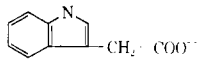
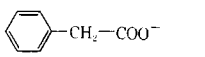
Equilibrium dialysis with [^{14}C]-β-phenylpropionate confirms this conclusion. At concentrations where the zinc enzyme binds 1 mole of the inhibitor, the apoenzyme binds none (Fig. 3A).

The extent to which the chemical properties of the metal ion determine the binding of β-phenylpropionate to the enzyme was explored by substituting Co^{2+} and Cd^{2+} for zinc. [^{14}C]-β-Phenylpropionate binds to the enzymes formed with these metal ions in the order $Zn^{2+} > Cd^{2+} > Co^{2+}$ (Fig. 3A). This is the same order in which the inhibitor prevents the exchange of these metal ions (Fig. 3B). Thus the metal ion is indispensible for β-phenylpropionate binding at the active center.

Peptidase activity of the zinc enzyme is inhibited to the very degree to which [^{14}C]-β-phenylpropionate is bound (Fig. 4). In accord with the less firm binding of β-phenylpropionate to cobalt carboxypeptidase, peptidase activity of the cobalt enzyme is inhibited less effectively than that of the zinc enzyme (Table I, line 1).

In contrast, inhibition of esterase activity of zinc, cadmium, and cobalt carboxypeptidases requires the same concentrations of inhibitor for equal inhibition (Table I, line 1). β-Phenylpropionate, 2 × 10^{-3} M, inhibits zinc, cobalt, and cadmium carboxypeptidase by about 50%, apparently independently of its affinity for the particular metal ion (Table I, line 1). This

TABLE I
EFFECT OF CARBOXYPEPTIDASE INHIBITORS ON METAL-ION EXCHANGE AT THE ACTIVE SITE AND THE INHIBITION OF PEPTIDASE AND ESTERASE ACTIVITIES[a]

Inhibitor[b]	Metal Ion	Half-Life of $*Me^{2+} \rightleftharpoons Me^{2+}$ Exchange[c] (hr)	Concentration of Agent at 50% Inhibition (M) Peptidase	Esterase
Control (no inhibitor)	Zn	5		
1. *Acetates* R—CH_2—COO^-				
	Zn	576	0.00006	0.0025
	Cd	320		0.0020
	Co	96	0.0003[d]	0.0025
	Zn	62	0.00007	0.015
	Zn	48	0.0003	0.05
	Cd	20		0.04
	Co	9	0.005[d]	0.06
I—CH_2—COO^-	Zn	37	0.0003	0.01
I—CH_2—$CONH_2$	Zn	5	no inhibition	
I—CH_2—CH_2—COO^-	Zn	32	0.0005	0.015
Br—CH_2—COO^-	Zn	9	0.01	[e]
CH_3—CH_2—COO^-	Zn	8	0.10	[e]
H—CH_2—COO^-	Zn	5	no inhibition	
2. *Amino acids*				
D-Phenylalanine	Zn	165	0.003	0.020
	Cd	64	[d]	0.025
	Co	20	0.02	0.015
L-Phenylalanine, *p*H 7.5	Zn	5	no inhibition	
*p*H 9.0	Zn	5	no inhibition	
N-Acetyl-D-phenylalanine	Zn	5	no inhibition	
D-Tryptophan	Zn	32	0.07	[e]
D-Leucine	Zn	12	0.50	[e]

[a] Carbobenzoxyglycyl-L-phenylalanine (CGP) was the peptide substrate and hippuryl-*dl*-β-phenyllactate (HPLA) was the ester substrate, both under standard assay conditions as "method." [b] The inhibitor concentration was 0.02 M in 1 M NaCl–0.05 M Tris, *p*H 8.0, 4°, in all exchange experiments. [c] $*Me^{2+} \rightleftharpoons Me^{2+}$ exchange refers to $^{65}Zn^{2+} \rightleftharpoons Zn^{2+}$, $^{115}Cd^{2+} \rightleftharpoons Cd^{2+}$, and $^{60}Co^{2+} \rightleftharpoons Co^{2+}$ exchanges, respectively. [d] No peptidase activity. [e] Not determined.

concentration is 30-fold higher than that required to inhibit the peptidase activity of the zinc enzyme to a comparable degree (Fig. 4).

A number of substituted acetates and D-amino acids are known to inhibit carboxypeptidase. Their effects on metal-ion exchange and on inhibition of peptidase and esterase activities are compared in Table I.

The degree of peptidase inhibition correlates well with the effects of inhibitors on metal-ion exchange. This is particularly apparent for the acetate group of inhibitors; on substitution of the acetate molecule both inhibition and retardation of exchange follow the order CH_3 < Br < I < phenyl < indole < benzyl. Throughout, the zinc enzyme is inhibited more rapidly than cobalt carboxypeptidase. Just as noted for β-phenylpropionate, comparison of peptidase and esterase inhibition shows that higher concentrations of all these agents are required to achieve comparable degrees of inhibition of esterase activity. The L-amino acids and N-substituted D-amino acids neither inhibit the enzyme nor do they retard exchange (Table I, line 2).

D-Histidine has been known to inhibit carboxypeptidase and the mechanism of inhibition was thought to be similar to that of the substituted acetates and amino acids in Table I. Figure 5 demonstrates, however, that both D- and L-histidine exert their effects on catalytic function by removing zinc from the enzyme, a process which is complete within 24 hours. The free α-amino group of histidine is required for this form of inhibition, since *N*-acetyl-L-histidine has no effect (Fig. 5).

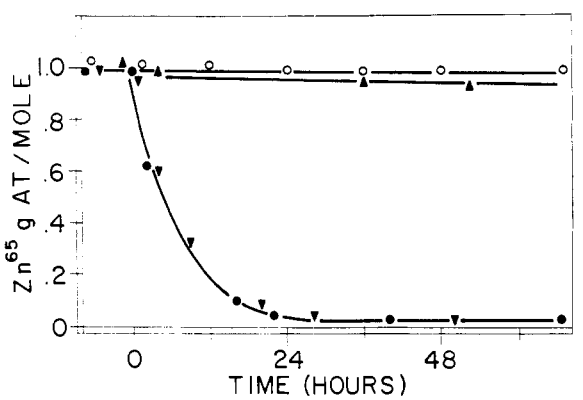


FIG. 5.—Effect of D- and L-histidine on removal of ^{65}Zn from [(CPD)^{65}Zn]. [(CPD)^{65}Zn], 1×10^{-5} M, was dialyzed against metal-free buffer (○), 2×10^{-2} M D-histidine (●), 2×10^{-2} M L-histidine (▼), and 2×10^{-2} M *N*-acetyl-L-histidine (▲) and enzyme-bound ^{65}Zn determined as a function of time as represented by the symbols. All dialyses were performed in 1 M NaCl–0.05 M Tris, *p*H 7.5, 4°, with 100-fold volume excess of dialysate.

DISCUSSION

The capacity of peptide substrates to prevent the binding of zinc or other metal ions to apocarboxypeptidase can be employed to demonstrate apoenzyme-substrate or inhibitor complexes (Figs. 1 and 2) (Coleman and Vallee, 1962a,b). While the *existence* of a complex can be demonstrated unambiguously in this manner, *failure* of a substrate or inhibitor to prevent metal binding cannot be considered unequivocal evidence *that a complex does not form.*

Like hippuryl-*dl*-β-phenyllactic acid, β-phenylpropionate does not prevent zinc binding, though it prevents the dissociation of the metal from the apoenzyme (Figs. 2A,B, 3B). On the basis of the criteria employed previously (Coleman and Vallee, 1962b) it

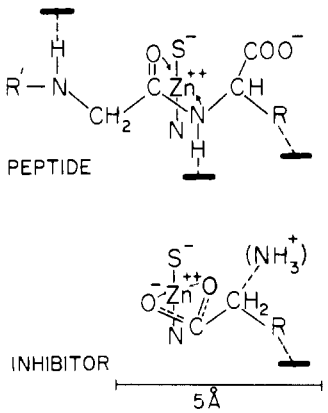


FIG. 6.—Model for inhibitor binding to the active center of carboxypeptidase compared to peptide substrate binding. *Peptide binding:* The representation is that previously proposed (Vallee *et al.*, 1963), the two -NH- functions and the R group of the substrate are shown as protein-binding groups; the complementary protein groups, unknown thus far, are indicated by the heavy lines. The carbonyl oxygen and peptide nitrogen act as donors to the Zn^{2+} ion. *Inhibitor binding:* The R group is represented as occupying the same binding position and orientation relative to the protein as the R group of the peptide. The α-carbon substituents are placed with stereochemistry *opposite* to that of the peptide (*vide supra*) bringing the carboxyl-donor group in position to coordinate to the Zn^{2+} ion. A free, charged amino group on the α-carbon is shown in brackets to show the analogy to D-amino acids which also act as inhibitors and from which the stereochemistry is deduced. Though the peptide is shown in the *trans* configuration, similar considerations of peptide and inhibitor binding would hold if the peptide were bound to the protein in the *cis* configuration.

was therefore concluded that β-phenylpropionate binds to the active site only when a metal is present, but it does not form complexes with the apoenzyme.

The direct binding studies with isotopically labeled β-phenylpropionate confirm the interpretation of results by means of Sephadex and isotope-exchange methods: One mole of [^{14}C]-β-phenylpropionate binds to metallocarboxypeptidases but none binds to the apoenzyme (Fig. 3A). These findings confirm earlier work in which [^{14}C]-β-phenylacetate was employed (Rupley and Neurath, 1960). These data do not eliminate the possibility, of course, that under some conditions, inhibitors or peptide and ester substrates might bind nonspecifically to enzyme sites without affecting metal-ion restoration or exchange.

Knowing that [^{14}C]-β-phenylpropionate binds to the metal atom at the active center of carboxypeptidase a number of enzymatic and physicochemical effects of this agent can now be interpreted. Thus, under appropriate conditions, β-phenylpropionate prevents carboxymethylation or alkylation of the single, zinc-binding cysteine residue of the enzyme (Walsh, *et al.*, 1962); or, alternately, it protects the two functional tyrosyl residues of the enzyme against acetylation which results in loss of peptidase and multifold increase in esterase activities (Simpson *et al.*, 1963; Riordan and Vallee, 1963; Vallee, 1964). Since the agent covers an area of 5 or 6 A in diameter, the protected groups may be presumed to be located within this radius about the zinc atom.

As might be expected, the dissociation constant governing the binding of β-phenylpropionate to the enzyme is a function of the specific metal ion at its active site (Fig. 3A). Under the experimental conditions employed the stability of the complexes decreases in the order $Zn^{2+} > Cd^{2+} > Co^{2+}$; the agent prevents the dissociation of these metal ions in the same order (Fig. 3B). When determined under identical conditions the apparent stability constants of the metallocarboxypeptidases also decrease in the order $Zn^{2+} > Cd^{2+} > Co^{2+}$ (Coleman and Vallee, 1961). This coincidence might imply that the stabilities of the β-phenylpropionate complexes are a direct function of the stability of the metalloenzyme. Data with a larger series of metallocarboxypeptidases would be required to examine the validity of this conjecture.

Comparison of these data obtained at equilibrium with the kinetic effects of β-phenylpropionate on peptidase and esterase activities reveals some interesting relationships. Although kinetics demonstrate typical competitive inhibition (Neurath and Schwert, 1950) equlibrium studies demonstrate a difference in the microscopic details of peptide and β-phenylpropionate binding (Coleman and Vallee, 1962a; Figs. 1B, 2A,B) which, however, is not reflected in the inhibition kinetics.

The active center binds β-phenylpropionate by a different mechanism and much more firmly than the peptide substrates, since both the dissociation constant, K_s, of apoenzyme-peptide substrate complexes and the Michaelis constant, K_m, of all peptide substrates measured so far are greater than 10^{-3} M. At a concentration of β-phenylpropionate in which 1 mole is bound to the enzyme (Fig. 3A), peptidase activity is almost completely abolished (Fig. 4).

Prior to the establishment of the essentiality of zinc to the action of carboxypeptidase the characteristics of a large number of competitive inhibitors similar in structure to β-phenylpropionate were investigated (Neurath and Schwert, 1950; Smith, 1951). The R group in substituted acetates was found to determine the degree of peptidase inhibition. This deduction is now fully confirmed by the capacity of these inhibitors to prevent metal-ion exchange (Table I). These substitutions produce compounds which prevent exchange in the same order in which they inhibit peptidase activity, i.e., CH_3 < Br < I < phenyl < indole < benzyl. Both effects would appear to be a function of the dissociation constant of the enzyme-inhibitor complex.

In the presence of β-phenylpropionate the exchange of Zn^{2+} takes 5–6 times as long as that of Co^{2+} (Table I, lines 1 and 2). Further, the dissociation constant of the [^{14}C]-β-phenylpropionate-cobalt-carboxypeptidase complex is about five times larger than that of the zinc-carboxypeptidase complex (Fig. 3A), and five times greater concentrations of β-phenylpropionate are required to inhibit the peptidase activity of the cobalt enzyme (Table I). Again, there is good agreement between physicochemical and enzymatic measurements, and similarly good correspondence for phenylacetate and D-phenylalanine has been observed.

About 30-fold greater concentrations of β-phenylpropionate are required to inhibit esterase than peptidase activity (Fig. 4). The other agents studied are similarly poorer inhibitors of esterase activity and require as much as 100-fold greater concentrations to obtain effects comparable to those on peptidase activity (Table I). In addition esterase inhibition does not vary as a function of the metal ion present at the active site (Table I). Though differences in the inhibitory effects of β-phenylpropionate on esterase and peptidase activities are obvious and marked, the data are not sufficient as yet to isolate specific parameters such as, for instance, the relative stability constants of metal-peptide, -ester or -inhibitor complexes, as being responsible for the observations. However, further work along these lines is in progress.

The halogenated acetates also inhibit carboxypeptidase, both iodoacetate and β-iodopropionate being highly effective. The resultant instantaneous inhibition is reversible on dilution and, on the basis of presently available data, cannot therefore be attributed to chemical modifications of the protein (Riordan and Vallee, 1963; Coombs *et al.*, 1964). It is conceivable that the iodine atom, analogous in dimensions to the aromatic R groups, binds to the protein also in a similar manner, perhaps through π—π or hydrophobic bonding.

In the course of studying the common features of inhibitors, the mode of action of D-histidine was examined also. D-Histidine, unlike all other D-amino acids, or the other inhibitors here discussed, inhibits the enzyme by removing zinc (Fig. 5), though *N*-acetyl-L-histidine fails to do so. Hence the participation of both the α-amino and imidazole nitrogens accounts for the successful competition of D-histidine with the enzyme for its zinc atom.

Tempting as it would be to draw detailed analogies between the binding of substrates and inhibitors, it is obvious that their structural differences are sufficiently great to preclude any but coarse comparisons. As has been emphasized, both the ester and β-phenylpropionate bind only to the metalloenzyme. Both the substrates and the inhibitors are substituted with a bulky R group on the α-carbon atom. The structural dissimilarities are almost as great as the identities, however, and the present data do not assist greatly in delineating the common mechanisms which result in binding, activity, or inhibition.

The limitations in drawing precise analogies between substrate and inhibitors are well illustrated by comparison of the essentiality of the free carboxyl group which is the specificity requirement of the enzyme. Its blockage by amidation abolishes hydrolysis of the peptide substrate glycyl-L-phenylalanine, but permits its binding (Coleman and Vallee, 1962b). Amidation of iodoacetate abolishes both binding and inhibition (Table I, line 1).

Stereochemical considerations further emphasize the difference in the mechanism of binding of inhibitors and substrates (Table I, line 2). In substrates the aromatic, branched aliphatic, or halogen side chains which are essential to binding must be in the L-configuration relative to the α-carbon (Coleman and Vallee, 1962a,b); but when the substituent side chain of a competitive inhibitor is asymmetric, e.g., phenylalanine, only the D isomer will bind to the active center (Table I, line 2) (Neurath and Schwert, 1950).

The metal-dependent binding of β-phenylpropionate is unexplained thus far. While a number of possible hypotheses could account for this phenomenon, it is attractive to consider that the inhibitor contributes a group to the coordination sphere of the metal ion which is not completely filled by the donor groups of the protein. The free carboxyl of β-phenylpropionate is the only group of this agent which, in fact, *can* act as a negative donor for the metal ion. The known stability constants of metal-carboxylate complexes cannot account for the magnitude of the dissociation constants of the enzyme-inhibitor complexes. Both the protein-ligand and ancillary binding groups of the inhibitor, presumably the phenyl ring, would also be expected to contribute to the binding affinity. Figure 6 depicts a hypothetical mechanism for the binding of an inhibitor such as β-phenylpropionate comparing it to that previously proposed for peptide binding (Fig. 6). The aromatic side chain of the inhibitor here occupies the same binding site as the C-terminal aromatic side chain of the substrate while the carboxyl group is oriented around the α-carbon with stereoisomerism opposite to that of the substrate. The continuing delineation of the comparative modes of substrate and inhibitor binding by physicochemical means should prove of considerable value in the discernment of the mechanism of action of carboxypeptidase.

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