Procarboxypeptidase A–Carboxypeptidase A Interrelationships. Metal and Substrate Binding*

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ABSTRACT: Apoprocarboxypeptidase combines with zinc and other transition metals to form stable metalloprocarboxypeptidases. Titrations with silver and *p*-mercuribenzoate indicate that in the zymogen as in carboxypeptidase, zinc is bound to a thiol group. Comparisons of the pH dependence of zinc binding and of the stability constants of metalloprocarboxypeptidases with those of metallocarboxypeptidases indicate that metals are bound significantly less firmly to the zymogen than to the enzyme. This is consistent with the absence of a nitrogen ligand in the zymogen, postulated to bind the metal in the enzyme. The magnitudes and sequence of the stability constants of transition metal–zymogen complexes confirm the involvement of a sulfur ligand in metal binding. In accord with these observations, at pH 7, complexometric titrations of apoprocarboxypeptidase with Zn^{2+} liberates only one proton, with a $pK_{app} = 8.9$. Thus, parallel studies of the metal binding of the two proteins indicate alterations in three-dimensional structure owing to their characteristic metal binding. Apparently, conformational changes incident to activation of the zymogen allow the formation of the specific, three-dimensional structure of the catalytically active enzyme. The esterase and peptidase activities observed are independent of the order in which metal substitution and tryptic activation are carried out, but a metal atom must apparently occupy the metal binding site of the zymogen for enzymatic activity to be observed on activation. Similar to carboxypeptidase, peptide and ester substrates, substrate analogs, and inhibitors retard exchange of ^{65}Zn in procarboxypeptidase, indicating the preexistence of a substrate-binding site in the zymogen. Significant features of this binding site seem to be present in the zymogen, but activation generates the final stereochemical specificity of the substrate binding site of the enzyme.

The activation of chymotrypsinogen, trypsinogen, or pepsinogen is achieved through hydrolysis of specific peptide bonds (Dreyer and Neurath, 1955; Rovery *et al.*, 1955; Davie and Neurath, 1955; Desnuelle and Rovery, 1961; Neurath, 1964), leading to an alteration of primary structure. The conformational reorientation (Pechère and Neurath, 1957; Fasman *et al.*, 1966) dictated by the resultant amino acid sequence is thought to generate the catalytically active center by properly juxtaposing functional residues.

The activation of procarboxypeptidase A differs from that of these other zymogens in several respects. It occurs as an aggregate of three subunits, I, II, and III, of which subunit I is the direct precursor of carboxypeptidase (Brown *et al.*, 1963a). The activation of subunit I requires the participation of an endopeptidase with chymotryptic-like activity generated from subunit II in the course of the activation process (Yamasaki *et al.*, 1963; Brown *et al.*, 1963a,b). Isolation of carboxypeptidase A incurs hydrolysis and destruction of subunits II and III, the latter a protein of unknown function. The resultant accumulation of peptide fragments renders the definition of the chemical and physical transformations accompanying activation difficult.

Procarboxypeptidase A contains significant amounts of zinc, as well as some iron and nickel (Neurath, 1955; Vallee *et al.*, 1960b; J. H. Freisheim, H. Neurath, and B. L. Vallee, to be published), but the relationship of these metals to the subunit structure of procarboxypeptidase has not been explored. The role of zinc in the activation process and its relationship to the formation of the catalytically active site has not been studied previously, but the very characteristics of the metal might serve to elucidate these processes.

The present study shows that activation of procarboxypeptidase A is accompanied by alterations in metal coordination which apparently result from and therefore reflect a conformational change in the protein. Further, metal-exchange data indicate the preexistence of substrate binding sites in the zymogen. A preliminary communication has been presented (Piras and Vallee, 1966).

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Materials and Methods

Procarboxypeptidase A was prepared from an acetone powder of bovine pancreas glands kindly supplied by Dr. H. Neurath, following the procedure of Yamasaki *et al.* (1963). The protein, having a sedimentation coefficient of 6S procarboxypeptidase A-S6 according to the nomenclature proposed by Neurath and co-workers (Yamasaki *et al.*, 1963; Brown *et al.*, 1963a,b) was used throughout this investigation. Sedimentation and electrophoretic analysis of the final lyophilized material indicated that the protein was 95% homogeneous; its properties corresponded to those previously described (Yamasaki *et al.*, 1963).

Trypsin was a twice-crystallized preparation containing 50% $MgSO_4$ (Worthington Biochemical Corp.). It was rendered metal free by exhaustive dialysis against 10^{-3} M HCl.

Buffers and reagent solutions were prepared from reagent grade chemicals. They were extracted with 0.1% dithizone in CCl_4 to remove contaminating metal ions. Throughout these studies special precautions were taken to prevent contamination by adventitious metal ions (Coleman and Vallee, 1960).

Apoprocarboxypeptidase was prepared by dialyzing procarboxypeptidase A (6–20 mg/ml) for 48 hr at 4° against three changes of 0.01 M Tris–HCl buffer, pH 7, containing 2×10^{-3} M 1,10-phenanthroline. The chelating agent was then removed by dialysis for an additional 48 hr against four changes of metal-free buffer; any insoluble material which appeared during this process was removed by centrifugation. The clear supernatant was then stored under a toluene atmosphere at 4° to prevent bacterial contamination. The apoprotein was obtained in approximately 80% yield and contained less than 8% of the initial zinc and no significant amounts of other metals.

Metal solutions were prepared from spectroscopically pure metal salts (Johnson Matthey Co., Ltd., London).

Dialysis tubing was Visking-Nojax casing appropriately treated in order to reduce metal binding (Brush *et al.*, 1963).

Protein concentrations were determined from the absorbance at 280 mμ, $\epsilon^{0.1\%}_{280\ m\mu}$ 1.9 (Yamasaki *et al.*, 1963), in a Zeiss PMQII spectrophotometer.

The pH was determined with a Radiometer pH M22 pH meter, equipped with a general purpose combined electrode.

Metal analysis was carried out by emission spectrography (Vallee, 1955). Zinc was also measured by atomic absorption spectrophotometry (Fuwa and Vallee, 1963), calcium by flame spectrophotometry, and iron with 1,10-phenanthroline (Sandell, 1959).

Sulfhydryl groups were determined by amperometric silver titration (Benesch *et al.*, 1955) or with *p*-mercuribenzoate (Boyer, 1954), both as previously described by Coombs *et al.* (1964).

Removal of zinc by dialysis at different pH values was carried out in a manner similar to that reported for carboxypeptidase (Coleman and Vallee, 1960; Wintersberger *et al.*, 1965).

Metalloprocarboxypeptidase stability constants were determined by equilibrium dialysis. For the exchange studies, apoprocarboxypeptidase was reconstituted to contain 1 g-atom of zinc/mole of zymogen. The stability constant for zinc procarboxypeptidase was established by the "direct method," and those for the other metals by the "competition method" (Coleman and Vallee, 1960, 1961). In each case, the concentrations of metal and zinc ions were adjusted such as to obtain approximately 50% exchange, at equilibrium. Since the stability constants of metalloprocarboxypeptidase are small (*vide infra*), the presence of excess zinc is necessary to assure the absence of any free apoprotein, a prerequisite for calculations of stability constants by means of the "competition method."

Prevention of metal exchange by different carboxypeptidase substrates, substrate analogs, and inhibitors was studied by the dialysis technique described for carboxypeptidase (Coleman and Vallee, 1962a,b, 1964). Procarboxypeptidase, 2.5×10^{-5} M, was dialyzed *vs.* an equimolar concentration of $^{65}Zn^{2+}$ until equilibrium was reached. The excess of radioactive zinc was then removed by repetitive dialyses *vs.* metal-free buffer. ^{65}Zn–procarboxypeptidase, 2.5×10^{-5} M, was equilibrated with 0.04 M inhibitor or substrate and Zn^{2+} was added. The exchange of ^{65}Zn for Zn^{2+} was followed as a function of time by measuring the radioactivity bound to the protein in a well-type scintillation counter (Tracerlab).

Proton Displacement from Apoprocarboxypeptidase by Zn^{2+}. A Radiometer TTT-1 pH-Stat, coupled to an Ole Dich recorder, was used for all the measurements. The electrode assembly consisted of the Radiometer GK-2021C combined electrode and plastic electrode vessels thermostated at 25°. In order to measure the protons released during the formation of the apoprocarboxypeptidase–metal complexes, separate solutions of the protein and of the metal, at identical pH values and ionic strengths, were prepared as follows. In one of the electrode vessels a solution of zinc chloride (5×10^{-3} M) and sodium citrate (5×10^{-3} M) was adjusted to the selected pH by means of the pH-Stat. The electrode was then carefully rinsed and dried, and placed in a second electrode vessel containing 3 ml of an unbuffered solution of apoprocarboxypeptidase ($1–1.1 \times 10^{-4}$ M). The pH was then adjusted to exactly that of the metal solution, and aliquots of the latter were added. The protons released were calculated from the recorded volume of 0.01 N NaOH required to restore the selected pH.

Assay Procedures. Procarboxypeptidase A was first activated by incubation with trypsin at pH 7 and 37° using a molar ratio zymogen:endopeptidase of 3:1. Maximal activity was achieved in 4–5 hr. Cobalt, nickel, and manganese carboxypeptidases were assayed in the presence of these respective metal ions at 10^{-4} M in order to ensure complete formation of the metalloenzymes under the assay conditions.

Esterase activity was determined by pH titration (Snoke *et al.*, 1948) with 0.1 N NaOH of the protons

released on hydrolysis of the substrate, using a pH-Stat and recorder. Assays were performed at 25°, with 3 ml of 0.01 M hippuryl *dl*-β-phenyllactate in 0.2 M sodium chloride–0.005 M Tris–HCl buffer, pH 7.5.

Peptidase activity was determined using carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as the substrate (Snoke and Neurath, 1949). The assays were performed at 0° in 1 M NaCl–0.02 M sodium Veronal buffer, pH 7.5, with an initial concentration substrate of 0.02 M. Activities were calculated from the linear portion of first-order reaction plots when hydrolysis did not exceed 15%.

Results

Metal Analyses of Procarboxypeptidase A. Procarboxypeptidase contains significant amounts of zinc (Neurath, 1955; Vallee *et al.*, 1960b). The stoichiometry of zinc, the content of other metals, and the effect of various conditions of isolation on their concentration will be reported in detail (J. H. Freisheim, H. Neurath, and B. L. Vallee, to be published). The metal content of the procarboxypeptidase preparations employed here was examined to provide the base line for the present metal-binding studies. In the final, purified material only zinc, iron, nickel, and calcium are found (Table I). In six preparations an average of 630 μg of zinc/g of protein was detected, corresponding to 0.84 ± 0.16 g-atom/mole of trimeric procarboxypeptidase (mol wt 87,000). In addition to zinc, iron and nickel are found in variable amounts averaging 0.14 and 0.05 g-atom/mole, respectively. An average of 0.83 ± 0.35 g-atom of calcium/mole is also present. The sum of the average zinc, iron, and nickel contents of these six preparations is 0.98 ± 0.12 g-atom/mole.

TABLE I: Metal Content of Procarboxypeptidase A-S6 (in g-atoms per mole).[a,b]

Prepn	Zn	Fe	Ni	Σ(Zn + Fe + Ni)	Ca
681	0.77	0.04	0.04	0.85	0.39
631	0.64	0.23	0.07	0.94	1.09
693	0.72	0.12	0	0.84	0.59
694	0.83	0.11	0.13	1.07	0.87
747	0.93	0.17	0.04	1.14	1.35
758	0.88	0.16	0	1.04	0.67

[a] Results are expressed as gram-atoms per mole of molecular weight of 87,000 (Yamasaki *et al.*, 1963). [b] Analyses for Al, Ba, Cd, Co, Cr, Li, Mn, Mg, Mo, Pb, Sn, and Sr detected only negligible amounts of these elements in the final purified material.

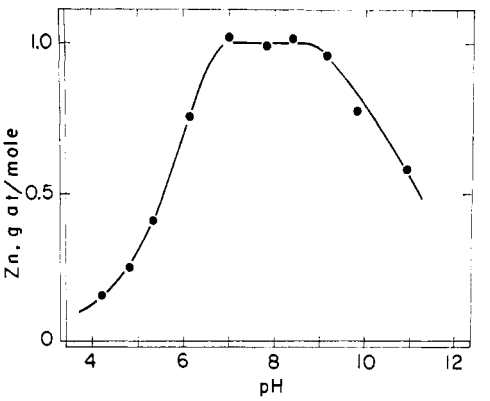

FIGURE 1: pH dependence of zinc binding to procarboxypeptidase A. Aliquots (1 ml) of procarboxypeptidase, 2.8 × 10^{-6} M, were dialyzed for 96 hr *vs.* 100 ml of 0.05 M Tris–HCl–0.05 M sodium acetate buffer, 4°, adjusted to the pH values indicated. Zinc remaining firmly bound to the protein was measured by atomic absorption spectroscopy.

Removal of Zinc by Dialysis at Different pH Values. Procarboxypeptidase is soluble in solutions of low ionic strength; thus equilibrium dialysis can be carried out in 0.05 M Tris–acetate buffers, without the high concentrations of NaCl required for similar experiments with carboxypeptidase (Coleman and Vallee, 1960). The effect of pH on the binding of zinc to procarboxypeptidase A, previously reconstituted to contain 1 g-atom of zinc/mole but no other metals, is shown in Figure 1. At equilibrium (96 hr), nearly 1 g-atom of zinc/mole remains firmly bound between pH 7 and 9. As the pH is decreased, however, zinc is lost rapidly, such that at pH 5.6, only 0.5 g-atom, and below pH 3.5 none. is bound. Zinc also dissociates above pH 9. Although this pH stability curve of procarboxypeptidase is similar to that previously reported for carboxypeptidase A, zinc is displaced more readily from the zymogen than from the enzyme.

Since the concentration of chloride ion in these experiments differed from that previously employed in similar studies of δ-carboxypeptidase (Coleman and Vallee, 1960) both proteins were dialyzed in 1 M NaCl, 0.05 M Tris–acetate, at 4°, to permit direct comparison (Figure 2). These conditions shift the dissociation curve of the zinc–procarboxypeptidase complex to more alkaline pH, so that now at pH 7 only 0.8 g-atom of zinc/mole is bound. Fifty per cent of the zinc of carboxypeptidase is lost at pH 5.2, but under these conditions 0.5 g-atom/mole is bound to the zymogen at pH 6.0. This shift of 0.8 pH unit indicates that the *apparent* stability constant of the zinc–zymogen complex is significantly lower than that of the zinc–enzyme.

Stability Constants of Metalloprocarboxypeptidase. The stability constants of a number of metallocarboxypeptidases have been determined in 1 M NaCl–0.05 M Tris–HCl, pH 8 (Vallee *et al.*, 1960b; Coleman and

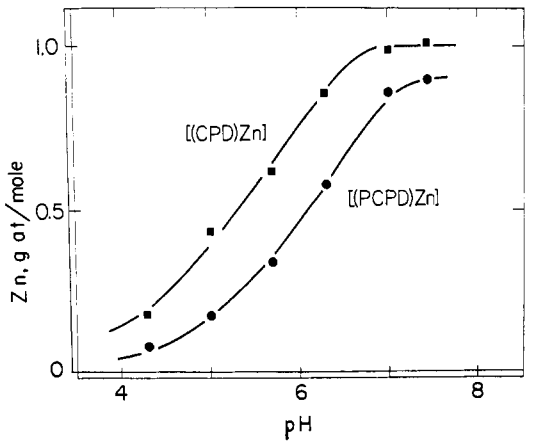

FIGURE 2: pH dependence of zinc binding to procarboxypeptidase and carboxypeptidase. Aliquots (1.4 ml) of procarboxypeptidase (●) and carboxypeptidase (■), 4×10^{-6} M, were dialyzed for 96 hr *vs.* 100 ml of 1 M NaCl–0.05 M Tris–HCl–0.05 M sodium acetate buffer, 4°, at the pH values indicated. Zinc remaining firmly bound to the proteins was measured by atomic absorption spectroscopy.

TABLE II: Stability Constants of Procarboxypeptidase A and Carboxypeptidase A.[a]

Metal	PCPD Log K_{app}[b]	PCPD Log K_{cor}[c]	CPD[d] Log K_{cor}	Δ Log K_{cor} (CPD − PCPD)
Mn	3.4	3.4	4.6	1.2
Co	4.2	5.4	7.0	1.6
Ni	3.4	5.9	8.2	2.3
Cu	2.5	8.1	10.6	2.5
Zn	6.8	9.0	10.5	1.5
Cd	5.5	8.4	10.8	2.4
Hg	3.9	18.3	21.0	2.8

[a] Procarboxypeptidase is symbolized as PCPD and carboxypeptidase as CPD. [b] Determined in 1 M NaCl, 0.05 M Tris–HCl, pH 8, 4°. [c] Corrected for the reduction of the concentration of free metal ions by complexation with the competing Tris and Cl^- ions (Coleman and Vallee, 1961). [d] Except for Mn, values from Coleman and Vallee (1961).

Vallee, 1961). In order to allow direct comparison with these previous data, the same conditions were used to determine the stability constants of the analogous metalloprocarboxypeptidases. A significant fraction of zinc dissociates from procarboxypeptidase under these circumstances permitting the use of the "direct method" (Coleman and Vallee, 1961) for the determination of the zinc–procarboxypeptidase stability constant. Once the latter is obtained, the absolute value of the stability constants for other metal complexes can be determined by the "competition method" (Coleman and Vallee, 1961). The resultant metalloprocarboxypeptidase stability constants, log K_{app}, are shown in Table II. An appropriate correction must be made for the complexation of the different metal ions to chloride and Tris present in the solutions (Coleman and Vallee, 1961). The log of the corrected stability constants, log K_{cor}, follows the order Hg $\gg$ Zn $\geqslant$ Cd $\geqslant$ Cu $>$ Ni $\geqslant$ Co $>$ Mn (Table II). The stability constants previously reported for δ-carboxypeptidase (Coleman and Vallee, 1961) are shown for comparison. Since the precision of the stability constants is not greater than 0.5 log unit, the sequences are essentially similar for both proteins. The value is highest for Hg; those for Cd, Zn, and Cu are close, followed by Ni, Co, and Mn. In general, however, the stability constants of the metal–zymogen complexes are smaller by one to three orders of magnitude. The difference is largest for Hg and smallest for Mn substitution.

Reaction of Apoprocarboxypeptidase with Silver. Silver titration of apoprocarboxypeptidase, containing 0.09 g-atom of residual zinc/mole, reveals the presence of 0.78 sulfhydryl group/mole (Table III). Addition of increasing amounts of zinc to the apoprotein, immediately prior to titration with silver, concomitantly decreases the mole fraction of thiol groups titrated. The sum of the mole fraction of zinc restored to the apozymogen and the mole fraction of sulfhydryl residues per mole titrated remains constant close to unity. Similar results have been obtained by titration with *p*-mercuribenzoate. The complementarity of the zinc and sulfhydryl contents is similar to that previously reported for carboxypeptidase A (Coombs *et al.*, 1964) and for carboxypeptidase B (Wintersberger *et al.*, 1965).

TABLE III: Complementarity of Ag^+-Titratable SH Groups and Zinc Content of Procarboxypeptidase A.[a]

Sample	Zn^{2+} (g-atom/mole)	SH (mole/mole)	Sum (SH + Zn^{2+})
1. (PCPD)	0.09	0.78	0.87
2. (PCPD) + 0.19 g-atom of Zn^{2+}	0.28	0.58	0.86
3. (PCPD) + 0.47 g-atom of Zn^{2+}	0.56	0.38	0.94
4. (PCPD) + 0.83 g-atom of Zn^{2+}	0.92	0.06	0.98

[a] Increments of Zn^{2+} were added first to 0.10–0.12 μmole of apoprocarboxypeptidase (PCPD) containing 0.09 g-atom of residual zinc and the remaining free SH was then titrated with Ag^+ (see Methods).

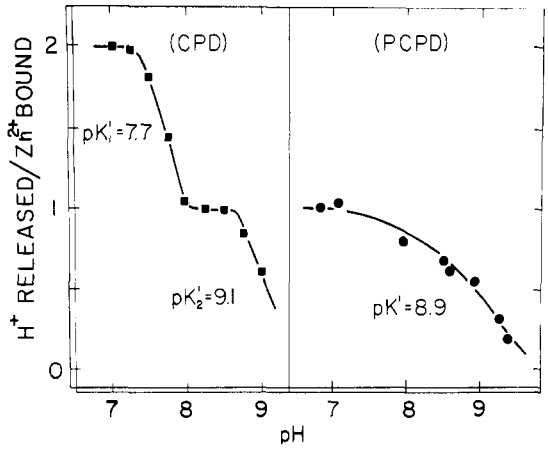

FIGURE 3: Displacement of protons from apoprocarboxypeptidase (●) and apocarboxypeptidase (■) by Zn^{2+}. Titrations were carried out as described under Methods. The data for apocarboxypeptidase are those of Coleman and Vallee (1961).

Displacement of Protons from Apocarboxypeptidase by Zn^{2+}. It has been shown previously (Coleman and Vallee, 1961) that at pH 7 complexometric titration of apocarboxypeptidase with zinc liberates two protons (Figure 3). The apparent pK values of the donor groups are 7.7 and 9.1; they have been attributed to the ionization of nitrogen and sulfur ligands, respectively. Similar titration of apoprocarboxypeptidase liberates only one proton at pH 7 (Figure 3). The apparent pK of the single donor group is 8.9, close to that of 9.1 in carboxypeptidase.

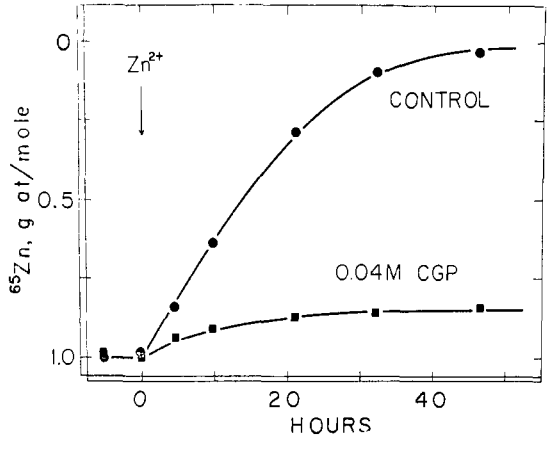

FIGURE 4: Effect of carbobenzoxyglycyl-L-phenylalanine (CGP) on exchange of ^{65}Zn–procarboxypeptidase. ^{65}Zn–procarboxypeptidase, 2.5×10^{-5} M, was equalibrated for 2 hr with 0.04 M carbobenzoxyglycyl-L-phenylalanine (■) in 0.05 M Tris–HCl buffer, pH 7.5, at 4°, and then exposed to 2.5×10^{-5} M Zn^{2+} at zero time. The control (●) was exposed directly to Zn^{2+}. ^{65}Zn bound to enzyme was measured as dialysis progressed.

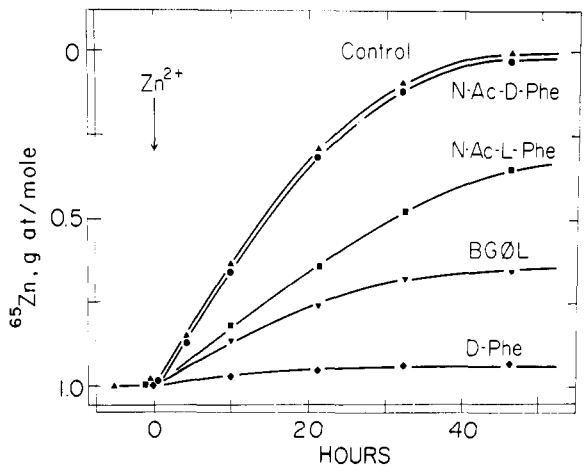

FIGURE 5: Effect of D-phenylalanine (D-Phe), *N*-acetyl-D-phenylalanine (*N*-Ac-D-Phe), *N*-acetyl-L-phenylalanine (*N*-Ac-L-Phe), and benzoylglycine phenyllactate (BGϕL) on exchange of ^{65}Zn in procarboxypeptidase. The experimental conditions are similar to those in Figure 4.

Metal Exchanges. In carboxypeptidase, substrates or inhibitors retard ^{65}Zn exchange by binding at or near the active site (Coleman and Vallee, 1960, 1962a,b, 1964). Similar studies have now been carried out with the zymogen. When ^{65}Zn–procarboxypeptidase is exposed to an equimolar concentration of stable zinc, 50% of the isotope exchanges in 12 hr (Figure 4). However, the presence of 0.04 M carbobenzoxyglycyl-L-phenylalanine, a typical carboxypeptidase substrate, markedly reduces the rate of exchange (Figure 4). Benzoylglycylphenyllactate, an ester substrate, also retards ^{65}Zn exchange (Figure 5). Further, while *N*-acetyl-L-phenylalanine retards exchange, the D

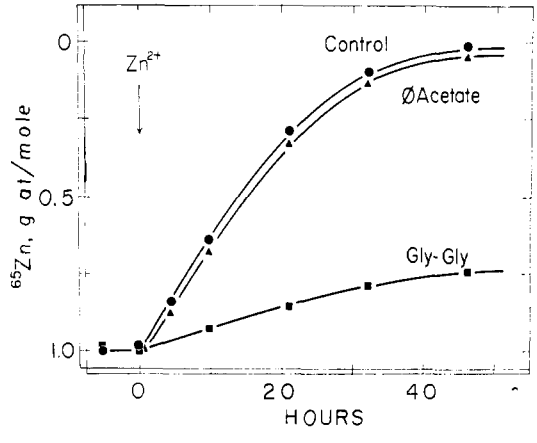

FIGURE 6: Effect of glycylglycine (Gly-Gly, ■) and phenylacetate (ϕ-acetate, ▲) on exchange of ^{65}Zn in procarboxypeptidase. The experimental conditions are similar to those in Figure 4.

isomer does not. Analogously, D-phenylalanine, a competitive inhibitor of the enzyme, completely inhibits metal exchange, while L-phenoylalanine is without effect (not shown). In general, the results for the zymogen and the enzyme are similar, but some distinct differences have been observed. Exchanges in the presence of glycylglycine and phenylacetate (Figure 6) differ from those previously reported for carboxypeptidase (Coleman and Vallee, 1962, 1964). The dipeptide retards the exchange in procarboxypeptidase, but not in carboxypeptidase. Phenylacetate, on the other hand, does not prevent exchange in the zymogen under the present conditions, while it is effective in preventing it for carboxypeptidase.

Effect of Metals on the Activation of the Zymogen. The enzymatic activities obtained when metal substitution precedes zymogen activation are similar to those obtained when metals are inserted directly into the enzyme, *i.e.*, when tryptic activation precedes metal substitution (Table IV). The resulting peptidase and esterase activities are independent of the order in which metal substitution and activation are carried out.

The effect on the activation process of the sequence of adding zinc and trypsin to apoprocarboxypeptidase was also examined. At 37° tryptic activation of the native metal containing zymogen is completed in 6 hr (Figure 7). When zinc is first added to apoprocarboxypeptidase to restore the precursor, and this is followed by tryptic activation, the resultant activity appears at a similar rate. In contrast, when apoprocarboxypeptidase is exposed first to trypsin, and activity is then measured on addition of zinc, the observed activation is reduced to very low levels. Under these conditions, the metal binding site of the zymogen must be occupied by a metal atom for activation to be observed.

TABLE IV: Enzymatic Activities of Trypsin-Activated Metalloprocarboxypeptidases and Metallocarboxypeptidases.[a]

	Peptidase		Esterase	
Metal	PCPD[b]	CPD[c]	PCPD[b]	CPD[c]
Zn	100	100	100	100
Mn	20	28	130	156
Ni	80	50	80	43
Co	210	214	140	114
Cd	0	0	170	143
Hg	0	0	30	86

[a] All activities in per cent of the zinc enzyme. Abbreviations are the same as in Table II. [b] The various [Me(PCPD)]'s were prepared by replacing zinc atoms with excess of other Me^{2+} ions by prolonged exchange. The [Me(PCPD)]'s, 1.9×10^{-5} M, were then activated with metal-free trypsin, 1×10^{-5} M, in 0.05 M Tris–HCl, pH 7.5, at 37°. Maximum activities were obtained in 4 hr. Assays were performed in the presence of 10^{-4} M excess Me^{2+} as described under Methods. [c] The [Me(CPD)]'s were obtained by substituting metals for zinc, directly in carboxypeptidase. The activities are those reported by Coleman *et al.* (1966), and obtained under conditions similar to *b*.

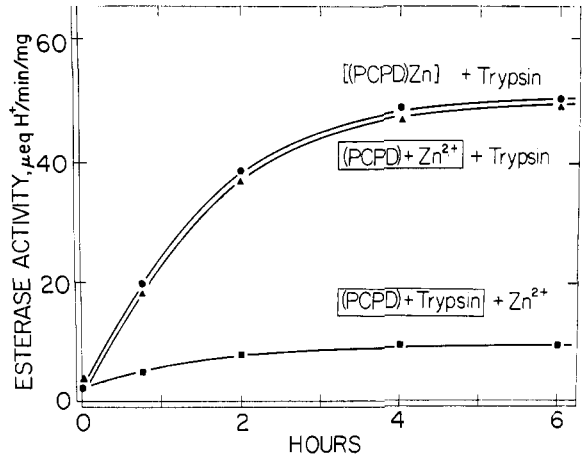

FIGURE 7: Tryptic activation of native (●), Zn reconstituted (▲), and apoprocarboxypeptidase (■). Procarboxypeptidase, 2.5×10^{-5} M, was activated with metal-free trypsin, 8×10^{-6} M, in 0.5 M NaCl, 0.05 M Tris–acetate buffer, pH 6.6, 37°. Assays were performed as described under Methods and the enclosures indicate the order of additions. Reconstitution with zinc was carried out with a sevenfold molar excess of metal ions over protein.

Discussion

Procarboxypeptidase A, prepared by the procedure of Keller *et al.* (1958), contains significant amounts of zinc, and also iron and nickel (Neurath, 1955; Vallee *et al.*, 1960b). The characteristics of procarboxypeptidase A-S6, the zymogen used throughout this study, are closely similar to those of previous preparations. Its physical-chemical characteristics and the amino acid composition of the subunits have been characterized (Yamasaki *et al.*, 1963). Metal analyses on the material employed in this study (Table I) are in agreement with more extensive studies to be reported (J. H. Freisheim, A. Neurath, and B. L. Vallee, to be published). The six preparations examined contain an average of 0.84 g-atom of zinc/mole. In addition, iron was consistently found, nickel was encountered more sporadically, and significant amounts of calcium were also detected. The presence of zinc, presumably as part of subunit I, the direct precursor of carboxy-

peptidase, is expected, but the occurrence and role of other metals requires comment.

Metal replacement studies in carboxypeptidase demonstrated earlier that both cobalt and nickel form active carboxypeptidases, though their stabilities are lower than that of the native zinc enzyme (Coleman and Vallee, 1961). It is conceivable that metals present in pancreatic tissue might mutually replace one another in binding to the zymogen either *in situ* or during isolation, depending on the conditions employed, especially since the stability constants of metalloprocarboxypeptidase are one to three orders of magnitude smaller than those of the corresponding metallocarboxypeptidases (*vide infra*). This could account for the small variations in zinc content of different preparations, and for the presence of variable but often significant amounts of iron and nickel, especially since the sum of these three metals comes close to 1 g-atom/molecular weight of 87,000 (Table I). Since procarboxypeptidase is also observed as a dimer of subunits I and II (Brown *et al.*, 1963b), reference to the appropriate molecular weight introduces sufficient uncertainty to preclude a precise delineation of the stoichiometry of each of the metals in the native material. Binding studies are, therefore, best carried out on material *reconstituted* to reflect precise stoichiometry, as in the present instance.

The roles of calcium, if any, in stabilizing procarboxypeptidase structure or in the enzymatic function of the endopeptidase with chymotryptic-like activity (subunit II) are unknown at present. Activation of the zymogen in the presence of 0.1 M calcium is known to proceed more rapidly and to a higher level of carboxypeptidase activity than in its absence. In the presence of calcium the activity rapidly declined after its maximum had been reached. These effects of calcium ions remain unexplained (Cox *et al.* 1964).

The removal of calcium and the other metals from procarboxypeptidase does not cause disaggregation of the zymogen, as demonstrated by sedimentation and electrophoretic analyses. Tryptic activation of apoprocarboxypeptidase reconstituted with zinc *alone* does not appear to differ from that of the native zymogen (Figure 7), suggesting that calcium is not required for the activation process. In addition, reconstitution of apoprocarboxypeptidase or prolonged exchange with excess metals, followed by dialysis *vs.* metal-free buffers to remove the excess metal, has not given indications that more than 1 g-atom of metal is bound per mole of zymogen. Thus, the results are consistent with the existence of one reversible metal binding site of subunit I. The relation, if any, of calcium to the as yet unknown enzymatic function of subunit III will require attention as the role of this fraction comes under scrutiny. Metal analyses of the individual fractions obtained after dissociation of procarboxypeptidase (Brown *et al.*, 1963a; Neurath and Freisheim, 1966) may well give indications in this regard.

The preparation of a stable, metal-free apozymogen, which can interact reversibly with metal ions to form 1:1 complexes, has allowed a study of the metal binding site of procarboxypeptidase, and comparison with that of the active enzyme has yielded information concerning the potential role of the metal in the process of activation.

Zinc is bound to a sulfur and nitrogen of carboxypeptidase, sulfur being the predominant ligand (Vallee *et al.*, 1960b). Involvement of a sulfur in metal binding in procarboxypeptidase was confirmed through amperometric titration with silver (Table III). The single thiol group of apoprocarboxypeptidase[1] is titratable as a function of the mole fraction of zinc bound, and the sum of zinc and sulfhydryl groups remains close to unity, an index previously employed to indicate metal binding to a thiol group (Coombs *et al.*, 1964; Wintersberger *et al.*, 1965).

The difference of 0.8 unit in the pH dependence of zinc binding to procarboxypeptidase and carboxypeptidase, respectively (Figure 2), suggests that the stability constant of the zinc–proenzyme is significantly lower than that of the zinc enzyme. In view of the demonstration of a metal mercaptide linkage in both proteins (*vide supra*) the magnitude of this shift indicates the absence of the other, presumably nitrogenous ligand, postulated in the enzyme. On this basis it would be expected that the stability constants for other transition metal–apozymogen complexes would be significantly lower than those of the corresponding metallocarboxypeptidase, as is indeed observed (Table II). The *differences* between the stability constants of the metal substitutions of carboxypeptidase and procarboxypeptidase follow a sequence (Figure 8) similar to that of nitrogenous ligands with a series of divalent metal ions (Williams, 1959). This is consistent with the postulated lack of participation of a nitrogenous ligand in binding metals to the zymogen. We have previously employed the comparison of the sequence and magnitudes of the stability constants of the metal complexes with bidentate ligands which vary in the nature of their donor atoms with those of the corresponding metallocarboxypeptidases as a guide to the identification of the metal binding site of this enzyme (Vallee *et al.*, 1960b; Coleman and Vallee, 1960). The reversible metal binding to apoprocarboxypeptidase provides a system, closely similar to but not identical with apocarboxypeptidase, which extends such comparisons and allows a test of their predictive value. The zymogen–enzyme pair provides a useful parallel in this relatively unexplored area which attempts to join pertinent features of protein and coordination chemistry.

[1] The metal-binding properties of the zymogen and its ability to generate an active product are not affected by the reversible removal of and reconstitution of procarboxypeptidase with zinc. One SH group can be detected in the apozymogen and none in the zinc-reconstituted protein. In native procarboxypeptidase 0.3–0.6 SH group/mole have been found variably, apparently unrelated to metal binding. This mole fraction of SH can be partially alkylated with iodoacetic acid, or with *N*-ethylmaleimide and treated with *p*-mercuribenzoate, without affecting metal binding. Such experiments performed with [^{14}C]iodoacetamide or *N*-ethylmaleimide, and *p*-mercuribenzoate, failed to give evidence that the label incorporated into the native zymogen could be found in the resultant carboxypeptidase.

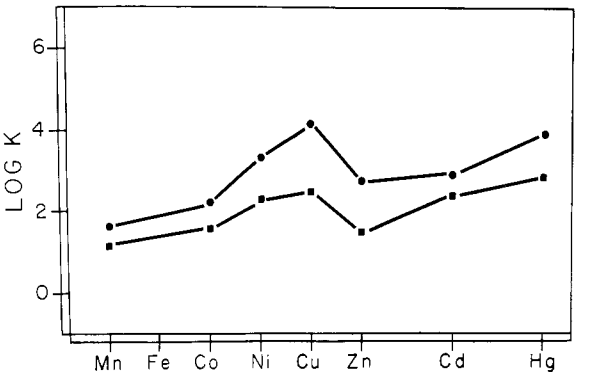

FIGURE 8: The log of the stability constants for metal–nitrogen complexes (●) compared with the difference between the corresponding complexes of metallocarboxypeptidases and metalloprocarboxypeptidases (■). The values of log K_1 are for NH_3 (Sillen and Martell, 1964), and those for Δ log K_{cor} are taken from Table II.

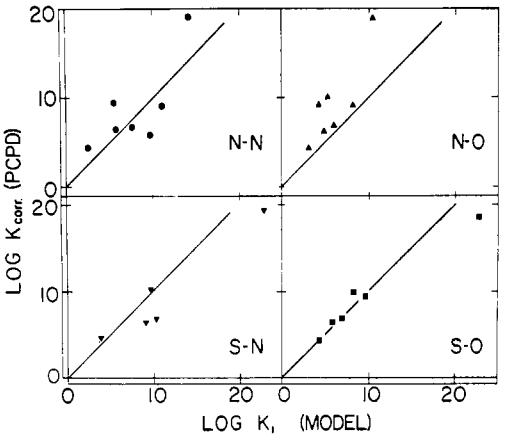

FIGURE 9: Correlation plots of log K_{cor} for procarboxypeptidase with log K_1 for bidentate model ligands. The values of log K_{cor} are taken from Table II, and corrected, in addition for Tris and chloride, for the ionization of the SH group of procarboxypeptidase. Log K_1 values are for ethylenediamine (●), glycine (▲), cysteine (▼), and mercaptoacetate (■) (Sillen and Martell, 1964).

Such comparisons of apparent stability constants suggest that in the zymogen the metal is bound to sulfur and to a ligand weaker than nitrogen. The apparent stability constants of metalloprocarboxypeptidases fail to correlate with those of metal mercaptides, of metal complexes with other monodentate ligands, or with metal complexes of bidentate ligands with nitrogen–nitrogen, nitrogen–oxygen, or sulfur–nitrogen binding sites (Figure 9). They do correlate well with those of sulfur–oxygen ligands, suggesting that an oxygen donor atom may coordinate with the metal in the zymogen. The present data do not reveal whether such an oxygen donor might bind to the metal in the enzyme, generating a tridentate rather than a bidentate binding site, or whether activation involves a O → N donor shift. The contribution of an oxygen donor to the stability constants of an S–N donor site, postulated in carboxypeptidase, could not be detected readily by the present procedure since the expected differences are within the limits of error of the method. However, the additional stability conferred on a monomercaptide by an oxygen donor is substantial due to the resulting chelating effect. The complexometric titrations would not reveal the dissociation of a proton from an oxygen donor site, *e.g.*, a carboxyl group, since its pK_{app} would be well below pH 7–9, the region in which the metal complex is fully formed, a prerequisite for unambiguous interpretation of this type of titration. Upon interaction of apocarboxypeptidase with zinc two protons are liberated at pH 7, and the pK values of the donor groups, 7.7 and 9.1, have been attributed to the ionization of the nitrogen and sulfur ligands, respectively (Coleman and Vallee, 1961). Since one of these ligands appears to be absent in procarboxypeptidase, the addition of Zn^{2+} would be expected to liberate only one proton at this pH,[2] a presumption in accord with the data (Figure 3). The apparent pK of the single donor group is 8.9, close to that of 9.1 found in carboxypeptidase.

Thus, the pH dependence of zinc binding, stability constants of different metallozymogen complexes, and displacement of hydrogen from the apozymogen by zinc are consistent with the interpretation that, compared to the enzyme, in the zymogen one less donor group is involved in binding metals. Comparisons of the stability constants of a series of metallocarboxypeptidases and chemical modifications have implicated an amino group as the nitrogenous ligand. The hypothesis that the zinc atom is bound to an α-amino group of carboxypeptidase was most consistent with data obtained by means of Edman degradation, dinitrophenylation, spectra of the pyridoxal-*P*–apocarboxypeptidase complex, and complexometric titrations (Coombs *et al.*, 1964). However, X-ray diffraction data on α-carboxypeptidase have been interpreted to show that the α-amino group is too far removed from the metal to be involved in binding in the crystalline state (Ludwig *et al.*, 1966). Acetylation of carboxypeptidase demonstrates that the acetyl zinc enzyme and the acetylapoenzyme differ by the reactivity of an ϵ-amino group toward fluorodinitrobenzene. Neither acetylation of the α-amino group nor of this ϵ-amino group prevents zinc binding, though the altered pH stability of the latter resultant product now resembles that of the weaker zinc binding in procarboxypeptidase where the SH group is the only one proven to bind the metal (Figures 1 and 2) (R. Piras,

[2] This titration had to be carried out in the absence of NaCl to achieve the binding of 1 g-atom of zinc to procarboxypeptidase at pH 7 (*vide supra*).

H. Kagan, and B. L. Vallee, in preparation). It remains difficult to assess the magnitude of the contribution by a nitrogenous donor group to the stabilization of the zinc–enzyme without further quantitative chemical data. The identification, by means of chemical modification in the presence and absence of zinc, of a nitrogen donor which binds the metal may be obscured by conformational changes *coincident* with the removal of the metal and by the lack of suitable models capable of mimicking the microscopic environment of the group in such proteins. The detailed analysis of this problem will be presented in a subsequent paper (R. Piras, H. Kagan, R. C. Davies, and B. L. Vallee, in preparation). Though past studies and present evidence do not implicate the "atypical sulfur residue" (Walsh *et al.*, 1962) in metal binding, its participation cannot be ruled out until more detailed information on its chemical identity is available.

Substitution of other metals for zinc induces different and characteristic enzymatic properties in the resultant metallocarboxypeptidases; cadmium and mercury carboxypeptidases do not hydrolyze peptides (Coleman and Vallee, 1961). This circumstance seemed to provide a means to examine in what manner metals might affect the activation process. Substitution of metals in the zymogen, followed by activation with trypsin, results in enzymatically active products, which exhibit activities characteristic of the particular metal (Table IV).

Interaction of metals with proteins has been postulated to stabilize protein structure (Fisher *et al.*, 1958; Rosenberg, 1960). The possibility exists that, in addition to imparting the final catalytic activity, the metal in procarboxypeptidase might also stabilize the protein during activation. Exposure of apoprocarboxypeptidase to trypsin, followed by reconstitution with excess zinc, does not yield an active product. Apparently, the nature of the metal does not modify the activation process (Table IV) but a metal atom must be present during tryptic activation to result in an active enzyme (Figure 7). Since trypsin hydrolyzes subunits II and III in addition to subunit I, it is at present difficult to study the details of this process with di- or trimeric procarboxypeptidase.

While zymogens are generally devoid of catalytic function, their capacity to bind substrates would seem to reflect enzymatic potential. Binding of substrates, substrate analogs, and inhibitors to chymotrypsinogen has been reported (Vaslow and Doherty, 1953; Erlanger, 1958; Weiner and Koshland, 1965; Glazer, 1965). More recently Deranleau and Neurath (1966) have presented evidence for the binding of substrate analogs both to chymotrypsinogen and to the active enzyme. Retardation of ^{65}Zn exchange in carboxypeptidase by substrates or inhibitors has been interpreted to indicate that they bind near to or at the active center (Coleman and Vallee, 1962a,b, 1964). Similarly, peptide or ester substrates of carboxypeptidase or inhibitors retard the exchange of ^{65}Zn–procarboxypeptidase (Figures 4 and 5), suggesting that significant features of the substrate binding site also exist in this zymogen. Neurath and Freisheim (1966) have arrived at similar conclusions independently and by different experimental means.

The characteristics of the compounds retarding this exchange in the enzyme are quite similar to those which are effective for the zymogen. In both proteins the substituted L-amino acids, *e.g.*, are more effective than the D isomers (Coleman and Vallee, 1963). However, the steric requirements of the procarboxypeptidase system seem to be less stringent since glycyl-D-leucine, *e.g.*, is ineffective for carboxypeptidase but prevents exchange with procarboxypeptidase. Collectively, these exchange data indicate that activation generates the final stereochemical specificity of the substrate binding site of the enzyme from the features that are already present in the zymogen.

In addition to metal substitutions in the zymogen, ultimately reflected in the activity of carboxypeptidase (Table IV), organic modifications of the zymogen, *e.g.*, acetylation, succinylation, also result in active enzymes with characteristics similar to those observed when the enzyme itself is chemically modified (Neurath and Freisheim, 1966). Thus, metal substitutions, organic modifications, substrate and inhibitor binding all indicate that significant aspects of the "active center" of carboxypeptidase are present in the zymogen. In this regard it is interesting to recall that procarboxypeptidase exhibits low esterase activity, even prior to tryptic activation (Yamasaki *et al.*, 1963). At present, the possibility cannot be dismissed that this activity is due to a fraction of molecules which are *partially* activated, even while retaining the molecular parameters of procarboxypeptidase A-S6. Alternatively, one might conjecture that some features of the active center of carboxypeptidase, already existent in the zymogen, allow the hydrolysis of some substrates, but that the ultimate capacity and specificity is acquired only on alterations of structure and metal coordination incident to activation. Studies of these problems with subunit I should further elucidate these questions.

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References

Benesch, R. E., Lardy, H. A., and Benesch, R. (1955), *J. Biol. Chem. 216*, 663.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.

Brown, J. R., Greenshields, R. N., Yamasaki, M., and Neurath, H. (1963a), *Biochemistry 2*, 867.

Brown, J. R., Yamasaki, M., and Neurath, H. (1963b), *Biochemistry 2*, 877.

Brush, J. S., Jensen, E. V., and Jacobsen, H. I. (1963), *Biochim. Biophys. Acta 74*, 688.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Coleman, J. E., and Vallee, B. L. (1961), *J. Biol. Chem.*

236, 2244.
Coleman, J. E., and Vallee, B. L. (1962a), *J. Biol. Chem. 237*, 3430.
Coleman, J. E., and Vallee, B. L. (1962b), *Biochemistry 1*, 1083.
Coleman, J. E., and Vallee, B. L. (1964), *Biochemistry 3*, 1874.
Coleman, J. E., Pulido, P., and Vallee, B. L. (1966), *Biochemistry 5*, 2019.
Coombs, T. L., Omote, Y., and Vallee, B. L. (1964), *Biochemistry 3*, 653.
Cox, D. J., Bovard, F. C., Bargetzi, J. P., Walsh, K. A., and Neurath, H. (1964), *Biochemistry 3*, 44.
Davie, E. W., and Neurath, H. (1955), *J. Biol. Chem. 212*, 515.
Deranleau, D. A., and Neurath, H. (1966), *Biochemistry 5*, 1413.
Desnuelle, P., and Rovery, M. (1961), *Advan. Protein Chem. 16*, 139.
Dreyer, W. J., and Neurath, H. (1955), *J. Biol. Chem. 217*, 527.
Erlanger, B. F. (1958), *Biochim. Biophys. Acta 27*, 646.
Fasman, G. D., Foster, R. J., and Beychok, S. (1966), *J. Mol. Biol. 19*, 240.
Fisher, E. H., Sumerwell, W. N., Junge, J. M., and Stein, E. A. (1958), *Proc. VIIIth Symp., IVth Intern. Congr. Biochem., Vienna, 1958.*
Fuwa, K., and Vallee, B. L. (1963), *Anal. Chem. 35*, 942.
Glazer, A. N. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 171.
Keller, P. J., Cohen, E., and Neurath, H. (1958), *J. Biol. Chem. 230*, 457.
Ludwig, M. L., Coppola, J. C., Hartsuck, J. A., Muirhead, H., Searl, J., Steitz, T. A., and Lipscomb, W. N. (1966), *Federation Proc. 25*, 346.
Neurath, H. (1955), *Discussions Faraday Soc. 20*, 257.
Neurath, H. (1964), *Federation Proc. 23*, 1.
Neurath, H., and Freisheim, J. H. (1966), *Federation Proc. 25*, 408.
Pechère, J. F., and Neurath, H. (1957), *J. Biol. Chem. 229*, 389.
Piras, R., and Vallee, B. L. (1966), *Federation Proc. 25*, 408.
Rosenberg, A. (1960), *Arkiv Kemi 17*, 25.
Rovery, M., Poilroux, M., Curmier, A., and Desnuelle, P. (1955). *Biochim. Biophys. Acta. 16*, 530.
Sandell, E. B. (1959), Colorimetric Determinations of Traces of Metals, 3rd ed, New York, N. Y., Interscience, p 522.
Sillen, L. G., and Martell, A. E. (1964), Stability Constants of Metal-ion Complexes, Special Publication No. 17, London, Chemical Society.
Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.
Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.
Vallee, B. L. (1955), *Advan. Protein Chem. 10*, 317.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1960a), *J. Biol. Chem. 235*, 64.
Vallee, B. L., Williams, R. J. P., and Coleman, J. E. (1960b), *Nature 190*, 633.
Vaslow, F., and Doherty, D. G. (1953), *J. Am. Chem. Soc. 75*, 928.
Walsh, K., Kumar, S., Bargetzi, J., and Neurath, H. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1443.
Weiner, H., and Koshland, D. E., Jr. (1965), *J. Biol. Chem. 240*, PC2764.
Williams, R. J. P. (1959), *Enzymes 1*, 391.
Wintersberger, E., Neurath, H., Coombs, T. L., and Vallee, B. L. (1965), *Biochemistry 4*, 1526.
Yamasaki, M., Brown, J. R., Cox, D. J., Greenshields, R. N., Wade, R. D., and Neurath, H. (1963), *Biochemistry 2*, 859.