Metallocarboxypeptidase-Substrate Complexes*

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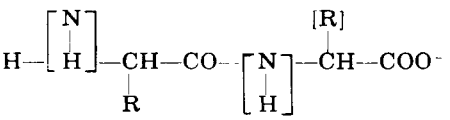
Unsubstituted and N-substituted dipeptides which are hydrolyzed by carboxypeptidase form stable apocarboxypeptidase-substrate complexes. Such peptide substrates prevent both the *association* and the *dissociation* of the metal ion at the active site. By means of a gel filtration technique, these studies have been extended to delineate the groups and stereospecificity of the substrate required for binding to the apoenzyme. Removal of any one of the three groups, indicated by brackets, from a typical dipeptide substrate of carboxypeptidase completely abolishes binding to the apoenzyme:



Such a dipeptide must be of the L-L configuration. The C-terminal free carboxyl group, an absolute requirement for hydrolysis of the peptide, is not required for formation of an apoenzyme-peptide complex. In marked contrast to such dipeptides, the ester and N-acylamino acid substrates for this enzyme do not prevent association of the metal ion; however, both prevent its *dissociation* as measured by kinetics and the effects of substrates on the exchange rates of metal isotopes. Each of these substrates lacks one of the two —NH— functions of the dipeptide. Thus, both of these —NH— functions seem involved in binding a substrate to the apoenzyme such that the resultant complex prevents association of the metal with its binding site.

Synthetic peptides which serve as substrates for native carboxypeptidase[1] interact with apocarboxypeptidase and prevent the restoration of activity by metal ions (Vallee, 1961; Felber *et al.*, 1962). These kinetic experiments have served as the model for a physicochemical method employing gel filtration to detect the existence of apoenzyme-peptide complexes and to measure their equilibrium constants (Coleman and Vallee, 1962a,b).

The present paper extends these studies to the interactions of apo- and metallocarboxypeptidases with a variety of other substrates. These experiments have defined the structural features of dipeptides which are required to form an apoenzyme complex. Esters and N-acylamino acids, however, form complexes only with the metalloenzyme, not with the apoenzyme. The free carboxyl group of a peptide substrate, by definition indispensible for hydrolysis by carboxypeptidase, is not an absolute requirement for its binding to the active center.[2]

MATERIALS AND METHODS

Beef-Pancreas Carboxypeptidase [(CPD) Zn][3].— Four-times recrystallized zinc carboxypeptidase was prepared from beef pancreas acetone powder[4] by the method of Allan *et al.*[5] The solution of the final crystals in 1.0 M NaCl–0.1 M Tris buffer, *p*H 7.5, was homogeneous in the ultracentrifuge and by moving boundary electrophoresis when examined in LiCl buffers of μ 0.3, *p*H 6.6 to 10.5. The proteolytic coefficient, *C*, of this preparation was 25 to 30 at *p*H 7.5, 25°. The esterase ac-

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[1] Carboxypeptidase and apocarboxypeptidase will refer to carboxypeptidase A and apocarboxypeptidase A throughout.

[2] The designation "active site" will refer specifically to the N—Me—S 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.

[3] The abbreviations used (in formulations only and when required for differentiation) are: [(CPD)-Zn], zinc carboxypeptidase, with (CPD) representing the apoenzyme and the brackets indicating the firm binding of zinc or other metals substituting for it, *e.g.* [(CPD)Cu].

[4] Kindly supplied by the Lilly Research Laboratories.

[5] B. J. Allan, P. J. Keller, and H. Neurath, unpublished.

tivity, expressed as a zero-order rate constant, k, was 1.15×10^3 μM H^+ per minute per mg of N at *p*H 7.5, 25°. The zinc to protein ratio was 1950 μg per g of protein or 1.03 g atom per mole, assuming a molecular weight of 34,300 for the protein (Smith and Stockell, 1954; Vallee and Neurath, 1955).

Peptidase activity was determined with carbobenzoxyglycyl-L-phenylalanine as described previously (Coleman and Vallee, 1960) and is expressed as an apparent proteolytic coefficient, C (Snoke and Neurath, 1949a; Coleman and Vallee, 1960). The assays were carried out at 25° in 0.05 M Tris buffer containing 1 M NaCl, *p*H 7.5; C was calculated from the linear portion of the first order reaction plots observed when hydrolysis did not exceed 15%. Initial substrate concentration was 0.02 M with an enzyme concentration of 0.00075 mg N/ml. The C values for the other peptide substrates mentioned in the text were obtained under similar conditions at *p*H 7.5, 25°, with an initial substrate concentration of 0.02 M. In experiments with the unsubstituted dipeptides the enzyme concentration was 0.075 mg N/ml. The peptides were chromatographically pure (Mann Research Laboratories, New York).

Esterase activity was determined as described (Snoke *et al.*, 1948; Coleman and Vallee, 1961). Assays were performed at 25° with 5 ml of 0.01 M hippuryl-*dl*-β-phenyllactate[6] in 0.1 M NaCl–0.005 M Tris buffer, *p*H 7.5. The activity, expressed as zero order velocity constants, k, with units of μM H^+ per minute per mg of N, was measured by titration of the hydrogen ions released on hydrolysis with 0.1 M NaOH with a *p*H Stat Titrator (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen).

Protein concentrations were measured either by 10% trichloroacetic acid precipitation followed by drying at 104° (Hoch and Vallee, 1953) or from absorbancy at 278 mμ (Davie and Neurath, 1955). The results of the two procedures were in excellent agreement. The Beckman model DU spectrophotometer was used throughout.

Zinc, cadmium, and copper carboxypeptidase were prepared as described (Coleman and Vallee, 1961). The spectrographically pure metals (Johnson Matthey Company, Ltd.) were dissolved in metal-free HCl and then diluted with metal-free buffer to the desired *p*H and molarity.

Metal Analyses.—Zinc, cadmium, and copper were determined by chemical methods which are specific for each (Vallee and Gibson, 1948; Saltzman, 1953; Adelstein *et al.*, 1956).

Metal Exchange by Equilibrium Dialysis.—The equilibrium dialysis technique, employed to exchange one metal ion for another at the active site of carboxypeptidase, has been described in detail (Coleman and Vallee, 1960, 1961). A metallocarboxypeptidase, 1×10^{-5} M, inactive against a particular substrate, was equilibrated against that substrate, 10^{-2} M. A second metal ion species, either radioactive or stable, was then added to start the exchange. The concentration of added metal was sufficient in each case to produce complete exchange as judged by the stability constants of the respective metallocarboxypeptidases and by a simultaneous control exchange in the absence of the substrate.

Gel filtration was carried out on 1 × 30 cm columns of G-25 Sephadex (Pharmacia, Uppsala) equilibrated with 1 M NaCl–0.05 M Tris buffer, *p*H 7.5, 4°. The flow rate of approximately 1 ml per minute was controlled by gravity, and 2 ml fractions were collected by an automatic fraction collector (Gilson Medical Electronics). The use of gel filtration both for the detection of apoenzyme-substrate complexes and for the determination of their equilibrium constants has been described (Coleman and Vallee, 1962b). Briefly, equimolar apocarboxypeptidase and Zn^{65++} ions instantaneously form zinc^{65} carboxypeptidase, and the complex is so stable that it does not dissociate on passage over the Sephadex column. On the other hand, if an appropriate peptide substrate is added to the apoenzyme prior to Zn^{65++} and the mixture is then passed over the column, very little [(CPD)Zn^{65}] is formed and the remainder of the Zn^{65++} appears as ionic zinc. The elution patterns for such mixtures were obtained as follows: 5×10^{-6} M apoenzyme was mixed with 10^{-2} M substrate, followed by the addition of 5×10^{-6} M Zn^{65++}, all dissolved in the buffer employed to equilibrate the column. The solution was mixed for 30 seconds, and a 1 ml aliquot applied to the column. Zn^{65++} was determined on the effluent by the counting of each fraction in a well-type scintillation counter (Tracerlab).

Results

Benzoylglycyl-L-phenylalanine, a typical N-substituted dipeptide substrate of carboxypeptidase, prevents the restoration of peptidase and esterase activities when added to apocarboxypeptidase prior to Zn^{++} ions, as shown for peptidase activity in Figure 1A. The prevention of the restoration of activity by the peptide substrate is a function of the concentration of the substrate in the incubation mixture. If the order of additions is reversed, and Zn^{++} ions are added to apocarboxypeptidase first, followed by benzoylglycyl-L-phenylalanine, activity is restored to that characteristic of the native enzyme which serves as the control.

The failure to restore activity in the presence of the peptide substrate is a consequence of the prevention of Zn^{++} binding at the active site of the enzyme, as can be shown under equilibrium conditions by means of the Sephadex technique (Fig. 1B). In the presence of 0.02 M benzoylglycyl-L-phenylalanine, only about 30% of the total apoenzyme, (CPD), combines with Zn^{65++} to form carboxypeptidase, [(CPD)Zn^{65}]. This effect is specific for many peptide substrates of

[6] Gift of Dr. H. Neurath.

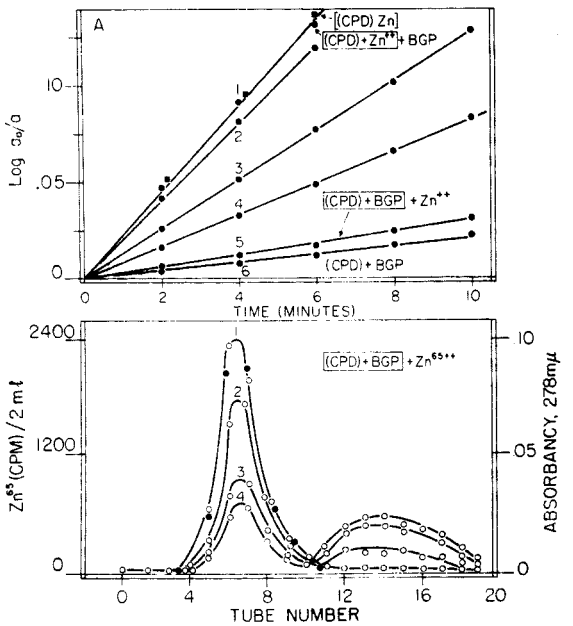


FIG. 1.—*A*, prevention of restoration of activity to apocarboxypeptidase, (CPD), by Zn^{++} ions as a function of the concentration of benzoylglycyl-L-phenylalanine (BGP). First-order progression curves: native zinc carboxypeptidase, [(CPD)Zn] (■); (CPD) + 0.04 M BGP (●, line 6) is the control; (CPD) + Zn^{++} + BGP (●, line 1) is (CPD) restored by equimolar Zn^{++} ions prior to the addition of 0.04 M BGP. Lines 2–5 represent restorations of activity when 0.005 M (line 2), 0.01 M (line 3), 0.02 M (line 4), and 0.04 M (line 5) BGP was added to (CPD) prior to Zn^{++} ions. (CPD), 5×10^{-6} M, plus BGP (concentrations as given), were preincubated for 2 minutes, then Zn^{++} ions, 5×10^{-6} M, were added. All components were in 1 M NaCl–0.05 M Tris, *p*H 7.5, 4°. Assays were started within 1 minute after mixing. Conditions: 0.10 ml aliquots of the incubation mixtures were assayed with 0.02 M carbobenzoxyglycyl-L-phenylalanine in 1 M NaCl–0.05 M Tris, *p*H 7.5, 4°. *B*, prevention of Zn^{65++} binding to apocarboxypeptidase, (CPD), by substrate, benzoylglycyl-L-phenylalanine (BGP). Samples of (CPD), 5×10^{-6} M, plus BGP (concentrations *vide infra*), were preincubated for 2 minutes, then Zn^{65++} ions, 5×10^{-6} M, were added. All components were in 1 M NaCl–0.05 M Tris, *p*H 7.5, 4°. After mixing for 1 minute, a 1-ml sample was passed over a 1 × 30 cm, G-25 Sephadex column at a flow rate of 1 ml per minute. Absorbancy at 278 mμ (●) and Zn^{65++} (○), counts per minute per 2 ml fraction, were determined on successive 2 ml fractions. BGP concentrations: curve 1: 0 (control, *no* substrate); curve 2: 0.005 M; curve 3: 0.01 M; curve 4: 0.02 M. Absorbancy at 278 mμ, similar in all experiments, is plotted only for curve 1, the control.

carboxypeptidase, but is not seen when the products of their enzymatic hydrolysis or their D isomers are used (Coleman and Vallee, 1962b).

Since the apoenzyme-substrate complex blocks the binding of Zn^{65++}, which combines only with the substrate-free apoenzyme, the degree of binding of Zn^{65++} to the apoenzyme as a function of substrate concentration is a measure of the position of the apoenzyme-substrate equilibrium (Fig. 1B). Zn^{65++} binding decreases as substrate concentration increases. Once the initial rapid reaction of Zn^{65++} with substrate-free apoenzyme has taken place, in the presence of 10^4 molar excess of substrate, further binding of Zn^{65++} is very slow during turnover of the apoenzyme-substrate complex. Additional significant Zn^{65++} binding does not take place during the first 30 minutes, as indicated by the linear reaction plots in Figure 1A. The initial Zn^{65++} binding is therefore a measure of the position of the apoenzyme-substrate equilibrium, and the equilibrium constant, K_s, for the apoenzyme-substrate complex can be calculated; for the benzoylglycyl-L-phenylalanine–apocarboxypeptidase complex it is 1.1×10^{-2} M (Fig. 2). Similarly, a binding constant, K_b, can be calculated from the activity data (Fig. 1A). The degree to which peptidase activity is restored should correspond to the amount of [(CPD)Zn^{65}] formed (Fig. 1B). Within the limits of the measurement, K_b determined in this manner is 1.1×10^{-2} M, identical with K_s (Fig. 2). Moreover, the same numerical value has been reported for the corresponding Michaelis constant, K_m, of the zinc enzyme (Snoke and Neurath, 1949a). Such data support the conclusion that the metal ion does not contribute significantly to the binding of L-L dipeptides (Coleman and Vallee, 1962a).

Carboxypeptidase also hydrolyzes the ester analogues of its synthetic peptide substrates (Snoke *et al.*, 1948) at approximately the same rate as the peptides (Table I). The restoration of activity to apocarboxypeptidase by Zn^{++} ions, however, is not affected by the sequence in which

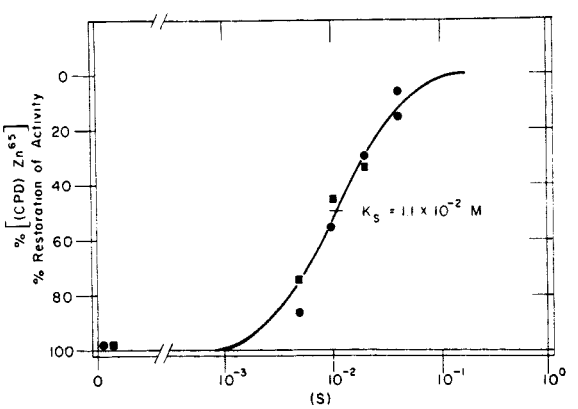


FIG. 2.—Determination of the equilibrium constant, K_s, for the apocarboxypeptidase-benzoylglycyl-L-phenylalanine complex. Per cent restoration of peptidase activity, ● (from Fig. 1A) and per cent formation of [(CPD) Zn^{65}], ■ (from Fig. 1B, calculated as described by Coleman and Vallee, 1962b) plotted as a function of benzoylglycyl-L-phenylalanine concentration, on a log scale. (CPD·Substrate)/(CPD) = 1 at a concentration of substrate = 1.1×10^{-2} M equal to the equilibrium constant, K_s. The Michaelis constant determined by Snoke and Neurath (1949a) was 1.1×10^{-2} M.

TABLE I

CARBOXYPEPTIDASE: SUSCEPTIBILITY OF ITS VARIOUS SUBSTRATES AND THEIR ANALOGUES TO HYDROLYSIS

All peptidase assays were performed with a substrate concentration of 0.02 M in 1 M NaCl–0.05 M Tris, *p*H 7.5, 25°. For the N-substituted dipeptides the enzyme concentration was 0.00075 mg N/ml and for the dipeptides and N-acetylphenylalanine it was 0.075 mg N/ml. For the substituted peptides the activity is expressed as the moles of substrate hydrolyzed per mole of enzyme in the first minute of reaction. For the dipeptides, the same per cent hydrolysis occurred only after 12 hours; the activities are expressed in terms of the hydrolysis occurring in 1 minute. Hydrolysis not detectable in 12 hours at an enzyme concentration of 0.075 mg N/ml is symbolized by 0.0.

Compound	Turnover Number[a] [(CPD)Zn] Moles S/ min/mole enz.
Carbobenzoxy-glycyl-L-phenylalanine	7100.0
Carbobenzoxy-glycyl-D-phenylalanine	0.0
glycyl-L-phenylalanine	1.2
L-leucyl-L-tyrosine	10.6
D-leucyl-L-tyrosine	10.2
N-acetyl-L-phenylalanine	2.2
N-acetyl-D-phenylalanine	0.0
Benzoyl-glycyl-L-phenylalanine	5200.0
Benzoyl-glycyl-L-phenyllactate (HPLA)[b]	5800.0
Glycyl-L-phenylalanine amide	0.0
Glycyl-L-tyrosine amide	0.0
Glycyl-glycine	0.0

[a] For purposes of comparison, the μmoles of free amino acid produced are expressed as moles substrate (*S*) per minute per mole enzyme. [b] The hydrolysis of benzoylglycyl-phenyllactate, HPLA (present in these experiments as the *dl* mixture) is restricted to one isomer (Snoke and Neurath, 1949a), presumably the L form.

the ester, hippuryl-β-phenyllactate, is added. Activity is fully restored whether the ester or the metal ions are added first (Fig. 3A), in contrast to the effect of the peptide analogue, benzoyl-glycyl-L-phenylalanine. Gel filtration confirms that hippuryl-β-phenyllactate does not affect the binding of Zn^{65++} to the apoenzyme (Fig. 3B).

Through substitution of groups in typical substrates, those which are essential for binding to the apoenzyme may be identified. The elution pattern for glycyl-L-phenylalanine (Fig. 4B) indicates that the removal of the carbobenzoxy group from carbobenzoxyglycyl-L-phenylalanine (Fig. 4A) has little effect on the ability of the peptide to prevent Zn^{65++} binding to the apoenzyme. This peptide also serves as a substrate for carboxypeptidase, although it is hydrolyzed over a thousand times more slowly than the carbobenzoxy derivative (Table I). However, the apoenzyme-substrate binding constants calculated for the two peptides show only a 2-fold

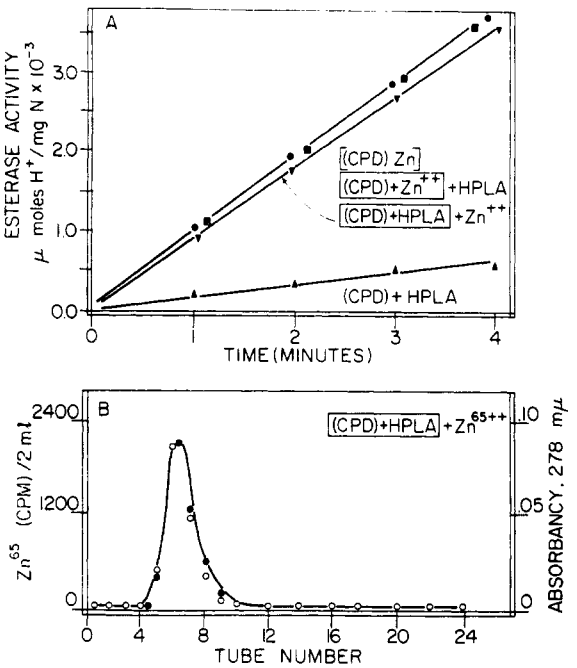


FIG. 3.—*A*, restoration of esterase activity to apocarboxypeptidase, (CPD), by Zn^{++} ions in the presence of hippuryl-β-phenyllactate (HPLA). Zero order progression curves: native zinc carboxypeptidase, [(CPD)Zn] (■); (CPD) + 0.01 M HPLA (▲) is the control; (CPD) + Zn^{++} + HPLA (●) is the apoenzyme restored by equimolar Zn^{++} ions prior to addition of 0.01 M HPLA to start the reaction; (CPD) + HPLA + Zn^{++} (▼) is the apoenzyme incubated for 1 minute with 0.01 M HPLA prior to the addition of Zn^{++} ions. Conditions of esterase assays: 1×10^{-6} M enzyme, 0.01 M *dl*-HPLA in 1 M NaCl–0.005 M Tris, *p*H 7.5, 25°. *B*, Zn^{65++} binding to (CPD) in the presence of HPLA. Conditions for preincubation as in Fig. 1B. A 1 ml sample of 5×10^{-6} M (CPD) plus 4×10^{-2} M *dl*-HPLA plus 5×10^{-6} M Zn^{65++} was passed over a 1 × 30 cm, G-25 Sephadex column at a flow rate of 1 ml per minute. Absorbancy at 278 mμ (●) and Zn^{65++} (○), counts per minute per 2 ml fraction, were determined on successive 2 ml fractions.

difference; 4.2×10^{-3} M and 9.5×10^{-3} M respectively.

In contrast to the removal of the carbobenzoxy group, removal of either the N-terminal amino group, as in N-acetyl-L-phenylalanine (Fig. 4C), or the C-terminal aromatic side chain, as in glycylglycine (Fig. 4D), completely abolishes the ability of these peptides to prevent binding of Zn^{65++} to the apoenzyme. Like other N-acylamino acids (Snoke and Neurath, 1949a), N-acetyl-L-phenylalanine is hydrolyzed, but glycylglycine is not (Table I).

In these experiments the formation of [(CPD)-Zn] from Zn^{++} ions and apocarboxypeptidase and its prevention have served as indices of substrate binding. The effect of substrates on the exchange of metals at the active site permits a different approach. It may be recalled that copper carboxypeptidase is completely inactive toward both

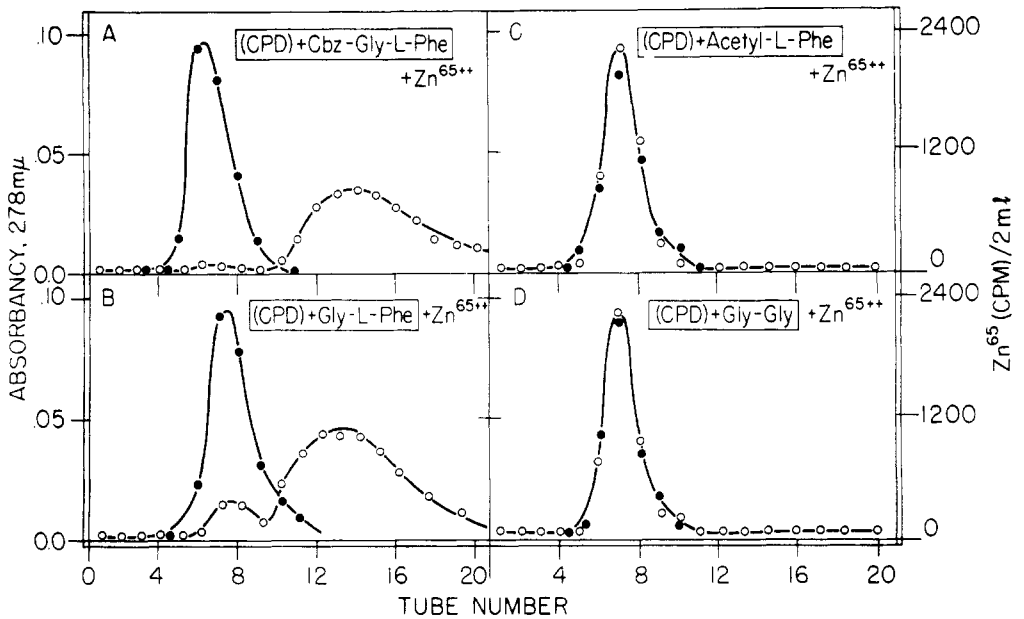


FIG. 4.—Prevention of Zn^{65++} binding to apocarboxypeptidase, (CPD), by carbobenzoxyglycyl-L-phenylalanine (CBz-Gly-L-Phe) and glycyl-L-phenylalanine (Gly-L-Phe) and binding of Zn^{65++} to apocarboxypeptidase in the presence of N-acetyl-L-phenylalanine (Acetyl-L-Phe) and glycylglycine (Gly-Gly). One ml samples of 5×10^{-6} M (CPD) plus 5×10^{-2} M Cbz-Gly-L-Phe plus 5×10^{-6} M Zn^{65++} (A); 5×10^{-6} M (CPD) plus 5×10^{-2} M Gly-L-Phe plus 5×10^{-6} M Zn^{65++} (B); 5×10^{-6} M (CPD) plus 5×10^{-2} M N-acetyl-L-Phe plus 5×10^{-6} M Zn^{65++} (C); and 5×10^{-6} M (CPD) plus 5×10^{-2} M Gly-Gly plus 5×10^{-6} M Zn^{65++} (D) were passed over a 1×30 cm, G-25 Sephadex column at a flow rate of 1 ml per minute. Absorbancy at 278 mμ (●) and Zn^{65++} (○), counts per minute per 2 ml fraction, were determined on successive 2 ml fractions. Conditions of preincubation as in Fig. 1B. Assays as described under "methods."

peptides and esters; cadmium, mercury, and lead carboxypeptidases only hydrolyze esters (Coleman and Vallee, 1961). When these enzymes are dialyzed against Zn^{++} or Zn^{65++} ions, both the appearance of peptidase activity and the time required for isotope replacement can measure the rate of exchange of these ions. The effects of substrates on these processes should reflect both in change of the rates at which activity is restored or the rates at which the isotopes of the elements will replace one another. When Zn^{++} ions are added to copper carboxypeptidase, activity is rapidly restored (Fig. 5). The addition of hippuryl-β-phenyllactate to the copper enzyme prior to that of Zn^{++} ions prevents the restoration of activity (Fig. 5). Since the ester does not affect the association of Zn^{++} with the apoenzyme (Fig. 3), it apparently prevents the *dissociation* of copper from the active site. Dialysis experiments were employed to examine this. Hippuryl-β-phenyllactate and N-acetyl-L-phenylalanine, both substrates for the native enzyme, prevent the dissociation of the inactive metal ion from the active site (Fig. 6A and 6B). Glycyl-glycine and the D isomer of N-acetyl-phenylalanine are not hydrolyzed by carboxypeptidase; they do not affect the dissociation of the metal ion (Fig. 6C and 6D).

The L configuration of the C-terminal residue

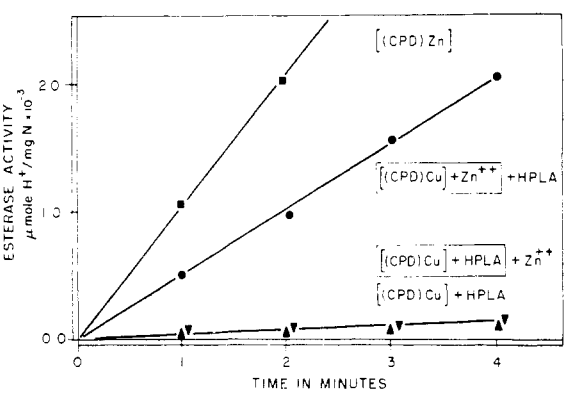


FIG. 5.—Prevention of restoration of esterase activity to copper carboxypeptidase, [(CPD)Cu], by Zn^{++} ions in the presence of hippuryl-β-phenyllactate, HPLA. Zero-order progression curves: native zinc carboxypeptidase, [(CPD)Zn] (■); copper carboxypeptidase plus 0.01 M *dl*-HPLA, [(CPD)Cu] + HPLA, (▲) is the control; [(CPD)Cu] + Zn^{++} + HPLA (●) is the copper carboxypeptidase incubated for 1 minute with equimolar Zn^{++} ions prior to the addition of 0.01 M *dl*-HPLA to start the reaction; [(CPD)Cu] + HPLA + Zn^{++} (▼) is the copper enzyme incubated for 1 minute with 0.01 M *dl*-HPLA prior to the addition of Zn^{++} ions to start the reaction. Esterase reactions were performed with 1×10^{-6} M enzyme, 0.01 M *dl*-HPLA in 1 M NaCl–0.005 M Tris, *p*H 7.5, 25°.

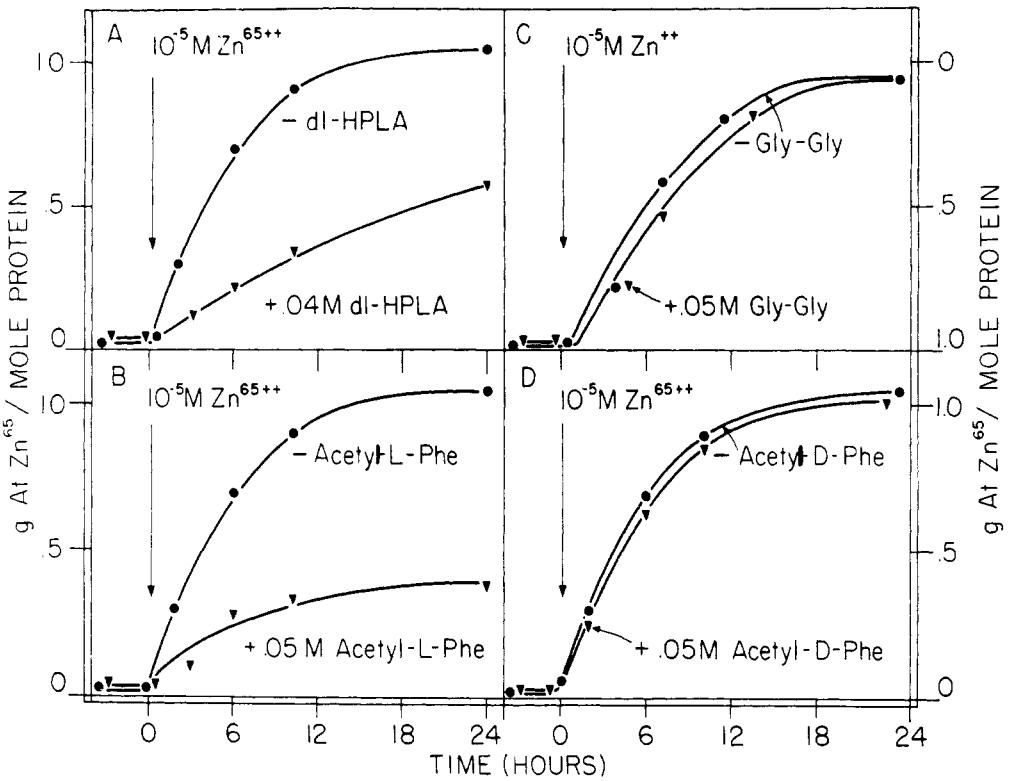


FIG. 6.—Exchange of Zn^{65++} for copper in [(CPD)Cu] or Zn^{++} for zinc^{65} in [(CPD)Zn^{65}] in the presence (+) and absence (−) of *dl*-hippuryl-β-phenyllactate (HPLA), N-acetyl-L-phenylalanine (Acetyl-L-Phe), glycylglycine (Gly-Gly), and N-acetyl-D-phenylalanine (Acetyl-D-Phe). As controls, 1×10^{-5} M [(CPD)Cu] (A, B, and D) or [(CPD)Zn^{65}] (C) were exposed to 1×10^{-5} M Zn^{65++} (A, B, and D) or Zn^{++} (C) at 0 time and the Zn^{65++} bound by the enzyme (●) measured as dialysis progressed. 1×10^{-5} M [(CPD)Cu] (A, B, and D) or 1×10^{-5} M [(CPD)-Zn^{65}] (C) were equilibrated for 2 hours with 4×10^{-2} M *dl*-HPLA (A), 5×10^{-2} M N-acetyl-L-Phe (B), 5×10^{-2} M N-acetyl-D-Phe (D), or 5×10^{-2} M Gly-Gly (C) followed by the addition of 1×10^{-5} M Zn^{65++} (A, B, and D) or 1×10^{-5} M Zn^{++} (C) at 0 time. Zn^{65++} bound by the enzyme (▼) was measured as dialysis progressed.

of the substrate, an absolute requirement for hydrolysis, is essential to prevent both the association and the dissociation of the metal ion at the active site (Coleman and Vallee, 1962b; Fig. 6B and 6D). In contrast, the configuration of the N-terminal residue is less critical. While both the L-L and D-L forms prevent dissociation of the metal ion from the binding site, only the L-L configuration prevents association of the metal ion as illustrated by the diastereoisomers L-leucyl-L-tyrosine and D-leucyl-L-tyrosine (Fig. 7A–D). The native enzyme hydrolyzes both compounds (Yanari and Mitz, 1957; Table I).

Although considerably less effective than glycyl-L-tyrosine,[7] glycyl-L-tyrosine amide, even though it is not hydrolyzed, still prevents the binding of a significant fraction of Zn^{65++} to apocarboxypeptidase (Table I) (Fig. 8A). The equilibrium constants, K_s, of this peptide and its amide derivative are 1.2×10^{-3} and 1.0×10^{-1} M respectively. Glycyl-L-phenylalanine amide increases the half-time for the exchange of stable Zn^{++} with the Zn^{65} of [(CPD)Zn^{65}] from 5 to 28 hours (Fig. 8B). By comparison, glycyl-L-phenylalanine increases the half-time of the exchange of Zn^{65++} for Cu^{++} of [(CPD) Cu] from 3 to over 100 hours.

[7] Gift of Dr. M. Hunter.

DISCUSSION

The synthetic dipeptide and carbobenzoxy dipeptide substrates of carboxypeptidase form stable apoenzyme-substrate complexes (Fig. 1 and 2; Table II, lines 1a and 2). The experimental and theoretical considerations leading to this conclusion have been detailed (Coleman and Vallee, 1962b). The results reported for benzoyl-glycyl-L-phenylalanine further document and detail the manner in which equilibrium constants of these apoenzyme-substrate complexes may be determined, through the measurement either of activity or of zinc binding (Fig. 1 and 2). The formation of a complex between the active center and L-L dipeptide substrates does not require a metal ion, though in its absence these substrates are not hydrolyzed, of course.

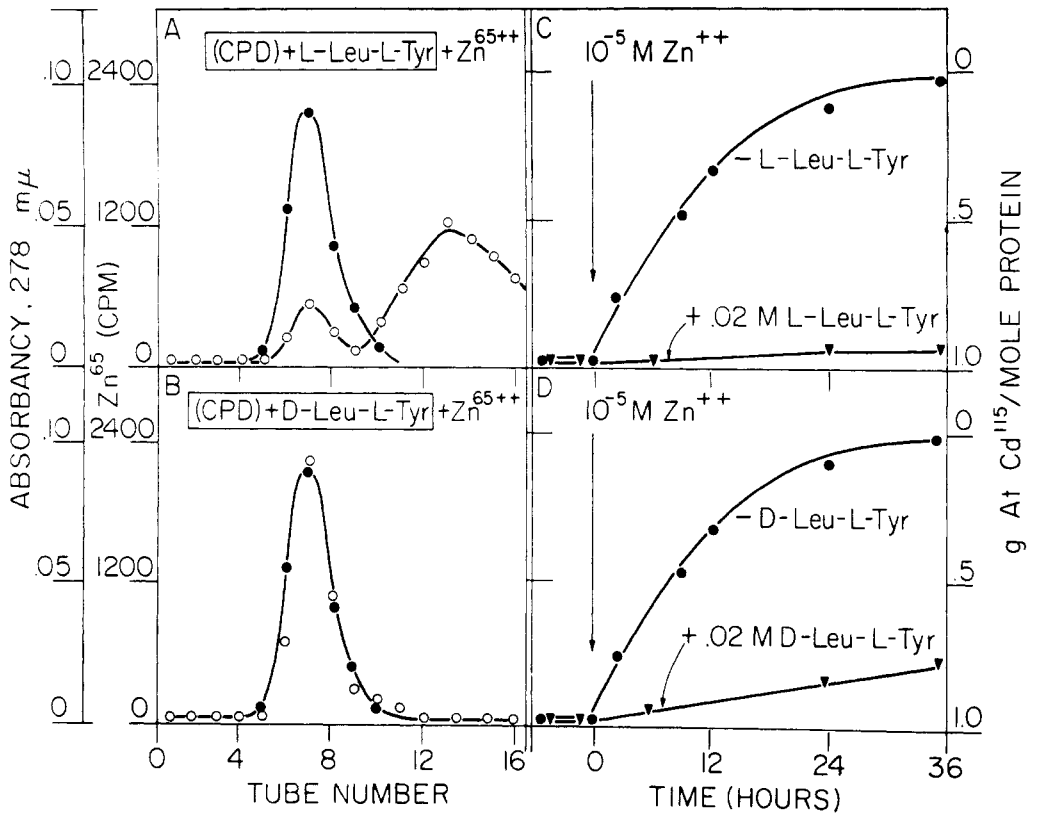


FIG. 7.—*A* and *B*, prevention of Zn^{65++} binding to apocarboxypeptidase, (CPD), by L-leucyl-L-tyrosine (L-Leu-L-Tyr) and binding of Zn^{65++} to apocarboxypeptidase in the presence of D-leucyl-L-tyrosine (D-Leu-L-Tyr). One ml samples of 5×10^{-6} M (CPD) plus 2×10^{-2} M L-Leu-L-Tyr plus 5×10^{-6} M Zn^{65++} (A); and 5×10^{-6} M (CPD) plus 2×10^{-2} M D-Leu-L-Tyr plus 5×10^{-6} M Zn^{65++} (B) were passed over a 1×30 cm, G-25 Sephadex column at a flow rate of 1 ml per minute. Absorbancy at 278 mμ (●) and Zn^{65++} (○), counts per minute per 2 ml fraction were determined on successive 2 ml fractions. Conditions of preincubation as in Fig. 1B. *C* and *D*, exchange of Zn^{++} for cadmium^{115} in [(CPD)Cd^{115}] in the presence (+) and absence (−) of L-Leu-L-Tyr and D-Leu-L-Tyr. As controls, 1×10^{-5} M [(CPD)Cd^{115}] was exposed to 1×10^{-5} M stable Zn^{++} at 0 time and the Cd^{115++} bound to the enzyme (●) measured as dialysis progressed. 1×10^{-5} M [(CPD)Cd^{115}] was equilibrated for 2 hours with 2×10^{-2} M L-Leu-L-Tyr (C) or 2×10^{-2}M D-Leu-L-Tyr (D) followed by the addition of 1×10^{-5} M Zn^{++} at 0 time. Cd^{115++} bound to the enzyme (▼) was measured as dialysis progressed.

Like other pancreatic peptidases, carboxypeptidase also functions as an esterase. An ester substrate of the enzyme, hippuryl-β-phenyllactate, a direct analogue of benzoylglycyl-L-phenylalanine, does not prevent the restoration of esterase or peptidase activities, and hence does not form an apoenzyme-substrate complex, on the basis of criteria employed here and previously[8]; in the ions. These rates may be measured either by isotopic or chemical determinations of the exchanging species or by the appearance or disappearance of the enzymatic activities, characteristically associated with the exchanging metal ions. The equilibrium dialysis techniques which are employed for such measurements have been previously described (Coleman and Vallee, 1960, 1961). Although a marked effect on metal binding appears to be a characteristic index of the formation of substrate complexes with the active center of carboxypeptidase, failure to detect a complex by these methods cannot be considered proof that one does not form. Although binding studies with labeled substrates of carboxypeptidase are not on record, experiments with C^{14} phenylacetate, a specific competitive inhibitor of the enzyme, set the pattern for the type of experiments which will likely prove conclusive. C^{14} phenylacetate only binds to the metalloenzyme, not to the apoenzyme (Rupley and Neurath, 1960). Experiments with the gel filtration method lead to the same conclusion (Coleman and Vallee, unpublished observations).

[8] Apoenzyme-substrate and metalloenzyme-substrate complexes are here defined in terms of the methods employed, *i.e.*, operationally. An *apocarboxypeptidase-substrate complex* is said to exist when the substrate measurably prevents either restoration of activity or metal binding to apocarboxypeptidase, as determined by means of the gel filtration technique previously described (Coleman and Vallee, 1962b). A *metallocarboxypeptidase-substrate complex* is said to exist when the substrate alters either the rate of dissociation of a metal ion from the active site or the rate at which it exchanges with a species of free metal

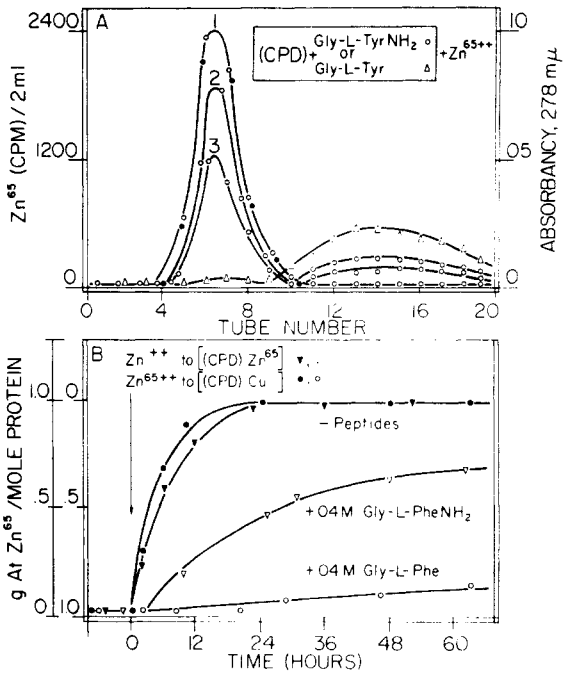


FIG. 8.—*A*, prevention of Zn^{65++} binding to apocarboxypeptidase, (CPD), by glycyl-L-tyrosine amide (Gly-L-TyrNH$_2$) and glycyl-L-tyrosine (Gly-L-Tyr). One ml of (CPD), 5×10^{-6} M, plus Zn^{65++}, 5×10^{-6} M, is the control (curve 1). The same concentration of (CPD) was exposed to gly-L-TyrNH$_2$, 0.05 M (curve 2) and 0.1 M (curve 3), and then to Zn^{65++}, 5×10^{-6} M. (CPD), 5×10^{-6} M, was also exposed to Gly-L-Tyr, 0.05 M, followed by the addition of Zn^{65++}, 5×10^{-6} M. All samples were passed over a 1×30 cm, G-25 Sephadex column and absorbancy at 278 mμ (●) and Zn^{65++} in the presence of Gly-L-TyrNH$_2$(○) or Gly-L-Tyr (△), counts per minute per 2 ml fraction, were determined on successive 2 ml fractions. Absorbancy at 278 mμ, similar in all experiments, is plotted only for the control. Conditions of preincubation as in Fig. 1B. *B*, exchange of Zn^{++} for zinc^{65} in [(CPD)Zn^{65}] or Zn^{65++} for copper in [(CPD)Cu] in the presence (+) and absence (−) of Gly-L-PheNH$_2$ and Gly-L-Phe. As controls, 1×10^{-5} M [(CPD)Zn^{65}] was exposed to 1×10^{-5} M Zn^{++} or 1×10^{-5} M [(CPD)Cu] was exposed to 1×10^{-5} M Zn^{65++} at 0 time, and the Zn^{65++} either remaining bound (▼) or being bound (●) by the enzyme was measured as dialysis progressed. Experiments in the presence of Gly-L-PheNH$_2$ (▽) and Gly-L-Phe (○) were conducted under identical conditions.

presence of this ester the Zn^{++} ion has free access to the metal binding site (Fig. 3A; Table II, line 3). Hippuryl-β-phenyllactate, however, does prevent the dissociation of the metal atom from the protein. The enzymatically inactive copper enzyme, for example, rapidly acquires enzymatic activity when exposed to Zn^{++} ions (Fig. 5), owing to exchange of Zn^{++} ions for copper at the active site (Fig. 6A); hippuryl-β-phenyllactate completely prevents this exchange (Fig. 5 and 6A). Apparently, esters only form complexes with metallocarboxypeptidases, though this does not necessarily demonstrate binding of the ester to the metal. Since the substitution of the ester oxygen for the —NH— group of the C-terminal residue is the only structural difference between benzoylglycyl-L-phenylalanine and hippuryl-β-phenyllactate, the —NH— group of the peptide must play a crucial role in preventing access of metals to their protein binding site.

The peptide, benzoylglycyl-L-phenylalanine, and the ester, hippuryl-β-phenyllactate, each represent one of the two characteristic specificities of the enzyme. It might, therefore, be surmised that these differences in the formation of peptide and ester complexes of the enzyme are also an expression of distinctive mechanisms which account for the characteristic specificities. However, the removal of the —NH— function at the N-terminal end of the peptide destroys the formation of an apoenzyme-substrate complex though the metalloenzyme-substrate complex forms in precisely the same manner as is observed on replacement of the peptide —NH— by an ester oxygen (Table II, lines 3 and 4). The removal of the R group substituted in the α-carbon of the C-terminus completely abolishes the formation of either complex (Table II, line 5). Simultaneously, all activity is destroyed.

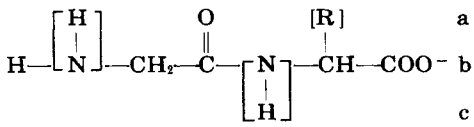
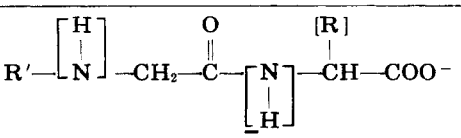
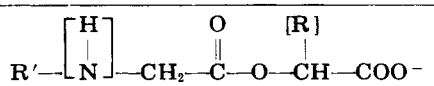
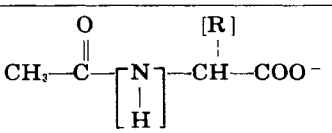
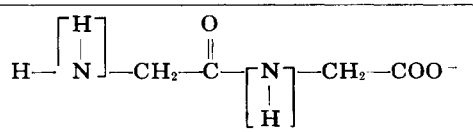
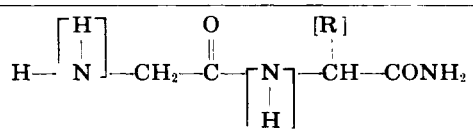
Bergmann, Fruton, Smith, Neurath, and their collaborators have previously delineated the specificity of carboxypeptidase on the basis of those structural and conformational features of synthetic substrates which are critical for their hydrolysis (Bergmann, 1935–36; Bergmann and Fruton, 1942; Stahmann *et al.*, 1946; Smith, 1948; Neurath and Schwert, 1950; Smith, 1951). In a similar manner those characteristics of substrates may be discerned which are required for the formation of the apoenzyme or metalloenzyme complexes or both. For any given substrate pairing of its rate of hydrolysis with the amount of its enzyme-complex formed demonstrates, moreover, that an apoenzyme or metalloenzyme complex does not necessarily result in hydrolysis. The magnitude of the binding constant, K_s, does not determine the rate of hydrolysis, of course.

The carbobenzoxy group, substituted in the N-terminal residue, is not required for binding to the enzyme; unsubstituted dipeptides bind virtually as well to the apoenzyme as their N-substituted derivatives (Fig. 4A and B; Table II, lines 1 and 2). However, the dipeptides are hydrolyzed at rates a thousand times less than the carbobenzoxy derivatives (Table I). Thus, the N-substitution profoundly affects the catalytic rather than the binding step. This same conclusion was originally reached on the basis of kinetic studies (Izumiya and Uchio, 1959).

Whereas L-L dipeptides form an apoenzyme complex, those in the D-L configuration bind only to the metalloenzyme; peptides of both configurations are hydrolyzed (Fig. 7A, B, D; Table I) (Yanari and Mitz, 1957). The N-acylamino acids behave completely analogously to the D-L dipeptides (Fig. 4C, 6B; Tables I and II, line 4).

TABLE II

APOCARBOXYPEPTIDASE- AND METALLOCARBOXYPEPTIDASE-SUBSTRATE AND SUBSTRATE ANALOGUE COMPLEXES[a] AND THEIR SUSCEPTIBILITY TO HYDROLYSIS AS A FUNCTION OF SUBSTRATE STRUCTURE AND CONFORMATION

	Compound	Structure[a]			Apo-enzyme-Substrate Complex	Metallo-enzyme-Substrate Complex	Susceptibility to Hydrolysis by [(CPD)Zn]
1	Dipeptide		a	L-L	+	+	+
			b	D-L	0	+	+
			c	L-D	0	0	0
2	N-Substituted dipeptide				+	+	+
3	Ester				0	+	+
4	N-Acylamino acid				0	+	+
5	Peptide without R				0	0	0
6	Amidated peptide				+	+	0

[a] R = aromatic or branched aliphatic side-chain. R′ = benzoyl or carbobenzoxy substituent.

Comparison of their structures suggests that the N-terminal $-NH_2$ group[9] plays a significant role in the formation of the apoenzyme-substrate complex, though it is not essential for hydrolysis (Table II, line 4). This circumstance, no doubt, accounts for the fact that the D-L peptides are hydrolyzed even though the N-terminal $-NH_2$ function is turned out of plane. The L configuration of the C-terminal substrate residue, in contrast, is an absolute requirement both for hydrolysis and formation of an enzyme complex (Coleman and Vallee, 1962b) (Fig. 6B, D; Table I).

Table II summarizes these facts. Three groups of the substrate are required minimally to form an apocarboxypeptidase-substrate complex: the —NH— function of the C-terminal residue, the —NH— function of the penultimate amino acid, and the R group on the α-carbon of the C-terminal residue. The abolition or substitution of either of the two —NH— functions results in the formation of a metalloenzyme-substrate complex and hydrolysis even though an apoenzyme complex can no longer be formed. Removal of the R group on the α-carbon, long known to be an absolute requirement for hydrolysis of any peptide by this enzyme (Neurath and Schwert, 1950), abolishes the formation of both complexes as well as activity.

It is of some interest that an enzyme-peptide complex can form even though the second fundamental structural requirement for enzymatic

[9] Studies of the binding of unsubstituted dipeptide substrates to the apoenzyme over the *p*H range 6.0 to 9.0 by the Sephadex method show little variation in the degree of formation of the apoenzyme-substrate complex from *p*H 6.0 to 8.0. This range suggests that the N-terminal group may be either in the NH_2 or NH_3^+ form without inducing a change in the affinity of the substrate for the apoenzyme. The hydrolytic rates of unsubstituted dipeptides, however, do exhibit a *p*H optimum which is shifted toward alkaline values (Izumiya and Uchio, 1959), suggesting a definite effect of charge on the hydrolytic mechanism. The close numerical similarity of the K_s values of N-substituted and unsubstituted peptides suggests that the —NH— function, common to both, is essential for the formation of their apoenzyme-substrate complexes.

specificity of this enzyme is violated: a free carboxyl group on the C-terminal residue (Waldschmidt-Leitz, 1934; Anson, 1937; Hofmann and Bergmann, 1940; Neurath and Schwert, 1950; Smith, 1951). Hydrolysis no longer occurs when the C-terminal carboxyl group is amidated (Table I); nevertheless such peptides form both apoenzyme and metalloenzyme complexes (Fig. 8; Table II, line 6). Thus, the free carboxyl group, an absolute requirement for the hydrolytic step, is not needed for the enzyme-"substrate" complex, though when amidated these peptides are bound less firmly (Fig. 8).

The importance of two consecutive —NH— functions of the peptide substrate in the formation of apoenzyme-substrate complexes suggests the participation of hydrogen bonding. On the basis of kinetic studies, several investigators have postulated such bonds for the formation of the α-chymotrypsin-substrate complex (Snoke and Neurath, 1949b; Kaufmann and Neurath, 1949; Bender and Turnquest, 1955). The elucidation of the complementarity of protein and substrate structure and conformation, now in progress, should permit precise delineation of this chemical process in carboxypeptidase. The present data do indicate the specific chemical structures of the substrate which are responsible for its interaction with the active center of carboxypeptidase.

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