Kinetics of Carboxypeptidase A. I. Hydrolysis of Carbobenzoxyglycyl-L-phenylalanine, Benzoylglycyl-L-phenylalanine, and Hippuryl-*dl*-β-phenyllactic Acid by Metal-Substituted and Acetylated Carboxypeptidases*

Richard C. Davies, James F. Riordan, David S. Auld,† and Bert L. Vallee

ABSTRACT: The hydrolysis of carbobenzoxyglycyl-L-phenylalanine (CGP) and benzoylglycyl-L-phenylalanine (BGP) is catalyzed by manganese, cobalt, nickel, and zinc carboxypeptidases but not by the cadmium, mercury, and copper derivatives. The active metallopeptidases are subject to both substrate activation and inhibition with CGP and to substrate activation with BGP. The ester analog, hippuryl-*dl*-β-phenyllactic acid (HPLA), is hydrolyzed by manganese, cobalt, nickel, zinc, cadmium, and mercury carboxypeptidases, and substrate inhibition is observed. Metal replacement alters the concentration range over which activation or inhibition by substrate is manifested, but it does not eliminate these effects. Hence, the anomalies do not appear to be attributable to the nature of the particular metal at the active site alone. The hydrolysis of CGP and HPLA by acetyl zinc and cadmium carboxypeptidases has been examined over an extended range of substrate concentration. Irrespective of the CGP concentration, up to 0.18 M, peptidase activity of the acetyl zinc enzyme is reduced to 3% of that for the native enzyme. The observed increase in esterase activity, six to seven times that of the native enzyme when assayed at 0.01 M HPLA, is characterized by a 30-fold increase in K_m, a 30% increase in V_{max}, and a displacement of substrate inhibition to a higher concentration range. The complex substrate–velocity profiles of the different metallo- and organically modified carboxypeptidases may relate to the particular dipeptide or ester substrates employed in the present kinetic analysis. The activation of peptidase and inhibition of esterase activities by products is reported.

Native carboxypeptidase A hydrolyzes both peptides and esters, akin to other pancreatic peptidases. The kinetics of the hydrolysis of both types of substrates deviate from classical Michaelis–Menten kinetics and are known to exhibit substrate inhibition and activation when assayed in solution (Elkins-Kaufman and Neurath, 1948, 1949; Lumry *et al.*, 1951; Lumry and Smith, 1955; McClure *et al.*, 1964; Bender *et al.*, 1965; Whitaker *et al.*, 1966; McClure and Neurath, 1966; Kaiser and Carson, 1964, 1965; Kaiser *et al.*, 1965; Carson and Kaiser, 1966; Awazu *et al.*, 1967). For carbobenzoxyglycyl-L-phenylalanine substrate inhibition has also been observed in the crystalline state (Quiocho and Richards, 1966). The observed kinetic anomalies may arise either from intrinsic features of the enzyme, the properties of the substrates employed, or both. We have begun to examine a number of inorganically and organically modified carboxypeptidases in which either the zinc atom has been replaced by other metals, the active center tyrosyl residues have been acetylated, or both, with the objective of identifying derivatives of the enzyme, substrates, and appropriate combinations of these which might be most suitable for further studies. Some of the properties of the various metallocarboxypeptidases and of acetyl and cadmium enzymes have been reported previously (Vallee, 1964; Coleman *et al.*, 1966). The kinetic parameters for these metalloenzymes have been examined and compared to those of the native zinc enzyme, since their characteristic activities were previously studied only at the substrate concentrations initially suggested (Snoke *et al.*, 1948) and since then conventionally employed for assay of the native enzyme (Coleman and Vallee, 1960). Preliminary reports of the present data have been made (Riordan *et al.*, 1965a; Vallee, 1967).

Materials and Methods

Beef Pancreas Carboxypeptidase.[1] Four-times-recrystallized zinc carboxypeptidase A was prepared from beef pancreas acetone powder by the method of Allan *et al.* (1964). The crystals were washed three times with deionized, distilled water and dissolved either

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received December 12, 1967.* This work was supported by Grant-in-Aid HE-07297 from the National Institutes of Health of the Department of Health, Education, and Welfare.

† American Cancer Society fellow.

[1] The abbreviations for the metalloenzymes are used in the formulation and figures only and when required for differentiation. [(CPD)Zn], zinc carboxypeptidase A; with (CPD) representing apoenzyme and the brackets indicating the firm binding of zinc or other metals to it, *e.g.*, [(CPD)Me] refers to the metallo

in 1 M NaCl–0.05 M Tris (pH 7.5) or in 1 M NaCl–0.02 M Veronal (pH 7.5) to yield stock solutions of about 1×10^{-4} M. The apparent proteolytic coefficients (C) of the preparations employed were 32 ± 2 at pH 7.5, 0° (corresponding to turnover numbers, V_0/e = 1380–1570/min), and the esterase activity, expressed as a turnover number or as a zero-order rate constant (k) was 6.6 ± 0.15 × 10^3 equiv of H^+/min per mole of enzyme at pH 7.5, 25°, when assayed using the standard substrates 0.02 M carbobenzoxyglycyl-L-phenylalanine (CGP) and 0.01 M hippuryl-*dl*-β-phenyllactic acid (HPLA). The zinc content of the native enzyme preparations varied from 0.96 to 1.00 g-atom per mol wt 34,600 (Bargetzi *et al.*, 1963).

Apocarboxypeptidase. Metal-free apocarboxypeptidase was prepared by dialysis against 1,10-phenanthroline (pH 7.5) followed by dialysis *vs.* metal-free buffer (Coombs *et al.*, 1964). All apoenzyme preparations employed contained less than 1% zinc, as measured by atomic absorption spectrophotometry (Fuwa *et al.*, 1964), and were essentially inactive toward either CGP or HPLA.

Metallocarboxypeptidases. Apoenzyme samples (10^{-4} M) in Visking-Nojax dialysis bags were equilibrated at 0° with a 100-fold volume excess of 10^{-3} M metal ion solution in either 1 M NaCl–0.02 M Veronal (pH 7.5) or 1 M NaCl–0.05 Tris (pH 7.5). The enzymes were stored in this manner until use. Enzyme was diluted to 10^{-5} M with metal solutions (10^{-3} M) in order to ensure greater than 99% formation of the metalloenzymes, as judged by their stability constants (Coleman and Vallee, 1961). Each metalloenzyme prepared in this fashion displayed activity characteristic of the particular metal substituted for zinc (Coleman *et al.*, 1966).

Acetyl Metallocarboxypeptidases. The zinc and cadmium carboxypeptidases (10^{-4} M) were acetylated with a 100-fold M excess of *N*-acetylimidazole in 0.02 M Veronal –1.0 M NaCl (pH 7.5) for 1 hr at 25°. Solutions were then diluted fivefold with 1 M NaCl–0.02 M Veronal buffer (pH 7.5) and the excess reagent and its breakdown products were removed by dialysis at 4° against three or four changes of a 20-fold volume excess of buffer during a 15-hr period. Under these conditions approximately five tyrosyl residues are known to be acetylated including two at the active center (Simpson *et al.*, 1963). Metalloenzymes acetylated in this manner have little or no peptidase activity but exhibit increased esterase activity when measured at 0.01 M HPLA, the concentration employed routinely for assay. With the acetylated enzymes Tris buffers were avoided because of their potential deacylating action on *O*-acetyltyrosyl groups (Riordan *et al.*, 1965b).

Metal Solutions. Standard solutions of cobalt, nickel, copper, and zinc chlorides were prepared by dissolving the spectrographically pure metals in dilute, metal-free HCl. Cadmium and mercury chlorides and manganese sulfate solutions were prepared from the spectrographically pure metal salts obtained from Johnson Matthey Co., Ltd. Solutions were diluted with metal-free buffer to give the desired pH and molarity and checked for possible zinc contamination before use.

Substrates. Carbobenzoxyglycyl-L-phenylalanine and benzoylglycyl-L-phenylalanine were purchased from Mann Chemical Co. as the free acids. Stock solutions (0.1 and 0.01 M) were prepared in 1 M NaCl–0.02 M Veronal (pH 7.5) or in 1 M NaCl–0.05 M Tris (pH 7.5) and extracted with dithizone dissolved in carbon tetrachloride before use. Hippuryl-*dl*-β-phenyllactic acid was purchased as the sodium salt (Cyclo Chemical Corp.). Stock solutions of 0.05 and 0.01034 M HPLA were prepared regularly in 0.05 M NaCl–0.005 M Tris (pH 7.5), extracted with dithizone, and stored at −15°. A fresh batch of solution was thawed every 2 days for use. All other materials were of reagent grade quality and were used without further purification.

Contamination from adventitious metal ions, especially zinc, present in trace amounts in the water, reagents, and substrates, as well as the reaction vessels and tubes, presents formidable problems in this type of work, since extremely low enzyme concentrations (1–3 × 10^{-9} M) are employed for the assays. Hence, where possible, all chemicals, substrates, and buffers were extracted with dithizone to remove contaminating metal ions. Polyethylene tubes and containers were employed throughout, and the cuvets were cleaned in acid and metal-free water as previously described (Coleman and Vallee, 1961). Moreover, assay mixtures were 10^{-4} M in the appropriate metal ions. During the assay, this large excess of metal ions ensures both full formation of the metalloenzyme and prevents incidental restoration of the zinc enzyme. Zn^{2+}, $<10^{-7}$ M, *i.e.*, <0.006 ppm, not readily detected analytically, could either combine with the apoenzyme resulting in activity or displace a more weakly bound metal atom from the enzymic active center during an assay.

Protein concentrations were measured by the absorbance at 278 mμ using the Zeiss PMQ spectrophotometer. A molar absorptivity of 6.42 × 10^4 M^{-1} cm^{-1} was used for all metalloenzymes and 5.92 × 10^4 M^{-1} cm^{-1} for the acetyl enzymes (Simpson *et al.*, 1963).

Activity Measurements. A stock solution of enzyme (10^{-5} M) containing 10^{-3} M metal ions was diluted tenfold each day and kept at 4°. Further dilutions to 10^{-7} or 6 × 10^{-8} M with buffer containing 10^{-4} M metal ions were then kept at 25°. These solutions, though stable for 2 hr, were used within 1 hr. Temperature was controlled to 25 ± 0.1° by means of a Haake thermostated circulator, since fluctuations of ±1° can cause a variation of ±6% in the activity measurements (McClure *et al.*, 1964).

Esterase activity of carboxypeptidase has not been found to depend markedly on changes in the ionic strength (Elkins-Kaufman and Neurath, 1948; McClure *et al.*, 1964; Bender *et al.*, 1965). The approximate constancy of k determined in ionic strengths of 0.08, 0.1, and 0.5 M for both 0.001 and 0.01 M HPLA would seem to confirm this. Consequently, a NaCl concentration of

carboxypeptidases, where Me may be Cd, Co, Hg, Mn, or Ni. [(AcCPD)Me], metallocarboxypeptidases acetylated with *N*-acetylimidazole; CGP, carbobenzoxyglycyl-L-phenylalanine; BGP, benzoylglycyl-L-phenylalanine; HPLA, hippuryl-*dl*-β-phenyllactate; TNM, tetranitromethane; DHT, diazo-1-H-tetrazole.

0.07 M (0.04 M from the buffer solutions and 0.03 M from the enzyme dilution) was employed for all esterase activities determined on the pH-Stat.

For substrate concentrations in the range of 0.1–0.001 M, activity was determined at 25° by titrating the protons released on hydrolysis of the ester (Snoke *et al.*, 1948). Titrations were performed on 3 ml of solution in 0.05 M NaCl–0.005 M Tris, at pH 7.5 with 0.1 M NaOH, using a pH-Stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen).

For ester concentrations below 0.001 M, hydrolysis was followed spectrophotometrically (Schwert and Takenaka, 1955; McClure *et al.*, 1964; Whitaker *et al.*, 1966) using a Cary Model 15 recording spectrophotometer and 3 ml of substrate solution in 0.05 M Tris (pH 7.5) in 1-cm silica cuvets in a thermostated cell compartment.

At higher substrate concentrations when activities were measured on the pH-Stat, zero-order kinetics were more readily apparent, and initial velocities could be measured *directly* from the recordings. For the spectrophotometric assays at low substrate concentrations, where direct determination of initial velocities was difficult, the data were fitted to first-order kinetics and initial velocities, expressed as turnover numbers, calculated from

$$\frac{V_0}{e} = \frac{2.303}{e}\frac{[S]}{2}\frac{\log a_0/a}{t} \qquad (1)$$

where V_0 = initial velocity; e = enzyme concentration in the reaction mixture; [S] = initial substrate concentration of the hippuryl-*dl*-β-phenyllactic acid; a_0 and a are concentrations of the *l* isomer of the substrate at time zero and time (t), and measured by ($A_{\text{final}} - A_{\text{start}}$) and ($A_{\text{final}} - A_{\text{time } t}$). All substrate concentration terms represented as [S] refer to hippuryl-*dl*-β-phenyllactic acid. Since only the *l* form of HPLA acid is hydrolyzed (Snoke and Neurath, 1949) and the *d* form does not seem inhibitory (McClure *et al.*, 1964; Whitaker *et al.*, 1966), the *dl* mixture of HPLA was employed, the true concentration of hippuryl-*l*-β-phenyllactic acid being [S]/2. At both 0.001 and 0.0002 M substrate the initial rates of hydrolysis were proportional to the enzyme concentration over the tenfold range employed (10^{-8}–10^{-9} M).

Peptidase Activity. In contrast to the esterase activity, peptidase activity of carboxypeptidase is more dependent on ionic strength, the activity rising with ionic strength and reaching a plateau at 1.0 M (Lumry *et al.*, 1951). The kinetic parameters of cobalt, zinc, and manganese carboxypeptidases at 0.1 and 1.0 M NaCl determined in this laboratory (unpublished observations) show that the apparent K_m, determined at high substrate concentrations, decreases as ionic strength increases.[2]

However, owing to the insolubility of carboxypeptidase at low ionic strengths, physical-chemical experiments requiring high concentrations of enzyme have necessitated the use of 1 M NaCl. Hence, in order to allow direct comparisons between data obtained by kinetics and at equilibrium all peptidase activities were measured in 1 M NaCl. Except for the zinc enzyme, all assays were conducted in the presence of 10^{-4} M metal ion to ensure complete formation of the metalloenzyme (*vide supra*) at 25° when the enzyme concentrations are 10^{-7}–10^{-8} M.

Assays using CGP or BGP over a concentration range 0.001–0.1 M were performed by a modification of the ninhydrin method (Snoke and Neurath, 1949). Either 3 or 6 ml of assay solution was used in polyethylene tubes at 25°, and 0.1- to 1.0-ml samples, depending on the substrate concentration under investigation, were withdrawn at 5-min intervals over the course of 20 min, blown into 1 ml of ninhydrin, and the color was developed at 100° for 20 min. After cooling and dilution to 25 ml with 50% propanol–water, the absorbance was read at 570 mμ on a Klett colorimeter and converted into micromoles of phenylalanine liberated. At the enzyme concentration employed only 5–10% of the substrate was hydrolyzed in 20 min and first-order plots of log a_0/a *vs.* time were constructed for each substrate concentration, where a_0 and a are the concentrations of substrate at times 0 and t, respectively. The initial velocity is calculated as a turnover number, in units of min^{-1}, given by eq 2. These hydrolyses were also allowed

$$V_0/e = \frac{2.303}{e}[S]\frac{\log a_0/a}{t} \qquad (2)$$

to go to completion as a check on substrate concentration. In order to span the much lower K_m value for BGP, assays below 10^{-3} M were performed spectrophotometrically using the same technique as for HPLA hydrolysis and as reported by Folk and Schirmer (1963). The molar absorptivity for BGP at 254 mμ is ε 2600 M^{-1} cm^{-1}, with an increase on complete hydrolysis of Δε 280 M^{-1} cm^{-1}, under the conditions used. Initial slopes were measured as above and duplicate measurements were made routinely. Peptidase activities are reported as turnover numbers per minute to allow direct comparison with the esterase activities.

Results

Peptide Hydrolysis. CARBOBENZOXYGLYCYL-L-PHENYLALANINE AND BENZOYLGLYCYL-L-PHENYLALANINE. Manganese, cobalt, nickel, and zinc carboxypeptidases are all active peptidases but the cadmium, mercury, and copper derivatives are not.

Effect of Substrate Concentration. The dependence of the *initial* reaction velocity upon changes in the concentration of CGP and BGP was studied over a 100- and a 1000-fold range of concentrations, respectively. The results are shown in Figure 1a,b for each metalloenzyme in 1.0 M NaCl. The velocity of CGP hydrolysis by cobalt carboxypeptidase increases markedly with increasing substrate concentration, reaching a maximum

[2] Values for the K_m for zinc, cobalt, and manganese carboxypeptidases at 0.1 M NaCl are 1.7, 1.6, and 2.2 × 10^{-2} M, respectively, when determined from 0.003 to 0.02 M concentrations of CGP.

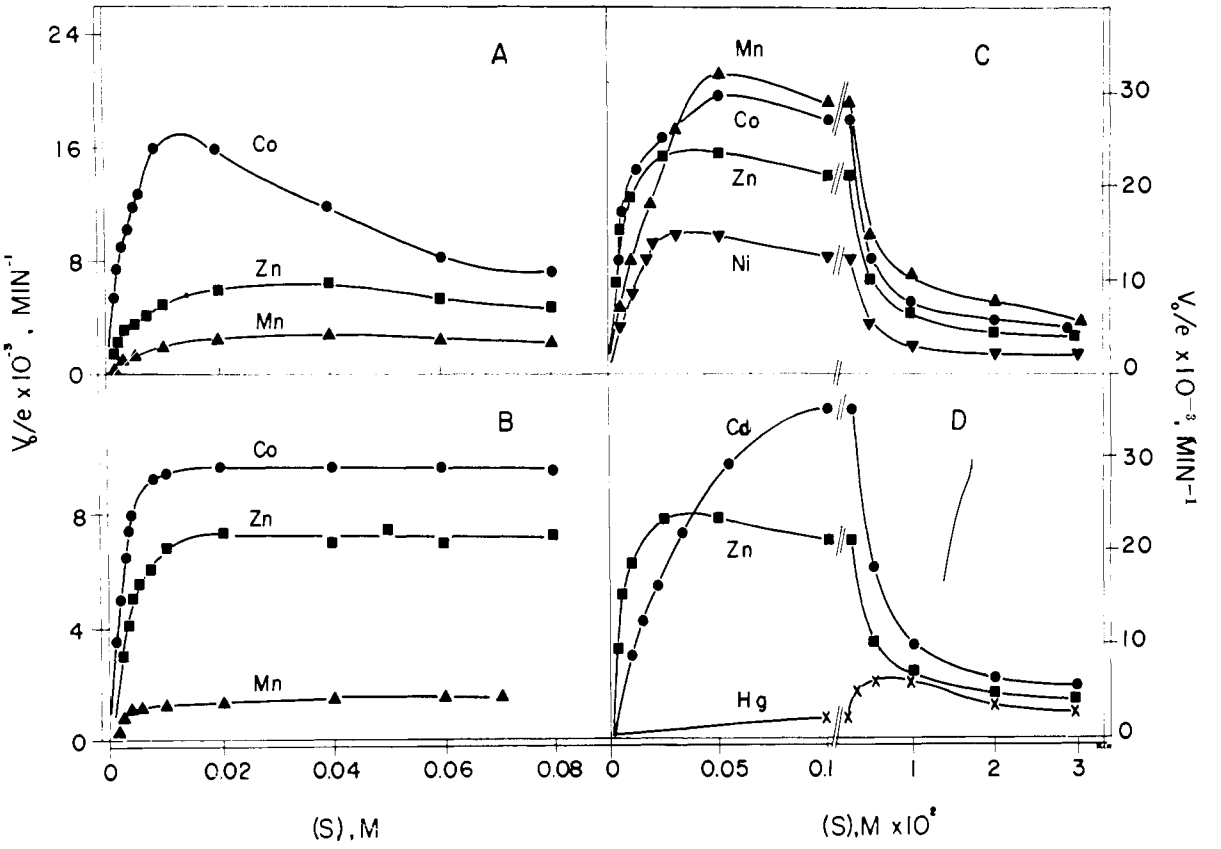

FIGURE 1: Activity *vs.* substrate concentration profiles of cobalt, manganese, nickel, cadmium, mercury, and zinc carboxypeptidases, when the substrates are CGP (A), BGP (B), and HPLA (C and D). See the text for the assay conditions.

at 1×10^{-2} M, decreases at higher substrate concentrations, and levels off at about 45% of the maximum when the substrate is 8×10^{-2} M. The profile for zinc carboxypeptidase is similar but the maximum velocity, observed at 4×10^{-2} M substrate, is only 40% of that for cobalt carboxypeptidase and decreases to about 60% of the maximum at 8×10^{-2} M substrate. The maximal activity of manganese carboxypeptidase similarly occurs at 4×10^{-2} M, and beyond this the decrease shows the same trend. Throughout the concentration range examined the relative velocities are Co > Zn > Mn (Figure 1a).

The activities and kinetic parameters for the peptidase activity of the nickel enzyme could not be determined accurately for either BGP or CGP. Above 3×10^{-3} M neither zero- nor first-order assay rates could be obtained even when only 10% of the substrate was hydrolyzed. At 2×10^{-2} M substrate, log a_0/a *vs.* time deviated positively from linearity at 25° but negatively at 0°, being linear at 10°. At 1×10^{-3} M substrate, however, the curves were zero order when up to 20% of the substrate is hydrolyzed. The detailed kinetics of the nickel enzyme are still under examination.

The metallocarboxypeptidases hydrolyze BGP in the same relative order as CGP, *i.e.*, Co > Zn > Mn. However, compared to CGP the apparent maximum rate of hydrolysis of BGP both by cobalt and manganese carboxypeptidases is decreased while that for the zinc enzyme is essentially unchanged. In contrast to CGP there is no significant decrease in the velocities at substrate concentration up to 0.08 M.

Kinetic Parameters. The double-reciprocal plots for the hydrolysis of CGP by all three metalloenzymes are not linear, but can be divided into three regions (Figure 2a). First, at low substrate concentration, $1\text{–}3 \times 10^{-3}$ M, there is a linear decrease in v^{-1} *vs.* S^{-1}. The slope changes rather abruptly at 3×10^{-3} M resulting in a second region of linear decrease which persists up to 1×10^{-2} M substrate. This latter concentration range is the one most conveniently examined and was therefore adopted widely in previous kinetic studies. This biphasic nature of the plot has been reported for the native enzyme (Whitaker *et al.*, 1966). In the third region, above 1×10^{-2} M substrate, v^{-1} increases rapidly, reflecting substrate inhibition. The Eadie plot for CGP clearly illustrates the anomaly of the first two regions (Figure 3).

The double-reciprocal plots for the hydrolysis of BGP by cobalt and zinc carboxypeptidases are similar, but with these concentrations of BGP, substrate inhibition is not apparent (Figure 2c). Over a substrate concentration range 1×10^{-4} to 1×10^{-3} M the relationship is linear. Above 1×10^{-3} M the plots begin to deviate from linearity but the change is less abrupt than with CGP, apparently increasing progressively with increasing substrate concentration. The double-reciprocal plot for the manganese enzyme is similar to that of these other metalloenzymes except that the plot is linear between 1×10^{-2} and 1×10^{-3} M begins to deviate below

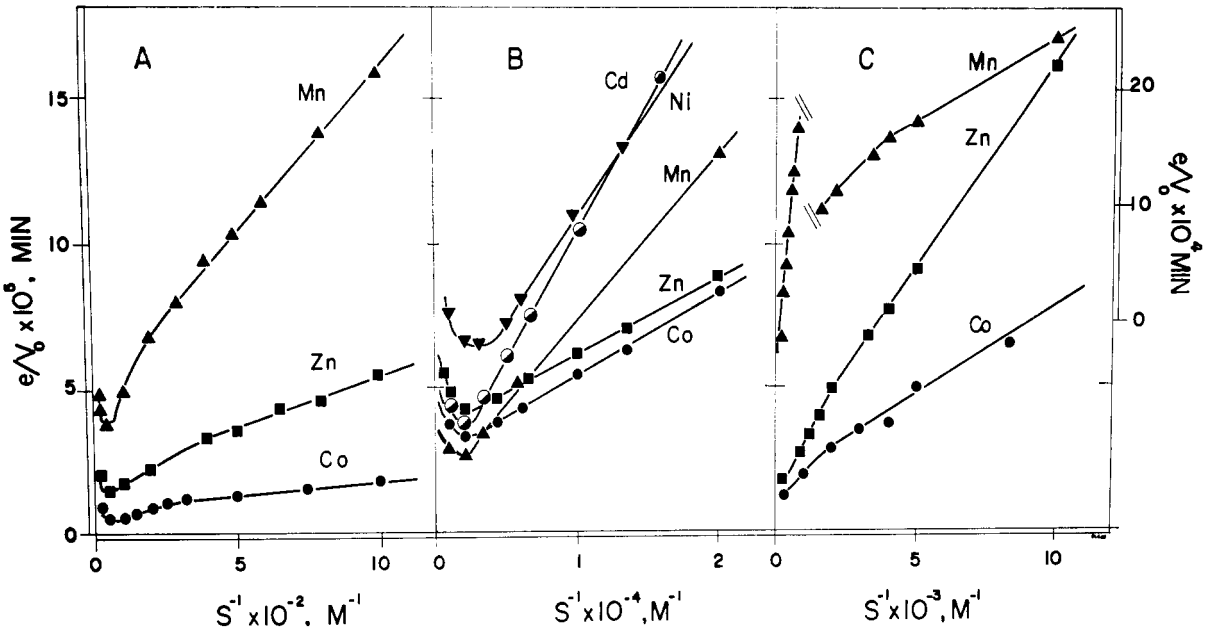

FIGURE 2: Lineweaver–Burk plots for metal-substituted carboxypeptidases: CGP (A), HPLA (B), BGP (C). The scale on the right-hand side pertains only to the hydrolysis of BGP by [(CPD)Mn].

TABLE I: Kinetic Constants for Peptide Hydrolysis by Zinc, Cobalt, and Manganese Metallocarboxypeptidases.[a]

Concn Range [(CPD)-Me]	CGP				BGP			
	1–4 × 10^{-3} M		0.4–4 × 10^{-2} M		1–4 × 10^{-4} M		0.1–1 × 10^{-2} M	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Zn	1.95	5.46	5.9	10.08	0.81	5.52	2.7	8.70
Co	1.17	12.30	4.4	22.02	0.48	7.38	1.35	10.68
Mn	2.29	2.28	6.0	3.24	0.11	0.45	1.55	1.56

[a] Assays performed in 0.02 M Veronal–1 M NaCl (pH 7.5), 25°, containing 1 × 10^{-4} M Me^{2+}. Units for K_m and V_{max} are M × 10^3 and min^{-1} × 10^{-3}, respectively.

1 × 10^{-3} M. Values of V_{max} and K_m calculated from the slopes and intercepts of the Lineweaver–Burk plots for the linear segment of both the lowest and the intermediate substrate concentration range of 3 × 10^{-3} to 10^{-2} M are shown in Table I. Based on either region of substrate concentration for these two substrates the relative orders of the observed catalytic activities and of V_{max} are Co > Zn > Mn.

The values of V_{max} and K_m for the native zinc enzyme [(CPD)Zn] are compared with literature data in Table II. It is well known that in addition to substrate concentration, ionic strength critically affects the kinetic parameters for this enzyme, as is also apparent from Table II. These variables largely account for any apparent differences between the results in the investigations cited.

Kinetic Orders. For the cobalt and zinc enzymes the first 10–15% of hydrolysis of both peptide substrates fits *first-order* kinetics at each of the substrate concentrations when examined at 25°; hence, initial velocities could be determined accurately between 8 × 10^{-2} and 1 × 10^{-3} M. Additionally, initial velocities for BGP could be measured directly from the spectrophotometer tracings at concentrations below 1 × 10^{-3} M.

Throughout the range of substrate concentration studied manganese carboxypeptidase acted like the zinc and cobalt enzymes on CGP. With BGP, however, in contrast to the first-order reactions for the zinc and cobalt enzymes, the rates for the manganese enzyme were zero order, *i.e.*, linear with time over at least 50% of the reaction, when BGP was varied from 10^{-4} to 10^{-3} M. Above 1 × 10^{-3} M the rate was first order, as for the zinc and cobalt enzymes. The activities were very low at concentrations of BGP below 5 × 10^{-4} M, making the extrapolation of "K_m" and "V_{max}" values very difficult for this region. Thus, for the hydrolysis of BGP, manganese carboxypeptidase behaves differently from the zinc and cobalt enzymes.

Ester Hydrolysis. HIPPURYL-*dl*-β-PHENYLLACTIC ACID (HPLA). *Effect of substrate concentration.* The hydrolysis of the ester, hippuryl-*dl*-β-phenyllactic acid, by zinc, cobalt, nickel, manganese, cadmium, mercury, and copper carboxypeptidases was studied over a 1000-fold range of concentration. The rate *vs.* substrate concentration profiles for zinc, cobalt, nickel, and manganese carboxypeptidases are shown in Figure 1c. For each, the velocity increases with increasing substrate concentration reaching a maximum at about 5×10^{-4} M, decreasing markedly thereafter to between 20 and 30% of the maximum velocities at 0.01 M, the concentration at which assays have been performed conventionally. The relative order of the activities of these metalloenzymes varies as a function of the concentration of substrate but is in the order Cd > Mn > Co > Zn > Ni > Hg at 5×10^{-4} M substrate where their activities are maximal.[3] Throughout the concentration range studied copper carboxypeptidase fails to hydrolyze HPLA.

Mercury carboxypeptidase, on the other hand, is the least active esterase with only about one-quarter of the activity of the zinc enzyme and one-sixth that of the cadmium enzyme. Its substrate concentration dependence rate profile has a broad maximum centered at about 5×10^{-3} M HPLA (Figure 1d).

Kinetic Parameters. For the zinc, cobalt, nickel, and manganese enzymes the Lineweaver–Burk plots are linear over a substrate concentration range from 3×10^{-5} to 3×10^{-4} M HPLA (Figure 2b), and for the cadmium enzyme from 1×10^{-4} to 1×10^{-3} M. The departures from linearity at high substrate concentrations reflect apparent substrate inhibition. Values for the apparent V_{max} and K_m, calculated from the linear segments, and the extrapolated intercepts of these plots are shown in Table III. The kinetic constants for the acetylated zinc and cadmium enzymes have been included for purposes of comparison. In this region ($<10^{-3}$ M) the esterase activity of mercury carboxypeptidase is very low. This circumstance together with the relatively large activity produced by very small amounts of contamination with zinc ions and the consequent presence of minute but measurable amounts of [(CPD)Zn], rendered precise measurements for the activities of this mercury enzyme unreliable and, hence, these constants are not included. The values of V_{max} and K_m for native zinc carboxypeptidase are compared to previous literature data for this enzyme (Table II).

Kinetic Orders. At substrate concentrations below 1×10^{-3} M the zinc, cobalt, nickel, and manganese enzymes exhibit *first-order* kinetics up to 50% hydrolysis; thereafter the plot of log a_0/a *vs.* time becomes steeper, as would be expected for kinetics with partial zero-order

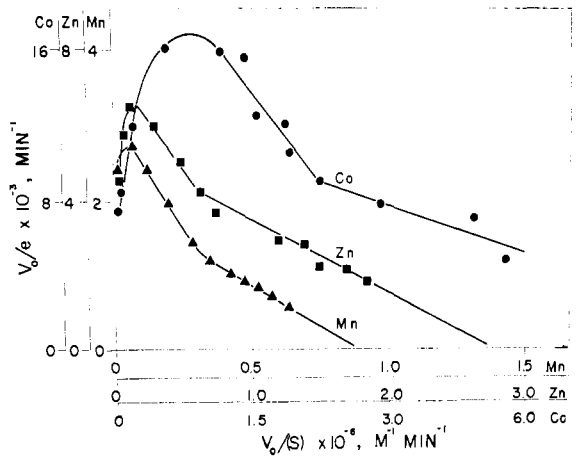

FIGURE 3: Eadie plots for the hydrolysis of CGP by cobalt, manganese, and zinc carboxypeptidases.

character (Figure 4). However, for cadmium carboxypeptidase, the first-order plots for concentrations of HPLA up to 10^{-3} M are linear up to 90% of hydrolysis, which could be consistent with a higher K_m than for the zinc enzyme (Figure 4), and a decrease in product inhibition. Above 10^{-3} M HPLA, inhibition is present and the zinc, cobalt, nickel, manganese, and cadmium en-

TABLE II: A Comparison of Literature and Present Values for the Kinetic Constants for Peptide Hydrolysis by Zinc Carboxypeptidase.

Substrate	μ (M)	K_m (M)	V_{max} (min^{-1} × 10^{-3})	Ref
CGP	LiCl (0.1)	37.0×10^{-3}	11.16	*a*
	NaCl (0.5)	5.83×10^{-3}	6.36	*b*
	NaCl (0.5)	14.0×10^{-3}	11.88	*b*
	NaCl (0.5)	16.6×10^{-3}	11.70	*c*
	NaCl (1)	1.95×10^{-3}	5.46[i]	*d*
	NaCl (1)	5.9×10^{-3}	10.08[i]	*d*
	NaCl (1)	6.5×10^{-3}	10.86	*e*
BGP	LiCl (0.1)	11.0×10^{-3}	10.86	*f*
	NaCl (0.5)	1.91×10^{-3}	6.72	*b*
	NaCl (0.5)	1.75×10^{-3}	7.86	*c*
	NaCl (1)	2.70×10^{-3}	8.70[i]	*d*
	NaCl (1)	0.81×10^{-3}	5.58[i]	*d*
HPLA	NaCl (0.07)	7.6×10^{-5}	28.6	*d*
	NaCl (0.1)	5.1×10^{-5}	28.0	*g*
	NaCl (0.5)	8.8×10^{-5}	34.7	*h*

[a] Elkins-Kaufman and Neurath (1948). [b] Whitaker *et al.* (1966). [c] Folk and Schirmer (1963). [d] Present work. [e] Lumry *et al.* (1951). [f] Snoke and Neurath (1949). [g] McClure *et al.* (1964). [h] Bender *et al.* (1965). [i] Calculated from linear portions of reciprocal plot in the *low* substrate concentration range. [j] Calculated from linear portions of reciprocal plots in the *intermediate* substrate concentration range.

[3] At optimal substrate concentration the activity of the manganese enzyme is much higher than the other metallocarboxypeptidases, a circumstance not apparent under assay conditions reported previously (Coleman and Vallee, 1961). In the earlier studies, substrate concentrations were suboptimal, and excess metal ions were not present in the reaction mixture, thus precluding complete formation of this derivative. The enhancement in rate is even more noticeable for the acetylated manganese enzyme, and this high activity has been reported (Coleman *et al.*, 1966).

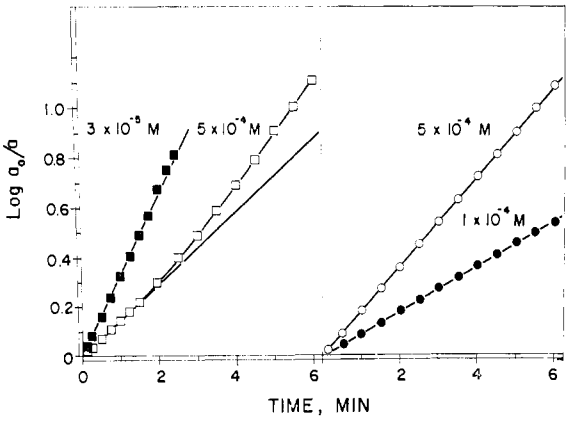

FIGURE 4: Time course of hydrolysis of HPLA by zinc (■, □) and cadmium (●, ○) carboxypeptidases.

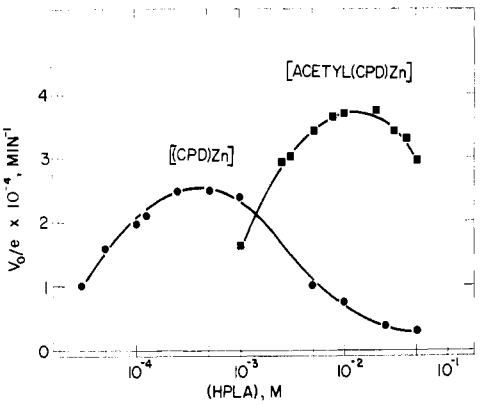

FIGURE 5: Esterase activities of native (●) and acetyl zinc carboxypeptidase (■) as a function of HPLA concentration at pH 7.5, 25°. Either pH-Stat or spectrophotometric measurement of rates were employed as detailed under Activity Measurements.

zymes follow *zero-order* kinetics while the mercury enzyme follows *first-order* kinetics.

Since the Lineweaver–Burk plots (Figure 1) are multiphasic it would seem likely that the extrapolated parameters "V_{max}" and "K_m" would be composite terms. Thus, it is of interest to note that there is an apparent ordering of the constants for the substrates regardless of the metal present, indicating that the causes of the kinetic complications may reside in part in the nature of the substrates used (Table IV).

Acetyl Zinc and Cadmium Carboxypeptidases. The hydrolysis of HPLA was studied over a 100-fold range of substrate concentration. Acetyl zinc carboxypeptidase hydrolyzes HPLA at progressively greater rates as the concentration of the substrate increases, becoming maximal at about 0.01 M. However, at even higher substrate concentrations the velocity decreases again; but this effect is displaced by about an order of magnitude in substrate concentration when compared with the native enzyme (Figure 5). The effects of acetylation on cadmium carboxypeptidases are analogous. Here, the rate of ester hydrolysis appears to approach a maximum at 0.1 M HPLA. More concentrated solutions of this substrate could not be prepared under the conditions employed, precluding further investigation. Acetylation apparently increases the maximal activity of the zinc but decreases that of the cadmium enzyme. At 0.01 M HPLA, the activities of the acetyl and unmodified cadmium enzymes are almost the same.

Kinetic Parameters. The Lineweaver–Burk plot of acetyl zinc carboxypeptidase departs from linearity at high substrate concentrations ($>2 \times 10^{-2}$ M). The values of K_m and V_{max} for both the zinc and cadmium enzymes calculated from these plots are shown in Table III. Acetylation increases the K_m of the zinc enzyme 30-fold and that of the cadmium enzyme 15-fold, while V_{max} *increases* from 28,600 to 42,000 min^{-1} for the zinc enzyme but *decreases* from 61,500 to 24,000 min^{-1} for the cadmium enzyme.

Discussion

The kinetics of hydrolysis of synthetic substrates by native carboxypeptidase A have been the subject of intensive investigation for the past 20 years. While a wide variety of substrates have been available, studies with inorganically or organically modified but enzymatically active carboxypeptidases have become possible only quite recently (Vallee, 1964; Riordan *et al.*, 1965a; Bender *et al.*, 1965; Riordan *et al.*, 1967; Sokolovsky and Vallee, 1967). While other substrates have been em-

TABLE III: Kinetic Constants for the Hydrolysis of HPLA by the Native Zinc Enzyme and Other Metallocarboxypeptidases.

[(CPD)Me]	K_m (M × 10^5)	V_{max} (min^{-1} × 10^{-3})
Zn	7.6	28.6
Co	9.8	37.7
Ni	21.0	27.6
Mn	32.0	56.8
Cd	55.0	61.5
[(AcCPD)Zn]	250	42.0
[(AcCPD)Cd]	830	24.0

TABLE IV: Relative Order of Kinetic Constants for Different Substrates as Function of the Metallocarboxypeptidases.

	Low Substrate Concentration	
	V_{max}	$1/K_m$
Zn	HPLA > CGP ≈ BGP	HPLA > BGP > CGP
Co	HPLA > CGP > BGP	HPLA > BGP > CGP
Mn	HPLA ≫ CGP > BGP	HPLA < BGP > CGP
	Intermediate Substrate Concentrations	
Zn	CGP > BGP	BGP > CGP
Co	CGP > BGP	BGP > CGP
Mn	CGP > BGP	BGP > CGP

ployed (Smith, 1952) many of the kinetic studies of the native enzyme have been performed with CGP and HPLA, substrates commonly used because of their high turnover numbers. In order to allow a direct comparison of ester and peptide hydrolysis and to account for possible steric factors, the peptide analog of HPLA, BGP, was also employed in these studies.

The present investigation was undertaken to examine the effect of substituting cobalt, manganese, nickel, cadmium, and mercury for the native zinc atom at the active site of the enzyme. The kinetic patterns of the native enzyme acting on these three substrates were employed as a basis for comparison.

The various anomalous features of the kinetics have long presented obstacles to the formulation of mechanistic schemes. In particular, the evaluation of K_m and V_{max} for different substrates has depended on the concentration range of substrate from which the values were extrapolated. Even when the range of inhibiting substrate concentration is neglected at least two sets of constants can be derived for CGP and BGP (Figure 2a and Table I) and, hence, conclusions based on such parameters require either a judgment as to the "proper" substrate range or a compromise. These kinetic ambiguities do not appear to result alone from the particular metal at the active site. Thus, while the range of concentrations where substrate and product effects appear are altered, the anomalies persist.

At low HPLA concentration, the maximum rates of hydrolysis vary in the order Cd > Mn > Co > Zn > Hg. Except for mercury the atomic radii of the metal ions substituted at the active site follow the same order as the hydrolysis of HPLA. Qualitatively, the relative rates of maximum hydrolysis for both CGP and BGP were in the order Co > Zn > Mn. No obvious explanation can be given for the rearrangement in the order for the hydrolysis of the peptides but the many varied factors of the physicochemical properties of the metals and their interaction with the active site, *e.g.*, coordination number, geometry, nature of the ligands, etc., would be expected to bear upon these events.

Every mercury and cadmium enzyme preparation exhibited a residual peptidase activity less than 5%, and frequently as low as 0.5% that of the native enzyme. The residual activity was always directly proportional to the percentage of zinc still present after preparation, as determined by atomic absorption spectrophotometry. Thus with these substrates, the enzymatic capacities of the cadmium and mercury are limited to ester hydrolysis, at rates characteristic of each. In the cross-linked, crystalline state mercury carboxypeptidase hydrolyzes CGP although its specific activity and that of crystals of the native enzyme is several 100-fold less than that of the native enzyme in solution (Bishop *et al.*, 1966). It cannot be discerned at this time whether or not this difference in behavior is a function characteristic of the two physical states of the enzyme examined.

All the catalytically active metalloenzymes examined exhibit substrate inhibition toward HPLA and substrate inhibition and activation, as well as product activation, toward CGP. Importantly, replacement of zinc by other metals displaces substrate inhibition to a different concentration range but does not abolish it. Substrate inhibition is most prominent for HPLA and independent of the metal at the active site. The binding of up to five molecules of HPLA at the active center has been found consistent with the kinetic data (McClure *et al.*, 1964).

TABLE V: Activation of Peptide (Carbobenzoxyglycyl-L-phenylalanine) and Inhibition of Ester (Hippuryl-*dl*-β-phenyllactic Acid) Hydrolysis by the Products Carbobenzoxyglycine and Benzoylglycine.

Product	M × 10^2	V_p/V_c (100)[a] CGP	HPLA
Carbobenzoxyglycine	0.5	134	73
	1.0	155	58
	2.0	181	40
	5.0	230	21
Benzoylglycine	1.25	252	85
	2.5	339	66
	3.55	370	62
	5.0	395	43

[a] Ratio of activities in the presence of product (V_p) to those in its absence (V_c) when CGP and HPLA concentrations are 10^{-3} M. The extent of such activation and inhibition also depends on substrate concentration. In addition a number of compounds, with hydrophobic characteristics similar to the blocking groups of such products, activate CGP while inhibiting HPLA hydrolysis, *e.g.*, benzoylglycineamide, benzamide, benzyl alcohol, cyclohexanol, and 3,3′-dimethylbutanol (R. C. Davies and B. L. Valee, in preparation).

While BGP has not been examined as extensively as some of the other peptide substrates of carboxypeptidase, available data indicate that in contrast to CGP this substrate displays neither activation nor inhibition (Whitaker *et al.*, 1966). In accord with these observations, in the present study, substrate inhibition was not detected up to 0.08 M substrate, but substrate activation was noted (Figure 2C). Further, one of the products of the reaction, benzoylglycine, inhibits esterase while activating peptidase activity. The reciprocal activation of peptide and inhibition of ester hydrolysis both by carbobenzoxyglycine and benzoylglycine are striking. The effects of these products are not restricted to activation of the hydrolysis of the parent peptides but can also be observed with others, observations which may likely be pertinent to the presumed mode of interaction of substrates with carboxypeptidase (Vallee, 1967). Similarly, benzoylglycineamide, benzamide, benzyl alcohol, cyclohexanol, and 3,3′-dimethylbutanol among others, with hydrophobic characteristics of the blocking group of such products, activate CGP while inhibiting HPLA hydrolysis (Table V). The hydrolyses differ in their details, since carbobenzoxyglycine activates BGP hydrolysis to a greater extent than does benzoylglycine; conversely benzoylglycine activates the hydrolysis of CGP more effectively

than does carbobenzoxyglycine. The detailed examination of these effects and the use of analogs of these products have revealed further differences between BGP and CGP as will be reported (R. C. Davies and B. L. Vallee, in preparation). Such studies bear importantly on the basic cause of the anomalous kinetic features of carboxypeptidase catalysis. Substrates differing in the N-terminal blocking groups, *e.g.*, CGP and BGP, may well interact with the native enzyme to different degrees.

Extensive studies of the kinetics of the acetyl metallocarboxypeptidases have now been performed. Acetyl zinc carboxypeptidases prepared with *N*-acetylimidazole or acetic anhydride have been examined previously (Simpson *et al.*, 1963; Whitaker *et al.*, 1966) and manganese, cobalt, nickel, copper, cadmium, and mercury enzymes have also been examined in this laboratory at one substrate concentration (Coleman *et al.*, 1966).

Irrespective of the CGP concentration employed, up to 0.18 M, peptidase activity of the acetyl zinc enzyme is reduced to 1–3% of that for the native enzyme. However, the esterase activity of acetyl zinc carboxypeptidase is increased 600–700% when assayed with 0.01 M HPLA. This substrate concentration, routinely employed for assays in the past (Snoke and Neurath, 1949), is in the region of substrate inhibition. Hence, at least part of the observed increase in activity can be attributed to the removal of inhibition (Riordan and Vallee, 1963; McClure *et al.*, 1964; Bender *et al.*, 1965) but, dependent upon the region of substrate concentration employed, either an increase, decrease, or no change in the rate of hydrolysis is noted. Qualitatively, acetylation displaces the rate *vs.* substrate profile into a range of substrate concentrations which is more accessible by conventional experimental means, and consequently V_{max} and K_m for esters are determined and investigated more readily under these circumstances. However, the observed increase in hydrolytic rate on acetylation is due *both* to a 30-fold increase in K_m and to an increase of V_{max} of about 30% and, therefore, acetylation appears to alter both binding of the ester and features of the catalytic step (McClure *et al.*, 1966). Consistent with these findings substrate inhibition is not abolished, but displaced to substrate concentrations higher than those generally employed for assays. In fact, this displacement of substrate inhibition resembles that seen on metal substitution (Figure 5). Further, as will be reported, the effect of the products and product analogs on the rate profile also persist, emphasizing the difficulties of obtaining kinetic data which yield unambiguous mechanistic implications with the substrates presently in use both when the native or these chemically modified enzyme derivatives are employed.

The number of ester substrates studied so far is much less extensive, of course, than that of peptides. Attempts at assigning specific substrate–enzyme surface interactions based on the structural features of the substrates alone would seem tenuous at this time. The emerging X-ray structure has already uncovered major conformation changes for the glycyl-L-tyrosine–carboxypeptidase complex (Lipscomb *et al.*, 1967; Steitz *et al.*, 1967). This observation suggests that the motility of the enzyme, perhaps altered to different degrees by different substrates, could represent an important variable of presently unknown dimensions in making comparisons between different enzyme–substrate complexes.

In this regard it is important to note, however, that although the cadmium and copper metallocarboxypeptidases are catalytically inactive toward peptides, peptides such as CGP can still bind to and inhibit the esterase activity of the cadmium enzyme very effectively. Many alternatives could account for this circumstance, *e.g.*, the peptide might fit the active center in such a way that the catalytic groups are not favorably arranged for its hydrolysis, or these groups might no longer be available for catalysis.

It would appear that the kinetic anomalies themselves reflect both the nature and organization of the active center as a whole as well as its interaction with the particular substrates used. Organic chemical modification of the enzyme, *i.e.*, acetylation and iodination (Simpson *et al.*, 1963), coupling with DHT (Sokolovsky and Vallee, 1967), or interaction with TNM (Riordan *et al.*, 1967), can alter some of the anomalies seen under conventional assay conditions. Specifically, the effects of products, peptides and other inhibitors on the kinetics of acetyl carboxypeptidase has already been examined (Vallee, 1967) and will be discussed further.

The kinetic anomalies would appear to reflect the inherent organization of the enzyme but relating importantly, perhaps, to the nature of the small synthetic substrates employed so far for the study of this single-chain hydrolytic enzyme. The hydrolyses of some other larger peptides currently under investigation are devoid of some of these complexities and may serve to map the characteristics of the active center, pertinent to hydrolysis. The possible elimination of some of the complexities outlined might assist in the recognition of the mechanistic details of catalysis to the extent that they can be revealed by kinetic approaches and, therefore, these studies have been extended to additional enzyme and enzyme derivative–substrate combinations which will be communicated.

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