shown (Bender *et al.*, 1962) to approach a maximum at *p*H 8.5 with no appearance of a normal bell-shaped profile. The *p*H dependence of α-chymotrypsin autolysis can be qualitatively obtained by subtracting the first titration curve from the second titration (Fig. 6). This gives the data shown in Figure 7. This curve indicates the amount of amino groups liberated as a function of time and *p*H since the titrations are performed from point to point at roughly 15-second intervals. The autolysis appears to increase with increasing *p*H. This could be explained by involving another center which dissociates at a higher *p*H. While this assumption is attractive in explaining the action of α-chymotrypsin against a protein substrate, considerably more quantitative data on the autolytic rate and the nature of the group involved must be obtained.

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Acetylcarboxypeptidase*

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Acetylation of carboxypeptidase with acetic anhydride increases esterase activity 6-fold and abolishes peptidase activity. These enzymatic changes are prevented by β-phenylpropionate and reversed by hydroxylamine. Acylation of the enzyme with other monocarboxylic acid anhydrides such as *i*-butyric, *n*-butyric, *n*-valeric, and propionic anhydrides also increases esterase and decreases peptidase activities. Though qualitatively the same the ensuing enzymatic changes are smaller than those observed with acetic anhydride. As in studies with acetylimidazole reported previously (Simpson *et al.*, 1963) the alterations in enzymatic activity correlate with the acetylation and deacetylation of two tyrosyl residues. Some kinetic and physical-chemical properties of Ac_A-carboxypeptidase are reported.

Previous studies from this laboratory have been concerned with the identification of those groups of the active site[1] of bovine pancreatic carboxypeptidase[2] which are involved in binding the catalytically essential zinc atom (Vallee *et al.*, 1960; Coleman and Vallee, 1961; Vallee *et al.*, 1961). Studies using site-specific reagents, the order and magnitude of the stability constants of a series of metallocarboxypeptidases, and complexometric titration data have indicated that the α-amino group of the N-terminal asparagine and the sulfhydryl group of the single cysteine residue of the enzyme constitute the metal binding site (Vallee *et al.*, 1960; Coleman and Vallee, 1961; Coombs and Omote, 1962).

While zinc is indispensable for activity, functional amino acid residues of the protein must also be essential in the catalytic process. Thus, peptide substrates of carboxypeptidase have been shown to form complexes with the metal-free apoenzyme (Coleman and Vallee, 1962a). Minimally, the aromatic or branched aliphatic side chain and the NH-function of the C-terminal amino acid as well as the NH-function of the penultimate amino acid are required for the formation of such a complex (Coleman and Vallee, 1962b). Clearly, these groups must interact with corresponding ones of the protein. In addition, the *p*H rate profile for peptidase activity suggests that a

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

[2] Carboxypeptidase will refer to carboxypeptidase A.

group on the protein with an apparent pK_a near 6.7 is essential for the hydrolysis of peptides (Neurath and Schwert, 1950).

The chemical nature of those groups of the enzyme involved in substrate binding, catalysis, or both has been investigated further by means of site-specific reagents. We have now found that chemical modifications of carboxypeptidase with monocarboxylic acid anhydrides result in marked increases in esterase and decreases of peptidase activity. These functional alterations can be prevented by a competitive inhibitor, β-phenylpropionate, and the consequences of acetylation can be reversed by hydroxylamine. Concomitant spectral studies demonstrate that the observed functional changes result from modification of tyrosyl residues at the active center.

Experimental

Materials

Five-times-recrystallized bovine pancreatic carboxypeptidase was prepared by the method of Allan *et al.*[3] Enzyme prepared by the method of Anson (1937) was obtained from Worthington Biochemical Corp., Freehold, N. J.. The zinc-to-protein ratio of all preparations was between 0.98 and 1.03 gram atoms/mw 34,300 (Smith and Stockell, 1954; Vallee and Neurath, 1955; Brown *et al.*, 1961). Apocarboxypeptidase was prepared as previously described (Vallee *et al.*, 1958 Felber *et al.*, 1962).

The chemicals employed in these experiments were of reagent grade and were used without further purification, with the exception of acetic anhydride which was redistilled with a consequent reduction in its Zn^{++} content to less than 0.01 ppm.

Methods

Peptidase Activity.—Determination of peptidase activity was made using carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as the substrate (Snoke and Neurath, 1949; Neurath and DeMaria, 1950). Activity is expressed as an apparent proteolytic coefficient C defined as log a_0/a/minute/mole enzyme, where a_0 and a represent the concentration of substrate at time zero and time t, respectively (Simpson *et al.*, 1963). The assays were performed at 0° in 0.02 M sodium Veronal–1.0 M NaCl buffer, *p*H 7.5, except where otherwise indicated. C was calculated from the linear portion of the first-order plots observed when hydrolysis did not exceed 15%.

Esterase Activity.—Determination of esterase activity was made as has been described (Snoke *et al.*, 1948). The activity was measured by titration with 0.1 N NaOH of the hydrogen ions released on hydrolysis with a Model TTT 1A *p*H-stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). Assays were performed at 25° with 5 ml of 0.01 M hippuryl-DL-β-phenyllactate in 0.2 M NaCl–0.005 M Tris HCl at *p*H 7.5. In some studies hippuryl-DL-indolyllactic acid[4] (HILA)[5] was employed as indicated in the results. Activities are expressed as zero-order velocity constants k, with units of μmoles H^+ per minute per μmole of enzyme.

Metal Analyses.—Zinc was determined either by atomic absorption spectroscopy (Fuwa and Vallee, 1963), or chemically as previously described (Vallee and Gibson, 1948).

[3] Allan, B. J., Keller, P., and Neurath, H., in preparation.

[4] We are indebted to Dr. Y. Omote for the synthesis of this ester.

[5] Abbreviations used in this paper: HILA hippuryl-DL-β-indolyllactic acid; CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-β-phenyllactic acid.

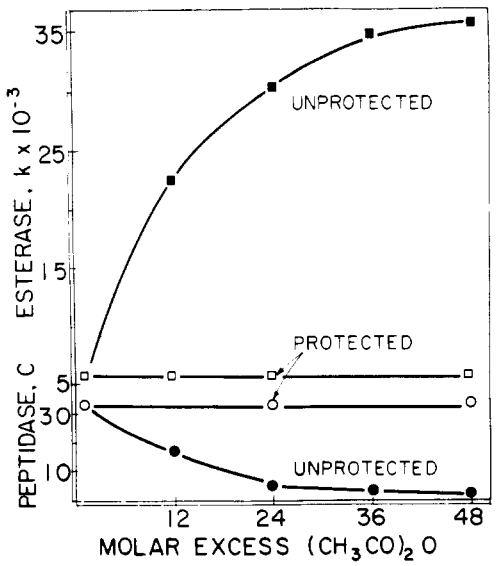

FIG. 1.—Effects of variation of the molar excess of acetic anhydride on esterase (■) and peptidase (●) activities of carboxypeptidase in the presence (○, □) and absence (●, ■) of 0.1 M β-phenylpropionate. Acetylations were performed at 0° in 0.02 M sodium Veronal–2 M NaCl, *p*H 7.5, for 30 minutes. The reaction mixture was dialyzed for 36 hours against at least three changes of the same buffer before activities were measured. Assays were carried out at *p*H 7.5 as under Experimental.

Protein Concentrations.—These were measured either gravimetrically, after 10% trichloroacetic acid precipitation followed by drying at 104° (Hoch and Vallee, 1953), or by the absorbancy at 278 mμ. The molar absorptivity of native carboxypeptidase is 6.45×10^4 liter $mole^{-1}$ cm^{-1}[6] while the values employed for acetylcarboxypeptidase acetylated with acetic anhydride under standard conditions (*vide infra*) in the presence or absence of β-phenylpropionate were 5.78 and 6.01 liter $mole^{-1}$ cm^{-1}. A Beckman DU spectrophotometer and a Cary Model 15 MS automatic recording spectrophotometer were employed. The *p*H was determined with a Radiometer *p*HM 22 *p*H meter equipped with a general purpose combined electrode. Precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957).

Acylations.—Concentrations of enzyme between 10^{-4} and 10^{-3} M in 0.02 M sodium Veronal–2 M NaCl buffer, *p*H 7.5, were employed for acylation by the various anhydrides listed in Table VI. *No* sodium acetate was added to the acylation mixtures in which the amount of anhydride varied from 12 to 72 times the protein concentration.[6] Except where indicated otherwise, acetylcarboxypeptidase was prepared by the addition of a 48-fold molar excess of acetic anhydride. The *p*H of the reaction was maintained at 7.5 by the addition of 1 N NaOH with a *p*H-stat. There was no further base uptake after 30 minutes at 0–4°. Excess anhydride was removed by exhaustive dialysis against the same buffer. The degree of modification of the free amino groups of carboxypeptidase was estimated by means of the ninhydrin reaction using phenylalanine as a standard (Moore and Stein, 1948).

[6] It was previously stated (Vallee, *et al.*, 1963) that the molar excess of acetic anhydride employed varied from 15 to 60. These concentrations were based on the absorptivity of the enzyme previously reported (Davie and Neurath, 1955). They are now corrected to the absorptivity values obtained by precipitation with trichloroacetic acid (Simpson *et al.*, 1963) and direct amino acid analysis of the enzme (Yamasaki *et al.*, 1963).

TABLE I

ZINC CONTENT AND FREE AMINO GROUPS OF CARBOXYPEPTIDASE ON ACETYLATION WITH ACETIC ANHYDRIDE

Molar Excess Anhydride	Gram Atoms Zinc/ Mole Enzyme	Equivalents Phenylalanine/ Mole Enzyme
0	1.02	13.8
24	—	8.0
48	0.95	4.7
72	0.79	4.4
160	0.76	1.6
320	0.37	1.2

Treatment with hydroxylamine was carried out for 10 minutes at 25° in 1.0 M NH_2OH in 0.02 M Veronal–2 M NaCl at *p*H 7.5, followed by dialysis. Labile acetyl groups were determined by a modification of the procedure of Balls and Wood (1956) using either 1 M hydroxylamine at *p*H 7.5, 0.02 M Veronal-2 M NaCl for 10 minutes at 25°, or 0.02 M hydroxylamine at *p*H 7.5, 0.001 M Tris–1 M NaCl for 200 minutes at room temperature.

RESULTS

Enzymatic Consequences of Acetylation.—Acetylation of carboxypeptidase with acetic anhydride for 30 minutes at *p*H 7.5, 4°, markedly alters the catalytic specificity and hydrolytic rates of the enzyme (Fig. 1). With carbobenzoxyglycyl-L-phenylalanine (CGP) as the substrate, peptidase activity decreases as the molar ratio of acetic anhydride to enzyme increases. However, esterase activity, measured with hippuryl-DL-β-phenyllactic acid (HPLA) increases dramatically. When the molar ratio of anhydride to enzyme is 48, the esterase activity of the acetylated enzyme is 600% greater than that of the control; simultaneously, peptidase activity is abolished completely, even though the acetylated enzyme prepared in this manner still contains one gram atom of zinc per mole (Table I, col. 2). The enzyme loses zinc and esterase activity and becomes insoluble when the concentration of acetic anhydride is increased further. These changes in activity during acetylation are accompanied by a decrease in free amino groups from 14 phenylalanine equivalents per mole of native enzyme to 4 per mole of acetylated enzyme (Table I, col. 3).

Acetylation of carboxypeptidase in the presence of 10^{-1} M β-phenylpropionate (Fig. 1), indoleacetate, iodoacetate, half-saturated sodium acetate (Table II, cols. 2 and 3), and certain peptide substrates essentially prevents these changes in specificity and hydrolytic rates. The degree of modification of the ε-amino lysine groups, however, is not significantly affected by the presence of these agents (Table II, col. 4). The competitive inhibitor β-phenylpropionate apparently protects enzyme groups other than these from being acetylated, since the full increase of esterase and decrease of peptidase activities is observed (Fig. 1) when such a protected enzyme is reacetylated after removal of this agent.

The enzymic changes coincident with acetylation are reversible. Incubation of Ac_A-carboxypeptidase[7] with 1 M hydroxylamine for 10 minutes at 25°, *p*H 7.5, fully restores both peptidase and esterase activities to those characteristic of the native enzyme. Thus, in a representative experiment, treatment with this nucleophilic reagent increases peptidase activity from 5% to 97%

[7] The prefixes Ac_A- and Ac_I will be used to denote acetylcarboxypeptidase prepared with acetic anhydride and acetylimidazole, respectively.

TABLE II

ACETYLATION OF CARBOXYPEPTIDASE IN THE PRESENCE OF INHIBITORS: ENZYMATIC ACTIVITIES AND FREE AMINO GROUPS. ACETYLATION WITH 48-FOLD MOLAR EXCESS OF ACETIC ANHYDRIDE AT *p*H 7.5, 0.02 M VERONAL–2 M NaCl, 0°, 30 MINUTES

Protective Agent	Esterase ($k \times 10^{-3}$)	Peptidase C	Equivalents Phenylalanine/ Mole Enzyme
None	35.4	1.7	5.0
β-Phenylpropionate, 0.1 M	6.3	34.3	4.7
Indoleacetate, 0.1 M	6.9	25.0	4.3
Iodoacetate, 0.1 M	7.6	32.7	—[a]
Sodium acetate, 7 M[b]	11.4	20.8	7.1

[a] Not measured. [b] Sodium acetate is known to be less effective as an inhibitor of carboxypeptidase than the other reagents listed. It was studied since it is frequently employed in acetylation reactions. Acetate ion–catalyzed hydrolyses of phenylacetate have been reported (Bender and Breslow, 1962). Therefore the "protective" effect of sodium acetate may be due in part to an acetate ion–catalyzed hydrolysis of *O*-acetyltyrosyl residues.

TABLE III

RESTORATION OF ACTIVITY TO Ac_A-CARBOXYPEPTIDASE [(Ac_ACPD)Zn] BY DEACETYLATION WITH HYDROXYLAMINE[a]

Enzyme	NH_2OH	Esterase ($k \times 10^{-3}$)	Peptidase C	Equivalents Phenylalanine/ Mole Enzyme
[(CPD)Zn]	0	6.60	35.0	13.8
[(CPD)Zn]	+	6.53	39.2	13.3
[(Ac_ACPD)Zn][b]	0	6.72	36.8	4.8
[(Ac_ACPD)Zn][b]	+	6.26	37.5	4.2
[(Ac_ACPD)Zn]	0	37.9	1.7	4.4
[(Ac_ACPD)Zn]	+	7.25	34.1	4.9

[a] (1 M NH_2OH, *p*H 7.5, 10 min., 25°.) The native enzyme [(CPD)Zn] and the acetylenzyme protected by β-phenylpropionate during acetylation with acetic anhydride are shown as controls for the deacetylation reaction with hydroxylamine. [b] β-Phenylpropionate protected.

of the unmodified control (Table III, lines 5 and 6). Simultaneously, esterase activity decreases from 570% to 110% of the control. In contrast, treatment of native carboxypeptidase or enzyme acetylated in the presence of β-phenylpropionate with hydroxylamine does not affect activities (Table III, lines 1 to 4). Again, on deacetylation, there is no change in the number of ninhydrin-reactive groups.

The effects of a 48-fold molar excess of acetic anhydride on peptidase and esterase activities of carboxypeptidase were studied as a function of *p*H (Fig. 2). The activities of each sample were measured at *p*H 7.5. As acetylation was performed at increasingly higher *p*H, the esterase acivity in this experiment progressively rose from 6.6×10^3 at *p*H 6 to 30×10^3 μmoles H^+/min/μmole of enzyme at *p*H 7.5, corresponding to 500% of the unmodified control value. Similarly, peptidase activity fell progressively from a *C* value of 27.4 at *p*H 6 to 1.5 at *p*H 7.5. Acetylation between *p*H 7.5 and 9 does not bring about further increases in esterase or decreases in peptidase activities.

Removal of zinc from Ac_A-carboxypeptidase by dialysis against 2×10^{-3} M 1,10-phenanthroline at *p*H 7.5, 4°, for 36 hours completely abolishes esterase activity: apoAc_A-carboxypeptidase is inactive. However, addi-

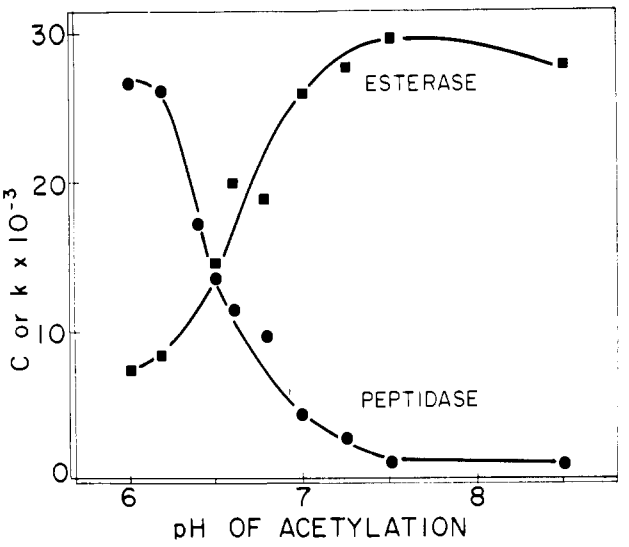

FIG. 2.—Effects of variation of the *p*H at which acetylation is performed on the esterase (■) and peptidase (●) activities of the resultant Ac_A-carboxypeptidase. Acetyltions were performed at the *p*H indicated on the abscissa. Each sample was then dialyzed for 36 hours against at least three changes of 0.02 M sodium Veronal–2 M NaCl, *p*H 7.5, buffer and assayed at *p*H 7.5 as under Experimental.

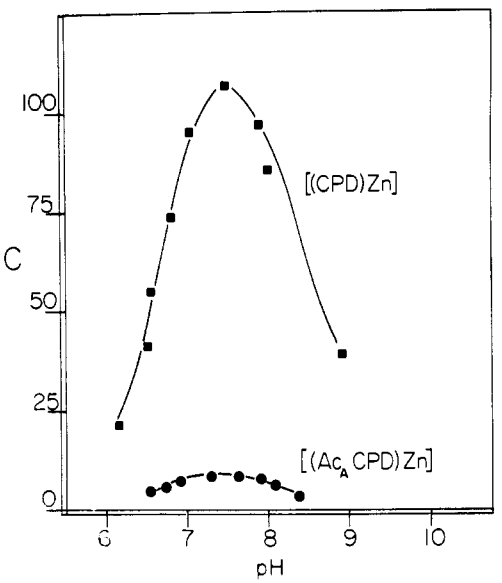

FIG. 3.—Peptidase *p*H-rate profiles for native (■) and Ac_A-carboxypeptidase (●). Activities were measured at 25° using carbobenzoxyglycyl-L-phenylalanine as substrate as described under Experimental. After completion of the assay the *p*H was determined by direct measurement on tl e experimental sample and on the control.

tion of 1 gram atom of Zn^{++} to apoAc_A-carboxypeptidase fully restores the esterase activity to that characteristic of Ac_A-carboxypeptidase though peptidase activity is not restored (Table IV). Thus, for the enzymatic consequences of acetylation to become apparent, zinc must be present at the active site. This is supported by the observation that acetylation of apocarboxypeptidase under metal-free conditions induces neither esterase nor peptidase activities.

Enzymatic Properties of Native and Ac_A-carboxypeptidase.—Assays were performed at *p*H values between 5.5 and 11.0 to gain insight regarding both the mechanism by which native carboxypeptidase hydrolyzes esters and by which chemical modification alters esterase and peptidase activities. The native enzyme exhibits optimal peptidase activity at *p*H 7.5 as previously reported (Neurath and Schwert, 1950) (Fig. 3). We have briefly indicated earlier (Vallee *et al.*, 1963) that the *p*H-rate profile of native carboxypeptidase, acting as an esterase, is changed considerably from that for peptidase activity. The points of inflection at *p*H 6.7 and *p*H 8.5 observed in the peptidase *p*H-rate profile are absent (Fig. 4). When the rate of HPLA hydrolysis is plotted against *p*H there is an initial rise

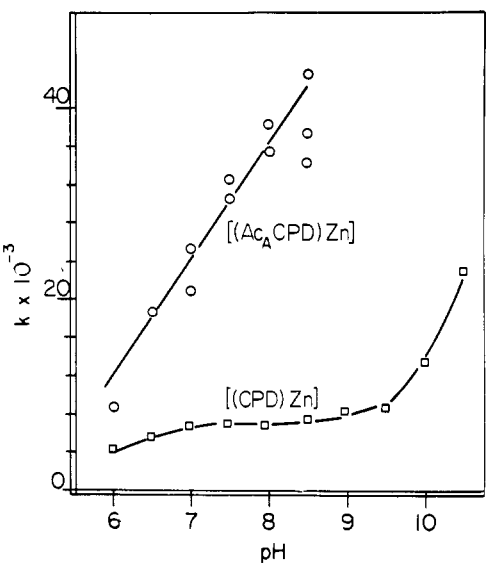

FIG. 4.—Esterase *p*H-rate profiles for native (□) and Ac_A-carboxypeptidase (○). Activities were measured using hippuryl-DL-β-phenyllactic acid for substrate, as described under Experimental, at the *p*H indicated on the abscissa.

TABLE IV

CATALYTIC ACTIVITY AND ZINC CONTENT OF ACETYLCARBOXYPEPTIDASE [(Ac_ACPD)Zn] AND APOACETYLCARBOXYPEPTIDASE (Ac_ACPD)[a]

Enzyme	Esterase ($k \times 10^{-3}$)	Peptidase C	Gram Atoms Zinc/Mole Enzyme
[(Ac_ACPD)Zn]	39.6	1.8	1.07
(Ac_ACPD)	4.2	0	0.05
(Ac_ACPD) + Zn^{++}	35.6	1.7	0.97

[a] Subsequent to acetylation of 10^{-4} M enzyme, zinc was removed by dialysis against three changes of 2×10^{-3} M 1,10-phenanthroline over a period of 36 hours at 4°. The metalloenzyme was reconstituted by addition of 10^{-4} M Zn^{++} ions.

between *p*H 5.5 and 7.0, a plateau between *p*H 7.0 and 9.0, followed by a second rise reaching a maximum at *p*H 10.5 and declining thereafter (not shown).

The individual rate measurements follow essentially zero-order kinetics up to about *p*H 10.5; beyond this the order of the reaction becomes mixed. This phenomenon, together with the greatly increased spontaneous rate of hydrolysis of the substrate, precluded studies beyond *p*H 10.5.

Throughout the *p*H range studied, acetylation reduces peptidase activity to less than 5% of the control (Fig. 3). The very small residual peptidase activity of the modified enzyme is not readily detected under standard assay conditions but it can be measured when the amount of enzyme is increased 10-fold and the assay is performed at 25° rather than at 0°. Under

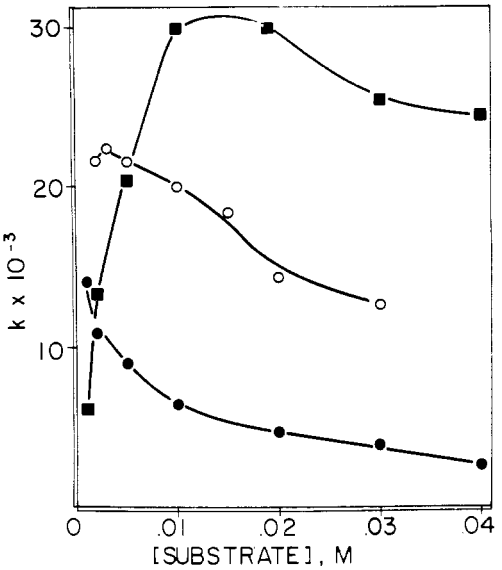

FIG. 5.—Dependence of the esterase activities on substrate concentration. Native (●) and Ac_A-carboxypeptidase (■) were assayed on the hippuryl-DL-β-phenyllactic acid, and native carboxypeptidase was studied with hippuryl-DL-β-indolyllactic acid (O) as the substrate.

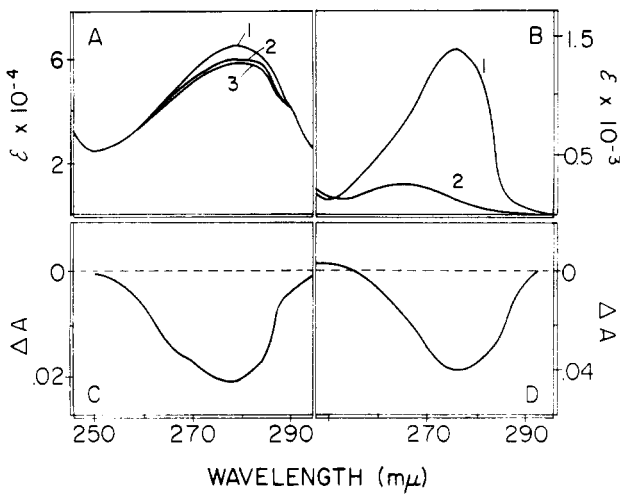

FIG. 6.—(A) Absorption spectra of carboxypeptidase (1) and Ac_A-carboxypeptidase acetylated in the presence (2) and absence (3) of β-phenylpropionate. (B) Absorption spectra of *N*-acetyltyrosine (1) and *N,O*-diacetyltyrosine (2). (C) Difference spectrum of β-phenylpropionate-protected and unprotected Ac_A-carboxypeptidase. (D) Difference spectrum of *N,O*-diacetyltyrosine and *N*-acetyltyrosine. The protein concentration in (C) was 1×10^{-5} M for both samples. The concentration in (B) and (D) was 3.2×10^{-6} M, identical for both. A Cary Model 15 recording spectrophotometer with 1-cm cells was employed. All spectra were obtained in 0.001 Tris–1 M NaCl, *p*H 7.5, buffer at room temperature.

these conditions, the peptidase *p*H-rate profile resembles that of the native enzyme, therefore the residual activity may be due to incomplete acetylation of the active center of the enzyme.

The esterase *p*H-rate profile of Ac_A-carboxypeptidase differs from that of the native enzyme. From *p*H 6 to *p*H 8.0 the increase in rate is linearly dependent upon *p*H and the magnitudes of the rates are increased over those for the native enzyme when HPLA is the substrate (Fig. 4). Difficulty was encountered in obtaining reproducible results above *p*H 8.0.

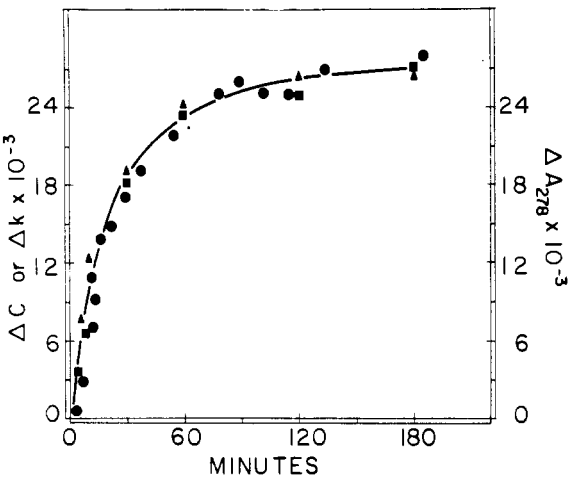

FIG. 7.—Deacetylation of Ac_A-carboxypeptidase with 0.02 M hydroxylamine in 0.001 M Tris–1 M NaCl, *p*H 7.5. The change of absorbance at 278 mμ (●) was followed in a Beckman DU spectrophotometer using 1-cm quartz cuvets. Enzyme acetylated in the presence of 0.1 M β-phenylpropionate served as the blank. Aliquots were removed from both cuvets at the indicated times and assayed for esterase (▲) and peptidase (■) activities as under Experimental. The activities plotted are those observed for the enzyme acetylated in the absence of β-phenylpropionate minus those obtained when acetylation was performed in the presence of the inhibitor. The left-hand ordinate refers to changes in peptidase (C) and esterase (*k*) activities. The right hand ordinate refers to changes in absorbance.

The dependence of the esterase activity of native carboxypeptidase on substrate concentration (Snoke *et al.*, 1948; Snoke and Neurath, 1949) was compared to that of Ac_A-carboxypeptidase (Fig. 5). As previously reported, the rate of hydrolysis of HPLA *decreases* progressively as the substrate concentration *increases* from 0.001 M to 0.04 M. Similar kinetics are observed when hippuryl-DL-β-indolyllactic acid (HILA) is the substrate, though no further increase in hydrolytic rate is seen below a concentration of 0.002 M HILA.

In contrast, the esterase activity of Ac_A-carboxypeptidase *increases* as the substrate concentration *increases*. The activity is maximal at 10^{-2} M, the concentration employed throughout these studies. At higher substrate concentrations activity is inhibited. These observations are quite analogous to similar ones of Walsh (Walsh, K. A., personal communication).

Spectral Properties of Ac_A-carboxypeptidase.—The molar absorptivity of native carboxypeptidase at 278 mμ decreases from $\epsilon_{278} = 6.42$ liter mole^{-1} cm^{-1} to 5.78 and 6.01 liter mole^{-1} cm^{-1} when Ac_A-carboxypeptidase is acetylated in the absence and presence of β-phenylpropionate, respectively. These data are quite analogous to those obtained with Ac_I-carboxypeptidase (Simpson *et al.*, 1963). Spectra of the native enzyme and of Ac_A-carboxypeptidase, prepared in the absence and presence of β-phenylpropionate, and the difference spectrum of the latter two, indicate the acetylation of tyrosyl residues (Fig. 6, A and C). As judged by the analogous spectra and difference spectrum for *N*-acetyltyrosine and *N,O*-diacetyltyrosine (Fig. 6, B and D), both the decrease in the absorptivity of the acetylated enzymes between 260 mμ and 290 mμ and the maximum change at 278 mμ are due to the *O*-acetylation of tyrosyl residues.

The number of *O*-acetyltyrosine residues in Ac_A-carboxypeptidase acetylated in the presence and absence of β-phenylpropionate can be calculated from the known molar absorptivities of *N,O*-diacetyl- and

TABLE V

LABILE ACETYL GROUPS OF β-PHENYLPROPIONATE-PROTECTED AND UNPROTECTED Ac_A-CARBOXYPEPTIDASE

Method[a]	Moles O-Acetyl/Mole Enzyme: Unprotected	Protected	Δ
Difference spectra	6.3	4.3	2.0
Acethydroxamate formation	7.2	5.4	1.8
Acethydroxamate formation in 4 M guanidine[b]	6.6	4.9	1.7

[a] Preliminary experiments with C^{14}-labeled acetic anhydride and acetylimidazole give analogous results. [b] *N,O*-Diacetyltyrosine has been reported to be deacetylated by hydroxylamine in 8 M urea (Cohen and Erlanger, 1961). Hence deacetylation in this solvent lends further support to the conclusion that acetylation of tyrosyl residues accounts for both the spectral and the enzymatic changes. Moreover, the transfer of acetyl groups from Ac_A-carboxypeptidase to hydroxylamine does not appear to be dependent on the conformation of the enzyme.

O-acetyltyrosine, as previously described for Ac_1-carboxypeptidase (Simpson *et al.*, 1963).

The spectral changes as well as the enzymic consequences of acetylation can be reversed by hydroxylamine. These can be correlated by simultaneous measurements of the increases of absorbance at 278 mμ, and of peptidase activity, as well as the decrease in esterase activity on incubation of Ac_A-carboxypeptidase with 0.02 M NH_2OH at *p*H 7.5, room temperature, for 200 minutes (Fig. 7). The concurrent changes both of activities and of the absorbance at 278 mμ confirm similar data obtained for Ac_1-carboxypeptidase, and demonstrate that the restoration of activity is due to the deacetylation of approximately 2 acetyl tyrosyl residues (Table V).

The number of moles of *O*-acetyltyrosine formed can also be determined by measuring the moles of acethydroxamate resulting from the deacetylation reaction. Incubation of Ac_A-carboxypeptidase, acetylated in the absence of β-phenylpropionate with 1 M hydroxylamine for 10 minutes at *p*H 7.5, results in the formation of 7.2 moles of acethydroxamic acid per mole of enzyme; 5.4 moles per mole are formed from the enzyme acetylated in the presence of β-phenylpropionate (Table V). The difference in the number of groups should represent the number of tyrosyl residues protected by the inhibitor.

Hydroxamate formation has also been carried out in the presence of 4 M guanidine. At this concentration of reagent the enzyme is inactivated completely and irreversibly. By this method the difference in the number of acetyl groups removed from the protected and unprotected enzyme is 1.7.

Some Physicochemical Properties of Acetylcarboxypeptidase.—While high concentrations of salt are required to solubilize native carboxypeptidase, acetylcarboxypeptidase is soluble in water. Thus only 6 μg of the enzyme prepared by the method of Anson (1937) is soluble in 1 ml of metal-free distilled water while under identical conditions at least 25 mg of acetylcarboxypeptidase dissolves.[8]

Acetylation with acetic anhydride at *p*H 7.5 does not significantly alter the sedimentation constant, $s_{20,w}$, the dispersion constant, λ_c, the specific optical rotation $[\alpha]_D^{10}$, or b_0 measured at this *p*H (Bethune and Ulmer, 1963). However, acetylation with a 48-fold molar excess of acetic anhydride produces significant changes in the electrophoretic properties of native carboxypeptidase. Two anionic fractions are formed, but both their mobilities differ from that of the native enzyme. Details of these studies will be reported elsewhere (Bethune, J. L., Ulmer, D. D., and Vallee, B. L., in preparation).

[8] Similar changes in solubility have been noted on acetylation of a number of globulins. Riordan, J. F. (1961), doctoral dissertation, Fordham University, New York.

TABLE VI

CATALYTIC ACTIVITIES AND FREE AMINO GROUPS FOR SEVERAL ACYLCARBOXYPEPTIDASES

Enzyme	Esterase ($k \times 10^{-3}$)	Peptidase C	Equivalents Phenylalanine/Mole Enzyme
Carboxypeptidase	6.6	35.0	13.8
Ac_A-carboxypeptidase	42.5	1.8	4.7
Propionylcarboxypeptidase	22.6	17.1	10.6
n-Butyrylcarboxypeptidase	12.6	22.4	12.3
n-Valerylcarboxypeptidase	10.7	28.0	13.3
i-Butyrylcarboxypeptidase	8.8	29.1	11.2

Other Acylcarboxypeptidases.—Other monocarboxylic acid anhydrides were also used as acylating agents under conditions identical to those for acetylation with acetic anhydride. The results obtained with propionic, *n*-butyric, *i*-butyric, and *n*-valeric anhydrides are listed in Table VI. The hydrolytic activities of these acylcarboxypeptidases are qualitatively similar to those observed for Ac_A-carboxypeptidase; in each instance the increase in esterase activity is accompanied by a commensurate decrease in peptidase activity. However, when a 48-fold molar excess of anhydride is employed, the magnitudes of the increases in esterase or decreases in peptidase activities of these acylenzymes are less than those of Ac_A-carboxypeptidase. These agents were also less effective than acetic anhydride in acylating ε-amino groups of lysine (Table VI). The concentrations of these anhydrides which could be employed for acylation without loss of zinc from the enzyme were higher than those of acetic anhydride. However, even under apparently optimal conditions for maximal acylation, neither the increases in esterase nor the decreases in peptidase activities ever reached the magnitudes of those observed with acetic anhydride.

Acylation of carboxypeptidase with increasing amounts of butyric anhydride serves as an example (Table VII). Both the degree of modification and

TABLE VII

EFFECT OF BUTYRIC ANHYDRIDE ON THE ENZYMATIC ACTIVITY AND FREE AMINO GROUPS OF CARBOXYPEPTIDASE

Excess of Anhydride	Esterase ($k \times 10^{-3}$)	Peptidase C	Equivalents Phenylalanine/Mole Enzyme
0	7.7	35.0	13.8
36	11.8	27.6	12.5
48	12.6	22.5	12.3
75	12.8	22.0	10.5
108	13.2	20.9	9.0
Acetylbutyryl-carboxy-peptidase[a]	17.2	19.8	7.3

[a] Butyrylcarboxypeptidase was prepared with a 48-fold molar excess of butyric anhydride, and a 24-fold excess of acetic anhydride was added directly to the butyrylation mixture.

esterase activity are augmented when more anhydride is employed, though the ensuing enzymatic changes are relatively small. An increase in the molar excess of butyric anhydride from 36- to 108-fold increases esterase activity from 154% to 170% of the control while the peptidase activity decreases from 79% to 60%. This is a minor change in activities in response to the large change in anhydride concentration, particularly when compared to the effects of acetic anhydride (Fig. 1). In accord with other instances in which the reactivity of higher chain anhydrides with enzymes has been studied (Dixon and Neurath, 1957), butyric and the other anhydrides (Table VII) are less reactive toward carboxypeptidase than is acetic anhydride. This is demonstrated by the further increase in esterase and decrease in peptidase activities when acetic anhydride is added to butyrylcarboxypeptidase (Table VII, line 6).

Discussion

These data document further the profound alteration in the catalytic specificity of carboxypeptidase previously reported (Riordan and Vallee, 1962; Simpson *et al.*, 1963; Vallee *et al.*, 1963; Riordan and Vallee, 1963): acetylation virtually abolishes peptidase activity when either CGP or one of a number of other synthetic peptides serves as the substrate,[9] constituting a dramatic curtailment of the dual specificity of this enzyme. Moreover, under the conditions here employed, the magnitude of the rate at which acetylcarboxypeptidase catalyzes the hydrolysis of the ester substrates HPLA and HILA is increased 6- to 7-fold over that of the native enzyme.

The transformation in catalytic specificity is a function of the molar excess of anhydride employed and is maximal when the molar ratio of anhydride to enzyme is 48 (Fig. 1). On increasing the ratio further, esterase activities decrease, presumably due to changes in physical properties or conformation (Table I). The decrease of activity in the presence of high concentrations of acetic anhydride is also observed when the enzyme is acetylated while protected by β-phenylpropionate. Whether the dissociation of zinc from the enzyme under these conditions precedes or results from these structural changes remains to be determined.

Apocarboxypeptidase is catalytically inactive (Vallee *et al.*, 1958), and hence zinc is an indispensable component of the active center of the enzyme. β-Phenylpropionate, like phenylacetate, binds only when zinc is present,[10] and binding is correlated directly to inhibition, indicating the interaction of β-phenylpropionate with the active center (Coleman, 1963). Further, the binding of this inhibitor masks protein groups of the enzyme which are critical to the catalytic mechanism, thereby preventing the enzymatic changes incident to acetylation. Apparently, these groups are located within the limited radius covered by β-phenylpropionate when bound at the zinc site of the native enzyme.

[9] Ac_A-carboxypeptidase exhibited greatly diminished activity relative to the native enzyme when assayed on benzoylglycyl-L-phenylalanine, carbobenzoxyglycyl-L-tyrosine, carbobenzoxyglycyl-L-valine, carbobenzoxyglutamyl-L-phenylalanine, L-leucyl-L-tyrosine, D-leucyl-L-tyrosine, glycyl-L-tyrosine, hippuryl-L-arginine, acetyl-L-phenylalanine, chloroacetyl-L-phenylalanine, and acetyl-DL-phenyllactate. The *p*H-rate profile for the hydrolysis of acetyl-DL-phenyllactate by native carboxypeptidase resembles that for carbobenzoxyglycyl-L-phenylalanine (Ogilvie, Riordan, and Vallee unpublished experiments).

[10] This is apparent both from kinetics and experiments with C^{14}-labeled β-phenylpropionate at equilibrium (Coleman, 1963).

Some insight concerning the presumable nature of the protected residues may be gained from considerations both of the enzymatic consequences of acetylation with monocarboxylic anhydrides and from the selectivity of their interaction with amino acid side chains of proteins. As zinc is bound to the —SH and α-NH_2 ligand sites of the protein (Vallee *et al.*, 1963), residues other than these must therefore be considered. Acetic and other monocarboxylic acid anhydrides may react with lysyl, aromatic and aliphatic hydroxyl, sulfhydryl, and perhaps histidyl residues of proteins (Fraenkel-Conrat, 1959),[11] and thus they afford only a limited degree of chemical selectivity. Additional reactions have been studied, therefore, to assist in the delineation of the group(s) which are modified (Vallee *et al.*, 1963).

Native zinc carboxypeptidase does not contain free sulfhydryl groups. The single sulfhydryl group, detected after removal of the metal (Vallee *et al.*, 1960) is not alkylated readily with reagents commonly employed for this purpose (Walsh *et al.*, 1962). The one atypical sulfur residue of carboxypeptidase can only be alkylated after reduction, but even then its alkylation does not affect activity (Walsh *et al.*, 1962), and β-phenylpropionate does not protect it against alkylation. Thus this group is not part of the active center and can be eliminated from consideration.

Diisopropylphosphorofluoridate does not react selectively with seryl groups of carboxypeptidase in the manner shown for some other proteolytic and esterolytic enzymes (Hartley, 1960). Exposure of the enzyme to this agent over a wide range of conditions has failed to demonstrate significant effects on catalytic activity. While this does not rule out reaction of acetic anhydride with seryl or threonyl hydroxyl groups, these residues, if acetylated, do not appear to be involved in activity.

Exposure of carboxypeptidase to iodoacetamide, iodoacetate, and bromoacetate have also failed to alter activity in the manner here described for acetic anhydride. These last two agents inhibit the enzyme instantaneously, competitively, and reversibly (Allan, B. J., Hoch, F. L., and Vallee, B. L., in preparation). Irreversible alterations of activity which might be expected on carboxymethylation of histidyl residues (Barnard and Stein, 1959; Gundlach *et al.*, 1959) have not been detected during periods of exposure up to 96 hours and over a range of *p*H from 5.4 to 9.0.

1-Fluoro-2,4-dinitrobenzene which reacts readily with ε-amino groups fails to alter either peptidase or esterase activities.[12] Similarly, amino groups are blocked by acetylation with acetic anhydride. However, neither β-phenylpropionate, which prevents the enzymatic changes, nor hydroxylamine, which reverses them, affects the acetylation of amino groups (Table I). Acetylimidazole moreover, which brings about even larger enzymatic changes than does acetic anhydride, apparently does not acetylate the amino groups (Simpson *et al.*, 1963). It would seem improbable that the acetylation of ε-amino groups would account for the enzymatic changes.

The results of succinylation and amidination support this view. These reactions modify free amino groups essentially to the same degree, but they do not alter the activity as observed with acetic anhydride (Riordan and Vallee, in preparation). Furthermore, iodination and photooxidation of carboxypeptidase, both of which increase esterase and abolish peptidase activities (Vallee *et al.*, 1963), do not modify amino or, in fact, aliphatic hydroxyl groups. Hence modification of these resi-

[11] Experiments with C^{14}-acetic anhydride have indicated approximately 32 residues are modified in the acetylated enzyme.

[12] Omote, Y., and Vallee, B. L., unpublished observations;

dues does not seem to be responsible for the enzymatic changes, either.

On the basis of these considerations, modification of tyrosyl or histidyl residues appears to be the most likely explanation for the changes observed on acetylation (*vide infra*).

The difference spectra in Figure 6 and the spectral changes accompanying deacetylation with hydroxylamine provide convincing evidence that *O*-acetylation of tyrosyl residues of carboxypeptidase are responsible for the alteration of catalytic activity. That two tyrosyl groups are affected can be calculated readily, and the data in Figure 7 which demonstrate direct correlation with activity are in complete accord with previous work employing acetylimidazole (Simpson *et al.*, 1963).

The participation of yet another group in the catalytic mechanism cannot be ruled out, however. When acetylation is performed at discrete values of *p*H between 6 and 7.5, the effect on enzymatic activities, assayed at *p*H 7.5, is altered markedly, although the capacity of acetic anhydride to act as an acetylating agent over this range of *p*H is presumably unchanged. The enzymatic consequences of acetylation when plotted as an activity profile bear close resemblance to the peptidase *p*H-rate profile of the native enzyme. Acetylation of a group which ionizes between *p*H 6 and 7 might be one possible explanation for these data. Alternatively such a group could play a catalytic role in the acetylation of the phenolic hydroxyl groups. It should be noted that difference spectra do not indicate the existence of an acetylhistidyl residue in the modified enzyme.

As previously documented (Neurath and Schwert, 1950), the *p*H-rate profile of peptide hydrolysis also exhibits an inflection between *p*H 6 and 7 and a maximum at *p*H 7.5, and falls off at higher values. The implications of these data to the mechanism of action of carboxypeptidase have been discussed (Vallee *et al.*, 1963).

The *p*H-rate profile for the esterase activity of native carboxypeptidase with HPLA as substrate when plotted as the log of the zero-order rate constant versus *p*H bears close resemblance to data of Agren *et al.* (1961) for the hydroxide ion–catalyzed hydrolysis of proteolytic esters and of Wolfenden (1963) for the hydrolysis of amino acyl RNA.[13] Above *p*H 10.5, esterase activity decreases, apparently due to loss of zinc from the active site. The over-all shape of the *p*H-rate profile suggests that the hydrolysis of HPLA by carboxypeptidase requires the participation of hydroxide ions. This view gains considerable support from the study of model systems in which the metal ion–catalyzed hydrolysis of esters has been found to be hydroxyl-ion dependent (Kroll, 1952).

Both the absolute rate and the rate of increase of esterase activity of Ac_A-carboxypeptidase as a function of *p*H are greater than those of the native enzyme up to *p*H 8.0. Variability of rate measurements cause difficulty above *p*H 8 as indicated by the scatter of points beyond this value (Fig. 4). Electrophoretic examination of Ac_A-carboxypeptidase indicates an active and an inactive component and the proportion of the latter increases with *p*H. This finding may account for the irreproducibility of activity measurements above *p*H 8.0 (Bethune and Ulmer, 1963).

These circumstances suggested that definitive kinetic studies extending those shown in Figures 3, 4, and 5 should be carried out with a preparation whose structure is unaltered by chemical modification and which does not contain inactive protein. Ac_1-carboxypeptidase seems to fulfill these requirements (Simpson *et al.*, 1963). Examination of the kinetics of Ac_1-carboxypeptidase should reveal the mode of action of the tyrosyl residues in the mechanism of action of this enzyme, in substrate binding, or both. Such studies are in progress.

[13] We are indebted to Dr. Richard Wolfenden for allowing us to see his manuscript prior to publication.

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The Amino Acid Composition of Bovine Pancreatic Carboxypeptidase A*

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The amino acid composition of bovine carboxypeptidase A_α has been determined for the twice-crystallized enzyme prepared from purified procarboxypeptidase A. Internal standards were used to enhance the precision of the estimations of the amino acid composition and to permit the direct determination of the weight concentration of the protein. The weight concentration has been used for calculations of the absorbancy index and of the specific enzymatic activity towards two substrates, carbobenzoxyglycyl-L-phenylalanine and hippuryl-DL-phenyllactate. Carboxypeptidase A_α contains 307 amino acid residues. Its amino acid composition is similar to those of bovine carboxypeptidase A_γ and A_δ, except for five to seven amino acids which are lacking in the latter two enzymes, apparently by the deletion of a fragment from the amino terminal region of these molecules.

Crystalline carboxypeptidase A was isolated by Anson (1937) from an exudate of thawing glands of bovine pancreas, and by Allan *et al.* (1964) from an extract of an acetone powder of the freshly-collected glands. Most of the work reported in the literature on carboxypeptidase A has been carried out with one or the other of these two preparations (Neurath, 1960; Vallee *et al.*, 1963). More recently, Cox *et al.* (1964) developed a method of isolation of the enzyme based on the activation of the partially purified zymogen. This method yields carboxypeptidase A of a high specific activity even after the first crystallization. The present paper describes some of the chemical and enzymatic properties of this enzyme.

A precise amino acid analysis is a fundamental prerequisite for the elucidation of the primary structure of any protein, and such an analysis has been performed in the present work with the necessary accuracy and reliability by means of the technique of ion-exchange chromatography (Spackman *et al.*, 1958) augmented by the use of internal standards, as suggested by Walsh and Brown (1962). These measurements have provided a reliable measure of the concentration of solutions of the protein, which in turn was used as a basis for the calculation of certain chemical, physical, and biological parameters of the enzyme. The amino acid composition of this enzyme (carboxypeptidase A_α) has been compared with those of carboxypeptidase A prepared by the method of Anson (1937) and Allan *et al.* (1964), respectively.[1]

A_α Amino terminal alanine (prepared according to Cox *et al.*, 1964).
A_β Amino terminal serine (occurs in variable yield in all preparations).

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[1] Bovine pancreatic carboxypeptidase A, prepared by the various methods of isolation, comprises several different chemical species of the enzyme. The following differences have been characterized (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1963):

A_γ Amino terminal asparagine (occurs as major component in preparation according to Anson, 1937). In contrast to A_δ, the apoenzyme of this chemical species cannot be fully reactivated by zinc (Vallee *et al.*, 1960).
A_δ Amino terminal asparagine (occurs as major component in preparations according to Allan *et al.*, 1964). The apoenzyme of this chemical species can be fully reactivated by zinc (Vallee *et al.*, 1960).

Experimental Procedure

Materials

Carboxypeptidase A_α was isolated from purified procarboxypeptidase by the method of Cox *et al.* (1964) and crystallized twice. The crystals were stored at 4° as a slurry in 0.001 M potassium phosphate buffer, *p*H 7.5. Before use, the crystals were washed three times with cold distilled water, and dissolved in 1 M NaCl–0.001 M Tris, *p*H 8.0. Carboxypeptidase A_γ prepared according to Anson (1937) as modified by Putnam and Neurath (1946) was obtained from the Worthington Biochemical Corporation. Carboxypeptidase A_δ was prepared by the method of Allan *et al.* (1964).

Chromatographically pure CGP[2] and β-thienyl alanine[3] were obtained from Mann Research Laboratories, Inc. Hippuryl-DL-phenyllactate was synthesized in this laboratory by a modification of the procedure of Snoke *et al.* (1948). α-Amino-β-guanidopropionic acid was a product of the California Corporation for Biochemical Research, and norleucine was obtained from the Nutritional Biochemical Corporation.

For hydrolysis of proteins, concentrated HCl was used from freshly opened bottles of Baker & Adamson reagent grade chemical. Tris was purified according to Fossum *et al.* (1951), and recrystallized from ethanol-water solutions. A 2 M solution at *p*H 8.0 was passed through a column of Chelex-100 (Bio-Rad Laboratories) in order to remove the contaminating heavy metal ions.

[2] The following abbreviations are used: CGP, carbobenzoxyglycyl-L-phenylalanine; HPLA, hippuryl-DL-phenyllactic acid; AGP, α-amino,β-guanido propionic acid.

[3] β-Thienylalanine appears on the chromatogram between norleucine and tyrosine.