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The Reaction of Carboxypeptidase A with Chromophoric Substrates *

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ABSTRACT: The reaction of bovine pancreatic carboxypeptidase A with a series of chromophoric substrates has been studied. Newly synthesized substrates used include the amides *N-trans*-cinnamoyl-L-phenylalanine, *N-trans*-3-(2-furylacryloyl)-L-phenylalanine, and *N-trans*-3-(3-indoleacryloyl)-L-phenylalanine as well as two ester analogs, *O-trans*-cinnamoyl-DL-β-phenyllactate and *O-trans*-3-(2-furylacryloyl)-DL-β-phenyllactate. Using ultraviolet absorbance spectrophotometry, the rates of carboxypeptidase-catalyzed hydrolyses of these substrates have been examined as functions of pH, ionic strength, temperature, and enzyme and substrate concentrations. The variation of reaction rates with enzyme and substrate concentrations was found to obey Michaelis–Menten kinetics, even when enzyme and substrate concentrations were approximately equal, as was the case in studies involving amide substrates. The activation parameters calculated from the effect of temperature upon the reaction rates are within the range previously reported for the action of carboxypeptidase on other substrates. Gel filtration on Sephadex G-50 has provided evidence for the existence of intermediates in reactions involving cinnamoylphenylalanine. A new assay for carboxypeptidase with furylacryloylphenyllactate as substrate has been developed.

The reaction of carboxypeptidase A with amide, ester, and protein substrates has been the object of intensive studies by several investigators (reviewed by Neurath and Schwert, 1950; Smith, 1951; Neurath, 1960). The time course of these hydrolytic reactions has been mostly measured by the ninhydrin reaction or by potentiometric titration at constant pH. In order to exploit more sensitive methods of measurements as are required for a detailed investigation of the mechanism of action of this enzyme, a series of substrates for carboxypeptidase A has been developed which allow the reaction to be followed by absorbance spectrophotometry in a region of the spectrum where the protein does not measurably absorb light. The substrates used for this purpose are derivatives of either phenylalanine or of β-phenyllactate wherein the amino or hydroxyl group has been acylated by one of a series of β-aroylacrylic acids (Bernhard *et al.*, 1965). By use of these substrates it has proven possible to examine the mechanism of carboxypeptidase-catalyzed reactions with particular attention to the presence of intermediates.[1]

Experimental Section

Commercial reagents of the highest available quality were obtained from the following sources: *N*-carbobenzoxyglycyl-L-phenylalanine, Mann Research Laboratories; L-phenylalanine, Nutritional Biochemical Corp.; cinnamoyl chloride and cinnamic acid, Eastman

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[1] Abbreviations used in this paper are: CiPhe, cinnamoylphenylalanine; FAPhe, furylacryloylphenylalanine; IAPhe, indoleacryloylphenylalanine; CiPLA, cinnamoylphenyllactate; FAPLA, furylacryloylphenyllactate; Ammediol, 2-amino-2-methyl-1,3-propanediol; and carboxypeptidase, bovine pancreatic carboxypeptidase A.

Organic Chemicals; L-phenylalanine methyl ester hydrochloride, Cyclo Chemical Corp.; DL-β-phenyllactic acid, Krishell Laboratories; furylacrylic acid and indoleacrylic acids, Aldrich Chemical Co.; dicyclohexylcarbodiimide, Schwartz Chemical Co.; dicyclohexylamine, J. T. Baker Chemical Co.; uniformly labeled [^{14}C]-L-phenylalanine (72.7 mc/mmole), Volk Radiochemicals.

[^{3}H]Cinnamic acid was prepared by the New England Nuclear Corp. by trifluoroacetic acid catalyzed exchange of ^{3}H into cinnamic acid. Solutions of [^{3}H]-cinnamoyl chloride were prepared from the tritiated acid by refluxing in $SOCl_2$.

The organic acids were recrystallized several times before use as spectrophotometric standards. Thionyl chloride was purified according to Fieser (1957). Elemental microanalyses were performed in duplicate by either G. Weiler and L. B. Strauss, Oxford, England, or A. Bernhard, Mulheim, West Germany. Melting points are in all cases reported as uncorrected values obtained using a Fisher-Johns apparatus. The sample was placed on the stage at a temperature of 10–15° below the melting point, and heating rates of 3–4°/min were not exceeded. Unless otherwise stated, all reactions and extractions were performed at room temperature. Although most of these substrates are light sensitive, it was not found necessary to avoid normal fluorescent overhead illumination during the syntheses. The substrates were protected from light during prolonged operations such as drying, and were stored dry in the dark in the presence of silica gel.

N-trans-Cinnamoyl-L-phenylalanine. In a typical preparation, L-phenylalanine (4.4 g, 27 mmoles) was dissolved in 1 equiv of 1.0 M NaOH and coupled at 5° with 3.33 g (20 mmoles) of cinnamoyl chloride dissolved in 20 ml of dry dioxane. The solution of acid chloride was added at intervals over a 20-min period while maintaining the pH between 10 and 11 by the periodic addition of 1.0 M NaOH. After stirring the reaction mixture for an additional 30 min, the aqueous phase was acidified with concentrated HCl to a strong congo blue reaction and the solvent was removed by rotary evaporation. The resulting pale yellow oil was crystallized twice from ethanol–water mixtures to produce a white material which was dried overnight *in vacuo* over P_2O_5 to yield 3.97 g of product (mp 176–178°).

Anal. Calcd for $C_{18}H_{17}NO_3$ (295.33): C, 73.20; H, 5.80; N, 4.74. Found: C, 73.06; H, 5.84; N, 4.60.

Cinnamoylphenylalanine preparations in which the cinnamoyl or phenylalanine portions were specifically labeled with ^{3}H or ^{14}C, respectively, were prepared using microscale modifications of the procedure used for the nonradioactive material. Purification of both radioactive substrates was effected by descending chromatography in *t*-amyl alcohol saturated with 3% ammonia. The two radioactive substrates prepared in this manner were found to possess chromatographic and electrophoretic mobilities identical with nonradioactive material. In addition, all three compounds displayed superimposable ultraviolet absorbance spectra and identical behavior in their reaction with carboxypeptidase.

N-trans-3-(2-Furylacryloyl)-L-phenylalanine. L-Phenylalanine methyl ester hydrochloride (1.0 g, 4.6 mmoles) was stirred vigorously in 20 ml of saturated aqueous K_2CO_3 and 25 ml of tetrahydrofuran. The organic phase was separated, dried over Na_2SO_4, chilled to 4°, and used to dissolve 0.64 g (4.6 mmoles) of 3-(2-furyl)acrylic acid and 0.95 g (4.6 mmoles) of *N,N'*-dicyclohexylcarbodiimide. The reaction mixture was incubated at 4° for 6 hr, after which time the solvent was removed by evaporation *in vacuo* and replaced with ethyl acetate containing one or two drops of acetic acid. The solution was chilled, freed of dicyclohexylurea by filtration, and washed successively with 0.2 M HCl, water, 50% saturated aqueous $NaHCO_3$, and water. After drying over Na_2SO_4 and removing the solvent by evaporation under vacuum, an oil was obtained and crystallized from methanol–water to yield a product which contained no phenylalanine and was discarded. The mother liquor of this undesired product was again taken to dryness and the resulting oil crystallized carefully from 2-propanol–water to yield tan needles of the desired material; yield 340 mg, mp 96.5–98.5°.

The ester (300 mg) was saponified with 1.3 ml of 1.0 M NaOH in a homogeneous system of dioxane and water. Upon complete saponification (25 min) the reaction mixture was acidified with HCl and extracted with ethyl acetate which was successively washed with 0.5 M HCl and water. The product was transferred from the ethyl acetate solution to saturated aqueous $NaHCO_3$ and returned to ethyl acetate after acidification. After drying over Na_2SO_4, the ethyl acetate solution was evaporated under reduced pressure to produce an oil which was crystallized three times from ethanol–water and dried overnight under vacuum over NaOH; yield 32%, mp 81–82°.

Anal. Calcd for $C_{16}H_{15}NO_4$ (285.29): C, 67.35; H, 5.30; N, 4.91. Found: C, 67.18; H, 5.37; N, 4.60.

N-trans-3-(3-Indoleacryloyl)-L-phenylalanine. L-Phenylalanine methyl ester hydrochloride (1.0 g, 4.6 mmoles) was stirred vigorously in a mixture of 20 ml of saturated aqueous K_2CO_3 and 25 ml of tetrahydrofuran. The organic phase was separated, dried over Na_2SO_4, warmed in order to dissolve 0.86 g (4.6 mmoles) of 3-(3-indole)acrylic acid, and chilled before the addition of 0.95 g (4.6 mmoles) of dicyclohexylcarbodiimide. The reaction mixture was allowed to stand overnight at 4°, acidified with a few drops of acetic acid, and incubated at 4° for an additional 2 hr. The crystalline residue was removed by filtration and discarded, after which crude indoleacryloylphenylalanine methyl ester could be crystallized from the filtrate by the careful addition of cyclohexane; yield, 85%, mp 163–166°. This material can be recrystallized from ethanol–water if desired; mp of the recrystallized product, 164–165°.

Due to acid lability of indoleacryloylphenylalanine, it was found convenient to prepare and use the dicyclohexylammonium salt of this compound. The crude ester

(560 mg, 1.6 mmoles) was dissolved in 10 ml of absolute methanol to which was added 1.0 ml of 2.0 M NaOH. After 90 min the solution was evaporated to dryness *in vacuo*. The resulting pale yellow solid was dissolved in 1 or 2 ml of ethanol, transferred to a separatory funnel, and diluted with some water. After adding diethyl ether to the funnel, cold 5% aqueous citric acid was introduced with continual shaking. When no further oil appeared upon acidification, the ether phase was separated, washed with water, dried over Na_2SO_4, and treated with 0.50 ml (2.5 mmoles) of dicyclohexylamine to produce a copious white oil which crystallized. The pure white crystals were separated, dried (802 mg, 97% yield), and recrystallized from ethanol–ether; mp 197–198°.

Anal. Calcd for $C_{32}H_{41}N_3O_3$ (515.70): C, 74.53; H, 8.01; N, 8.15. Found: C, 74.29; H, 7.95; N, 8.13.

*O-trans-Cinnamoyl-*DL*-β-phenyllactic Acid.* A solution of 1.0 g (6.0 mmoles) of cinnamoyl chloride dissolved in 5 ml of tetrahydrofuran was chilled to 5° and mixed with a cooled solution of 1.0 g (6.0 mmoles) of DL-β-phenyllactic acid in an equal volume of the same solvent. Over a 5-min period, aliquots of pyridine totaling 1.2 ml (14 mmoles) were added to the reaction mixture and the system was stirred at 5° for 60 min. After an additional 60 min of stirring at room temperature the solvent was removed on a rotary evaporator and the clear colorless oil dissolved in chloroform. The chloroform solution was extracted with water, dried over Na_2SO_4, and evaporated *in vacuo* to yield an oil which was crystallized three times from ethanol–water and dried over P_2O_5 overnight *in vacuo;* mp 130.4–131.5°; mixture melting range with cinnamic acid 108–127°; yield 81%.

Anal. Calcd for $C_{18}H_{16}O_4$ (296.31): C, 72.96; H, 5.44. Found: C, 73.47; H, 5.50.

*O-trans-3-(2-Furylacryloyl)-*DL*-β-phenyllactic Acid.* This compound was synthesized using furylacryloyl chloride (Sasake, 1910) and a modification of the procedure introduced by Bartlett and Ross (1947) for the synthesis of furylacryloyl esters.

A solution of 2.0 g (14.5 mmoles) of furylacrylic acid in 25 ml of thionyl chloride was gently refluxed for 45 min. The solvent was removed by distillation and the dark brown oil dissolved in diethyl ether which was then removed by distillation at reduced pressures. After repeating the ether entrainment a second time, the product was dissolved in 20 ml of ether and treated with 1.6 ml (19 mmoles) of pyridine. The resulting mixture was added in small aliquots to 1.57 g (9.5 mmoles) of β-phenyllactic acid in 50 ml of diethyl ether and allowed to react for 15 min. After removing the solvent by evaporation *in vacuo*, the resulting dark mass was thoroughly extracted with boiling ethyl acetate. The ethyl acetate extract was washed successively with 0.5 M HCl and water, then extracted with saturated aqueous $NaHCO_3$ until no further yellow color was transferred to the aqueous phase. The $NaHCO_3$ solution was acidified with concentrated HCl to produce a white oil which was transferred to ethyl acetate. The ethyl acetate solution was dried over Na_2SO_4 and evaporated at reduced pressures to yield a light tan oil which solidified upon trituration with petroleum ether (mp 30–60°) and was recrystallized three times from cyclohexane; yield 64%, mp 97–98°.

Anal. Calcd for $C_{16}H_{14}O_5$ (286.27): C, 67.12; H, 4.93. Found: C, 67.78; H, 5.07.

The optical purity of the substrates was determined by enzymic hydrolysis with carboxypeptidase. In all cases the absorption spectrum of the final reaction mixture was within ±2% of the spectrum expected for complete hydrolysis of the susceptible isomer. In addition, thin layer chromatography of enzymic hydrolysates of the amides indicated complete conversion of the substrate into phenylalanine and the acid corresponding to the parent acyl group.

Buffers. In the following description, the term standard buffer will be used to define solutions of 0.1 M ionic strength in buffer salts with NaCl added to a total ionic strength of 1.10 M. The buffer salts employed are: pH 4.6–6.7, sodium acetate–acetic acid; pH 7.0–8.3, Tris–HCl; and pH 8.5–9.8, Ammediol–HCl. Buffers containing guanidine hydrochloride were made by titrating appropriate amounts of guanidine carbonate with concentrated HCl until complete conversion of the chloride was obtained and incorporating the resulting solution into the desired buffer. All other buffers are as stated in the text.

Substrate Solutions. Substrate solutions were made by weight from the appropriate compounds in buffers of known composition. Unless otherwise stated, the concentrations given refer to the concentration of the susceptible isomer. Due to the fairly rapid spontaneous hydrolysis of the ester substrates, substrate solutions were not kept for periods longer than two to three days. Before use, the spectrum of each substrate solution was routinely checked to ensure that no changes had taken place.

Enzyme. Carboxypeptidase A_γ[2] was purchased from the Worthington Biochemical Corp., Freehold, N. J., stored as a crystalline suspension under water saturated with toluene vapor, and used without further purification. Several lots were used in the present study, all of which possessed the same specific activity and solubility characteristics. Before use, crystals of enzyme were washed twice with distilled water and dissolved in 3.0 M NaCl. The solutions were centrifuged and stored at 4° in concentrations of about 30 mg/ml. Such solutions exhibit no change in activity over periods of several months. Working solutions were prepared daily by diluting the stock solutions to the desired concentration with 1.0 M NaCl. In no case were any enzyme solutions kept for longer than 5–7 days.

Enzyme concentrations were measured by absorbance at 278 mμ (Beckman DU spectrophotometer) and using a molar extinction coefficient of 6.49×10^4 l./mole cm (Bargetzi *et al.*, 1963). It was found that carboxy-

[2] The three methods of preparation and the four chemical species of carboxypeptidase are summarized by Bargetzi *et al.* (1963).

peptidase exhibits a negative deviation from Beer's law at absorbances >1 unit, and for this reason all solutions were diluted to produce a final absorbance between 0.2 and 0.5 unit.

DFP treatment of the enzyme was routinely carried out by use of 10^{-3} to 10^{-4} M DFP in solutions buffered at pH 7.5. The reaction was usually allowed to proceed for 30 min at 4°, then for an additional 30 min at room temperature with occasional readjustment of the pH to 7.5, if necessary. Phenylmethanesulfonyl fluoride (Fahrney and Gold, 1963) and tosyl-L-phenylalanine chloromethyl ketone (Schoellman and Shaw, 1963) were also used as reagents to inhibit adventitious endopeptidases present in the enzyme preparations used. These inhibitors were found to be without effect on carboxypeptidase activity.

Measurements of enzyme activity were either made in the pH-stat using hippurylphenyllactate (Bargetzi *et al.*, 1963) or the chromophoric substrate furylacryloylphenyllactate. The latter assay was followed in the spectrophotometer at 320 mμ at an initial substrate concentration of 3.5×10^{-5} M in pH 7.5 standard buffers at 25°. The rate constants for this reaction (*cf.* Table III) correspond to a value of $v_0/E_0 = 9.87$ sec^{-1}.

Spectral Data. Infrared spectra were obtained in chloroform solution in a Perkin-Elmer Model 21 recording double beam infrared spectrophotometer. Routine measurements of ultraviolet absorbance were performed in either a Beckman DU or a Zeiss PMQ-II spectrophotometer. In either case, slit widths of 0.5 mm were not exceeded. Kinetic and spectral measurements were performed as described by McClure *et al.* (1964), utilizing a Perkin-Elmer Model 350 recording spectrophotometer equipped with time drive and repetitive scan devices. Extinction coefficients in a given solvent were evaluated from the variation of absorbance with the concentration of the relevant compound. In all cases the Beer–Lambert law was obeyed within the concentration range examined.

Kinetics. Kinetic data were obtained either by measuring initial velocities directly or by utilizing the fact that

$$v_0 = S_0 \left\{ \lim_{t \to 0} \left[\frac{\partial \ln S}{\partial t} \right] \right\} \qquad (1)$$

In all cases plots of log S *vs.* time (t) were linear beyond 50% completion of the reaction. When used, initial velocities evaluated by use of eq 1 agreed to within 2 to 3% with those obtained by visual examination. Rate constants taken from semilogarithmic presentations of S *vs.* t or evaluated by dividing initial velocities by the appropriate concentrations are considered *observed* constants. These constants will be represented by the symbols k_1^{obsd} for an observed first-order constant and k_2^{obsd} for an observed second-order constant. The symbols lacking the superscript obsd will be associated with specific steps in a given mechanism. In order to avoid confusion, k_1^{obsd} will be taken with respect to either substrate concentration or time, but never with respect to enzyme concentration. The symbol (v_0/E_0) is used for the latter parameter. In all cases, kinetic parameters have been extrapolated to time 0 in order to avoid interference with back reactions, product inhibition, or both.

All the data obtained have been subjected to refinement by one of two further treatments. If a plot of the data yielded a straight line, the slope, intercept, and standard deviations of both quantities were determined using the method of least squares. If the data did not fit a straight line but were believed to fit a postulated function, the agreement was examined using digital computation and numerical analysis based upon the Newton–Raphson method (Scarborough, 1962). Equations which could be recast to yield a linear function such as the Michaelis–Menten equation were generally tested by both procedures. No significant differences were found between the two methods in such cases.

Radioactive Counting. ^{14}C was determined using either the Nuclear-Chicago Model 183 scaler equipped with a D-47 gas-flow counter and automatic sample changer or a Packard Tri-Carb liquid scintillation counter. Efficiencies realized using the two instruments were 31–33 and 80–83%, respectively, and were determined with each run in order to serve as a basis for comparison from run to run. Liquids were counted by placing a 1.0-ml sample in a scintillation vial, adding 10 ml of scintillation liquid, chilling for 2 hr or more at 4°, and counting. Radioactive samples on paper cut from chromatograms were diced into a scintillation vial and allowed to steep in 1.0 ml of a suitable eluent for 15 min, after which time 10 ml of scintillation liquid was added and the vials were chilled and counted. Paper spots handled in this way yielded precise results if the elution was complete. Tritium was counted only in the scintillation counter, utilizing the methods given for ^{14}C. Efficiencies varied between 20 and 22%.

Chromatography. Amino acids were separated either in descending 4:1:5 1-butanol–glacial acetic acid–water (Katz *et al.*, 1959) or in the two-dimensional system of Richmond and Hartley (1959). Cinnamic acid and cinnamoylphenylalanine were fractionated by descending chromatography in *t*-amyl alcohol saturated with 3% aqueous ammonia. It was found that reproducible R_F values with this system could be obtained only by washing the chromatography paper in solvent to which 10^{-4} M disodium ethylenediaminetetraacetic acid had been added. Washing was accomplished by placing papers in the usual chromatography troughs and allowing solvent to elute over them for 2–3 days. They were then dried and stored in the dark at 4° until needed. Sephadex G-50 columns were prepared according to the directions of Coleman and Vallee (1962b).

Results

Typical spectra obtained during the carboxypeptidase-catalyzed hydrolysis of furylacryloylphenyllactate and indoleacryloylphenylalanine are given in Figure 1. For both the ester and the amide, hydrolysis is accompanied by a marked change in absorbance and a shift of the absorbance maximum to shorter wave-

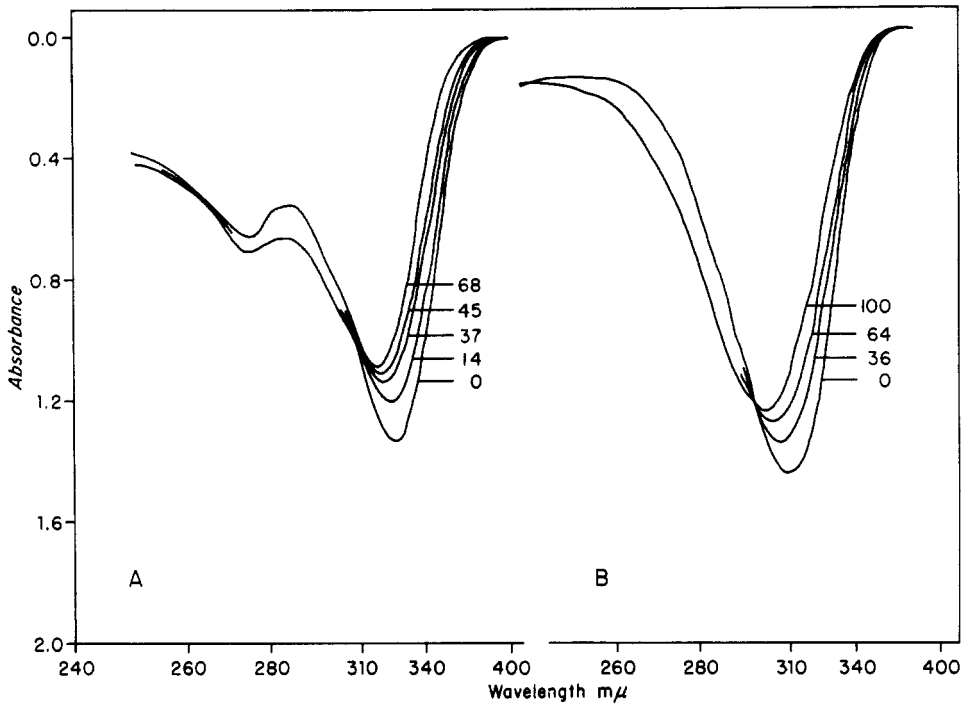

FIGURE 1: The spectral changes occurring during hydrolysis in IAPhe (A) and FAPLA (B). The figures represent the extent of the reaction in per cent. For (A): $E_0 = 3.0 \times 10^{-5}$ M, $S_0 = 5.5 \times 10^{-5}$ M. For (B): $E_0 = 6.4 \times 10^{-9}$ M, $S_0 = 6.2 \times 10^{-5}$ M (total concentration). Both reactions were performed in 0.1 M ionic strength Tris–HCl, pH 7.5.

TABLE I: Ultraviolet Absorbance Characteristics of Some β-Arylacrylic Acids and Derivatives.[a]

Compd	λ_{max} (mμ)	ϵ_m ($\times 10^{-4}$)
CiPhe	277	2.57
CiPLA	281	2.17
Cinnamate	269.5	2.03
FAPhe	303	2.56
FAPLA	308.5	2.34
Furylacrylate	294	2.30
IAPhe	327	2.43
	285[b]	0.918
	275	1.12
	250[b]	0.788
Indoleacrylate	313	1.88
	282[b]	1.06
	273	1.20
	245[b]	0.56

[a] All data were obtained in pH 7.5 standard buffers, 25°. [b] Minimum.

lengths. These changes are typical of all the substrates examined at all ionic strengths (0.1–1). The well-defined isosbestic points observed in these two reactions are also characteristic of the reactions involving each of the remaining substrates. The carboxypeptidase-catalyzed hydrolysis of each substrate was found to obey first-order kinetics when initial substrate concentrations of less than about 5×10^{-5} M were employed (Figure 2). The data of Figure 2 document the fact, observed for all the substrates at all ionic strengths (0.1–1), that the first-order rate constant for the reaction is independent of the wavelength of observation. Relevant spectral data concerning not only the substrates but also the hydrolysis products, β-arylacrylic acids, are given in Table I.

TABLE II: Changes in Ultraviolet Absorbance Occurring during the Hydrolysis of Substrates.[a]

Substrate	λ_{opt}[b] (mμ)	$\Delta\epsilon_M$[c] ($\times 10^{-4}$)	Isosbestic Pt[d]
CiPhe	295	1.01	261
CiPLA	290	1.00	273
FAPhe	310	1.04	289, 256
FAPLA	320	1.44	298, 240
IAPhe	345	0.975	312, 262

[a] All data were obtained in pH 7.5 standard buffers, 25°. [b] λ_{opt} represents the wavelength at which the greatest change in absorbance is observed during the reaction. [c] $\Delta\epsilon_m$ represents the change in extinction coefficients observed at λ_{opt}, based upon the concentration of the susceptible isomer. [d] Observed during the carboxypeptidase-catalyzed conversion of substrate to products.

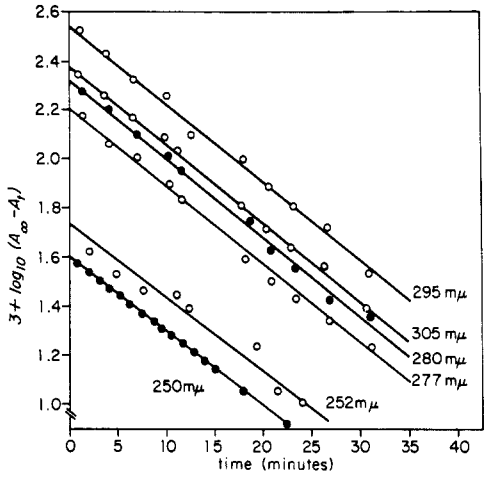

FIGURE 2: Progress curves for the carboxypeptidase-catalyzed hydrolysis of CiPhe, taken from data similar to that of Figure 1 and plotted in terms of first-order kinetics as functions of the absorbance values observed at time t (A_t) and time infinity (A_∞). The 250-mμ data are replotted from an experiment in which expanded transmission scales were employed. $E_0 = 3.6 \times 10^{-5}$ M, $S_0 = 3.6 \times 10^{-5}$ M, in 0.1 M ionic strength Tris–HCl, pH 7.5, 25°.

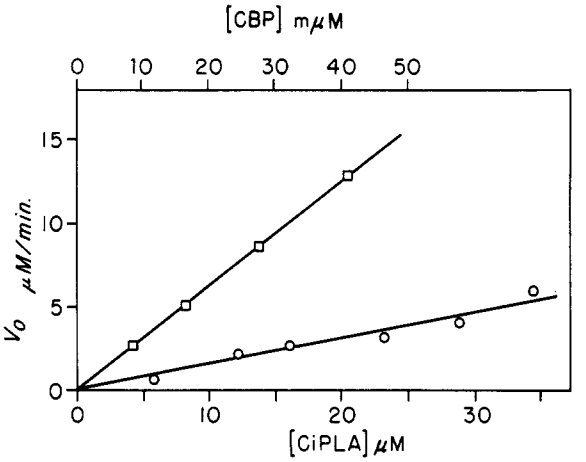

FIGURE 3: The variation in initial rates of hydrolysis of CiPLA with enzyme (□) and substrate concentration (○) in pH 7.5 standard buffer. In the upper curve (□) the substrate concentration was maintained at 1.75×10^{-5} M; in the lower (○) the enzyme concentration was maintained at 8.2×10^{-9} M.

The wavelengths corresponding to the maximum changes in absorbance during the reactions are listed in Table II, along with the associated changes in extinction coefficients. The wavelengths given in Table II have been routinely used to obtain the progress curves of reactions in the work that follows. That the reaction actually being observed in these systems is the carboxypeptidase-catalyzed hydrolysis of the expected bond in the substrate was indicated by the following experiments. (1) If the reaction between the enzyme and any of the substrates was allowed to proceed to completion the spectrum of the products agreed exactly with the spectrum of a 50:50 mixture of substrate and the relevant acid.[3] (2) Thin layer chromatography of the products of reactions involving the amide substrates revealed the presence of only phenylalanine and the expected β-arylacrylic acid. (3) If the reaction was followed by both absorbance changes and by chemical methods, the observed rates were equal. Thus when cinnamoylphenylalanine (1.12×10^{-4} M) was treated with carboxypeptidase (1.2×10^{-5} M) and the reaction followed simultaneously by the release of ninhydrin-positive material (Matheson *et al.*, 1961) and by the change in absorbance at 295 mμ, the initial rates of reaction were found to be 6.27 and 6.20×10^{-8} mole/l. sec, respectively. (4) Experiments in which the carboxypeptidase-catalyzed hydrolysis of cinnamoyl-L-[^{14}C]-phenylalanine was followed by means of chromatographic separation of the products also gave equal rates for the appearance of free phenylalanine and the disappearance of cinnamoylphenylalanine. These latter results also demonstrate that the reaction between cinnamoylphenylalanine and carboxypeptidase is in a steady state in the initial phase.

These *N*-acylated amino acids are relatively poor substrates for carboxypeptidase. For example, the reaction of the enzyme with cinnamoylphenylalanine, a typical member of the series, is characterized by an observed turnover number of about 0.2 min^{-1} ($S_0 = 1.0 \times 10^{-4}$ M). The possibility that these slow reactions were catalyzed by a contaminating enzyme was excluded by the observation that the hydrolysis of cinnamoylphenylalanine was completely inhibited if the enzyme was first exposed to 1,10-phenanthroline (1×10^{-3} M) for 120 min in standard buffer, pH 8.0, at 25°. Under these conditions, the hydrolysis of 0.01 M hippuryl-phenyllactate is reduced to less than 1% of the initial rate. The hydrolysis of cinnamoylphenylalanine was also inhibited by β-phenylpropionate, a competitive inhibitor of carboxypeptidase (Elkins-Kaufman and Neurath, 1949).

In contrast to the amide substrates, the ester analogs are excellent substrates for carboxypeptidase. The turnover numbers are relatively high (>2000 min^{-1}), and as in the case of the amides, enzymatic hydrolysis is inhibited by 1,10-phenanthroline as well as by β-phenylpropionate.

Dependence of Initial Velocities on Concentration of Reactants. Preliminary experiments indicated that pH 7.5 and an ionic strength of 1.0–1.1 were experimentally convenient conditions to study the hydrolysis of the present substrates since in this range hydrolysis rates were insensitive to variations of ionic strength. In all

[3] Due to their low extinction coefficients, neither phenyllactic acid nor phenylalanine contribute appreciably to the absorbance of these reaction mixtures.

TABLE III: Kinetic Parameters for the Carboxypeptidase-Catalyzed Hydrolysis of Chromophoric Substrates.[a]

Substrate	k_2^{obs}[b] (l./mole sec)	K_m (mole/l.)	k_2 (sec^{-1})
CiPhe	33.0	$\sim 6 \times 10^{-4}$[c]	...
FaPhe	37.8	...	...
IAPhe	2.27	$5.84 \pm 0.65 \times 10^{-4}$	$1.38 \pm 0.08 \times 10^{-3}$
CiPLA	3.12×10^5	...	...
FAPLA	3.56×10^5	$1.32 \pm 0.07 \times 10^{-4}$	47.0 ± 1.4

[a] All data were obtained in pH 7.5 standard buffers, 25°. [b] Extrapolated to 0 substrate concentration. It can be shown that eq 4 yields $k_2^{obsd} = k_2/(K_m + E_0)$ in the limit of 0 substrate concentration; accordingly, the value of k_2^{obsd} given for indoleacryloylphenylalanine has been calculated by this means. [c] This value has been approximated from gel filtration experiments using Sephadex G-50, assuming the existence of a one-to-one complex between CiPhe and carboxypeptidase and employing the data of Figure 9.

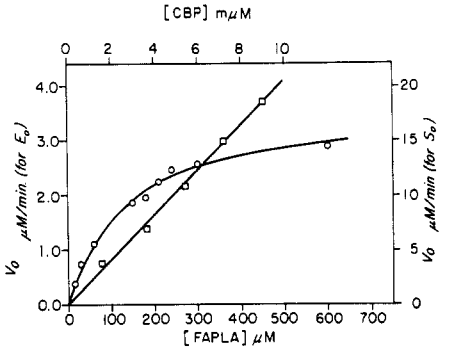

FIGURE 4: The variation in initial rates of hydrolysis of FAPLA with enzyme (□) and substrate (O) concentrations in pH 7.5 standard buffer. $S_0 = 3.05 \times 10^{-5}$ M for the curve representing variations in the enzyme concentration (□); $E_0 = 6.4 \times 10^{-9}$ M for the curve representing variations in the substrate concentration (O).

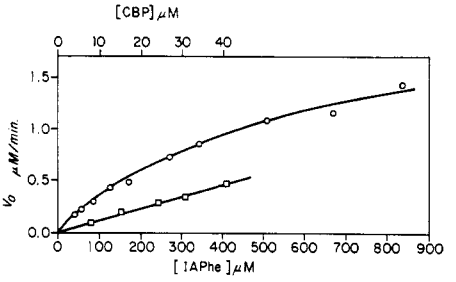

FIGURE 5: The variation in steady-state rates of hydrolysis of IAPhe with enzyme (□) and substrate (O) concentrations in pH 7.5 standard buffers. In the lower curve (□), the substrate concentration was maintained at 9.2×10^{-5} M; in the upper (O), the enzyme concentration was maintained at 2.9×10^{-5} M. [CBP] denotes carboxypeptidase.

instances, initial reaction rates were directly proportional to enzyme concentration. This was true of ester as well as of amide substrates, the latter reacting with approximately equal concentrations of enzyme. Typical data are given in Figures 3–5.

The variation of reaction rates with substrate concentrations was more complex. In low substrate concentrations ($<5 \times 10^{-5}$ M) the initial velocity was proportional to substrate concentration. Data of this type were obtained for cinnamoylphenylalanine, furylacryloylphenylalanine, and cinnamoylphenyllactate. The relationship found for the hydrolysis of cinnamoylphenyllactate (Figure 3) is typical of these three substrates. The corresponding rate constants, obtained by least-squares extrapolations, are presented in Table III.

Initial rates of the enzyme-catalyzed hydrolysis of the ester substrate furylacryloylphenyllactate were measured over a 38-fold range (1.6×10^{-5} M to 6.0×10^{-4} M) and are presented in Figure 4, along with the variation of the reaction rate with the enzyme concentration for the same substrate. These data gave a linear relation when plotted according to Eadie (1942) and were analyzed by means of digital computation in order to obtain the reaction constants given in Table III.

The reaction between carboxypeptidase and indoleacryloylphenylalanine was measured over a 21-fold range of substrate concentrations (4.2×10^{-5} M to 8.7×10^{-4} M). Data representing the variation of initial rates with both substrate and enzyme concentration are given in Figure 5. Despite the fact that the enzyme concentration is not insignificant in comparison to substrate concentration, these rates also varied with substrate concentration according to a rectangular hyperbola. The reaction parameters obtained by treatment of the data using digital computation (see Discussion) are given in Table III.

Variation of Reaction Rates with pH, Ionic Strength, and Temperature. Representative data for the pH dependence of reaction rates are given in Figure 6 for the hydrolysis of two analogous substrates, furylacryloylphenylalanine and furylacryloylphenyllactate.

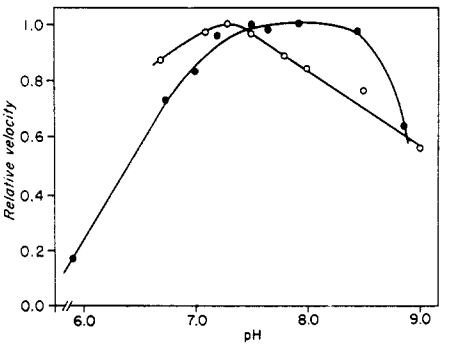

FIGURE 6: The variation of initial velocities with pH for the carboxypeptidase-catalyzed hydrolyses of FAPhe (○) and FAPLA (●). For FAPhe: $E_0 = 3.2 \times 10^{-5}$ M, $S_0 = 5.4 \times 10^{-5}$ M. For FAPLA: $E_0 = 3.8 \times 10^{-9}$ M, $S_0 = 3.15 \times 10^{-5}$ M. The reactions were carried out in standard buffers of pH 7.5 at 25°.

These data are also typical of those obtained with the other compounds. In the hydrolysis of the amide substrates the optimum is near pH 7.5, as is also true for the carboxypeptidase-catalyzed hydrolysis of carbobenzoxyglycyl-L-phenylalanine and related substrates (Neurath and Schwert, 1950). The pH–rate profile of cinnamoylphenyllactate is similar to that given in Figure 6 for furylacryloylphenyllactate. The peaks observed with the ester substrates are broader than those found with the amides, and the esters possess slightly higher pH optima. In order to facilitate comparison, the effects of ionic strength and temperature were all measured at pH 7.5.

Representative data of the effect of ionic strength, adjusted by the addition of NaCl, on hydrolysis rates are shown in Figure 7 for the carboxypeptidase-catalyzed hydrolyses of cinnamoylphenylalanine, indoleacryloylphenylalanine, furylacryloylphenylalanine, and furylacryloylphenyllactate. It is apparent that in all cases increasing ionic strength progressively decreases hydrolysis rates up to *ca.* 0.5 M. Beyond this concentration, the rates of hydrolysis are nearly independent of ionic strength. In view of these findings, standard conditions of 1.1 M ionic strength were chosen, 1.0 M being contributed by NaCl and 0.1 M by the buffer salts.

The effect of varying temperatures on initial rates was determined for each substrate in pH 7.5 standard buffers. In each case, the measured rates were determined at five to eight different temperatures. Substrate concentrations were $<5.0 \times 10^{-5}$ M, in order to stay within the range of first-order kinetics (see above). Under these conditions, the temperature effect for each substrate could be adequately described by the Arrhenius equation. Representative data for cinnamoylphenylalanine are presented in Figure 8. Activation parameters calculated according to the absolute rate theory (Eyring and Stearn, 1939) are presented in Table IV. The values are

TABLE IV: Activation Parameters for the Carboxypeptidase-Catalyzed Hydrolyses of Some Chromophoric Substrates.[a]

Substrate	ΔE_{act} (cal/mole)	ΔF^* (cal/mole)	ΔH^* (cal/mole)	ΔS^* (cal/mole deg)
CiPhe	19.2	15.4	18.6	10.7
FAPhe	16.3	15.3	15.7	1.4
IAPhe	16.9	17.0	16.3	−2.4
CiPLA	4.7	10.0	4.1	−19.8
FAPLA	8.6	9.5	8.0	−5.4

[a] Obtained at 25° in pH 7.5 standard buffer.

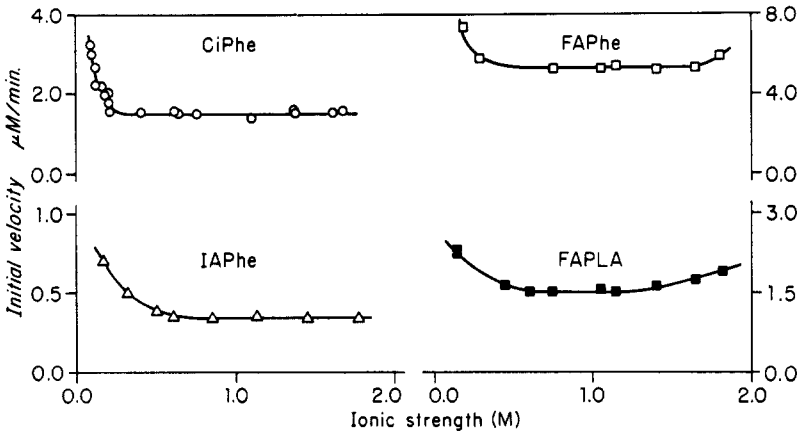

FIGURE 7: The variation of reaction velocity with ionic strength for the carboxypeptidase-catalyzed hydrolyses of the amide substrates CiPhe (○), FAPhe (□), IAPhe (Δ), and the ester substrate FAPLA (■). Conditions: 25° in pH 7.5 Tris–HCl buffers of ionic strength 0.1 M. For CiPhe, $E_0 = S_0 = 2.7 \times 10^{-5}$ M; for FAPhe, $E_0 = 4.3 \times 10^{-5}$ M, $S_0 = 5.4 \times 10^{-5}$ M; for IAPhe, $E_0 = 5.0 \times 10^{-5}$ M, $S_0 = 5.3 \times 10^{-5}$ M; for FAPLA, $E_0 = 2.8 \times 10^{-9}$ *M*, $S_0 = 3.5 \times 10^{-5}$ M.

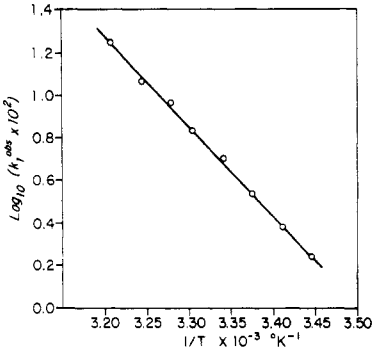

FIGURE 8: The effect of temperature on the carboxypeptidase-catalyzed hydrolysis of CiPhe. $S_0 = 3.0 \times 10^{-5}$ M, $E_0 = 2.4 \times 10^{-5}$ M, pH 7.5 standard buffer.

in the range of those generally encountered with carboxypeptidase (Neurath and Schwert, 1950; Lumry *et al.*, 1951).

Binding of Substrates to Carboxypeptidase. The preceding experiments delineate the kinetic behavior of these chromophoric substrates toward carboxypeptidase. Since some of these react at measurable rates with the enzyme in nearly equimolar concentrations, it was deemed of interest to attempt the demonstration of enzyme–substrate intermediates, using the gel filtration method as described by Coleman and Vallee (1962b). To this end, a solution containing both enzyme and substrate was prepared and, after a suitable reaction period, subjected to fractionation by gel filtration on small columns of Sephadex G-50. By this means fractions of high molecular weight material representing enzyme and enzyme–substrate complexes should be separated from smaller molecules (buffer, unreacted substrate, reaction products, etc.). If a radioactive substrate is employed, the presence of enzyme–substrate complexes can be detected by the appearance of radioactivity in the enzyme fractions. The system cinnamoyl-[^{3}H]-L-phenylalanine–carboxypeptidase was investigated in this manner. The reactants were incubated in 0.1 M Tris–HCl buffers, pH 7.5, for 30 sec before the entire reaction mixture was applied to a Sephadex G-50 column. The results of three experiments in which the pH of the eluting buffers was varied from 4.4 to 7.5 and 9.4, respectively, are shown in Figure 9. The pH at which the separation is performed clearly affects the amount of cinnamoyl residue which is associated with the enzyme fractions. While a small amount of tritium is found when elution is carried out at pH 7.5, at pH 9.4 the enzyme fraction contained no more ^{3}H than the control. Gel filtrations performed at pH 4.4 suggest much more extensive complex formation. At this pH the binding of tritium to the enzyme was unaffected by changing the flow rates, the column lengths, or both, even though the times of separation were thereby varied over a fivefold range. These findings indicate that the complex responsible for the appearance of ^{3}H in the enzyme-containing fractions is stable at pH values near 4.4.

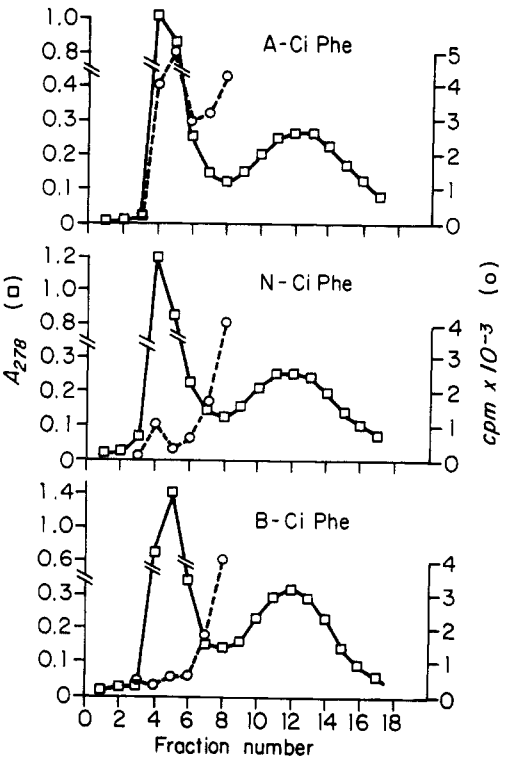

FIGURE 9: Separation of mixtures of [^{3}H]cinnamoyl-L-phenylalanine (9.8×10^{-5} M, 2.9×10^6 cpm/μmole) and carboxypeptidase (7.3×10^{-5} M) following a 30-sec incubation at pH 7.5, 25°. A sample of 1.0 ml was applied to a 1.5 × 10 cm column of Sephadex G-50 and eluted with 0.1 M buffers of the appropriate pH (4.4, 7.5, and 9.4, respectively). The enzyme-containing fractions emerged from the column between 3 and 5 min after mixing the enzyme and substrate. □, A_{278}; ○, counts per minute per milliliter of eluate.

Although the pH at which gel filtration is carried out strongly influences the composition of the enzyme-containing fractions, the pH of the incubation before the separation seems to be less important in this regard. This point is illustrated by the data of Figure 10 which demonstrate the filtration of a reaction in which both the preincubation and the separation were performed at pH 4.4. The binding of ^{3}H to the enzyme is the same as that shown by filtrations in which the preincubation was performed at pH 7.5 (Figure 9, top).

The binding demonstrated by these experiments can be prevented by incorporation of 0.1 M β-phenylpropionate into the separation buffers. An experiment in which this has been done, which in all other respects is exactly parallel to that of the top diagram of Figure 9, is given in Figure 11. It is clear that no substrate radioactivity is found in the fractions containing high molecular weight material. Guanidine hydrochloride can also prevent this binding. Control experiments were carried out to show that 0.1 M acetate buffers, pH 5.0, containing 6.0 M guanidine hydrochloride will inactivate carboxypeptidase very quickly (<5 sec) with the

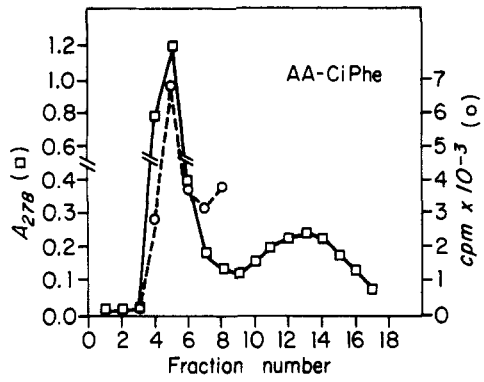

FIGURE 10: As in Figure 9, except both the reaction and the separation were carried out at pH 4.4. Specific activity of CiPhe, 2.9 × 10^6 cpm/μmole. □, A_{278}; ○, counts per minutes per milliliter of eluate.

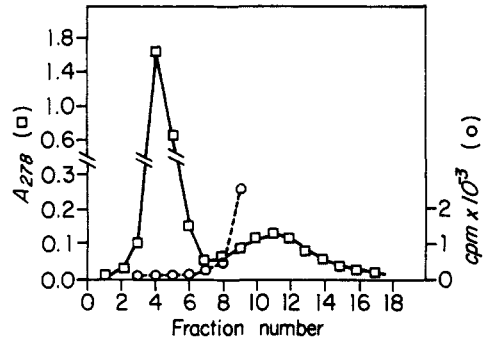

FIGURE 11: Gel filtration as in Figure 9, top diagram, except that the separation buffers contained also 0.1 M β-phenylpropionate. Specific activity of CiPhe, 2.9 × 10^6 cpm/μmole. □, A_{278}; ○, counts per minute per milliliter of eluate.

formation of a soluble product. Using this solvent as an eluent, a reaction similar to that shown in the top diagram of Figure 9 was performed. As was the case when β-phenylpropionate was employed, no radioactivity was observed in the enzyme-containing fractions.

Control experiments of this same type were carried out using tritiated cinnamic acid and carboxypeptidase. In a series of reactions in which both the preliminary incubation and the separation were carried out at pH 4.4, radioactive cinnamate was again observed to emerge from the column in the fractions containing the enzyme. A similar effect was observed if the filtration was carried out at pH 7.5, but at this pH the amount of cinnamate bound to the enzyme was markedly reduced. In the light of these results, it was of interest to examine the carboxypeptidase-catalyzed hydrolysis of furylacryloylphenyllactate in the absence and presence of 0.005 M cinnamate in pH 7.5 standard buffers. The results of such an experiment, given in the upper portion of Figure 12, indicate strong inhibition of the reaction

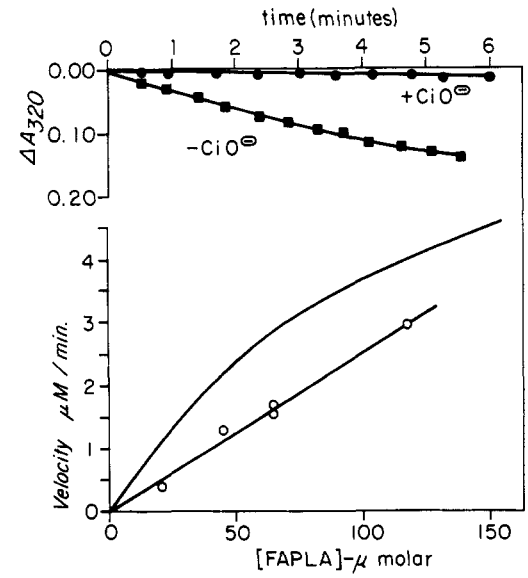

FIGURE 12: The effect of cinnamic acid on the hydrolysis of FAPLA. Upper curve: progress curves for FAPLA hydrolysis in the presence (●) and absence (■) of 0.0051 M cinnamate. E_0 = 3.0 × 10^{-9} M, S_0 = 4.1 × 10^{-5} M in pH 7.5 standard buffer. Lower curve: velocity–substrate relationships for a similar reaction. The experimental points were obtained in the presence of 3.1 × 10^{-3} M cinnamate; the smooth curve is calculated from the observed data for K_m and k_2 for FAPLA. E_0 = 3.0 × 10^{-9} M, pH 7.5 standard buffer.

by cinnamate. If the substrate concentration is varied at constant concentrations of enzyme and cinnamate, the results presented in the lower portion of Figure 12 are obtained. In this presentation, the solid line has been calculated from the observed kinetic parameters for the uninhibited hydrolysis of furylacryloylphenyllactate.

Discussion

The reactions of carboxypeptidase with its substrates have been investigated by a variety of classical techniques. The results presented herein have demonstrated the useful qualities of substrates which can be studied by means of ultraviolet absorbance spectrophotometry in wavelength regions where the enzyme practically does not absorb. The substrates introduced in this investigation fall into two classes, one composed of amides and based upon a series of *N*-β-arylacryloyl-L-phenylalanine derivatives, and a second one composed of the analogous esters, based upon a series of *O*-β-arylacryloyl-DL-β-phenyllactate derivatives. The reaction of all these substrates has been shown to involve the carboxypeptidase-catalyzed hydrolysis of the amide or ester linkage, respectively.

The effect of the enzyme concentration on the initial rates of the carboxypeptidase-catalyzed hydrolysis of each substrate has been determined. As shown by the

representative data presented above, in each case the reaction rates are directly proportional to the enzyme concentration.

The dependence of the reaction velocity upon the substrate concentration was also determined for each substrate. Of the five substrates examined, only the reaction involving furylacryloylphenyllactate was found to obey the usual form of Michaelis–Menten kinetics (Michaelis and Menten, 1913). In this scheme, the mechanism is defined by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_1 \xrightarrow{k_2} E + P \qquad (2)$$

which yields, upon invoking a steady state for C_1 and the fact that $S_0 \gg E_0$, a rate law of

$$v_0 = \frac{k_2 E_0 S_0}{K_m + S_0} \qquad (3)$$

when S and E refer to the substrate and enzyme concentrations, respectively, v_0 is the velocity of the reaction, $K_m = (k_2 + k_{-1})/k_1$, and the subscript 0 refers to quantities evaluated at 0 time. The agreement between eq 3 and the data obtained for the carboxypeptidase-catalyzed hydrolysis of furylacryloylphenyllactate has been examined by both the standard analytical methods (Eadie, 1942) and by numerical analysis based upon the Newton–Raphson method to produce the values given in Table III.

The reactions using the second ester substrate, cinnamoylphenyllactate, were studied over a smaller range of substrate concentrations. Within this range (3.0×10^{-6} M to 3.4×10^{-5} M) the reaction velocity is proportional to the substrate concentration. Although the observed first-order nature of this reaction does not require a mechanism as given in eq 2, it is clear that eq 3 can describe the data if the substrate concentration is sufficiently small with respect to K_m.

Turning now to the reactions involving the amide substrates, a difficulty at once arises. Although the assumption that $S_0 \gg E_0$ was justified when the ester substrates were studied, experiments with the amides were carried out under conditions of *ca.* equal substrate and enzyme concentrations. Under these conditions, it is not valid to apply eq 3 to the data. In considering this problem, it is of interest to examine the rate laws which can be obtained from the mechanism of eq 2 without making any assumption about the relative magnitudes of the enzyme and substrate concentrations. Using only the steady-state assumption, solution of this mechanism yields (Reiner, 1959)

$$v_0 = \frac{k_2}{2} \times \{[K_m + S_0 + E_0] - \sqrt{[K_m + E_0 + S_0]^2 - 4E_0S_0}\} \qquad (4)$$

in which the symbols are defined as given above. After rearranging, the term under the radical may be modified using the binomial expansion to obtain

$$v_0 = \frac{\varphi k_2}{2}\left\{\frac{1}{2}\left[\frac{4E_0S_0}{\varphi^2}\right] + \frac{1}{8}\left[\frac{4E_0S_0}{\varphi^2}\right]^2 + \frac{3}{48}\left[\frac{4E_0S_0}{\varphi^2}\right]^3 + \ldots\right\} \qquad (5)$$

when $\varphi = (E_0 + S_0 + K_m)$.

If both E_0 and S_0 are much less than K_m, eq 5 reduces to

$$v_0 = (k_2/K_m)E_0S_0 \qquad (6)$$

which predicts that, under these conditions, reaction velocities will be first order with respect to both enzyme and substrate concentrations even if $E_0 \simeq S_0$.

Before returning to a consideration of the experimental data, let us consider some further properties of eq 5 which will be of value in the subsequent discussion. In particular, we require an expression for the error involved if a system which actually obeys the quadratic form of eq 4 is interpreted in terms of the Michaelis–Menten form of eq 3. Let v_m and v_q equal the velocities calculated using eq 3 and 5, respectively; then, if we define the error, ϵ, with respect to v_m and set $x = 4E_0S_0/(K_m + E_0 + S_0)^2$, we may derive

$$\epsilon = \left[\frac{1}{4}x + \frac{1\cdot3}{4\cdot6}x^2 + \frac{1\cdot3\cdot5}{4\cdot6\cdot8}x^3 + \frac{1\cdot3\cdot5\cdot7}{4\cdot6\cdot8\cdot10}x^4 + \ldots\right] \qquad (7)$$

which can be expressed as

$$\epsilon = \sum_{i=1}^{\infty} \frac{\prod_{j=1}^{i}(2j-1)}{\prod_{j=1}^{i}(2j+2)}x^i \qquad (8)$$

The quantity ϵ is the error introduced if a Michaelis–Menten system in which the enzyme and substrate concentrations are approximately equal is treated as though $S_0 \gg E_0$. If E_0 is held constant, eq 8 yields a maximal ϵ at $S_0 = K_m + E_0$.

Returning to an analysis of the experimental data, consider the reactions in which cinnamoylphenylalanine and furylacryloylphenylalanine were used as substrates. In both these cases, the reaction velocities display a first-order dependence upon the substrate concentration within the concentration range examined (*ca.* 5×10^{-6} to 5×10^{-5} M) and, therefore, conform to eq 6. Although these data can be explained by means of the assumptions involved in deriving eq 6, this fact alone does not require the participation in these reactions of the mechanism given in eq 2. However, unless a complex, present at low concentrations, is formed between the two reactants it is difficult to explain the fact that these reactions are first order with respect to time when second-order kinetics would be predicted.

Data obtained in reactions which employ indoleacryloylphenylalanine as substrate are well described by the classical form of eq 3 even though the assumptions invoked in the derivation of this equation render its application invalid in this case. In an attempt to explain this anomaly, the agreement between the data and eq 4 was studied by means of iterative techniques using digital computation. It was found that the errors involved were the same using either equation; *e.g.*, K_m is subject to an error of 11.2% when calculated according to either eq 3 or 4, and an error of 6.1% must be invoked in k_2 with either equation. The use of the error function presented in eq 8 provides a satisfactory answer to this apparent contradiction, for it predicts a maximal error of 1.23% in the velocities predicted using these two equations. In this case, then, the data are not of sufficient precision to enable the distinction between eq 3 and 4 to be made purely on the basis of a velocity–substrate concentration relationship. Due to the theoretical difficulty in accepting eq 3 as a valid description of this system, the values of K_m and k_2 given in Table III for the reaction with indoleacryloylphenylalanine are those calculated using eq 4.

The rate constants for the reactions of these substrates have been collected in Table III. In operational terms of the amount of enzyme needed to conduct a measurement under optimal conditions, the two ester substrates compare favorably with hippuryl-DL-β-phenyllactate (McClure *et al.*, 1964), the most susceptible ester substrate of the enzyme. This suggests their use as assay substrates, in which capacity furylacryloylphenyllactate has been routinely employed during these experiments. This substrate is particularly useful due to its long wavelength absorbance which allows studies to be conducted in the presence of carboxypeptidase inhibitors, many of which possess strong electronic transitions near 260 mμ.

The effect of ionic strength on the reaction has been studied by several workers (Gorini and Labouesse-Mercouroff, 1954; Davie *et al.*, 1959; Lumry *et al.*, 1951). Reactions involving both carbobenzoxyglycyl and benzoylglycyl amino acids have been reported to show increased reaction rates with increasing ionic strength, but complete inhibition has been shown by Davie *et al.* for the reaction of carboxypeptidase with β-lactoglobulin. The curves for all these cases exhibit a plateau at ionic strengths of 0.3 and 0.5 M, depending upon the substrate employed. The results given in this paper show a marked inhibition of the reaction with each substrate, but in no case is the nearly total inhibition found by Davie *et al.* encountered here. Since carboxypeptidase-catalyzed hydrolyses require the anionic form of the substrate carboxyl group, the ionic strength effects observed with these simple *N*-acyl amino acids can be rationalized by an electrostatic attraction between a negatively charged substrate and a positive charge within the active site of the enzyme (Neurath and de Maria, 1950). The varying results found with more complicated substrates, such as *N*-acyl dipeptides or proteins, may be due to ionic strength effects on secondary binding sites or to ionic strength induced changes in the conformation of either enzyme or substrate.

The variation of reaction rates with temperature for each of these substrates is adequately described by the Arrhenius equation, using activation parameters which are similar to those previously reported for carboxypeptidase-catalyzed hydrolyses (Neurath and Schwert, 1950; Lumry *et al.*, 1951). At the low substrate concentrations used for these measurements, the observed rates are a function of (k_2/K_m). If K_m is independent of temperature in the hydrolysis of these compounds, as it is for other substrates (carbobenzoxyglycyl amino acids, Lumry *et al.*, 1951; Labouesse, 1958; hippuryl-phenyllactate, McClure *et al.*, 1964) the temperature dependence of (k_2/K_m) would refer to the breakdown of the enzyme–substrate complex. However, even this interpretation is tenuous for k_2 itself is probably a collection of constants related to a more detailed mechanism.

This study was initially undertaken in order to examine these reactions for the presence of intermediates. The methods of detecting such intermediates by spectrophotometric methods have been well demonstrated by reactions with catalase (Chance, 1947), α-chymotrypsin (Hartley and Kilby, 1954; Bender *et al.*, 1962; Kézdy *et al.*, 1964; Bernhard *et al.*, 1965), trypsin (Bernhard and Gutfreund, 1965), and in the imidazole-catalyzed transamination of α-aminophenylacetic acid and pyridoxal (Bruice and Topping, 1963). The kinetic results obtained in the reactions presented in this study do not reveal any of the anomalies which would be expected if spectrophotometrically operable intermediates were present. For example, the isosbestic points found in each reaction are well defined during the course of the reaction, indicating that any intermediates which may be present do not measurably perturb the absorbance of the system at these wavelengths. In addition, the first-order rate constants for a given reaction are independent of the wavelength of observation, again implying the existence of a mechanism with no demonstrable intermediates.

Although spectrophotometric techniques have not allowed the detection of intermediates, the presence of at least one enzyme-containing complex has been inferred by means of gel filtration. In these experiments, the formation of a complex involving carboxypeptidase and the cinnamoyl moiety of cinnamoylphenylalanine has been demonstrated by the appearance of cinnamoyl-bound ^3H in the enzyme-containing fractions obtained by elution from Sephadex G-50 columns. The complex has been shown to decompose while filtration is taking place if filtration is carried out at pH 9.4 or 7.5, but a stable product can be obtained by performing the filtration at pH 4.4 or 5.0. The relative rates of hydrolysis of analogous esters and amides make it unlikely that the observed stable complex is an acylated form of the enzyme. The observed second-order rate constants for the reactions with furylacryloylphenylalanine and its ester analog favor the hydrolysis of the ester by 9400-fold, as do the equivalent quantities for the two cinnamoyl derivatives. If an acylated enzyme is present

in these systems, its steady-state concentration in the reactions with analogous substrates must vary by the same factor, making it unlikely that an acylated enzyme would appear at detectable concentrations in the amide hydrolyses. A more specific argument concerning the structure of this complex can be based upon the pH effects on its formation. It has been shown that the pH at which the filtration is carried out strongly influences the stability of the complex. In particular, filtrations carried out at pH 4.4 or 5.0 result in the formation of a complex which undergoes no apparent breakdown during the fractionation. Experiments in which filtrations at pH 4.4 were preceded by incubation of the reaction mixtures at either pH 4.4 or pH 7.5 revealed no differences, despite the fact that carboxypeptidase is essentially inactive at pH 4.4. These data support the conclusion that this complex is formed in a rapid pH-dependent association of the enzyme and unreacted substrate, and is not necessarily any more complicated than the Michaelis complex. These results with the native enzyme and an acylated amino acid extend those reported by Vallee *et al.* (1963), who observed that binding of dipeptide substrates to the apoenzyme is unaffected by pH variations between 6.0 and 7.5, but falls off at higher values. A similar result has been obtained by Barman and Gutfreund (1965) with a reaction involving trypsin and benzoyl-L-arginine ethyl ester.

The effect of β-phenylpropionate on the reaction is also in keeping with the hypothesis that the Michaelis complex is being observed in these experiments. β-Phenylpropionate is known to behave as a simple competitive inhibitor of carboxypeptidase (Elkins-Kaufman and Neurath, 1949), and probably binds at the active site of the enzyme. It would then be expected that, as observed, formation of the complex would be prevented by the presence of the inhibitor. The effect of guanidine hydrochloride in blocking the formation of the complex is probably due to a nonspecific disruption of the enzyme conformation, implying that, as to be expected, the native structure of the enzyme is necessary in order to observe this binding.

The binding between carboxypeptidase and cinnamoylphenylalanine has a counterpart in that observed, by the same methods, between carboxypeptidase and cinnamate anion. This interaction probably also occurs at the active site of the native enzyme, for it is also prevented by the presence of β-phenylpropionate or guanidine hydrochloride. In addition, cinnamate ion has been shown to inhibit the rate of the carboxypeptidase-catalyzed hydrolysis of furylacryloylphenyllactate. This inhibitory action of cinnamate was not observed in a previous investigation (Smith *et al.*, 1951), in which *N*-carbobenzoxyglycyl amino acids were employed as substrates. It is possible that the different substrates used in the two studies are responsible for the qualitative differences in results just as qualitative differences in the behavior of the apoenzyme toward these two classes of substrates have been shown to occur (Coleman and Vallee, 1962a).

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The Reduction of Folate by Borohydride*

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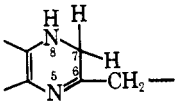
ABSTRACT: After reduction of folate by borohydride, the products have been examined by chromatography on columns of DEAE-cellulose. In addition to unreacted folate, the only compounds observed under a variety of conditions were tetrahydrofolate and dihydrofolate; the latter product could be obtained in yields as high as 30%. Contrary to our previous results, dihydrofolate produced by this method appeared to be identical with the material resulting from the reduction of folate with hydrosulfite. Experiments with tritium-labeled borohydride provided evidence that the hydride ion adds first to carbon atom 7 of the pyrazine ring during reduction of folate to dihydrofolate, and then to carbon atom 6 during reduction of dihydrofolate to tetrahydrofolate. In this manner, dihydrofolate-7-[^{3}H] and tetrahydrofolate-6-[^{3}H], -7-[^{3}H], and -6,7-[^{3}H] have been prepared. Labeled tetrahydrofolates have been used for the preparation of 5-methyltetrahydrofolate-6-[^{3}H] and -7-[^{3}H]. When 5-methyltetrahydrofolate-7-[^{3}H] was oxidized with O_2 or with H_2O_2 in the presence of peroxidase, the 5-methyldihydro product had a much lower specific activity than its precursor. Conversely, oxidation of 5-methyltetrahydrofolate-6-[^{3}H] was accompanied by no loss of label. Thus, when 5-methyltetrahydrofolate is oxidized under these conditions, the product appears to be 5-methyl-5,6-dihydrofolate.

The pyrazine ring of folic acid can be reduced by treatment with hydrosulfite or borohydride and by catalytic hydrogenation. In each instance, the specific reaction conditions employed determine whether the end product is dihydrofolate or tetrahydrofolate. Thus, when folate is treated with hydrosulfite at room temperature, dihydrofolate is obtained (Futterman, 1957; Blakley, 1960), but if the reaction is carried out at 75°, tetrahydrofolate is the main product (Silverman and Noronha, 1961). Similarly, dihydrofolate and tetrahydrofolate, respectively, are obtained when folate is hydrogenated over platinum oxide in alkaline or acidic solutions (O'Dell *et al.*, 1947; Hatefi *et al.*, 1960). Mathews and Huennekens (1963) demonstrated that borohydride readily reduced dihydrofolate, but not folate. By modifying the original reaction conditions of Mathews and Huennekens (1963), we were able to achieve a reaction of folate with borohydride (Smith *et al.*, 1963). A product, isolated by precipitation at pH 2.5, was postulated to be an isomer of dihydrofolate not identical with the hydrosulfite-produced material. Hillcoat and Blakley (1964), however, found that reduction of folate by borohydride yielded primarily tetrahydrofolate along with a small amount (*ca.* 3%) of dihydrofolate. The latter material was indistinguishable by high voltage paper electrophoresis from the product of hydrosulfite reduction. Evidence has been presented by Pastore *et al.* (1963) and by Hillcoat and Blakley (1964) that hydrosulfite-reduced dihydrofolate is the 7,8-isomer (I).



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We have now reinvestigated the reduction of folate by borohydride under various conditions. The reaction

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