The Kinetics of Some Carboxypeptidase A and Acetylcarboxypeptidase A Catalyzed Hydrolyses*

John R. Whitaker,† Fred Menger,‡ and Myron L. Bender

ABSTRACT: The kinetic constants, k_{cat} and K_m(app), have been determined for the carboxypeptidase A and acetylcarboxypeptidase A catalyzed hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine, hippuryl-L-phenylalanine, and hippuryl-L-phenyllactic acid. Acetylcarboxypeptidase A was prepared by treatment of carboxypeptidase A with *N*-acetylimidazole; the most extensively acetylated enzyme showed 9% of the activity of the unmodified enzyme on a standard assay using benzyloxycarbonylglycyl-L-phenylalanine. The (major) substrate inhibition seen in the carboxypeptidase A catalyzed hydrolysis of hippuryl-L-β-phenyllactic acid and the (minor) substrate activation seen in the carboxypeptidase A catalyzed hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine are not observable in the hydrolysis of the substrates by acetylcarboxypeptidase A. With the substrate benzyloxycarbonylglycyl-L-phenylalanine, acetylation of the enzyme decreased k_{cat} by a factor of two but increased the K_m(app) by 15-fold. With the exact structural analogs, hippuryl-L-phenylalanine and hippuryl-L-β-phenyllactic acid, acetylation of the enzyme decreased the k_{cat} of the peptidase reaction 7-fold, whereas the k_{cat} of the esterase reaction went up about 2.5-fold. On the other hand, acetylation of the enzyme increased the K_m(app) of the esterase reaction by 40-fold, whereas that for the peptidase reaction did not change. Thus, no clear-cut mechanistic implications may be drawn from these kinetic results.

Vallee and co-workers have found that various treatments of carboxypeptidase A, such as acylation with carboxylic acid derivatives, photooxidation, iodination, or replacement of the zinc ion of the enzyme by either cadmium or mercury ions, *increase the esterase activity* of the enzyme, as measured by a standard assay using hippuryl-DL-β-phenyllactic acid or hippuryl-DL-β-indolyllactic acid, but *decrease the peptidase activity*, as measured by a standard assay using benzyloxycarbonylglycyl-L-phenylalanine or several other peptide substrates (Vallee *et al.*, 1963; Simpson *et al.*, 1963; Riordan and Vallee, 1963; Vallee, 1964a,b; Bethune *et al.*, 1964; Riordan and Vallee, 1964). Thus, chemical modification of carboxypeptidase A leads to particularly intriguing results. It is of interest to examine the modifications of carboxypeptidase A with a view to finding out whether such modifications exert changes in bindings, K_m(app), in the catalytic process, V_{max}, or in other factors which affect the rate.

A priori, chemical modification of an enzyme such as carboxypeptidase A could manifest itself in a modified rate of hydrolysis through one or more of the following causes. (1) The catalytic rate constant, k_{cat}, could change. (2) The apparent Michaelis constant, K_m(app), could change. (3) K_i of an inhibitor or substrate could be altered. (4) In a multistep enzymatic process (*e.g.*, if the reaction involved an acyl-enzyme intermediate) the steps could be affected differently; in the extreme case there could be a change in the rate-limiting step. (5) The dependence of active center groups on medium effects (pH, ionic strength, dielectric constant) could be altered. (6) K_{diss} of the metal–protein complex could change. (7) A contaminant, either another enzyme or an impurity in the substrate (such as a D isomer), could be selectively modified or show a selective response to the modification. It is obvious that all of these possibilities cannot be separated by the determination of the Michaelis parameters of the modified enzyme, but some of the possibilities may be eliminated by this approach.

The kinetics of the hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine by carboxypeptidase A have been studied in detail by Lumry *et al.* (1951) and by Elkins-Kaufman and Neurath (1948). However, the two groups did not agree on the value of K_m(app). Kinetic constants have also been reported for the hydrolysis of hippuryl-DL-phenylalanine by carboxypeptidase A (Snoke and Neurath, 1949; Labouesse, 1958). The hydrolysis of hippuryl-L-β-phenyllactic acid by carboxypeptidase A has been studied by several groups (Snoke *et al.*, 1948; Snoke and Neurath, 1949; Riordan and Vallee, 1963). More recently, the kinetics of this reaction were examined in detail (McClure *et al.*, 1964; Bender *et al.*, 1965). The system was found to be characterized by inhibition by excess substrate. Acetylation of the enzyme led to a loss of this substrate inhibition (Bender *et al.*, 1965).

In this paper, the effects on the kinetic constants of

* From the Department of Chemistry, Northwestern University, Evanston, Illinois. *Received July 22, 1965; revised October 20, 1965.* This research was supported by grants from the National Institutes of Health.

† NIH Special Postdoctoral Fellow on leave from the Department of Food Science and Technology, University of California, Davis, Calif., 1963–1964.

‡ NIH Postdoctoral Fellow. Present address: Department of Chemistry, Emory University, Atlanta, Ga.

amide hydrolysis by acetylation of carboxypeptidase A are examined. For this purpose, the peptide substrates, benzyloxycarbonylglycyl-L-phenylalanine and hippuryl-L-phenylalanine (an exact structural analog of hippuryl-L-β-phenyllactic acid), were used. Hippuryl-L-β-phenyllactic acid was also used in order to confirm previous work which had employed hippuryl-DL-β-phenyllactic acid (McClure *et al.*, 1964; Bender *et al*, 1965).

Experimental Section

Carboxypeptidase A. Five-times-crystallized carboxypeptidase A (Mann Research Laboratories, Lot No. C2560, water suspension) was prepared for use as previously described (Bender *et al.*, 1965). The enzyme would be largely carboxypeptidase A_γ (Bargetzi *et al.*, 1963).

Acetylcarboxypeptidase A. Acetylation of carboxypeptidase A with *N*-acetylimidazole followed the procedure of Simpson *et al.* (1963). Preparation and activities of the unmodified and acetylated enzymes, using a standard assay procedure employing benzyloxycarbonylglycyl-L-phenylalanine as substrate, have been reported previously (Bender *et al.*, 1965). The activities for the unmodified enzyme (enzyme 1) and the acetylated enzymes (enzymes 2 and 3) were 112, 36, and 9 sec^{-1} (10.6 sec^{-1} for a second preparation), respectively. The reason that enzyme 2 contained a significantly lower number of acetyl groups/molecule than enzyme 3 is that enzyme 2 was acetylated, dialyzed, and stored in a Tris buffer whereas enzyme 3 was prepared and stored in a Veronal buffer. The nucleophilic character of Tris may account for these results. The reproducibility of the acetylation in Veronal buffer (enzyme 3), as determined by enzymatic activity, is very satisfactory. The kinetics using the acetylated enzymes were carried out within 4–7 days of their preparation, since a slow deacetylation occurs on standing. However, during the period of the kinetic determinations, no significant change in activity of the enzymes occurred, as checked using the standard benzyloxycarbonylglycyl-L-phenylalanine assay.

Substrates. Benzyloxycarbonylglycyl-L-phenylalanine (Mann, Lot No. F3916, chromatographically pure) was used without further purification, mp 131–132°, lit. mp 125–126° (Hofmann and Bergmann, 1940; Neurath *et al.*, 1947). It was a pure L compound as determined by its complete enzymatic hydrolysis. Hippuryl-L-phenylalanine (Mann, Lot No. N1054, chromatographically and analytically pure) was used without further purification, mp 146–147°. L-Phenylalanine (Mann, Lot No. 3630, chromatographically pure, α_D 33.9 in water (*c* 1.9); benzyloxycarbonylglycine (Mann, Lot No. K2464), mp 119–120°, lit. mp 120–121° (Hofmann and Bergmann, 1940); and benzoylglycine (Mann, Lot No. K2252, Cp) were used without further purification.

L-β-Phenyllactic acid was prepared from L-phenylalanine by deamination, recrystallized twice from water, and dried over P_2O_5; mp 125.5–126.0°, lit. mp 125.5° (Eiduson *et al.*, 1950). Hippuryl-L-β-phenyllactic acid was prepared by treatment of L-β-phenyllactic acid with 2-phenyl-5-oxazolone according to the method of Kaiser and Carson (1965). Its initial crystallization was carried out in benzene–1,2-dichloroethane. Subsequently it was recrystallized from 1,2-dichloroethane and from carbon tetrachloride–chloroform; mp 117–118°.[1] The material was analytically pure and was shown to be a pure L compound by the fact that a given amount of this material gave twice as much enzymatic hydrolysis product as the same amount of the DL compound.

Buffers. Tris–HCl buffers were prepared from Tris (Sigma Chemical Co., primary reagent), 1 M hydrochloric acid (Fisher standardized reagent), and reagent grade potassium chloride. For enzymatic assays the buffer was 0.05 M, pH 7.50, μ = 0.50 (total, including substrate), adjusted with potassium chloride. The same results were obtained with 0.005 M Tris–HCl, μ = 0.50, buffer or 0.05 M Tris–HCl buffers containing 0.50 M lithium chloride, 1.00 M potassium chloride, or 2.00 M potassium chloride. The initial rate of hydrolysis of 0.02 M benzyloxycarbonylglycyl-L-phenylalanine in 0.05 M, pH 7.50, phosphate buffer, μ = 0.50, was decreased 41% over that in Tris–HCl buffer, which is in agreement with the results of Lumry *et al.* (1951). pH measurements were made on a Radiometer 4C pH meter standardized against pH 6.5 Radiometer standard buffer. At the end of each kinetic run the pH of the reaction solution was again measured.

Enzyme Concentration. The protein content and enzymatic activity under standard conditions were determined each day that kinetic analyses were performed. Since the concentration of enzyme needed for an assay is low (10^{-7}–10^{-9} M), it was sometimes found convenient to determine the protein concentration at 222.5 mμ where the molar extinction coefficient is eight times larger than at 278 mμ. The following extinction coefficients were used: carboxypeptidase A, ϵ_{278} 6.41 × 10^4 M^{-1} cm^{-1}, $\epsilon_{222.5}$ 5.27 × 10^5 M^{-1} cm^{-1}; and acetylcarboxypeptidase A, ϵ_{278} 5.9 × 10^4 M^{-1}, $\epsilon_{222.5}$ 5.28 × 10^5 M^{-1} $cm.^{-1}$. Extinction coefficients at 278 mμ are those reported by Simpson *et al.* (1963). The enzymatic activity was determined using 3.23 × 10^{-4} M benzyloxycarbonylglycyl-L-phenylalanine at pH 7.50 and 25.0°, with a Cary Model 14 spectrophotometer at 222.5 mμ. The second-order rate constant, $k_1'(k_1/E_0)$, using these conditions was 2.38 ± 0.06 × 10^4 M^{-1} sec^{-1} (15 determinations) for carboxypeptidase A and did not change significantly over a 3-month period.

Kinetic Measurements. The kinetics of hydrolysis of the substrates by carboxypeptidase A and by acetylcarboxypeptidase A were determined using a Cary Model 14 PM recording spectrophotometer equipped with a thermostated cell compartment. The wavelengths used in following the various reactions were: benzyloxycarbonylglycyl-L-phenylalanine, 220–236 mμ; hippuryl-L-β-phenyllactic acid, 250–285 mμ; and

[1] The authors are indebted to Professor E. T. Kaiser and Dr. F. N. Carson, University of Chicago, for the preparation of these two derivatives.

TABLE I: Kinetics of Hydrolysis of Unmodified and Acetylated Carboxypeptidase A.[a]

Carboxypeptidase A (enzyme 1)		Acetylcarboxypeptidase A (enzyme 2)		Acetylcarboxypeptidase A (enzyme 3)	
$S_0 \times 10^4$ (M)	$V_0/E_0 \times 10^{-2}$ (sec^{-1})	$S_0 \times 10^3$ (M)	$V_0/E_0 \times 10^{-2}$ (sec^{-1})	$S_0 \times 10^3$ (M)	$V_0/E_0 \times 10^{-2}$ (sec^{-1})
		Benzyloxycarbonylglycyl-L-phenylalanine			
494	1.46	98.3	0.647	100	0.258
197	1.12	59.0	0.575	60.2	0.197
177	1.23	39.3	0.500	59.9	0.210
118	0.929	19.8	0.357	50.2	0.176
98.4	0.785	9.83	0.265	40.1	0.157
88.6	0.768			39.9	0.157
68.0	0.661			20.0	0.0913
59 4	0.571			20.0	0.0889
59.0	0.568				
56 1	0.577				
43.6	0.461				
39.4	0.445				
19.7	0.273				
11.8	0.189				
7.86	0.127				
		Hippuryl-L-phenylalanine			
507	1.15	51.1[b]	0.431	60.8	0.177
304	1.18	30.7	0.394	42.6	0.163
203	1.09	20.4	0.382	30.4	0.170
152	1.07	10.2	0.327	18.2	0.151
101	1.01	6.13	0.239	12.2	0.148
60.9	0.852	5.11	0.205	6.08	0.125
50.7	0.845			6.08	0.127
30.4	0.698			3.04	0.103
20.3	0.613			3.04	0.0996
14 2	0.495				
14.2	0.465				
10.1	0.412				
10.1	0.412				
		Hippuryl-L-β-phenyllactic Acid			
40.0	1.88			4.08	6.87
28.6	2.44			2.86	6.16
20.4	2.83			2.04	5.56
12.1	3.48			2.04	5.41
8.16	3.81			1.22	4.02
4.04	3.67			0.816	2.95
4.04	3.81			0.612	2.44
2.04	3.35			0.408	1.75
2.04	3.30			0.306	1.50
0 808	2.51				
0.808	2.40				
0.642	2.35				
0.642	2.25				
0.385	1.76				
0 385	1.84				
0.321	1.62				

[a] In aqueous solution at 25.0°; $\mu = 0.5$; pH 7.50; Tris-HCl buffer. [b] The enzyme used in these determinations was enzyme 3 which had deacetylated on standing to give an enzyme showing 32% of the activity of the unmodified enzyme by the standard assay.

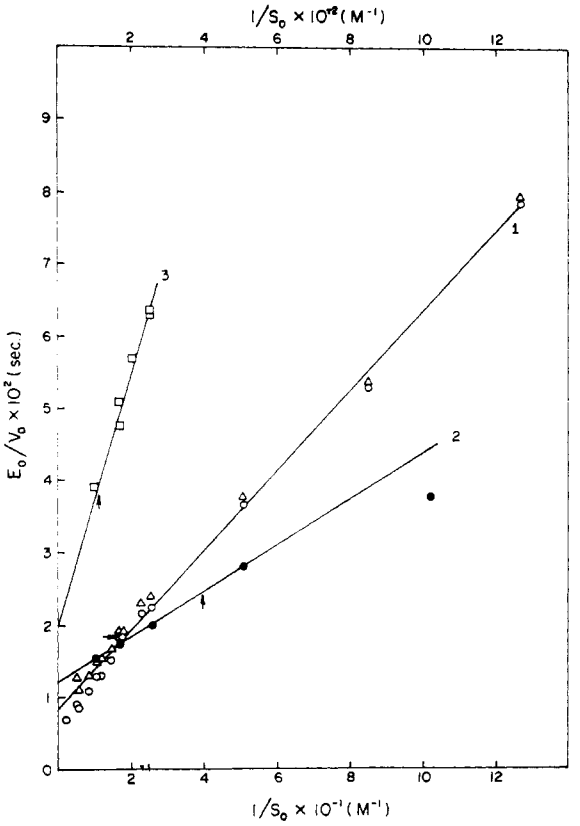

FIGURE 1: Effect of benzyloxycarbonylglycyl-L-phenylalanine concentration on the rate of its hydrolysis by enzyme 1 (○, △, top scale), enzyme 2 (●, bottom scale), and enzyme 3 (□, bottom scale) at 25.0°, pH 7.50, Tris–HCl buffer, μ = 0.5. The △ corresponds to data corrected for substrate activation (see text). Arrows denote K_m(app) of each reaction.

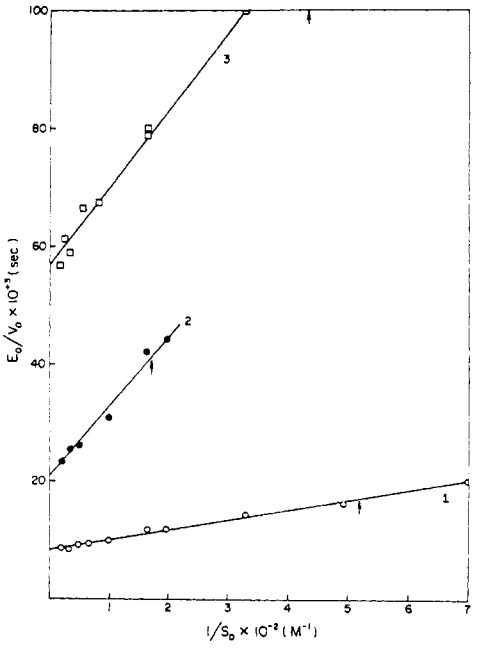

FIGURE 2: Effect of hippuryl-L-phenylalanine concentration on the rate of its hydrolysis by enzyme 1 (○), enzyme 2 (●), and enzyme 3 (□) at 25.0°, pH 7.50, Tris–HCl buffer, μ = 0.5. The enzyme 2 used in this plot was prepared from enzyme 3 by allowing it to stand in the refrigerator for several weeks. Arrows denote K_m(app) of each reaction.

hippuryl-L-phenylalanine, 281–289 mμ. Infinity values were determined for each reaction solution in order to check the stoichiometry.

For benzyloxycarbonylglycyl-L-phenylalanine and hippuryl-L-phenylalanine, the reaction cuvet contained 3 ml of a solution of the substrate which had been dissolved in the 0.05 M, pH 7.50, Tris–HCl buffer by the addition of an equivalent amount of 1 M sodium hydroxide, and the pH was adjusted to 7.50. The blank cuvet contained 3 ml of buffer. The solutions were equilibrated at 25.0° in the compartment of the spectrophotometer. A wavelength was selected such that the absorbance difference betweeen substrate and buffer solutions was of the order of 1.8. After establishing a base line, 50 μl of enzyme solution was added and mixed, and recording was started within 5–10 sec. The reaction was followed to completion and the absorbance data were converted to rate data by use of the molar absorption coefficient determined on the same reaction solution. The hydrolysis of hippuryl-L-β-phenyllactic acid was measured as previously described (Bender *et al.*, 1965).

Amide hydrolyses catalyzed by acetylcarboxypeptidases A (enzymes 2 and 3) utilized 20-fold larger enzyme concentrations than hydrolyses catalyzed by carboxypeptidase A. Although enzyme 3 was stored in Veronal buffer, the enzymatic hydrolyses were always carried out in Tris buffer because of blanking problems. Since initial slopes were usually taken for only 10 min in the rate experiments, this time of contact with Tris buffer was much too small to affect the kinetics.

All reported kinetic constants were obtained from initial reaction rate data.

Results

The effect of initial substrate concentration on the initial rates of hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine, hippuryl-L-phenylalanine, and hippuryl-L-β-phenyllactic acid by carboxypeptidase A and by two acetylcarboxypeptidases A is given in Table I. Lineweaver–Burk plots of these data are shown in Figures 1–3.

The Lineweaver–Burk plot of the initial velocity *vs.* initial substrate concentration for the carboxypeptidase A catalyzed hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine gave a continuous curve rather than the usual simple straight line (Figure 1). The nature of the curve led us to suspect that the substrate or the products might be perturbing the kinetics. Therefore, the effects

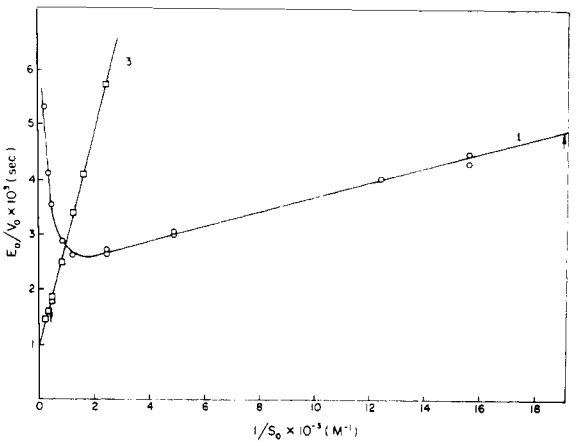

FIGURE 3: Effect of hippuryl-L-β-phenyllactic acid concentration on the rate of its hydrolysis by enzyme 1 (○) and enzyme 3 (□) at 25.0°, pH 7.50, Tris-HCl buffer, μ = 0.5. Arrows denote K_m(app) for each reaction.

of the hydrolysis products, L-phenylalanine and benzyloxycarbonylglycine, on the rate of the reaction were determined. L-Phenylalanine was found to inhibit competitively the reaction whereas benzyloxycarbonylglycine was found to increase the rate of the reaction. A linear relationship was found between y, the slope of the line (E_0/V *vs.* $1/S$), and the initial benzyloxycarbonylglycine concentration. The data are described by: $y = 8.0 \times 10^{-5}$ M sec $- (1.2 \times 10^{-3}$ sec)[CBZgly]$_0$. Since benzyloxycarbonylglycine increased the rate of hydrolysis, it is reasonable to assume that the substrate, benzylcarbonylglycyl-L-phenylalanine, also increased the rate. Making the assumption that these two compounds activate the enzyme by the same mechanism and to the same extent, the observed initial velocities

$$V_0 = V_0(\text{obsd})[y/(8.0 \times 10^{-5}\ \text{M sec}^{-1})] \qquad (1)$$

were corrected by eq 1 where y and 8.0×10^{-5} M sec^{-1} refer to the benzyloxycarbonylglycine data. The corrected data give a linear Lineweaver–Burk plot (Figure 1). The Michaelis–Menten formulation for other than initial rates must be expanded to include the product inhibition (by L-phenylalanine) and product activation (by benzyloxycarbonylglycine) as well as the substrate activation found in this system.

Although an apparent substrate activation was found in the hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine, resulting in a convex Lineweaver–Burk plot, a normal linear Lineweaver–Burk plot was observed in the carboxypeptidase A catalyzed hydrolysis of the related peptide, hippuryl-L-phenylalanine.

In the carboxypeptidase A catalyzed hydrolysis of hippuryl-L-β-phenyllactic acid a marked substrate inhibition is found, resulting in a concave Lineweaver–Burk plot. Essentially, the same results were obtained with hippuryl-L-β-phenyllactic acid as with hippuryl-DL-β-phenyllactic acid (McClure *et al.*, 1964; Bender *et al.*, 1965).

The acetylcarboxypeptidase A catalyzed hydrolyses of all three substrates are well behaved. That is, there is no evidence for substrate activation of the hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine nor is there evidence for the extensive substrate inhibition of the hydrolysis of hippuryl-L-β-phenyllactic acid under the conditions studied. In determining the kinetic constants of the acetylcarboxypeptidase A reactions, the highest substrate concentration was always as large as or larger than the highest used with the unmodified enzyme.

Discussion

The kinetic constants, k_{cat} ($= V_{max}/E_0$) and K_m(app), for the carboxypeptidase A and acetylcarboxypeptidase A catalyzed hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine, hippuryl-L-phenylalanine, hippuryl-DL-β-phenyllactic acid, and hippuryl-L-phenyllactic acid are summarized in Table II.

Carboxypeptidase A. For the carboxypeptidase A catalyzed hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine, k_{cat} and K_m(app) have been reported to be 181 sec^{-1} and 6.5×10^{-3} M (Lumry *et al.*, 1951) and 186 sec^{-1} and 3.7×10^{-2} M (Elkins-Kaufman and Neurath, 1948), whereas the k_{cat} in Table II is significantly smaller. However, if k_{cat} and K_m(app) are calculated using only the uncorrected high substrate concentration data of Table I, values of 198 sec^{-1} and 1.40×10^{-2} M, respectively, are found. Thus, the present values are in agreement with previous results, except that we have taken into account the slight substrate activation found in this reaction.

The kinetic constants for the carboxypeptidase A catalyzed hydrolysis of hippuryl-L-β-phenyllactic acid are almost identical with those obtained when hippuryl-DL-β-phenyllactic acid was used (McClure *et al.*, 1964; Bender *et al.*, 1965). Although the presence of the D isomer has been reported to have no effect on the kinetics of hydrolysis by carboxypeptidase A (Snoke and Neurath, 1949), it was felt desirable to redetermine the kinetic constants in the absence of the D isomer for comparison with the kinetic constants of acetylcarboxypeptidase A. The agreement between the two sets of kinetic constants validates the use of the DL mixture and the assumption that the D isomer has no effect on the reaction. The same conclusion was reached by comparison of kinetic constants obtained from initial and complete reaction rate data. This conclusion is further substantiated by the fact that both the DL mixture and the L isomer exhibit identical substrate inhibition phenomena.

The kinetic constants of the carboxypeptidase A catalyzed hydrolysis of hippuryl-L-phenylalanine are quite similar to those obtained for the hydrolysis of the similar peptide, benzyloxycarbonylglycyl-L-phenylalanine, with the exception that no substrate activation is found in the former reaction. These results are in agreement with those obtained with hippuryl-DL-

TABLE II: Kinetic Constants of the Hydrolysis of Some Peptides and Esters by Unmodified and Acetylated Carboxypeptidase A.[a]

Substrate	Carboxypeptidase A (112 sec^{-1})[b] (enzyme 1) k_{cat} (sec^{-1})	K_m(app) (10^3 M)	Acetylated Enzyme (36 sec^{-1})[b,c] (enzyme 2) k_{cat} (sec^{-1})	K_m(app) (10^3 M)	Acetylated Enzyme (10 sec^{-1})[b,d] (enzyme 3) k_{cat} (sec^{-1})	K_m(app) (10^3 M)
Benzyloxycarbonylglycyl-L-phenylalanine	106	5.83	82.2	25.7	50.0	89.1
Hippuryl-DL-β-phenyllactic acid[e]	578	0.088	797	0.82	1090	1.78
Hippuryl-L-phenylalanine	118	1.91	47.6[f]	5.81[f]	17.6	2.31
Hippuryl-L-phenyllactic acid	424	0.053			1120	2.24

[a] In aqueous solution at 25.0°; μ = 0.5; pH 7.50; Tris–HCl buffer. [b] Activity on benzyloxycarbonylglycyl-L-phenylalanine under standard conditions (Bender *et al.*, 1965). [c] This enzyme corresponds to enzyme 2 of Table I. [d] This enzyme corresponds to two preparations of enzyme 3 of Table I, which gave activities of 9.0 and 10.6 sec^{-1} on the standard assay. The enzyme showing an activity of 9.0 sec^{-1} was used with the first two substrates while the enzyme showing an activity of 10.6 sec^{-1} was used with the last two substrates. [e] Data of Bender *et al.* (1965). [f] The enzyme used in these determinations was enzyme 3 which had deacetylated on standing to give an enzyme showing an activity of 36 sec^{-1} by the standard benzyloxycarbonylglycyl-L-phenylalanine assay.

phenylalanine (Snoke and Neurath, 1949; Labouesse, 1958).

When comparing the kinetic constants of the carboxypeptidase A catalyzed hydrolysis of the two peptides and the ester listed in Table II, the most striking relation is that the k_{cat} values of the ester and peptides are within a factor of five of one another but the K_m(app) of the ester is 20- to 110-fold less than that of the peptides. In chymotrypsin kinetics, such a difference in K_m(app) values is found between comparable ester and amide substrates. However, it is there accompanied by a sizable difference in k_{cat} values between ester and amide substrates. Therefore, our data do not permit us to invoke the argument for a two-step mechanism involving an acyl-enzyme intermediate which has been used to explain such differences in α-chymotrypsin reactions.

Acetylcarboxypeptidase A. Kinetic differences are seen between the unmodified enzyme and the two acetylated enzymes. The most interesting change occurs in the kinetics of the hydrolysis of hippuryl-L-β-phenyllactic acid; when the enzyme is acetylated, the substrate inhibition disappears (Table I and Figure 3) (see Bender *et al.*, 1965; Riordan *et al.*, 1965). While it cannot be categorically stated that substrate inhibition is lost completely, it certainly is depressed to the extent that it is not seen up to a substrate concentration of 3–4 × 10^{-3} M, which is a 10-fold higher concentration than the first appearance of substrate inhibition with the unmodified enzyme. Likewise, the substrate activation seen in the hydrolysis of benzyloxycarbonylglycyl-L-phenylalanine with the unmodified enzyme disappears in the acetylated enzyme. The disappearance of substrate inhibition overshadows all other phenomena brought about by the acetylation of carboxypeptidase A. A possible explanation of this phenomenon is that part of the active site leading to nonproductive binding is blocked on acetylation whereas that part of the active site leading to productive binding is not affected by acetylation.

When carboxypeptidase A was acetylated, the k_{cat} of the peptidase reaction with benzyloxycarbonylglycyl-L-phenylalanine decreased by a factor of 2 while that of the esterase reaction with hippuryl-L-β-phenyllactic acid increased by approximately a factor of 2 (Table II). On the other hand, on acetylation of the enzyme, the K_m(app) of both peptidase and esterase reactions increased by 15- to 20-fold. Thus, the most extensive effect of acetylation toward these substrates is seen in the K_m(app), presumably related to binding, rather than in the k_{cat}, presumably related to the catalytic process. Coleman *et al.* (1964) have reported that acetylcarboxypeptidase A will not bind benzyloxycarbonylglycyl-L-phenylalanine but will still bind hippuryl-L-β-phenyllactic acid. Of course, none of these numerical changes expresses the fact pictorially evident in Figure 3 that the most profound change upon acetylation of the enzyme is the loss (or displacement to an experimentally inaccessible region) of the substrate inhibition in the hydrolysis of hippuryl-L-β-phenyllactic acid.

The substrates used previously to compare the peptidase and esterase activity of carboxypeptidase A are not optimally suited for a comparison of the unmodified and acetylated enzymes because these substrates have slightly different backbones. The exact

structural analogs of one another, hippuryl-L-phenylalanine and hippuryl-L-β-phenyllactic acid, offer a more suitable comparison. When carboxypeptidase A was acetylated, the k_{cat} of the peptidase reaction went down about 7-fold whereas the k_{cat} of the esterase reaction went up about 2.5-fold (and the substrate inhibition disappeared). The effect of enzyme acetylation is slightly greater with hippuryl-L-phenylalanine than with benzyloxycarbonylglycyl-L-phenylalanine. On the other hand, on acetylation of the enzyme, the K_m(app) of the esterase reaction went up by about 40-fold while that for the peptidase reaction did not change. Thus, for this apparently comparable set of substrates, the behavior produced by acetylation of the enzyme is not easily rationalized.

In general, no clear-cut mechanistic implications may be drawn from these kinetic results. The structural requirements of the active site have been changed by the extensive acetylation, presumably of two tyrosine residues (Simpson *et al.*, 1963), as reflected in the increase in K_m(app) of two of the three substrates tested. This increase in K_m(app) could indicate either that the tyrosines are involved in the binding of the substrate to the active site or that acetylation simply introduces steric hindrance to the site. The small effects of acetylation on k_{cat} are also difficult to interpret mechanistically.

Enzyme 2, with an intermediate degree of acetylation, gave changes in k_{cat} and K_m(app) which were intermediate between the results obtained with the unmodified enzyme and the more extensively acetylated enzyme 3. These results indicate that an enzyme more extensively acetylated than enzyme 3 should show even more extensive changes in k_{cat} and K_m(app) than those changes reported here.

References

Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Bender, M. L., Whitaker, J. R., and Menger, F. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 711.

Bethune, J. L., Ulmer, D. D., and Vallee, B. L. (1964), *Biochemistry 3*, 1764.

Coleman, J. E., Pulido, P., and Vallee, B. L. (1964), *Federation Proc. 23*, 423.

Eiduson, S., Camien, M. N., and Dunn, M. S. (1950), *Arch. Biochem. 29*, 302.

Elkins-Kaufman, E., and Neurath, H. (1948), *J. Biol. Chem. 175*, 893.

Hofmann, K., and Bergmann, M. (1940), *J. Biol. Chem. 134*, 225.

Kaiser, E. T., and Carson, F. W. (1965), *Biochem. Biophys. Res. Commun. 18*, 457.

Labouesse, J. (1958), *Biochim. Biophys. Acta 28*, 341.

Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Am. Chem. Soc. 73*, 4330.

McClure, W. O., Neurath, H., and Walsh, K. A. (1964), *Biochemistry 3*, 1897.

Neurath, H., Elkins, E., and Kaufman, S. (1947), *J. Biol. Chem. 170*, 221.

Riordan, J. F., Davies, R. C., and Vallee, B. L. (1965), *Federation Proc. 24*, 410.

Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.

Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.

Snoke, J. E., Schwert, G. W., and Neurath, H. (1948), *J. Biol. Chem. 175*, 7.

Vallee, B. L. (1964a), *Federation Proc. 23*, 8.

Vallee, B. L. (1964b), Abstracts, 6th International Congress of Biochemistry, New York, N. Y., p. 255.

Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 109.

CORRECTION

In the paper entitled Catalysis of the H_2–HTO Exchange by Hydrogenase. A New Assay for Hydrogenase, by S. R. Anand and Alvin I. Krasna, in Volume 4, No. 12, p 2747, December 1965, the following corrections should be made: *All* tritium activities in units of mv reported in the text, Figures 2, 3, and 4, and Table I are to be decreased by a factor of 100. This does *not* cause any change in any other values reported in the paper.