was obtained by preparative vpc on a 5-ft SF-96 on Chromosorb W column at 210°.

Anal. Calcd for $C_{15}H_{19}NO_2$: C, 73.33; H, 7.80. Found: C, 71.76; H, 7.84.

Spectral data confirming the structure of X are detailed in the Discussion.

The ether solution of neutral products was analyzed by vpc and contained *ca.* 5% of recovered 4,7-diethyl-1-indanone, 34% of 4-ethyl-7-vinyl-1-indanone (XI), and 61% of isomeric lactams. An analytical sample of XI was obtained by preparative vpc using a 5-ft 20% Versamide 900 on Chromosorb W column at 200°.

Anal. Calcd for $C_{13}H_{14}O$: C, 83.83; H, 7.58. Found: C, 83.71; H, 7.76.

The minor lactam, whose structure was substantiated by nmr and infrared, was shown to be 5,8-diethyl-3,4-dihydrocarbostyril, λ_{max}^{EtOH} 253 mμ (ε 8900).

Anal. Calcd for $C_{13}H_{17}NO$: C, 76.80; H, 8.43. Found: C, 76.98; H, 8.57.

The major lactam was 5,8-diethyl-3,4-dihydroisocarbostyril, λ_{max}^{EtOH} 239 mμ (ε 7000) and 290 mμ (ε 2000). The extinction coefficients are uncertain here because of minor impurities.

Anal. Calcd for $C_{13}H_{17}NO$: C, 76.80; H, 8.43. Found: C, 76.49; H, 8.31.

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pH Dependence of the Hydrolysis of O-Acetyl-L-mandelate Catalyzed by Carboxypeptidase A. A Critical Examination[1,2]

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Abstract: The pH dependences of the kinetic constants for the hydrolysis of O-acetyl-L-mandelate catalyzed by carboxypeptidase A were studied over the range pH 5.5 to 9.0 at 25°. The data were analyzed by computer using an original statistical analytical method for the determination of competitive product inhibition constants. The value of k_{cat}/K_m was found to depend on a base of $pK_a \sim 6.9$ and on an acid of $pK_a \sim 7.5$ while the corresponding acid dissociation constants on which k_{cat} depends are 7.2 and 7.9, respectively. Possible ionizing groups in this enzyme responsible for these observations are discussed. The results also are examined in relation to previously proposed mechanisms and models for the esterolytic and proteolytic actions of carboxypeptidase A.

Carboxypeptidase A is a pancreatic, zinc-containing metalloenzyme which mediates the hydrolysis of peptides having a free carboxyl function at the terminal α-amino acid moiety and which must be of the L configuration. L-α-Acyloxycarboxylic acids also have been found to be substrates for this enzyme. Recently Vallee and co-workers[4,5] and Neurath and co-workers[6] have found a marked difference between the pH–rate profiles for ester and peptide substrates. In addition, the former group has found[4,5,7-9] that various treatments of the enzyme such as acylation, iodination, etc., lead to an increase in the esterase activity toward O-(N-benzoylglycyl)-DL-3-phenyllactic acid and to a decrease in the peptidase activity toward N-(N-carbobenzyloxyglycyl)-L-phenylalanine. These results led to the proposal that carboxypeptidase A hydrolyzes esters and peptides by different mechanisms.[4,8] If this difference were borne out by subsequent studies, it would constitute the first case in which the esterolytic and proteolytic actions of a hydrolytic enzyme could not be assumed to follow common pathways.

However, Bender, *et al.*,[10] have shown that the increased esterase activity of acetylated carboxypeptidase A is primarily the result of a striking decrease in substrate inhibition of the catalytic hydrolysis of O-(N-benzoylglycyl)-DL-3-phenyllactate under the conditions of assay used by Vallee's group; this decrease far outweighs an increase in the apparent Michaelis constant and causes the observed "activity" increase. From this study the conclusion was reached that no clear-cut mechanistic implications were provided by the available kinetic data.

Previous work from this laboratory[11] indicated that the pH–rate profile for the carboxypeptidase A catalyzed hydrolysis of O-acetyl-L-mandelate was markedly different from the profile reported[4-6] for O-(N-benzoylglycyl)-DL-3-phenyllactate but was similar to that reported[4,5,12] for the peptide substrate N-(N-carbobenzyloxyglycyl)-L-phenylalanine. A detailed analysis of pH effects, however, requires the determination of both k_{cat} and k_{cat}/K_m as functions of pH in order to separate the influence of hydrogen ion conentration on the free enzyme from that on the enzyme–substrate complex. This paper reports the results of such an

(1) This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

(2) Paper III in the series Studies on the Esterase action of Carboxypeptidase A. For papers I and II of this series see ref 11 and 14, respectively.

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analysis of the O-acetyl-L-mandelate–carboxypeptidase A system, which did not display the complicating effects of substrate inhibition or substrate activation found with certain other substrates although strong product inhibition by L-mandelate was observed.

Experimental Section

Materials. O-Acetyl-L-mandelic acid was obtained by the method described previously,[11] mp 97–99°.[13]

O-(N-Benzoylglycyl)-L-mandelic acid was prepared as partially described in a preliminary communication.[14] A mixture of 7.61 g (50 mmoles) of L-mandelic acid and 8.06 g (50 mmoles) of 2-phenyl-5-oxazolone[15] (mp 89.0–90.0°) in 100 ml of benzene was heated at reflux under anhydrous conditions for 1.5 hr to give 14.78 g (94%) of crude O-(N-benzoylglycyl)-L-mandelic acid, mp 166–167.5°. The product was recrystallized from 8:1 v/v 1,2-dichloroethane–acetonitrile yielding beautiful white crystals: mp 167.0–168.1°, equiv mol wt 314.2 phenolphthalein end point (equiv mol wt 313.3 calcd), $[\alpha]^{24}D$ +104° (*c* 3.135, dimethylformamide).[16]

Anal. Calcd for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.83; N, 4.47. Found: C, 65.00; H, 5.00; N, 4.28.

Buffers, solvents, and salts were all of reagent grade quality and the water used was distilled and was then demineralized with a mixed-bed ion exchange column (Continental Demineralization Service). The normalities of standard hydrochloric acid and sodium hydroxide solutions (Fisher Scientific Co.) were checked against each other and the latter solutions were checked against potassium hydrogen phthalate.

Carboxypeptidase A was purchased as a suspension of crystals in toluene–water from Sigma Chemical Co. (lot No. C91B-80 and 123B-1590). All enzyme preparations used in this work were obtained from bovine pancreas by the method of Anson[17] as modified by Putnam and Neurath[18] and have been designated carboxypeptidase A_γ by Bargetzi, *et al.*[19] Stock solutions in 1 *M* sodium chloride, 0.05 *M* 2-amino-2-(hydroxymethyl)-1,3-propanediol–HCl buffer, pH 7.50, were prepared as described earlier[11] using Visking-Nojax dialysis casings boiled in 5% sodium carbonate for 10 min and then treated as recommended by Klotz and Hughes.[20] Each stock solution had an enzyme concentration between 5×10^{-5} and 1×10^{-4} *M* and was stored in a refrigerator at 4°. Spectrophotometrically determined concentrations were always consistent with the activity toward either 0.04 *M* O-acetyl-L-mandelate or 0.01 *M* (N-benzoylglycyl)-L-mandelate at pH 7.50 and 25.00° as described in the next section. The activity remained unchanged over a 3-month period, the maximal time during which a given preparation was used. Preparations of carboxypeptidase A from a different source (Worthington Biochemical Corp., lot No. COA708, two times crystallized) or enzyme crystallized after treatment with diisopropyl phosphofluoridate[21] to destroy endopeptidase activity (Sigma Chemical Co., lot No. 83B-0330-1) had activities toward O-acetyl-L-mandelate within 15% of that of a standard preparation. The standard preparations had activities which agreed with each other within 10%.

Activity determinations were performed as described previously[11] using an automatic titrator. The enzyme concentration was calculated using the spectrophotometrically determined absorbance of the stock solution at 278 mμ and the value of 6.42×10^4 l. mole^{-1} cm^{-1} as the extinction coefficient[7] of carboxypeptidase A at this wavelength. For O-acetyl-L-mandelate the 5-ml aqueous reaction mixtures contained 0.04 *M* substrate, 0.55 *M* sodium chloride, 0.005 *M* 2-amino-2-(hydroxymethyl)-1,3-propanediol, 5×10^{-6} to 1×10^{-5} *M* enzyme, and 1.35% (v/v) dimethylformamide. The measurements were made at pH 7.50 and 25.00 ± 0.02° using 0.1000 *N* sodium hydroxide as the titrant. Under these conditions the initial reaction velocity normalized to 1 *M* enzyme ($v_0/(E)_0$ or $\bar{v}_0$) was 11.9 ± 0.7 min^{-1} (20 determinations). Under similar conditions (0.50 *M* sodium chloride, 2.5×10^{-4} *M* 2-amino-2-(hydroxymethyl)-1,3-propanediol, 5×10^{-8} to 1×10^{-7} *M* enzyme, and 0.77% (v/v) dimethylformamide) 0.01 *M* O-(N-benzoylglycyl)-L-mandelate had $\bar{v}_0$ equal to 1.73 ± 0.10 $\times 10^3$ min^{-1} (31 determinations).

Kinetic Measurements. Hydrolyses of O-acetyl-L-mandelate and O-(N-benzoylglycyl)-L-mandelate were followed by automatic titration at constant pH employing a Radiometer Type TTTlb titrator in conjunction with a Type SBR2c titrigraph using a combined glass and calomel electrode in the manner described previously.[11] The reaction cell was thermostated at 25.00 ± 0.02° and reactions were run in a nitrogen atmosphere above pH 7. In each run with O-acetyl-L-mandelate, 500 μl of stock enzyme solution was used regardless of the enzyme concentration in order to ensure that the final concentrations of sodium chloride and Tris would always be the same. The data were corrected for the variable enzyme concentrations. Generally, the reactions were followed for either 200 or 400 min, and 20 to 25 points separated by 8- or 16-min time intervals were read from each continuous trace of titrant consumed *vs.* time.

Base Catalysis. The rates of hydrolysis of O-acetyl-L-mandelate and O-(N-benzoylglycyl)-L-mandelate under the conditions described above were followed in the absence of enzyme at pH values from 9 to 11. At a given pH the reactions were pseudo first order in substrate. The pseudo-first-order rate constants were found in turn to be directly proportional to the hydroxide ion concentration. Thus, the second-order rate constant, k_2, for base catalysis was straightforwardly calculated to be 4.70 ± 0.10 l./mole/min for O-acetyl-L-mandelate and to be 62.2 ± 1.0 l./mole/min for O-(N-benzoylglycyl)-L-mandelate. These results were used to correct the data for nonenzymatic hydrolysis of the substrate.

Product Buffering Effect. It has been pointed out[11,22] that the consumption of titrant may not be a direct measure of the extent of reaction if either or both the substrate and products are not fully dissociated at the pH of the reaction. The pK_a of mandelic acid is 3.4[23] and the pK_a values of both O-acetyl-L-mandelic acid and O-(N-benzoylglycyl)-L-mandelic acid were found to be even smaller. Thus, protonation of either the substrate or L-mandelate product could be neglected above pH 5. However, acetic acid, which has a pK_a of 4.76,[24] exerts a significant buffering effect in the low pH region. The effect for N-benzoylglycine, having a pK_a of 3.65,[25] was negligible in this study. Correction for both product buffering[11] and base-catalyzed hydrolysis of the substrate was made with the equation

$$v = v_{exptl}[1 + (H^+)/K_a] - k_2(OH^-)(S) \qquad (1)$$

where v is the corrected velocity in moles/l./min, v_{exptl} is the measured rate of titrant consumption, K_a is the dissociation constant of either acetic acid or N-benzoylglycine at 25°, and (S) is the concentration of substrate.

Organic Solvent Effects. Since solutions of the substrates in dimethylformamide were used for the introduction of acylated mandelic acids into the reaction vessel, effects of this solvent on both the pH and the catalytic reaction were checked. Changing the concentration of dimethylformamide from 0 to 10% by volume in 0.5 *M* sodium chloride, 0.005 *M* Tris at pH 7.50 caused no apparent change in the observed pH. A change of 0.01 pH would have been readily detectable. This result is in agreement with the expectation that organic solvents should have no effect on the observed pH of Tris buffer when measured with the glass electrode.[26]

Several reactions of O-acetyl-L-mandelate in which the concentration of dimethylformamide was increased from less than 1% to a maximum of 2% (v/v) (the concentration of dimethylformamide in the standard reaction mixtures was $^1/_3$% per $^1/_{100}$ *M* of substrate) either prior to the introduction of the enzyme or after the reaction had commenced were run at pH 7.5. In each case the reaction velocities did not change by more than 10% which was

(13) All melting points are uncorrected.
(14) E. T. Kaiser and F. W. Carson, *Biochem. Biophys. Res. Commun.*, **18**, 457 (1965).
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about the variability of the standard runs. In addition, the time courses of the reactions were unchanged by the increase in organic solvent, the traces of titrant consumed *vs.* time being approximately superimposable at a given substrate concentration.

Ion Effects. The effects of various ions on the hydrolysis of O-acetyl-L-mandelate by carboxypeptidase A were checked by adding 250-μl aliquots of a 2 *M* solution of the species to standard reaction mixtures in which the enzymatic reaction had been proceeding for 1 hr at pH 7.5. Comparison of the reaction velocities before and after addition of the aliquot indicated that sodium acetate, sodium chloride, and 2-amino-2-(hydroxymethyl)-1,3-propanediol (each final concentration being increased by about 0.1 *M*) diminished the reaction velocity by only a slight amount as would be expected from the dilution effect alone. In contrast, 0.1 *M* sodium L-mandelate drastically inhibited the reaction virtually instantaneously.

Addition of the titrant to the reaction mixture has two effects. The first, changes in the ionic strength of the reaction medium, were shown above to be negligible. The second, an increase in volume of the reaction mixture, must be taken into account. Since the empirical evaluation procedure of Booman and Niemann[27] was used to obtain the initial velocity of each reaction it follows that the reaction rate evaluated at 0 time is also the rate for the reaction at the initial volume. Arguments leading to the conclusion that dilution by the titrant produces an insignificant error in the initial velocity have been presented in some detail by Niemann and co-workers.[28]

However, the apparent time course of the reaction would be systematically affected by the volume increase attending addition of the titrant. The actual concentration of species Z in solution at a particular time denoted by the subscript s (see Elmore, *et al.*;[29] s is directly proportional to the time) is given by

$$(Z)_s = \frac{(Z)_{app,s}V_0}{V_0 + y_sC} \tag{2}$$

where V_0 is the initial volume of the reaction mixture, y_s is the experimental parameter which is proportional to the volume of titrant consumed, C is a constant which converts y_s to the same units as V_0, and $(Z)_{app,s}$ is either the initial concentration of the enzyme, $(E)_0$, or the apparent concentration of the product, $(P)_{app,s} = \bar{y}_sA$, or the apparent concentration of the substrate, $(S)_{app,s} = (S)_0 - \bar{y}_sA$. A is a constant which transforms $\bar{y}_s$ (the value of y normalized so that $\bar{y}_0 = 0$)[30] into units of moles/l.

Thus, the rate equation for an enzymatic reaction involving competitive product inhibition, which applies to the carboxypeptidase A-catalyzed hydrolysis of O-acetyl-L-mandelate,[11] is

$$\frac{v_s}{(E)_0} = \frac{k_{cat}(S)_{app,s}\left[\frac{V_0}{V_0 + y_sC}\right]^2}{K_m\left[1 + \frac{(S)_0}{K_I}\left(\frac{V_0}{V_0 + y_sC}\right)\right] + \left(1 - \frac{K_m}{K_I}\right)(S)_{app,s}\left(\frac{V_0}{V_0 + y_sC}\right)} \tag{3}$$

where $K_m = (k_{-1} + k_{cat})/k_1$ in the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \tag{4}$$

and $K_I = k_{-I}/k_I$, the competitive product inhibition constant for L-mandelate (P_2) in the equilibrium

$$E + P_2 \underset{k_{-I}}{\overset{k_I}{\rightleftharpoons}} EP_2 \tag{5}$$

As will be seen from the results, in general $(S)_0 > K_I$ so that eq 3 can be written approximately as

$$\frac{v_s}{(E)_0} \approx \frac{k_{cat}(S)_{app,s}\left(\frac{V_0}{V_0 + y_sC}\right)}{K_m(S)_0/K_I + (1 - K_m/K_I)(S)_{app,s}} \tag{6}$$

Thus, a plot of $(E)_0/v_s$ *vs.* $1/(S)_{app,s}$ should show concave curvature upward to an extent dependent on the actual values of y_sC/V_0. Experimentally, the maximal dilution caused by the titrant was 5% so that the maximal value of y_sC/V_0 was 0.05. This implies that a linear fit of a plot of $(E)_0/v_s$ *vs.* $1/(S)_{app,s}$ should have a slope biased in the positive direction by a maximum of about 2.5%.

The rate expression for a reaction involving competitive product inhibition in which no dilution of the reaction mixture occurs is given by

$$(E)_0/v = \frac{K_m}{k_{cat}}[1 + (S)_0/K_I]\frac{1}{(S)} + \frac{1}{k_{cat}}(1 - K_m/K_I) \tag{7}$$

If the slope and ordinate intercept of a plot of $(E)_0/v$ *vs.* $1/(S)$ are termed B_1 and B_0, respectively (*cf.* Figure 2), it follows that for a given initial substrate concentration

$$K_I = (S)_0/(k_{cat}B_1/K_m - 1) \tag{8}$$

or

$$K_I = K_m/(1 - k_{cat}B_0) \tag{9}$$

or

$$K_I = \frac{K_m[B_1 + B_0(S_0)]}{[B_1 - B_0K_m]} \tag{10}$$

By inspection of eq 8–10 it can be seen that any one of these methods of calculating K_I from a positively biased fit will lead to numerical values of the inhibition constant which are *smaller* than the true constant by a percentage somewhat smaller than the bias in the fit. Thus, the K_I values calculated by a statistical method which enforces a straight-line fit of $(E)_0/v$ *vs.* $1/(S)$ for a given run should lead to K_I values 1–3% smaller than the unbiased K_I. Indeed, it was found by extensive high-speed computer analysis of the data that the K_I values calculated from each of eq 8–10 were all in agreement and that the bias was either negligible or of the same magnitude as the experimental error.[30]

Denaturation Studies. The irreversible effects of extremes of pH as well as temperature on the activity of carboxypeptidase A were studied in the following manner. Standard reaction mixtures of 8.41×10^{-6} *M* enzyme at pH 7.50 which did not contain any substrate were made up and split into control and test samples. The test samples were adjusted to pH 5.50 or 9.00 by slow addition of 2 *N* hydrochloric acid or 2 *N* sodium hydroxide with rapid stirring. The corresponding control samples were diluted with similar volumes of 2 *M* sodium chloride. One control sample contained 0.2 *M* sodium L-mandelate at pH 7.5. All samples were then maintained at 25.0° in capped vials except for one control sample which was stored in a refrigerator at 4°. The activity of each sample was determined at measured times by withdrawing 10-μl aliquots and measuring the catalytic activity toward 5 ml of 1.00×10^{-3} *M* sodium O-(N-benzoylglycyl)-L-mandelate in 0.50 *M* sodium chloride at pH 7.50 and 25.00° using 1.000×10^{-2} *N* sodium hydroxide as the titrant. The reaction mixtures required about 1 or 2 min to equilibrate due to the differences in pH between some of the enzyme aliquots being tested and the final pH of reaction. Consequently, time zero was arbitrarily set at 4 min after the introduction of the enzyme and 5 more points at 2-min intervals from each run were used to obtain relative activities by the polynomial curve fitting procedure described elsewhere.[27,29,30] In each of these cases a second-order polynomial was required to fit the data. A white precipitate was noted in the enzyme sample at pH 5.5 after 1 day. All of the test enzyme samples were found to have unchanged pH after 2 days at 25.0°.

Product Analysis. Reaction mixtures containing the products of reaction of carboxypeptidase A and O-acetyl-L-mandelate from about 100 runs were saved and acidified to pH 1 with concentrated hydrochloric acid. White precipitate, presumably denatured enzyme, was removed by filtration and the clear, colorless filtrate was saturated with sodium chloride and was then continuously extracted with ether for 3 days. The ethereal extract was dried with sodium sulfate and split into two portions.

The first portion was analyzed by gas chromatography using a stainless steel column packed with 20% of a mixture of 3:1:4

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isophthalic acid–Versamid 900–Carbowax 4000 terminated with terephthalic acid on 45–60 mesh Chromosorb W (Wilkens Instrument and Research, Inc.). The results of comparisons with authentic samples were consistent with the presence of acetic acid, dimethylformamide (the solvent used to introduce the substrate), racemic or optically active mandelic acid, and possibly O-acetyl-L-mandelic acid, but the latter two compounds decomposed on the column under the conditions employed.

The second portion of the ethereal extract as well as samples of acetic, formic, DL-mandelic, L-mandelic, and O-acetyl-L-mandelic acids in ether were treated with an ethereal solution of diazomethane prepared from N-methylnitrosourea by a standard procedure.[31] The methylation reaction selectively forms the methyl esters[32] of hydroxy acids and these could be analyzed by gas chromatography using the technique of Williams.[32] In this case a stainless steel column packed with 10% ethylene glycol adipate on 45–60 mesh acid-washed Chromosorb W was employed in the analysis.

The results indicated that acetic acid and mandelic acid are the sole products of the reaction under consideration. In order to settle the question of the optical properties of the mandelic acid, a sample of the reaction product extract was evaporated under high vacuum to a pale yellow oil of $[\alpha]^{24}D$ +127° (*c* 5.990, acetone) as compared to $[\alpha]^{27}D$ +160° (*c* 5.350, acetone) for pure L-mandelic acid. Thus, the mandelic acid in the product was no less than 90% of the L configuration. A 100-mg sample of the oil eventually solidified to give white crystals, of which 50 mg was isolated after suspension in, and washing with, cold benzene. The crystals had mp 131–133°; a mixture melting point with authentic L-mandelic acid was 131–133.5°.

High-Speed Computer Analysis of the Data. Booman and Niemann[27] have described an empirical method for computing the initial velocity of a reaction from experimental data recording the progress of the reaction at equal time intervals. This method involves fitting of the data to orthogonal polynomials by least-squares approximations and is based upon the treatment given by Milne.[33] Subsequently, Elmore, *et al.*,[29] adapted this procedure to the calculation of kinetic parameters by a weighted least-squares statistical analysis. In the present work, a program following the mathematical exposition of the latter group was written (with some small modifications and additions) in machine language for an IBM 7094 computer. This program should be adaptable for use with most IBM 7094 computers.

In the first phase of the calculations, data (y_s, given as per cent reaction) which have been taken at equally spaced time intervals (8 or 16 min in the present case) are fed into the computer and fitted to a polynomial of the form

$$y_s = a_0 + a_1 s + a_2 s^2 + \ldots + a_m s^m \qquad (11)$$

In the second phase a weighted least-squares method is used to fit the initial velocities to the Lineweaver–Burk equation. In phase three the K_m and k_{cat} values obtained in this way are used to calculate K_I from the time course of each reaction using eq 10. Further details are given elsewhere.[30]

Results

Kinetic Variables. It was found that most of the variables which *might* have influenced the catalysis of hydrolysis of O-acetyl-L-mandelate by carboxypeptidase A had no measurable effect under the conditions employed. These included the concentrations of dimethylformamide (the solvent used to introduce the substrate), acetate (one of the products), sodium chloride, and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris buffer). In addition, comparable results were obtained with various enzyme preparations, and activity changes during storage at 4° were insignificant. The product buffering effect at low pH as well as the effect of base-catalyzed hydrolysis of the substrate are taken account of by means of eq 1.

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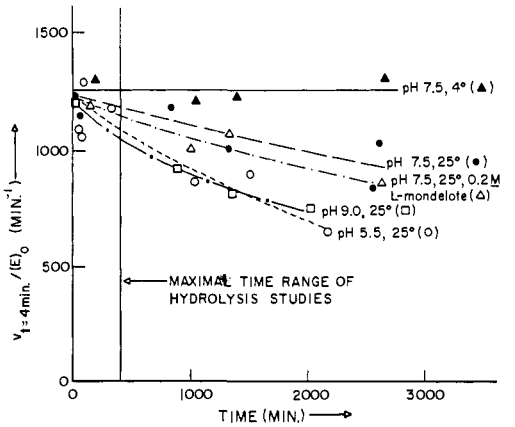


Figure 1. Denaturation of carboxypeptidase A by temperature and by extremes of pH.

Effects which are not negligible and which will be discussed in turn are (1) denaturation of the enzyme by acid, base, and heat; (2) dilution of the reaction mixture by the titrant; (3) product inhibition by L-mandelate; and (4) pH effects on kinetic parameters.

Denaturation. The time-dependent decrease in activity of solutions of carboxypeptidase A adjusted to pH values 5.5, 7.5, and 9.0 and maintained at 25° is shown in Figure 1. In addition, results for one solution containing 0.2 *M* sodium L-mandelate at pH 7.5 and 25° and one control at pH 7.5 which was kept in a refrigerator at 4° are shown. The test solutions were prepared so as to approximate the reaction conditions usually employed except that the substrate was not present. No activity losses occurred in the pH 7.5 solution stored at 4°. However, similar solutions at pH 7.5 and 25°, either in the presence or absence of sodium L-mandelate, lost about $^1/_6$ of their activity over a 24-hr period. The samples maintained at pH 5.5 or 9.0 at 25° lost about $^1/_3$ of their activities during the same period. The slowness of denaturation and the scatter in the activity determinations (~10%) necessitated the rather long time scale of these studies and make it difficult to ascertain the kinetic course of the initial denaturation. The denaturation process at pH 5.5 seems to be particularly complex since some precipitate appeared during the test period.

If the denaturation process be assumed to be first order in enzyme with rate constant k_1', then the observed reaction velocity at any time would be lower than the velocity in the absence of denaturation by the factor $\exp(-k_1't)$. From Figure 1 it can be seen that this correction factor would maximally be about 6% for reactions run at pH 7.5 and about 13% for reactions run at pH values of 5.5 or 9.0. Following the arguments concerning titrant dilution given in the Experimental Section, the initial velocities should not be significantly affected by slow enzyme denaturation whereas values of K_I derived from linear fits of plots of $(E)_0/v$ *vs.* 1/(S) should be smaller than the true constants by somewhat less than 3% at pH 7.5 and by less than 7% at the extremes of pH. It will be shown in the discussion of L-mandelate inhibition that both this bias and the bias produced by titrant dilution do not severely influence the results.

It is interesting to compare the denaturation results found here with those observed by Rupley and Neu-

rath.[34] They found that the esterase activity of carboxypeptidase A was unchanged after dialysis against buffers of pH 4.6 to 11.3 for 62 hr at 0° provided that zinc (a component necessary for catalytic activity) was added to the test solutions to replace any losses incurred in dialysis. Thus, the denaturation observed here must be very slow at 0°, in agreement with our observation that stock solutions of enzyme were stable for months at 4°. Bethune, *et al.*,[35] have reported that the esterase activity of this enzyme was unchanged after dialysis against buffers of pH 6 to 9 for 18 hr. A direct comparison of the present results with their results is not possible since they did not report the temperature of dialysis.

A report[36] that 3-phenylpropionate, a competitive inhibitor of both peptidase[37] and esterase[14] action, protected carboxypeptidase A against denaturation suggested the experiment in which L-mandelate, another competitive inhibitor of the esterase action of this enzyme,[11,38] was added to one of the test solutions. However, the present results indicate that 0.2 *M* L-mandelate had no effect on the rate of denaturation of carboxypeptidase A at pH 7.5 and 25°.

The nature of the precipitate appearing in the enzyme solution maintained at pH 5.5 is not clear although it is known that the zinc-free enzyme is less soluble than the native enzyme,[36] particularly at low pH.[34] Other experiments[39] showed that this precipitation reaction was quite rapid at pH 5.0 or less, but the relative rate was decreased by adding Zn(II) to the enzyme solutions or by dilution with 1 *M* sodium chloride. This suggests that the precipitate appearing at low pH was probably the zinc-free apoenzyme. As a result of this problem and since the enzyme concentrations required for the study of O-acetyl-L-mandelate were close to the highest practicable in kinetic studies of this sort ($\sim 10^{-5}$ *M*), the lower limit of pH for which accurate results could be obtained was 5.5. The upper limit was set by base-catalyzed hydrolysis of O-acetyl-L-mandelate, which contributed a major portion of the observed rates above pH 9.0.

Titrant Dilution. The effects of titrant dilution and enzyme denaturation on the time course of the hydrolysis of O-acetyl-L-mandelate catalyzed by carboxypeptidase A were discussed from a predictive standpoint under the headings Ion Effects and Denaturation, respectively. It was concluded that the sum of these two effects should lead to a bias of about 4–6% in K_I values at pH 7.5 and of about 8–10% in the values of K_I at pH 5.5 or 9.0.

The direction of the bias was confirmed by using only the first few points in each run to calculate K_I since the magnitude of the effect is time dependent. That is, in forcing a straight line fit of $(E)_0/v$ *vs.* $1/(S)$ for a given run the value of K_I increased with increasing time. It was found that K_I values calculated using all of the points were 7 to 30% larger than they would have been if no curvature were present. The results were close to those predicted between pH 7 and 9 but the bias was larger than expected at pH 6.5 and less. Whatever the reason for this it is clear that, after taking account of known biasing factors in the data, eq 7 describes the time course of individual runs for all of the data within 20% over the pH range 5.5 to 9.0.

Product Inhibition. It was shown previously that the hydrolysis of O-acetyl-L-mandelate at pH 7.5 catalyzed by carboxypeptidase A is subject to competitive product inhibition.[11] The equation describing the time course of the reactions was found to be

$$\frac{(S)_0 - (S)}{t} = A\frac{\ln (S)_0/(S)}{t} + B(E)_0 \qquad (12)$$

where (S) is the concentration of the substrate, $(E)_0$ is the initial concentration of the enzyme, t is the time, A is a linear function of $(S)_0$, and B is a constant. The initial velocities of the reactions were given by the equation

$$v_0 = -[d(S)/dt]_0 = \frac{C(E)_0(S)_0}{D + (S)_0} \qquad (13)$$

where C and D are constants. The simplest interpretations of these results are the schemes shown in eq 4 and 5, where P_1 is acetate and P_2 is L-mandelate. The complex EP_2 is assumed to be catalytically inactive.

The product analysis in the present investigation showed by gas chromatography that acetate and mandelate were the only products of the reaction and the optical rotation of an oil isolated from the reaction mixtures, which eventually crystallized to pure L-mandelic acid, showed that the reaction produces mandelate which is at least 90% of the L configuration. This is the first case in which direct evidence for the stereochemistry of the products of ester hydrolyses by carboxypeptidase A has been presented. In addition, sodium acetate concentrations far in excess of those produced in the reactions being studied did not significantly affect the rate while added sodium L-mandelate at concentrations similar to those produced in the reactions drastically reduced the reaction rate. Thus, the assignment of P_2 as L-mandelate is entirely correct and no inhibitory role need be assigned to acetate. The lack of acetate inhibition found here is analogous to the results of Coleman and Vallee[40] and of Smith, *et al.*[41]

Assuming the correctness of schemes shown in eq 4 and 5, eq 12 and 13 may be rewritten as

$$\frac{(S)_0 - (S)}{t} = \frac{K_m[K_I + (S)_0]}{K_m - K_I}\frac{\ln (S)_0/(S)}{t} + \frac{k_{cat}(E)_0}{1 - K_m/K_I} \qquad (14)$$

and

$$v_0 = \frac{k_{cat}(E_0)(S)_0}{K_m + (S)_0} \qquad (15)$$

where $K_m = (k_{-1} + k_{cat})/k_1$, and $K_I = k_{-I}/k_I$. Equation 14 is the integrated form of the Michaelis–Menten velocity equation for competitive product inhibition,

(34) J. A. Rupley and H. Neurath, *J. Biol. Chem.*, **235**, 609 (1960).
(35) J. L. Bethune, D. D. Ulmer, and B. L. Vallee, *Biochemistry*, **3**, 1764 (1964).
(36) K. Imahori, H. Fujioka, and T. Ando, *J. Biochem.* (Tokyo), **52**, 167 (1962).
(37) E. Elkins-Kaufmann and H. Neurath, *J. Biol. Chem.*, **178**, 645 (1949).
(38) E. T. Kaiser and F. W. Carson, unpublished data on the inhibition of the hydrolysis of O-(*N*-benzoylglycyl)-L-mandelate catalyzed by carboxypeptidase A.
(39) E. T. Kaiser and F. W. Carson, unpublished data.
(40) J. E. Coleman and B. L. Vallee, *Biochemistry*, **3**, 1874 (1964).
(41) E. L. Smith, R. Lumry, and W. J. Polglase, *J. Phys. Colloid Chem.*, **55**, 125 (1951).

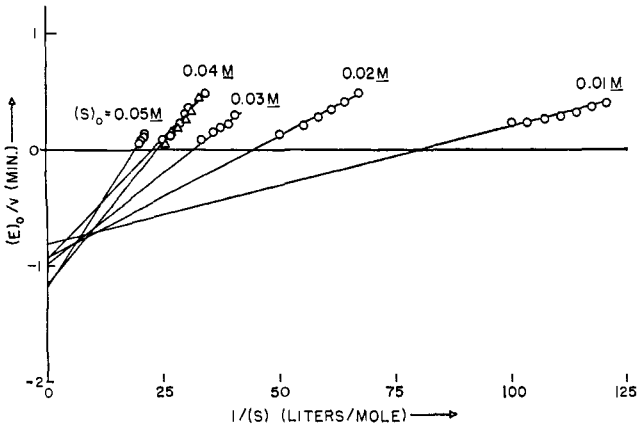


Figure 2. Lineweaver–Burk plots of individual runs for the hydrolysis of O-acetyl-L-mandelate by carboxypeptidase A at pH 7.5, 25°.

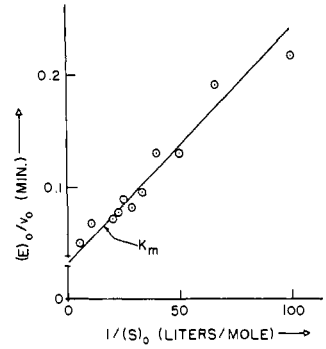


Figure 3. Lineweaver–Burk plot of initial points for the hydrolysis of O-acetyl-L-mandelate by carboxypeptidase A at pH 7.5, 25°.

the use of which has been described in detail by Foster and Niemann.[42] The derivative of eq 14 is

$$v = \frac{k_{cat}(E)_0(S)}{K_m + (S) + K_m(I)/K_I} \qquad (16)$$

where (I) = $(S)_0 - (S)$, and eq 15 is the special case of eq 16 in which (I) = 0. It is obvious that if an additional term for nonenzymatic hydrolysis of the substrate were added to eq 16, then integration would no longer give a simple linear relationship between the experimentally measurable parameters. Since base-catalyzed hydrolysis of O-acetyl-L-mandelate is not negligible at high pH, eq 7 (a rearrangement of eq 16), with the appropriate correction for nonenzymatic hydrolysis, has been the basis for the calculation of kinetic constants in this work. The detailed statistical treatment is given elsewhere.[30]

Figure 2 displays a plot of $(E)_0/v$ *vs.* 1/(S) for some of the typical data obtained at pH 7.5. $(E)_0/v$ represents the reciprocal of the reaction velocity corrected for the product buffering effect and for nonenzymatic hydrolysis normalized to 1 *M* enzyme concentration. At pH 7.5 the corrections were negligible and v was just the observed reaction velocity. It is apparent that the plots are essentially linear although the slight upward concavity caused by titrant dilution and enzyme denaturation is noticeable. The straight lines drawn through the points were those calculated by the computer using linear regression equations.[30]

Since the ordinate intercept

$$B_0 = \frac{1}{k_{cat}}(1 - K_m/K_I) \qquad (17)$$

B_0 should be constant and this is shown to be the case within ±20% in Figure 2. K_I values calculated from eq 8–10 using B_0 and B_1 values from Figure 2 agreed within experimental error with both the computer value of K_I (obtained by a statistical analysis[30]) for all of the data at pH 7.5 and the previously reported value,[11] which did not depend on a knowledge of k_{cat} or K_m.

pH Effects. Alberty[43] has pointed out that a number of pitfalls exist in the determination of the ionization constants of groups in the active site from kinetic studies. Factors which must be considered are: (1) substrate ionization, (2) substrate activation or inhibition, (3) buffer effects, (4) width of pH range, (5) denaturation (irreversible or slowly reversible), (6) tertiary structure changes, and (7) electrostatic effects. The first possible complication, changes in the ionization of the substrate, may be eliminated since the pK_a of O-acetyl-L-mandelate was found to be less than 3.4.[11] Thus, the substrate is fully ionized throughout the pH range 5.5 to 9.0. Second, neither substrate activation nor substrate inhibition was observed in any case. Lineweaver–Burk[44] plots of $(E)_0/v_0$ *vs.* $1/(S)_0$ were linear at any given pH in the range 5.5 to 9.0. Since substrate inhibition is an important factor in the carboxypeptidase A catalyzed hydrolysis of O-(N-benzoylglycyl)-L-mandelate[14] and of O-(N-benzoylglycyl)-DL-3-phenyllactate[6,10] at concentrations of substrate somewhat above K_m, data at pH 7.5 for O-acetyl-L-mandelate were extended to $(S)_0 = 0.2$ *M*. A plot of $(E)_0/v_0$ *vs.* $1/(S)_0$ for the reaction at pH 7.5 is shown in Figure 3. No substrate inhibition is evident up to a concentration more than three times the value of K_m, which is 0.06 *M*. The third possibility, activation or inhibition by the buffer, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), was discounted by the following experiments. Varying the buffer concentration over the range 0.005 to 0.1 *M* did not change the initial velocity of reactions at pH 7.5; a tenfold variation of the enzyme concentration at 0.005 *M* buffer and pH 7.5 gave initial velocities which were directly proportional to the enzyme concentration for a given substrate concentration. The fourth problem, studying a broad enough pH range to observe significant variations in the kinetic constants, was unimportant since five- to tenfold variations in k_{cat} were found between the optimum pH and the pH extremes. Fifth, no "instantaneous" irreversible enzyme denaturation occurred since enzyme solutions at either pH 5.5 or 9.0 had the same activities as solutions at pH 7.5 on readjusting the pH to 7.5 (*cf.* Figure 1) after a short time. The slow denaturation observed would have a negligible effect on the initial velocities as discussed under Denaturation.

The sixth problem is not presently resolvable in entirety. Various physical properties of carboxypeptidase A such as electrophoretic mobility, diffusion, sedimentation, and optical rotatory dispersion have been

(42) R. J. Foster and C. Niemann, *Proc. Natl. Acad. Sci. U. S.*, **39**, 999 (1953); *cf.* also R. R. Jennings and C. Niemann, *J. Am. Chem. Soc.*, **77**, 5432 (1955), and references cited therein.

(43) R. A. Alberty, *J. Cellular Comp. Physiol.*, **47**, 245 (1956).

(44) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

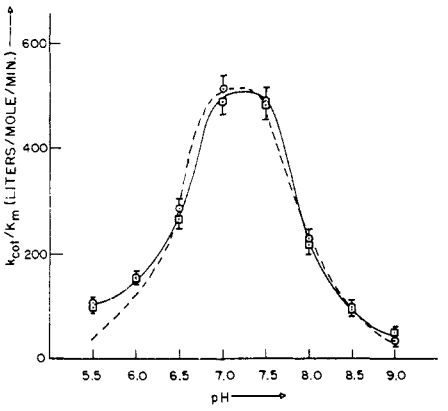


Figure 4. k_{cat}/K_m *vs.* pH for the hydrolysis of O-acetyl-L-mandelate catalyzed by carboxypeptidase A at 25°: ———, experimental curve; – – – –, calculated curve using $K_{a1E} = 1.25 \times 10^{-7}$, $K_{a2E} = 3.34 \times 10^{-8}$; ⊙, results calculated using all of the points in each run; ⊡, results calculated using the first ten points in each run; ⊥ or ⊤, one standard deviation.

studied[18,34–36,45–48] but information pertaining to conformational changes on complexation of the enzyme is sparse. The constancy of the specific optical rotation at 0°[34] and of both the dispersion constant, λ_c, and b_0[35] of the Moffitt equation at 10° with changes in pH suggest that little change in the helical content (estimated to be 70[34] or 80%[36]) of carboxypeptidase A occurs in varying the pH. On the other hand Fujioka and Imahori[49] found that 3-phenylpropionate, a competitive inhibitor of carboxypeptidase A, produced no change in b_0 for the enzyme in 1 *M* sodium chloride at pH 7.5 and room temperature but induced a small Cotton effect near 295 mμ.[49] Finally, quantitative comparison[30] of various optical results[34–36,49,50] suggests that the helical content of carboxypeptidase A depends on the salt concentration.

Taken together, these results suggest that in the present investigation conformational differences between the free enzyme species at various pH values probably were not very great. However, this conclusion should be qualified with the statement that even small changes in conformation near ionizing groups involved in catalysis might lead to large perturbations of their dissociation constants. Regrettably, it is not known what the effect of either substrate or product on conformation of the enzyme would be and the results of the Japanese workers[36] imply that they might be large.

A further complication to compound the problem may arise from quaternary structural changes with variation of pH. The recent sedimentation data of Bethune indicated that carboxypeptidase A forms rapidly equilibrating polymers at high ionic strength (2.5 *M* sodium chloride) at pH 7.5.[51] He also found that 3-phenylpropionate induces a split boundary in the sedimentation pattern, although certain other inhibitors and peptide substrates do not have this effect.[52] Until further information becomes available, the possible contribution of such phenomena to the interpretation of our results must be unknown.

The last problem, the importance of electrostatic effects which could influence the apparent pK values of ionizing groups in the enzyme, is impossible to solve without a detailed knowledge of the nature of the active site. Since firm binding of zinc(II) to the enzyme is a prerequisite for hydrolytic activity[53–55] it might seem that the electrostatic field of the metal could perturb the pK values of groups involved in catalysis. However, it is entirely possible that the zinc ion is remote from the actual catalytic site. This possibility is not abrogated by the fact that competitive inhibitors of carboxypeptidase stabilize the zinc–enzyme complex.[40,52,56]

For example, inhibitors might induce a conformational change leading to stabilization of the zinc–enzyme complex. In addition, depending on the experimental conditions, cationic and nonionic detergents cause conformational changes in the enzyme while increasing the activity, and anionic detergents result in a decrease in activity accompanying conformational changes.[36,57] The implication of these results is that certain changes in the properties of carboxypeptidase A such as activation, inhibition, and stabilization may arise indirectly from conformational changes, rather than from direct interaction with the active site. It is not yet possible, however, to make clear-cut distinctions between the two possibilities in most cases.

If, on the other hand, zinc(II) is located near or at the site of substrate hydrolysis, it is not clear how it would influence observed pK values since the titrimetric data of Coleman and Vallee[58] indicate that two protons are released when zinc binds to the apoenzyme. As a consequence the charge on zinc may be largely neutralized by the chelating ligands. The nature of other charged groups which might be near the active site is wholly unknown. Thus, it should be understood that the interpretation of the results presented in this paper must be given within the context of the limited knowledge of the effects of pH on conformational changes of carboxypeptidase A and the dearth of information on charged groups near the active site.

In this work it was found that Lineweaver–Burk plots of $(E)_0/v_0$ *vs.* $1/(S)_0$ were linear throughout the pH range 5.5 to 9.0. The variation of the reciprocals of the slopes of these plots, k_{cat}/K_m, with pH is shown in Figure 4. The values were calculated by computer for 10 to 25 runs at each pH. The results using either the first ten points in each run or all the points (20–25) are essentially the same indicating that the weighting procedure used[30] produces consistent results independent of the number of points in each run to be fitted to a smooth curve. In addition, all of the results were plotted and checked by eye to assure that no unusual Lineweaver–Burk fits were calculated by the computer.[59] The bell shape of Figure 4 may most easily

(45) E. L. Smith, D. M. Brown, and H. T. Hanson, *J. Biol. Chem.* **180**, 33 (1949).
(46) P. J. Keller, E. Cohen, and H. Neurath, *ibid.*, **223**, 457 (1956).
(47) B. Jirgensons, *Arch. Biochem. Biophys.*, **85**, 532 (1959).
(48) B. Jirgensons, *ibid.*, **96**, 314 (1962).
(49) H. Fujioka and K. Imahori, *J. Biol. Chem.*, **237**, 2804 (1962).
(50) H. Fujioka and K. Imahori, *J. Biochem.* (Tokyo), **53**, 341 (1963).
(51) J. L. Bethune, *J. Am. Chem. Soc.*, **85**, 4047 (1963).
(52) J. L. Bethune, *Federation Proc.*, **23**, 161 (1964).
(53) B. L. Vallee and H. Neurath, *J. Am. Chem. Soc.*, **76**, 5006 (1954).
(54) B. L. Vallee, J. A. Rupley, T. L. Coombs, and H. Neurath, *ibid.*, **80**, 4750 (1958).
(55) B. L. Vallee, J. A. Rupley, T. L. Coombs, and H. Neurath, *J. Biol. Chem.*, **235**, 64 (1960).
(56) J. E. Coleman and B. L. Vallee, *ibid.*, **237**, 3430 (1962).
(57) J. Labouesse, *Biochim. Biophys. Acta*, **28**, 341 (1958).
(58) J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, **236**, 2244 (1961).
(59) It is conceivable that two or three unusually well-fitted kinetic runs could lead to virtual ignorance of the other runs since a weighting

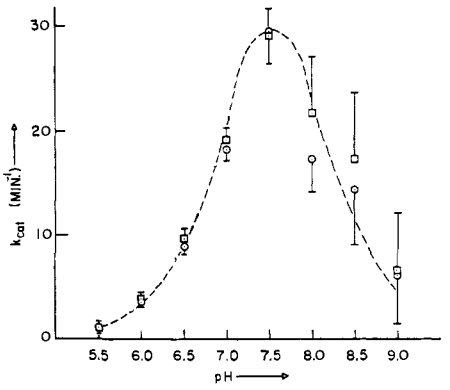


Figure 5. k_{cat} *vs.* pH for the hydrolysis of O-acetyl-L-mandelate catalyzed by carboxypeptidase A at 25°: ----, calculated curve using $K_{a1ES} = 6.18 \times 10^{-8}$, $K_{a2ES} = 1.28 \times 10^{-8}$; ⊙, results calculated using all of the points in each run; ⊡, results calculated using the first ten points in each run; ⊥ or ⊤, one standard deviation.

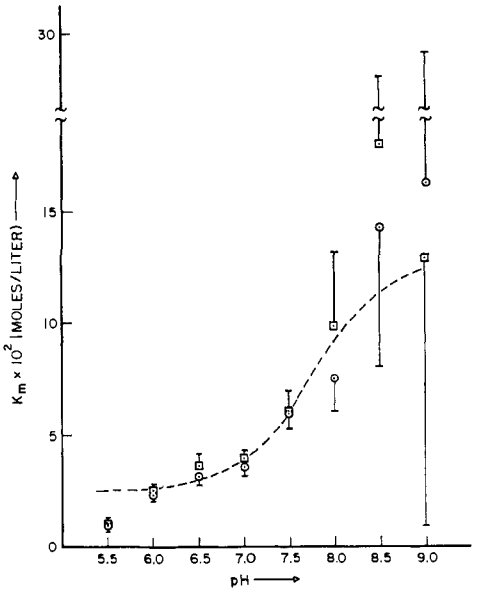
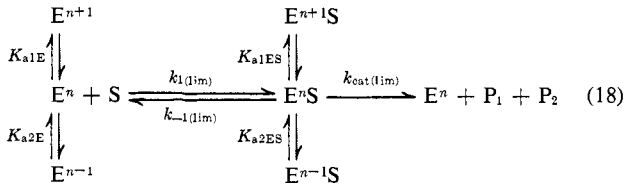


Figure 6. K_m *vs.* pH for the hydrolysis of O-acetyl-L-mandelate catalyzed for carboxydase A at 25°: ----, calculated curve; ⊙, results calculated using all of the points in each run; ⊡, results calculated using the first ten points in each run; ⊥ or ⊤, one standard deviation.

be interpreted in terms of k_{cat}/K_m depending on a base of low pK_a and on an acid of somewhat higher pK_a. A minimal elaboration of eq 4 which might fit the observed pH dedendence is



(18)

where n represents the net charge on the active form of the enzyme and (lim) refers to the limiting values which would be obtained were all of the enzyme in the active form. The K_a values are acid dissociation constants for the ionizing groups affecting catalytic activity. Alberty and Massey[60] have treated this scheme in detail. It can be shown that

$$K_m = K_{m(lim)}\left[\frac{1 + (H^+)/K_{a1E} + K_{a2E}/(H^+)}{1 + (H^+)/K_{a1ES} + K_{a2ES}/(H^+)}\right] \quad (19)$$

$$k_{cat} = \frac{k_{cat(lim)}}{1 + (H^+)/K_{a1ES} + K_{a2ES}/(H^+)} \quad (20)$$

and

$$k_{cat}/K_m = \frac{k_{cat(lim)}/K_{m(lim)}}{1 + (H^+)/K_{a1E} + K_{a2E}/(H^+)} \quad (21)$$

Thus, eq 21 would be useful in obtaining pK_a values of catalytic groups on the free enzyme while eq 20 gives results for the ES complex. Using Alberty's method[60,61] the acid dissociation constants of the groups involved in catalysis were calculated from the equations

$$K_{a1} = (H^+)_{a,1/2max} + (H^+)_{b,1/2max} - 4(H^+)_{max} \quad (22)$$

and

$$K_{a2} = (H^+)^2_{max}/K_{a1} \quad (23)$$

where the subscripts, a,$^1/_2$max and b,$^1/_2$max refer to the hydrogen ion concentration at which the parameter being considered attains half its maximal experimental value. It was found that k_{cat}/K_m depended on a base of pK_{a1E} = 6.9 and on an acid of pK_{a2E} = 7.5. Using these values, the dashed line in Figure 4 was calculated from eq 21. The calculated values fit the experimental results rather well except at pH 5.5 and 6.0.

A qualitatively similar curve was obtained in the plot of $k_{cat}(V_{max}/(E)_0)$ *vs.* pH shown in Figure 5. The rather large error range at high pH, which was not seen in the k_{cat}/K_m results, arose solely because the slopes of the Lineweaver–Burk plots at high pH were large and the ordinate intercepts were small. Thus, a not unreasonable experimental error of 10 or 15% in $(E)_0/v_0$ led to much larger *percentage* errors in $1/k_{cat}$, the ordinate intercept. Using the same approach as with the k_{cat}/K_m results it was found that k_{cat} depends on a base of pK_{a1ES} = 7.2 and on an acid of pK_{a2ES} = 7.9. The dashed line in Figure 5 was calculated from these values and provides an excellent fit of all the data, even at low pH.

The remarkable similarity between Figures 4 and 5 suggests that the base of pK_a = 6.9 in the free enzyme is the same as that of pK_a = 7.2 in the enzyme–substrate complex. Similarly the acidic group of pK_a = 7.5 may have a perturbed pK_a of 7.9 in the complex. This *increase* in the apparent pK_a on complexation is in the direction expected if the complex were more negatively charged than the free enzyme. It is quite probable that the anionic form of the substrate is the reactive species and the results given here are in full accord with such a presumption. This increase in pK_a values attending complexation may be significant; it should be pointed out, however, that small errors in the determination of $(H^+)_{1/2max}$ lead to large errors in calculated pK_a values when the two pK_a values are not well separated.

A plot of K_m *vs.* pH is shown in Figure 6. Although the results are not very meaningful at high pH due to the unavoidable error discussed above, it can be seen

procedure was used in the calculations. However, it was found that at any given pH the weights of all runs were comparable and no such effect was produced.

(60) R. A. Alberty and V. Massey, *Biochim. Biophys. Acta*, **13**, 347 (1954).

(61) M. Dixon and E. C. Webb, "Enzymes," 2nd ed, Academic Press Inc., New York, N. Y., 1964, pp 116–145.

that K_m shows a definite upward trend with increasing pH. The dashed line was calculated from eq 19 using the four K_a values determined above. As expected, the calculated line fits the data quite well except at pH 5.5.

It was hoped that a comparison of the variations of K_m and K_I with pH would provide some insight into the modes of binding of substrate and inhibitor. Table I records the variation of K_I with pH and it is not qualitatively the same as the variation of K_m shown in Figure 6.

Table I

pH	$K_{I,20-25} \times 10^3$ (M)[a]
5.5	0.54 ± 0.15
6.0	2.31 ± 0.53
6.5	3.39 ± 0.68
7.0	1.57 ± 0.25
7.5	1.79 ± 0.40
8.0	2.12 ± 0.64
8.5	5.34 ± 2.61
9.0	6.88 ± 5.82

[a] Calculated using all of the points in each run.

Unfortunately the changes in K_I over the pH range investigated are not large and it is impossible to draw any clear-cut conclusions about differences or similarities between the variations of K_m and K_I with pH.[62]

Discussion

In enzymatic reactions the primary problem in acquiring the information necessary for the development of a mechanistic picture arises from the great structural complexity of enzymes. Since most enzymes will catalyze reactions of substrates which are of small size compared to proteins the notion of the "active site," that region of the protein *directly* involved in the formation of the transition state, has developed. By virtue of the over-all complexity of enzymes, however, most data about the functional groups involved in catalysis have been of an indirect nature. The only direct approach thus far has been the detection of the formation of covalent enzyme–substrate compounds (*e.g.*, acylchymotrypsin[63] and phosphoglucomutase[64]) followed by degradative structure determinations.

In the case of carboxypeptidase A all evidence pertaining to the nature of groups involved in catalysis has been of the indirect type involving correlations of enzyme activity and/or spectral characteristics with chemical changes in the environment. In brief these studies indicate the following. (a) One gram-atom of firmly bound zinc per mole of enzyme in native carboxypeptidase A is required for full activity.[53] Other transition metals may replace zinc but the resultant activity is strongly dependent on the nature of the cation; activity is directly proportional to the metal content of the enzyme.[54,55,58,65] (b) The reaction of the metal-free apoenzyme with thiol-specific reagents,[4,66,67] the spectrum of the cobalt enzyme,[65,68] and the sequence and magnitude of stability constants of metallocarboxypeptidases[58,65] all suggest that one of the ligands binding zinc to the native enzyme is a mercaptide anion[58] of a half-cystine. (c) The amino acid sequence at the zinc-binding half-cystine is ...Gly-Lys-Ala-Gly-(Ala,Ser)-Ser-(Pro,Ser,CySH)-Ser-Glu-Thr-Tyr...[69–71] (d) The sequence and magnitude of stability constants of metallocarboxypeptidases,[58,66] the results of complexometric titrations,[58] activity losses observed on reaction with nitrogen-specific reagents,[4,67] and the spectra of pyridoxal phosphate–enzyme complexes[67] all suggest, with some qualifications,[74] that the α-amino group of N-terminal asparagine supplies a second chelating ligand for zinc.[67] (e) The N-terminal amino acid sequence of carboxypeptidase A_γ is Asp-NH_2-Tyr-Ala-Thr-Tyr-His-Thr-Leu-Asp-Glu-Ileu-Tyr-Asp-Phe-Met.... The main difference between this enzyme[17,18] and that from other preparations[76,77] seems to reside in the length of the N-terminal portion of the peptide chain[78] resulting in variation of the N-terminal amino acid.[79,80] (f) Two tyrosyl residues seem to be intimately associated with the catalytic process; thus the spectral and kinetic data of Simpson, *et al.*,[7] indicate that several groups on the enzyme may be acetylated, but only after acetylation of these particular tyrosines do the catalytic properties of the enzyme change. In addition, binding of 3-phenylpropionate affects the chemical and physical properties of these subunits, for this competitive inhibitor prevents acetylation of two tyrosines,[7] makes at least one tyrosyl more and at least one less susceptible to iodination,[81] and causes spectral changes

(62) Other inhibition studies are being performed in this laboratory which may give more definitive information than was possible in this study.

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(70) K. S. V. Sampath Kumar, K. A. Walsh, J. P. Bargetzi, and H. Neurath in "Aspects of Protein Structure," G. N. Ramachandran, Ed., Academic Press Inc., New York, N. Y., 1963, p 319.

(71) Abbreviations for amino acids used here are the same as those given by Greenstein and Winitz[72] which are based on the proposal of Brand.[73] In the metalloenzyme the proton of the half-cystine sulfhydryl group has been displaced by the metal.[67]

(72) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1961, pp 767–770.

(73) E. Brand, *Ann. N. Y. Acad. Sci.*, 47, 189 (1946); *cf.* also F. Sanger, *Advan. Protein Chem.*, 7, 1 (1952).

(74) Ando and Fujioka[75] found that acylation of carboxypeptidase A with acetic anhydride for a short time completely acetylated the α-amino group of N-terminal asparagine while the peptidase activity remained unchanged. While the first observation, that asparagine becomes acetylated, is not in *experimental* disagreement with the findings of other workers it is surprising that acetylation of a group chelating the metal should cause no change in either the initial velocity or in the time course of the activity assay. The second observation, that acetylation produced no change in the peptidase activity of carboxypeptidase A, is in apparent disagreement with the subsequent results of Vallee's group[4,6,7] as well as of Bender, *et al.*,[10] who found that the peptidase activity decreased sharply on acetylation of the enzyme. It is possible that the discrepancies arose from differences in the buffers employed in acylations since Bender, *et al.*,[10] reported the degree of acetylation by *N*-acetylimidazole to be buffer dependent.

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(76) D. J. Cox, F. C. Bovard, J. P. Bargetzi, K. A. Walsh, and H. Neurath, *Biochemistry*, 3, 44 (1964).

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(79) J. P. Bargetzi, E. O. P. Thompson, K. S. V. Sampath Kumar, K. A. Walsh, and H. Neurath, *J. Biol. Chem.*, 239, 3767 (1964).

(80) K. S. V. Sampath Kumar, K. A. Walsh, J. P. Bargetzi, and H. Neurath, *Biochemistry*, 2, 1475 (1963).

in the enzyme which might be interpretable in part as perturbation of the tyrosine absorption.[49] (g) Imahori and co-workers found that ultraviolet irradiation of carboxypeptidase A partially inactivates the enzyme and destroys tryptophyl (and tyrosyl) residues while altering the spectral changes and inhibition caused by compounds which are competitive inhibitors of the native enzyme.[50] These facts, combined with the observation of a small Cotton effect at 295 mμ, close to the absorption maximum of the indole chromophore, on adding 3-phenylpropionate to the normal enzyme suggested the possibility that tryptophyl residues may influence such binding. The authors felt, however, that these results were mainly suggestive and their conclusions were tentative. (h) Photooxidation of carboxypeptidase A in the presence of methylene blue causes destruction of histidine with a commensurate decrease in peptidase activity.[67]

Correlation of the above results with the pK values found in this investigation poses a difficult problem. The activity changes accompanying alteration of a given group could be due to a variety of causes including the development or diminution of steric hindrance, conformational changes of the enzyme (which need not be large), or actual participation in the catalytic reaction. A second problem arises in that without detailed knowledge of the structure of the enzyme it is hard to predict the dissociation constants of groups affecting the catalysis. To make this point clear consider fumarase which has $pK_{a1E} = 6.3$ and $pK_{a2E} = 6.9$ while pK_{a1EI} and pK_{a2EI} vary from 5.7 to 7.4 and from 7.1 to 7.8, respectively, depending on the nature of the carboxylic acids used as inhibitors.[82] If such large effects on apparent ionization constants can result from inhibitor binding then surely perturbations of the same order of magnitude could arise intramolecularly, *i.e.*, from polar groups on the enzyme itself.

In addition, it should be borne in mind that the scheme presented in eq 18 is a relatively simple one which fits our data, but it is quite possible that the detailed mechanism for the hydrolysis of esters is more involved than our present results would indicate and that there are several enzyme–substrate complexes which intervene sequentially as intermediates in the hydrolytic process. Zerner and Bender have shown that in such a case the Alberty treatment based on eq 18 is valid only if the degree of protonation of the first-formed enzyme–substrate complex corresponds to that of the product-forming complex.[83]

Another point which should be made is that in studies on chymotrypsin it has been found that the pH dependencies of the catalytic rate constants for hydrolytic reactions may be correlated with the pH dependence of a rate-controlling acylation or deacylation step.[84,85] When acylation of the enzyme is rate controlling, a bell-shaped dependence is found, and when deacylation is rate controlling, a sigmoid dependence is observed. In view of the lack of information concerning the intermediates in carboxypeptidase A catalyzed ester hydrolyses, it seems simplest to employ the Alberty treatment here, but our conclusions regarding the significance of the pH dependence of the apparent rate parameters may need to be modified in the future when additional mechanistic information is available. Finally, the whole question of the meaning of bell-shaped curves such as Figures 4 and 5 has been reconsidered. Kirkwood[86] proposed that these curves could be interpreted in terms of the interaction of the fluctuating protein charge with the substrate dipole. Although this interpretation has been criticized for not fitting the data except in the region of the pH optimum[87] such interactions may be partially responsible for the variation of kinetic parameters with pH. This theory assigns the pH optimum as the pK_a of the vicinal group or groups on the protein responsible for interaction and in the present case a pK_a of about 7.5 would obtain.

The ionizing groups available on most proteins and their usual pK_a values are carboxyl (3.0–4.7), imidazolium of histidine (5.6–7.0), α-amino (7.4–8.4), sulfhydryl of half-cystine (8.3–8.6), hydroxyl of tyrosine (8.5–10.9), ε-amino of lysine (9.4–10.6), guanidinium of arginine (11.6–13.3), and hydroxyl of serine (~13.6).[88–90] Half-cystine may be eliminated from consideration since of the two in carboxypeptidase A one binds zinc as a mercaptide anion,[4,58,66,67] and the sulfhydryl group of the other which appears after reduction may be carboxymethylated with no resultant change in enzymatic activity.[69] The only α-amino group of carboxypeptidase A_γ resides on N-terminal asparagine;[78–80] one line of evidence indicates that this group plays no part in the catalytic process[75] and the other indicates that this group is always zincated and does not protonate or deprotonate,[58] at least in the free enzyme. Thus, despite the above-mentioned controversy regarding the function of this group,[74] both conclusions indicate that the α-amino group plays no part in the catalytic mechanism *via* ionization and it will not be further considered. The remaining ionizing functions are each present to the extent of between 8 and 53 residues per molecule.[19] At present the supporting evidence required to make a choice between them is lacking and only speculation is possible. An obvious possibility for the basic group of $pK_{a1E} = 6.9$ and $pK_{a1ES} = 7.2$ (assuming both pK_{a1} values to be due to the same group) is imidazolium of histidine. The only support for this choice is, as mentioned under h, that photooxidative destruction of histidine proportionally reduces the activity of the enzyme.[67] This is a situation found with a number of other enzymes[91] and the involvement of the imidazolium function in the mechanism of α-chymotrypsin action is particularly well documented.[92] The most likely choices for the subunit bearing the acidic group having $pK_{a2E} = 7.5$ and $pK_{a2ES} = 7.9$ are histidine, tyrosine, and possibly lysine. Although 2,4-dinitrofluorobenzene reacts with lysyl residues on the enzyme without alteration of

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(85) M. L. Bender, G. E. Clement, F. J. Kezdy, and B. Zerner, *ibid.*, **85**, 358 (1963).
(86) J. G. Kirkwood, *Discussions Faraday Soc.*, **20**, 78 (1955).
(87) K. J. Laidler, *ibid.*, **20**, 254 (1955).
(88) Reference 61, p 144.
(89) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press Inc., New York, N. Y., 1958, p 534.
(90) T. C. Bruice, T. H. Fife, J. J. Bruno, and N. E. Brandon, *Biochemistry*, **1**, 7 (1962).
(91) E. A. Barnard and W. D. Stein, *Advan. Enzymol.*, **20**, 51 (1958).
(92) *Cf.* M. L. Bender and F. J. Kezdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964), and references cited therein.

catalytic activity,[8] the possibility that lysine is involved in the catalytic process cannot be excluded unless it be shown that all of the lysyl groups react with this reagent. The effects of modifications of tyrosine and histidine on enzymatic activity were discussed under f and h, respectively; the evidence is suggestive but by no means establishes a connection between either of these subunits and the higher pK_a function found in this study.

The variation of the competitive product inhibition constant, K_I, with pH is relatively smaller in the region pH 6.0 to 8.0 than that found for K_m. The dependence of the calculation of K_I on both k_{cat} and K_m resulted in large enough experimental errors so that it cannot be said that any of the values in the region pH 6 to 9 differ significantly from a value of about 2×10^{-3} M. It is noteworthy that K_I is always more than one order of magnitude smaller than K_m at a given pH, but no definitive explanation of this result can be given at the present time. Although product inhibition was found to be competitive and the product is structurally similar to the substrate, it is not known whether binding occurs at the same site for both compounds. Indeed, the possibility that binding site(s) on the enzyme in addition to the active site may exist is suggested by the facts that substrate inhibition has been observed for both ester and peptide substrates,[6,10,14,93-95] substrate activation has been observed for both peptide and ester substrates,[96,97] activation by *N*-carbobenzyloxyglycine has been observed for both peptide and ester hydrolyses,[96,97] and brief mention of activation of peptide hydrolyses by D-peptides has been made.[95] The tenability of this hypothesis of multiple binding sites on carboxypeptidase A and the answer to the related question of whether or not the enzyme is allosteric in character must await further studies of these intricate processes.[97]

Competitive product inhibition has also been found by Yanari and Mitz[93] in the carboxypeptidase-catalyzed hydrolysis of the peptide substrate N-(N-carbobenzyloxyglycyl)-L-phenylalanine at pH 9.0. They concurred with Elkins-Kaufman and Neurath[98] that the inhibition was primarily due to the anionic form of L-phenylalanine.[99] There has been some disagreement in the literature as to the kinetic constants for the enzyme-catalyzed hydrolysis of N-(N-carbobenzyloxyglycyl)-L-phenylalanine at pH 7.5 and 25°.[12,56,57,94,95,98,102-104] However, most of the discrepancies probably arise from ionic strength differences, which profoundly influence the catalytic activity,[94] and from specific buffer effects. For example, orthophosphate causes both time dependent[104] and instantaneous inhibition[94] of carboxypeptidase A. Recently, Bender[96] has found that appreciable competitive product inhibition occurs in this reaction at pH 7.5 and 25°. Thus, the limited information available indicates that both peptide and ester substrates produce products of like configuration which competitively inhibit the enzyme.

From a mechanistic standpoint little can be said about the mode of binding of α-hydroxy acids to carboxypeptidase A. Coleman and Vallee[40] proposed a general binding mechanism for inhibition which differed stereochemically from the binding mechanism of substrates. This model was proposed to account for reports that D-amino acids inhibit the enzyme while L-amino acids do not.[37,40,98] However, Bender's finding of inhibition by L-phenylalanine does not fit the model. Furthermore, our results indicate that L-mandelate is at least 100 times more effective than D-mandelate as an inhibitor and also could not fit the model. Thus, it must be concluded that no simple similarities or differences between the mode of binding of substrates and inhibitors to carboxypeptidase A can yet be said to have been found.

It would be desirable to compare the present results for the esterolytic action of carboxypeptidase A with similar studies on peptide hydrolyses. Neurath and Elkins-Kaufman[105] found that both K_m and k_{cat} for the hydrolysis of N-(N-carbobenzyloxyglycyl)-L-phenylalanine decrease with increasing pH from pH 6.5 to minima at pH 7.5 to 7.8, rise to maxima at ~pH 8.3, and fall again up to pH 9.[9] In contrast Lumry, Smith, and Glantz[94] later found that K_m increased only slightly and k_{cat} did not change over the range pH 7.5 to 8.3 for the same substrate. Lumry and coworkers[94,95] found similar results with both N-(N-carbobenzyloxyglycyl)-L-tryptophan and N-(N-carbobenzyloxyglycyl)-L-tyrosine, and extension of the pH range under investigation showed that K_m decreased between pH 6.6 and 7.5 and was then constant to pH 8.5 while k_{cat} increased sharply from pH 6.6 to 7.2 and then slowly increased up to pH 8.5.[95] The above contradictions make it hazardous to compare our results with these studies on peptide substrates. Several rather subtle complicating effects on the carboxypeptidase A catalyzed hydrolysis of N-(N-carbobenzyloxyglycyl)-L-phenylalanine which may account for these disparities have become evident. These are: (1) ionic strength effects,[94] (2) specific buffer effects,[94,95,104] (3) inhibition by high concentrations of substrate,[93,94,98] and (4) substrate activation at lower concentrations of substrate.[96] In the case of O-acetyl-L-mandelate neither substrate inhibition, nor substrate activation, nor specific buffer effects was apparent. Unfortunately, however, considerable product inhibition by L-mandelate occurred and also the rate of hydrolysis of the ester was so slow that the reproducibility was affected and 10 or 20% effects on observed velocities might not be noticeable. The present study of the esterase action of carboxypeptidase A cannot be ade-

(93) S. Yanari and M. A. Mitz, *J. Am. Chem. Soc.*, 79, 1154 (1957).
(94) R. Lumry, E. L. Smith, and R. R. Glantz, *ibid.*, 73, 4330 (1951).
(95) R. Lumry and E. L. Smith, *Discussions Faraday Soc.*, 20, 105 (1955).
(96) M. L. Bender, *et al.*, Northwestern University, personal communication. We thank Professor Bender for providing us with his results on carboxypeptidae A catalyzed peptide hydrolyses prior to publication.
(97) E. T. Kaiser, S. Awazu, and F. W. Carson, *Biochem. Biophys. Res. Commun.* 21, 444 (1965). Substrate activation was found in the hydrolysis of O-hippurylglycolate catalyzed by carboxypeptidase A, and the kinetic results obtained with this substrate suggest that the enzyme may possess allosteric character.
(98) E. Elkins-Kaufmann and H. Neurath, *J. Biol. Chem.*, 175, 893 (1948).
(99) It has generally been assumed that carboxypeptidase A stereospecifically releases amino acids of the L configuration from peptides; in some hydrolyses of acylated tyrosine, tryptophan, and phenylalanine substrates this has been substantiated by the isolation of optically pure L-amino acid products from the reaction mixtures.[100,101]
(100) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, 134, 225 (1940).
(101) J. B. Gilbert, V. E. Price, and J. P. Greenstein, *ibid.*, 180, 473 (1949).
(102) J. E. Snoke and H. Neurath, *ibid.*, 181, 789 (1949).
(103) H. Neurath and G. de Maria, *ibid.*, 186, 653 (1950).
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quately compared with the peptidase action of this enzyme until a complete kinetic study of all of the above-mentioned effects as a function of pH is performed for a peptide substrate. Furthermore, it would be extremely desirable to have more information about the nature of any intermediates formed during the catalytic process. On the basis of the unusual pH–activity behavior displayed by the ester substrate O-(N-benzoylglycyl)-L-3-phenyllactate[106] in comparison with the peptidase substrate N-(N-carbobenzyloxyglycyl)-L-phenylalanine, the proposal has been made that these two substrates are hydrolyzed by different mechanisms.[4,5,8] In particular it was suggested that some nucleophile, B, on the enzyme catalyzes peptide hydrolyses while hydroxide ion replaces B as a nucleophile in ester hydrolyses. The present results invalidate the basis for this proposal (at least as a general mechanistic hypothesis) since the pH dependences of k_{cat}, k_{cat}/K_m, and the activity of carboxypeptidase A toward O-acetyl-L-mandelate throughout the substrate concentration range are all qualitatively similar to the pH–activity curve found for N-(N-carbobenzyloxyglycyl)-L-phenylalanine. The qualitative differences between hydrolyses of O-acetyl-L-mandelate and of O-(N-benzoylglycyl)-L-3-phenyllactate could be due to different rate-determining steps in a common mechanism or to a variety of other causes, including some of the complicating factors mentioned above. It is our opinion that the data presently available are insufficient to make any firm distinctions between the mechanisms of peptide and ester hydrolyses catalyzed by carboxypeptidase A.

(106) Vallee and co-workers found that the pH–activity profile for the carboxypeptidase A catalyzed cleavage of this substrate rose between pH 5.5 and 7.0, exhibited a plateau between pH 7.0 and 9.0, rose again to a maximum at pH 10.5, and then declined;[4,5] these results were confirmed at other substrate concentrations in the region pH 7.65–9.70 by McClure, *et al.*;[8] in contrast, the pH–activity profile for N-(N-carbobenzyloxyglycyl)-L-phenylalanine was bell shaped, being approximately symmetrical about pH 7.5.[4,5,12]

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Amide Hydrolysis. Superimposed General Base Catalysis in the Cleavage of Anilides[1]

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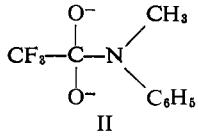
Abstract: The base-catalyzed hydrolysis of 2,2,2-trifluoro-N-methylacetanilide (I) at pH 9.5–10 follows the kinetic law $-d[I]/dt = [I]\{k_0 + [HO^-](k_1 + \sum_i k_i[B_i])\}$ which corresponds to the superimposition of general base catalysis upon specific hydroxide ion catalysis. The results are in accord with a rate-determining elimination of N-methylaniline from an intermediate adduct or with a less likely general base catalyzed attack by hydroxide ion in a concerted displacement reaction.

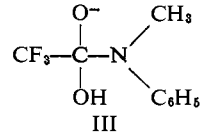
Biechler and Taft[2] and Bruylants and Kèzdy[3] have shown that 2,2,2-trifluoro-N-methylacetanilide (I) and other acyl-activated amides undergo hydrolysis according to the kinetic law of eq 1. A similar, but

$$-d[I]dt = [I](k_1[HO^-] + k_2[HO^-]^2) \quad (1)$$

much more complex, expression has recently been given by Mader[4] for 2,2,2-trifluoroacetanilide. Biechler and Taft[2] attributed the term second order in hydroxide ion to intermediacy of the dinegative ion II, but the data are equally consistent with the general base catalyzed decomposition of III, the k_1 term representing solvent catalysis and the k_2 term hydroxide ion catalysis. As part of a general investigation of catalysis in these and related systems, we have examined this system for general catalysis. Solvent isotope effects will be reported at a later time.



II



III

Results

Table I and Figure 1 show the dependence of the observed first-order rate constant for hydrolysis of I on sodium glycinate concentration in glycine–sodium glycinate buffers. The hydroxide ion dependences of the slopes and intercepts of these lines (Table II) allow determination of rate constants for the individual bases.

These data are consistent with eq 2, the values of the

$$-d[I]/dt = [I]\{k_0 + [HO^-](k_1 + \sum_i k_i[B_i])\} \quad (2)$$

(1) This research was supported by the National Institutes of Health under Research Grant No. GM-12477-01. For further details, see G. W. Zuorick, M.S. Thesis, University of Kansas, Aug 1965. A preliminary report of this work has appeared: R. L. Schowen and G. W. Zworick, *Tetrahedron Letters*, 3839 (1965).

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(3) A. Bruylants and F. Kèzdy, *Record Chem. Progr.*, **21**, 213 (1960).

(4) P. M. Mader, *J. Am. Chem. Soc.*, **87**, 3191 (1965).