into a 10-ml. flask and dissolved in 1 ml. of dioxane. Another 10-ml. dioxane solution containing *p*-nitrophenol (0.24170 g., 0.00173 mole) and tri-*n*-butylamine (0.42 ml., 0.00173 mole) was prepared. To initiate the reaction, 9 ml. of the *p*-nitrophenol and tri-*n*-butylamine solution was pipetted into the oxazolone solution. The final ratio of *p*-nitrophenol:amine:oxazolone was 10:10:1.

5. **Reaction between Oxazolone and Phenylalanine Methyl Ester.**—Phenylalanine methyl ester (0.67126 g., 0.00375 mole) was weighed into a 5-ml. volumetric flask to which 4 ml. of dioxane was added. A 2-ml. dioxane solution of 2-phenyl-4-benzyloxazolin-5-one (0.04631 g., 0.000189 mole) was prepared. To initiate reaction, 1 ml. of the oxazolone solution was added to the 4 ml. solution of phenylalanine methyl ester. The ratio phenylalanine methyl ester:oxazolone was 41:1.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CHICAGO, CHICAGO 37, ILL.]

Studies on the Esterase Action of Carboxypeptidase A. Kinetics of the Hydrolysis of Acetyl-L-mandelate[1,2]

BY E. T. KAISER AND F. W. CARSON

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O-Acyl esters of mandelic acid have been found to be excellent substrates for kinetic measurements on the esterase activity of carboxypeptidase A. A detailed study of the carboxypeptidase-catalyzed hydrolysis of O-acetylmandelate has been carried out, using primarily the optically pure L-compound. The value of K_m for the hydrolysis of acetyl-L-mandelate is 0.070 ± 0.014 mole/liter at pH 7.5. At this pH competitive inhibition of the hydrolysis by one of the products, L-mandelate, is observed with a K_i of 1.76 ± 0.16 × 10^{-3} mole/liter. The pH–rate profile for the hydrolysis of acetyl-L-mandelate exhibits a maximum near pH 7.5, a value close to the optimal pH reported previously for peptidase activity. Other α-acyloxy acids have also been examined as potential substrates for carboxypeptidase A with a view toward determining the specificity and reactivity requirements of the enzyme as an esterase.

Introduction

This paper is the first of a projected series on the esterase action of carboxypeptidase A. A zinc-containing metalloenzyme with a molecular weight of about 34,300,[3-5] carboxypeptidase A is known to catalyze the hydrolysis of peptides in which a free carboxyl group is situated α to the hydrolytically labile amide linkage.[6] Its catalysis of the hydrolysis of some α-acyloxycarboxylic acids has also been reported.[6-8] However, although the specificity and the kinetics of the hydrolytic action of carboxypeptidase A on peptide substrates have been investigated in great detail, considerably less attention has been devoted to the examination of a number of the most basic features of its action on ester substrates. A survey of the literature reveals a lack of a thorough kinetic analysis of the carboxypeptidase A-catalyzed hydrolysis of an ester. The effect of pH upon esterase activity, the effects of inhibitors, and the relationships between the structure and stereochemistry of substrates and their hydrolytic reactivity all need clarification. In this paper the results of a kinetic study of the hydrolysis of a relatively uncomplicated ester, O-acetylmandelate, will be discussed, together with several experiments designed to delve into the specificity requirements of carboxypeptidase A.

Our choice of O-acetylmandelate as a suitable compound for study was motivated by the knowledge that mandelic acid can be readily resolved and that the preparation of its O-acyl esters in optically pure form can be accomplished without difficulty. Fortunately, since the hydrolysis of O-acetylmandelate catalyzed by carboxypeptidase A proceeds at a rate which is conveniently measurable using an automatic titrator, this substrate is particularly amenable to kinetic investigation.

Experimental

Materials. O-Acetyl-DL-mandelic Acid.—Racemic O-acetylmandelic acid was obtained from Aldrich Chemical Co. The commercial product was apparently partially hydrated. Recrystallization from hexane gave pure anhydrous O-acetyl-DL-mandelic acid, m.p. 80.5–81.5° (lit.[9] m.p. 79–80).

L-Mandelic Acid.—Mandelic acid was resolved through its strychnine salt. A mixture of 152 g. (1 mole) of racemic mandelic acid and 167 g. (0.5 mole) of strychnine in 1500 ml. of water was heated on a steam bath and enough concentrated ammonium hydroxide was added to give a clear yellow solution. The solution was seeded with a crystal of pure strychnine L-mandelate and was allowed to cool to room temperature. Recrystallization of the precipitated salt from 1 l. of water and air drying gave colorless needles of strychnine L-mandelate with indefinite m.p. in the range 107–116° (lit.[10] m.p. 115–116°). (This material was probably a hydrate since exhaustive desiccation raised the m.p. to 128–131°.) The salt was treated with hot water and excess ammonium hydroxide and, after cooling, the strychnine was collected. The filtrate was saturated with sodium chloride and was acidified with hydrochloric acid. Repeated extraction of the filtrate with ether and evaporation of the solvent from the dried extracts gave 30 g. (39%) of crude L-mandelic acid which was suitable for the preparation of acylated derivatives. The yield of recovered material could be increased substantially by using

(1) This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, and the Block Fund of the University of Chicago. Part of the work reported here was performed in the Department of Chemistry, Washington University, St. Louis 30, Mo.

(2) Presented in part at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept. 8–13, 1963, Abstracts, p. 98C.

(3) B. L. Vallee and H. Neurath, *J. Biol. Chem.*, **217**, 253 (1955).

(4) E. L. Smith and A. Stockell, *ibid.*, **207**, 501 (1954).

(5) J. R. Brown, D. J. Cox, R. N. Greenshields, K. A. Walsh, N. Yamasaki, and H. Neurath, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1544 (1961).

(6) See H. Neurath in "The Enzymes," Vol. 4, 2nd Ed., Academic Press, Inc., New York, N. Y., 1960, p. 11, for a comprehensive review.

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

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

(9) F. K. Thayer in "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1941, p. 12.

(10) A. McKenzie, *J. Chem. Soc.*, 964 (1899).

the mother liquors in subsequent resolutions. Recrystallization of a sample from acetone–benzene gave white needles, m.p. 131–133° (lit.[10-12] m.p. 132–133°, 133.5°, 133.5°) and $[\alpha]^{27}D$ +154° (*c* 2.047, water) (lit.[10] $[\alpha]^{6}D$ +158.7°).

D-Mandelic Acid.—D-Mandelic acid was prepared *via* the (−)-ephedrine salt according to Roger[13] and had m.p. 131–133°, $[\alpha]^{27}D$ −151° (*c* 2.023, water) (lit.[12] $[\alpha]^{20}D$ −156.9° (*c* 2, water)).

O-Acetyl-L-mandelic Acid.—A mixture of 10 g. (0.066 mole) of crude L-mandelic acid and 25 ml. (0.0266 mole) of acetic anhydride was heated on a steam bath for 3 hr. The excess acetic anhydride was stripped off by rotary evaporation *in vacuo* and a colorless oil was obtained. Addition of ether and water to the oil and re-evaporation gave 8 g. (62%) of white crystals of crude O-acetyl-L-mandelic acid. After two recrystallizations from water the product had m.p. 54–55° (lit.[14] m.p. of monohydrate 56°) and exhaustive desiccation over phosphorus pentoxide *in vacuo* with warming gave the anhydrous acid, m.p. 97–99° (lit.[11,15] m.p. 96.8°, 96.5–98°) and $[\alpha]^{25}D$ +153° (*c* 2.044, acetone) (lit.[11,15] $[\alpha]^{20}D$ −157° (*c* 2.4, acetone) and $[\alpha]^{15}D$ −153.7° (*c* 2.085, acetone) for the anhydrous D-isomer).

O-Acetyl-D-mandelic acid was prepared from crude D-mandelic acid in the same manner as described for the L-isomer except that exhaustive desiccation was omitted. The product had m.p. 54–55°, equiv. mol. wt. 212 bromothymol blue end point (equiv. mol. wt. calcd. for monohydrate: 212), and $[\alpha]^{23}D$ −150° (*c* 2.123 in acetone) (lit.[14] $[\alpha]D$ −156.4° (*c* 3.33, acetone) for the monohydrate).

Acetoxyacetic acid was purchased from Aldrich Chemical Co. and was recrystallized from benzene; m.p. 63–66.5° (lit.[16,17] m.p. 67–68°, 66–68°).

O-Acetylbenzilic acid was prepared by the method of LaMer and Greenspan[18]; m.p. 108.5–109.5° (lit.[18] m.p. 104.5–104.8°) after exhaustive desiccation *in vacuo* over phosphorus pentoxide; equiv. mol. wt. 270 phenolphthalein end point (equiv. mol. wt. calcd.: 270).

Carboxypeptidase A was obtained as a suspension of crystals in toluene–water from Sigma Chemical Co. Standard enzyme solutions were prepared by dialyzing the suspension at 4° against 1 *M* sodium chloride buffered with 0.1 or 0.05 *M* 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) at pH 7.5. The buffer components were of reagent grade quality, and the water was distilled and demineralized with a mixed-bed ion exchange column. The dialysate was centrifuged and the supernatant liquid was filtered under pressure through a Schleicher and Schuell medium membrane. An aliquot of the filtrate was withdrawn and diluted to a known volume, and the absorbance at 278 mμ was determined by means of either a Beckman Model DU spectrophotometer or a Cary Model 14 recording spectrophotometer. Taking the value of 6.42×10^4 l. mole^{-1} cm.$^{-1}$ as the extinction coefficient[19] of carboxypeptidase A at this wave length, the original filtrate was then diluted with dialysis buffer to a calculated concentration of 1.00×10^{-4} *M*. Stock solutions thus prepared appeared to be quite stable for at least 3 months when stored at 4° since the activity toward acetyl-L-mandelate at pH 7.5 was unchanged during that period; in addition, there was good agreement between the titrimetric activity and the spectrophotometrically determined concentrations of all enzyme preparations.

Kinetic Measurements.—The hydrolyses of acylated α-hydroxy acids were followed by automatic titration at constant pH employing either an International Instrument Co. automatic titrator equipped with a Leeds and Northrup pH meter and recorder or else a Radiometer Type TTTlb titrator in conjunction with a Type SBR2c titrigraph. The consumption of basic titrant recorded by the pH-Stat is a direct measure of the amount of acetic acid released in the hydrolysis of O-acetyl-L-mandelate to produce acetic acid and L-mandelate provided that the products do not buffer the reaction mixture. Since mandelic acid has a pK_a of 3.4[20] while that of acetylmandelic acid was found to be even smaller, the error introduced in the titrimetric measurements by association of this product with hydrogen ions present in solution would be less than 3% at pH 5, and it would be completely negligible at higher pH. However, acetic acid which has a pK_a of 4.76[21] exerts a nonnegligible buffering effect in the low pH region. The appropriate correction is given[22] by the equation

$$v = v_{exp}[1 + (H^+)/K_a]$$

where v is the velocity of the reaction in moles/liter/min. corrected for the product buffering effect, v_{exp} is the measured rate of titrant consumption, and K_a is the dissociation constant of acetic acid at 25°. This type of correction was applied to all of the data taken below pH 7 in order to reduce the buffering effect error to less than 1%. Infinity titers obtained were always in accord with the values expected from the stoichiometry of the hydrolysis reactions.

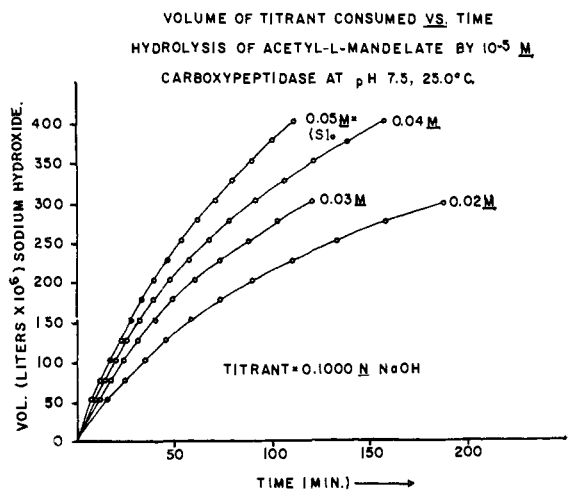

Figure 1.

All solvents and buffer components were of reagent grade quality, and demineralized water was used in preparing solutions. The titrant was Fisher 0.1000 *N* sodium hydroxide, and the reaction vessel was thermostated at 25.00 ± 0.02°.

In a typical run the instrument was first standardized against a buffer of pH 7.40 ± 0.02. The titrating mechanism was set at pH 7.50 and sufficient 0.500 *M* sodium chloride to give a final volume of 5.00 ml. was added to the reaction vessel and allowed to equilibrate for 10 min., while being magnetically stirred. Then 100 μl. of 2.000 *M* O-acetyl-L-mandelic acid in either dimethylformamide or acetonitrile was pipetted in simultaneously with 100 μl. of 2.0 *N* sodium hydroxide. At this point 500 μl. of 1.00×10^{-4} *M* stock carboxypeptidase solution was added, and the pH was quickly adjusted to 7.50. Thus the initial concentrations of all materials for this type of run were: 2% dimethylformamide or acetonitrile, 0.53 *M* sodium chloride, 0.005 or 0.01 *M* Tris, 1.00×10^{-5} *M* carboxypeptidase, and 0.04 *M* sodium O-acetyl-L-mandelate. The automatic titrating mechanism maintained the set pH during the hydrolytic reaction by adding increments of base as small as 0.2 μl. and also continuously recorded the amount of base consumed *vs.* time.

In other runs the volume, temperature, concentration of enzyme, concentration of sodium chloride, and concentration of buffer were the same while the pH was varied, the concentration of substrate varied from 0.01 to 0.05 *M*, and the concentration of acetonitrile or dimethylformamide varied from 0.5 to 2.5%. Above pH 8 the reactions were run in a nitrogen atmosphere.

Results and Discussion

Kinetics of the Hydrolysis of O-Acetyl-L-mandelate at pH 7.5.—The results of four typical runs measuring the rates of hydrolysis of O-acetyl-L-mandelate at a variety of concentrations catalyzed by carboxypeptidase A at pH 7.5 are illustrated in Fig. 1. The points

(11) W. R. Angus and R. P. Owen, *J. Chem. Soc.*, 227 (1943).
(12) R. H. F. Manske and T. S. Johnson, *J. Am. Chem. Soc.*, **51**, 1906 (1929).
(13) R. Roger, *J. Chem. Soc.*, 1544 (1935).
(14) P. Walden, *Z. Physik. Chem.*, **17**, 713 (1895).
(15) A. McKenzie and H. B. P. Humphries, *J. Chem. Soc.*, 1105 (1909).
(16) M. S. Kharasch, H. N. Friedlander, and W. H. Urry, *J. Org. Chem.*, **16**, 533 (1951).
(17) R. Anshutz and W. Bertram, *Ber.*, **36**, 466 (1903).
(18) V. K. LaMer and J. Greenspan, *J. Am. Chem. Soc.*, **56**, 956 (1934).
(19) R. T. Simpson, J. F. Riordan, and B. L. Vallee, *Biochemistry*, **2**, 616 (1963).
(20) G. Kortum, W. Vogel, and K. Andrussow, *J. Pure Appl. Chem.*, **1**, 410 (1960).
(21) Reference 20, p. 241.
(22) A. N. Kurtz and C. Niemann, *Biochemistry*, **1**, 238 (1962).

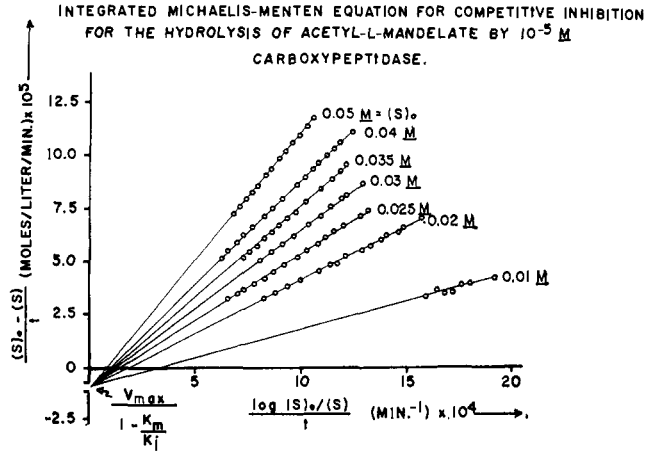

Figure 2.

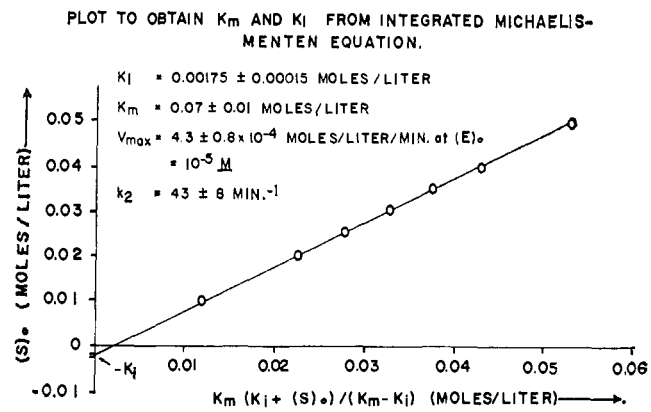

Figure 3.

shown are those which were taken from the continuous record of the titrator for the purposes of calculation. The curves of these plots do not follow a simple kinetic order, and the sharp decrease of the rates of hydrolysis with time suggests that product inhibition of enzymatic catalysis is important here. Indeed, the addition of 0.01 to 0.08 M sodium L-mandelate to the reaction mixture for runs employing 0.04 M O-acetyl-L-mandelate as the substrate caused such drastic inhibition of hydrolysis that the rates of reaction became too slow to be measured conveniently and accurately.

The scheme which fits the kinetics of the carboxypeptidase A-catalyzed hydrolysis of O-acetyl-L-mandelate is shown below, together with the corresponding integrated Michaelis–Menten rate expression[23] (E = carboxypeptidase A, S = O-acetyl-L-mandelate P_1 = acetate, and P_2 = L-mandelate). The L-mandelate, P_2, produced during hydrolysis acts as a competitive inhibitor.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P_1 + P_2$$

$$E + P_2 \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EP_2$$

Integrated Michaelis–Menten equation

$$\frac{[S]_0 - [S]}{t} = \frac{K_m(K_i + [S]_0)}{K_m - K_i} \ln \frac{[S]_0/[S]}{t} + \frac{V_{max}}{1 - K_m/K_i}$$

$$= a \ln \frac{[S]_0/[S]}{t} + \frac{V_{max}}{1 - K_m/K_i}$$

where $K_m = (k_{-1} + k_2)/k_1$, $K_i = k_{-i}/k_i$, and

$$a = \frac{K_m(K_i + [S]_0)}{K_m - K_i}$$

The integrated Michaelis–Menten equation for competitive inhibition by a product applies to the kinetic data obtained at pH 7.5 very satisfactorily. This is illustrated by Fig. 2 in which $([S]_0 - [S])/t$ is plotted on the ordinate against $(\log [S]_0/[S])/t$ on the abscissa. The results obtained were highly reproducible, and most of the lines are made up of points taken from more than one run at a given substrate concentration. The linearity of the plots thus obtained indicates that competitive product inhibition is involved since the lines would curve for other types of product inhibition.

The intercept on the ordinate of Fig. 2 is a function of V_{max}, K_m, and K_i, and the slopes of the straight lines, a, are related to K_m, K_i, and $[S]_0$ by the equation

$$[S]_0 = a(1 - K_i/K_m) - K_i$$

Thus, in order to separate the various kinetic parameters, in Fig. 3 the values of $[S]_0$ have been plotted against the respective slopes, a, found from Fig. 2. The intercept along the $[S]_0$ axis is $-K_i$, and, with this in hand, the values of K_m and V_{max} can also be calculated. They are given in Fig. 3 for pH 7.5. It should be noted that K_i is considerably smaller than K_m, indicating strong competitive inhibition by the product, L-mandelate.

As a check on the application of the integrated Michaelis–Menten equation and the kinetic constants computed by means of it, K_m and V_{max} have also been determined by the Lineweaver–Burk procedure.[24] Using this procedure, reaction velocities have been measured as approximately zero-order rates for the initial phases of each run before 5% hydrolysis has occurred and before significant product inhibition has taken place. From Fig. 4 it can be seen that the Lineweaver–Burk plot of the kinetic results is linear. Any scatter observed is due primarily to the difficulty encountered in attempting to equilibrate the reaction system in the short times (usually less than 1 min.) which were necessary in these measurements in order to avoid noticeable product inhibition.

The agreement between kinetic parameters calculated by the Michaelis–Menten and Lineweaver–Burk procedures at pH 7.5 is quite good, as indicated in Table I, where the results are summarized. All of the kinetic data were treated by the method of least squares.

TABLE I

Kinetic parameter	Calculated by Lineweaver–Burk method	Calculated from integrated Michaelis–Menten equation
K_m	0.082 ± 0.011	0.070 ± 0.014 mole/l.
K_i	...	0.00176 ± 0.00016 mole/l.
V_{max}	$3.3 \pm 0.4 \times 10^{-4}$	$4.3 \pm 0.8 \times 10^{-4}$ mole/l./min.
k_2	33 ± 4	43 ± 8 min.$^{-1}$

pH Effects.—Studies on the pH dependence of K_m, K_i, and V_{max} for the hydrolysis of O-acetyl-L-mandelic acid are now in progress, and when completed

(23) 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.

(24) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).

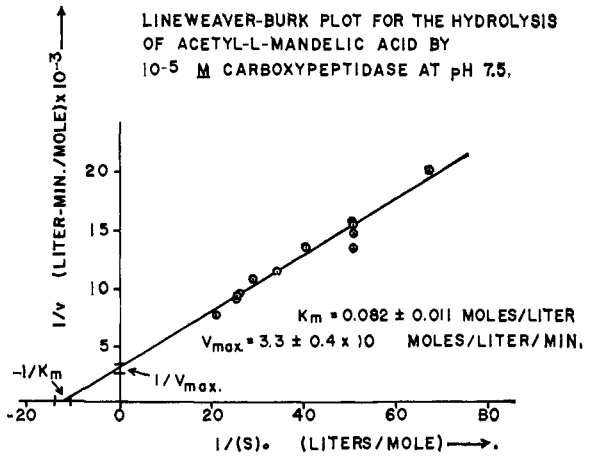

Figure 4.

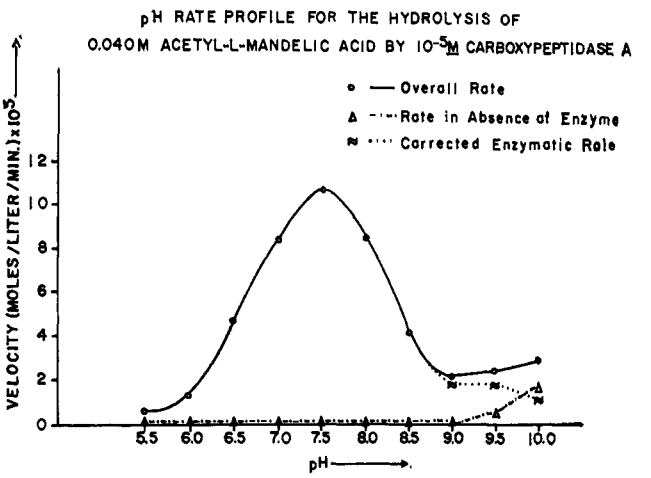

Figure 5.

they should provide valuable information concerning the catalytic sites in carboxypeptidase A. Some data on the effect of pH on the initial velocities of hydrolysis of the L-ester are shown in Fig. 5. The rate maximum observed occurs near pH 7.5, and the pH–rate profile is reminiscent of that reported previously[7,25] for the peptidase activity of carboxypeptidase A which exhibits a maximum at pH 7.5. Since the substrate concentration for the data illustrated in Fig. 5 is low compared with K_m, pH changes will affect the kinetics through the ionization of both the enzyme–substrate complex and the free enzyme. Thus a detailed analysis of pH effects requires the determination of the pH variation of V_{max}, the ionization of only the enzyme–substrate complex being influenced by the pH in this case. Our preliminary results indicate that V_{max} varies in a manner which is qualitatively similar to the curve shown in Fig. 5. It should be noted that the pH–rate profile for the hydrolysis of another ester, O-acetyl-DL-β-phenyllactic acid, has also been recently reported to resemble that for a peptide.[26]

From Fig. 5 it can be seen that the rate of the hydroxide-ion catalysis of the hydrolysis of O-acetyl-L-mandelate becomes appreciable above pH 8.5 and must be corrected for in computing the rate of the enzymatic reaction. Above pH 9 accurate rate measurements are difficult not only because the correction for hydroxide-ion catalysis is large, but also because protein precipitation during the course of a run in the alkaline solution is important.[27]

The pH behavior observed for the carboxypeptidase A-catalyzed hydrolysis of O-acetyl-L-mandelic acid contrasts with the behavior noted by Vallee and his coworkers for the hydrolysis of O-hippuryl-DL-β-phenyllactic acid (HPLA).[7,8] They found that the rate of the enzyme-catalyzed cleavage of HPLA rose with increasing pH between pH 5.5 and 7.0. Between 7.0 and 9.0 the pH–rate profile exhibited a plateau and this was followed by a second rise which reached a maximum beyond 10.5 and then declined. When the logarithms of the zero-order rate constants determined in the alkaline range for the hydrolysis of HPLA were plotted against pH, the pH–rate profile was found to resemble known data on the hydroxide-ion catalyzed hydrolysis of protolytic esters.[28] Because of this resemblance, it has been suggested that hydrolysis of HPLA catalyzed by carboxypeptidase A requires the participation of hydroxide ions.[7,8] The further elaboration of the reasons for the difference in the pH dependence of the hydrolyses of O-acetyl-L-mandelic acid and HPLA poses an intriguing problem whose solution may provide valuable information about the esterase action of carboxypeptidase A.

Specificity Requirements of the Esterase Action of Carboxypeptidase A and Mechanistic Considerations.—In view of the success attained in the use of O-acetyl-L-mandelic acid as a substrate for the kinetic investigation of carboxypeptidase A, it is of interest to determine what structural and stereochemical features are required for an ester to be a substrate for the catalytic action of the enzymes. Structural variations in the alcohol moiety of the esters will be considered first. One basic requirement which is commonly assumed to hold is that the substrate must possess a free carboxyl group α to the alkoxide portion of the ester bond which is to be hydrolyzed. On the other hand, the location of the phenyl group in the alcohol moiety does not seem to be as critical; thus in O-acetyl-L-mandelic acid it is α to the carbonyl carbon atom, but in O-acetyl-DL-β-phenyllactic acid it is β. Indeed, the question arises as to whether the phenyl group is needed at all. In order to answer this question the enzymatic hydrolysis of 0.01 M α-acetoxyacetic acid was attempted. At pH 7.5 and a concentration of 10^{-5} M carboxypeptidase, no catalytic effect of the enzyme on the hydrolysis of the ester could be observed.

Since a phenyl substituent is apparently an important structural feature of an ester substrate, it is of interest to determine if additional phenyl substituents cause enhanced reactivity. A test of this possibility with O-acetylbenzilic acid quickly indicated that in this type of system further α-phenyl substitution does not increase reactivity. In fact, at pH 7.5, a substrate concentration of 0.01 M, and a concentration of 10^{-5} M carboxypeptidase, no catalysis of hydrolysis by the

(25) H. Neurath and G. W. Schwert, *Chem. Rev.*, **46**, 69 (1950).

(26) Unpublished experiments of Ogilvie, Riordan, and Vallee cited in ref. 8.

(27) Although the confidence which can be placed in the results obtained above pH 9 is clearly a great deal less than in results found at lower pH, the data presented in Fig. 5 have been extended as far into the alkaline range as our measurements permit. This has been done in order to provide as wide a comparison as possible with the pH–rate data described in the literature for other substrates.

(28) A. Ågren, V. Hedsten, and B. Jonsson, *Acta Chem. Scand.*, **15**, 1532 (1961).

enzyme was found. It is not clear at present whether the lack of a catalytic effect exercised by carboxypeptidase is due to the absence from O-acetylbenzilic acid of a proton on the carbon atom at the alkoxide link or else to a steric factor.

The stereochemistry of the substrate has received relatively little attention as yet in the study of the specificity of carboxypeptidase as an esterase. A preliminary examination of the hydrolysis of O-acetyl-D-mandelate in the presence of 10^{-5} M enzyme at pH 7.5 reveals no enzymatic catalysis. The stringency of the stereochemical requirements of carboxypeptidase are being investigated further at present, and the results of this work should be of importance to the development of mechanistic hypotheses for the action of the enzyme.[29]

In conclusion, although considerable information on the kinetic behavior of carboxypeptidase A as an esterase is now available, the mechanism of its action clearly remains to be elucidated. Work on many mechanistic aspects of carboxypeptidase action is underway in our laboratory and will be reported in due course.

(29) The variations in the structures of the substrates discussed so far have been in the alcoholic residue of the ester. Studies have also been started on other esters such as O-chloroacetylmandelic acid in which the acyl part of the ester is changed. A comparison of the rates of the carboxypeptidase-catalyzed hydrolysis of O-chloroacetyl-DL-mandelate and O-acetyl-DL-mandelate at reactant concentrations of 0.02 M shows that the chloroacetyl compound reacts about forty times faster than the acetyl ester at pH 7.5. Should this rate enhancement for the chloroacetyl compound still hold true when V_{max} values are compared, it will suggest that nucleophilic attack on the ester carbonyl group is involved in a kinetically important step of the ester hydrolyses. We are now in the process of unraveling the kinetic parameters for the hydrolysis of O-chloroacetylmandelate.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH AND THE DEPARTMENT OF BIOCHEMISTRY, THE UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

Studies on Polynucleotides. XXXI.[1] The Specific Synthesis of $C_{3'}$–$C_{5'}$-Linked Ribopolynucleotides (6).[2] A Further Study of the Synthesis of Uridine Polynucleotides[3]

BY C. COUTSOGEORGOPOULOS AND H. G. KHORANA

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Acetylation of 5′-O-monomethoxytrityluridine 3′-phosphate followed by an acidic treatment gave 2′-O-acetyluridine 3′-phosphate in practically quantitative yield. Treatment of a mixture of pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate and 2′-O-acetyluridine 3′-phosphate with dicyclohexylcarbodiimide in dry pyridine followed by a work-up involving successive acetic anhydride–pyridine and ammoniacal treatments gave homologous uridine polynucleotides. Members up to the hexanucleotide were isolated pure and characterized. Higher homologs up to the decanucleotide were also demonstrated to be present in the polymeric mixture. Several of the side products encountered were also characterized.

In an earlier paper the synthesis of uridine oligonucleotides containing exclusively the $C_{3'}$–$C_{5'}$ internucleotidic linkages was reported.[2c] At that time, however, 2′-O-acetyluridine 3′-phosphate, the key intermediate, was available in limited quantities, and the study of the polymerization reaction was necessarily of a preliminary nature with only the di- and trinucleotides being characterized definitively as the products. Further work on the synthesis of uridine polynucleotides is reported in this paper. Added interest in these polymers accrued from the fact that at the time the present work was undertaken, enzymatically synthesized high molecular weight polyuridylic acid was the only homopolymer known to stimulate the formation of polyphenylalanine in a cell-free amino acid incorporation system.[4] It was hoped that the availability of the short-chain uridine polynucleotides of known size and end groups would facilitate further study of the polypeptide-synthesizing system and perhaps indicate direction of further work on the synthesis of specific ribopolynucleotides of interest in the study of the amino acid code.[5]

The method used for the preparation of pyridinium 2′-O-acetyluridine 3′-phosphate (V) is illustrated in the structures I–V. 5′-O-Monomethoxytrityluridine 3′-phosphate (I) may be prepared directly from uridine 3′-phosphate in good yield.[2d] Treatment with acetic anhydride in the presence of an excess of tetraethylammonium acetate results in the quantitative acetylation of the 2′-hydroxyl group,[2d] the initial product in this reaction being the mixed anhydride II. The decomposition of anhydrides of this type is routinely effected by a short aqueous pyridine treatment of the acetylation mixture.[6] However, II was found to have rather high stability in aqueous pyridine, the hydrolysis being apparently inhibited by the presence of the bulky monomethoxytrityl group[7] and by the large excess of acetate ions.[8] Because of the restriction on the duration of the aqueous pyridine treatment of II imposed by the lability of the 2′-O-acetyl group,[9] the procedure

(1) Paper XXX: T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964).

(2) Earlier papers which deal directly with this topic: (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, **84**, 430 (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); (d) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (e) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963).

(3) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, the National Science Foundation, and the Life Insurance Medical Research Fund, Rosemont, Pa.

(4) (a) M. W. Nirenberg and J. H. Matthaei, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1588 (1961); (b) P. Lengyel, J. F. Speyer, and S. Ochoa, *ibid.*, **47**, 1936 (1961).

(5) F. H. C. Crick in "Progress in Nucleic Acid Research," Vol. I, J. N. Davidson and W. E. Cohn, Ed., Academic Press, Inc., New York, N. Y., 1963, p. 163.

(6) (a) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958); (b) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).

(7) Thus the mixed anhydride linkage in II is more stable than that in 2′,5′-di-O-acetyluridine 3′-phosphate.[2c]

(8) A similar case is the inhibition of the hydrolysis of acetic anhydride by pyridine in buffers containing acetate ions [A. R. Butler and V. Gold, *J. Chem. Soc.*, 4362 (1961)].

(9) The group owes its lability to the presence of the adjacent phosphomonoester group. The 2′-O-acetyl groups in nucleosides involved in 3′→5′ phosphodiester linkage are much more stable.