phosphorus pentachloride a still more impure product was obtained.

Dimesityl Ketimine Hydrochloride (VI).—To a stirred solution of mesitylmagnesium bromide (prepared on a 0.1-mole scale as described by Barnes[17]) was added 14.3 g. (0.10 mole) of mesitonitrile in 100 ml. of toluene. After distillation of the ether, the reaction mixture was refluxed for 12 hours, and then decomposed with iced hydrochloric acid. The precipitate was collected on a funnel and dried to give 22.8 g. (76%) of dimesityl ketimine hydrochloride (light tan) melting at 257–263°. A sample, recrystallized from a mixture of ethanol and ethyl acetate, melted at 264–267°.

Anal. Calcd. for $C_{19}H_{24}NCl$: N, 4.64; Cl, 11.75. Found: N, 4.73; Cl, 12.22.

The free dimesityl ketimine VIII was obtained by treatment of the hydrochloride VI with 10% sodium hydroxide; after recrystallization from 95% ethanol, it melted at 130°.

Anal. Calcd. for $C_{19}H_{23}N$: N, 5.28. Found: N, 5.27.

Treatment of 10.0 g. (0.033 mole) of dimesityl ketimine hydrochloride with 3.5 g. (0.05 mole) of hydroxylamine hydrochloride and 6.7 g. (0.083 mole) of sodium acetate in 140 ml. of 70% ethanol (refluxed for 9 days) failed to produce the oxime, 96% of dimesityl ketimine (m.p. 122–124°) being recovered on adding water.

Heating a solution of 3.0 g. of dimesityl ketimine hydrochloride, 1.5 g. of hydroxylamine hydrochloride and 2.0 g. of sodium acetate in 30 ml. of 70% ethanol in a sealed Carius tube at 170° for 24 hours failed to give the oxime, 55% of the ketimine (m.p. 123–124°) being recovered.

Dimesityl Ketimine N,N-Dimethyl Iodide (VII).—This compound was prepared in 92% yield from 2.8 g. (0.011 mole) of dimesityl ketimine, 10.0 g. (0.07 mole) of methyl iodide and 20 ml. of 5% sodium bicarbonate employing the procedure described by Fuson[15] for the preparation of duryl phenyl N-methyl ketimine methiodide. The product (VII) melted at 256–259° dec.

Anal. Calcd. for $C_{21}H_{28}NI$: N, 3.33. Found: N, 3.14.

(17) R. P. Barnes, *Org. Syntheses*, **21**, 77 (1941).

Treatment of quaternary salt VII with hydroxylamine hydrochloride and sodium acetate in 70% ethanol failed to give the ketoxime after 12 hours of refluxing or after 24 hours in a sealed tube at 185°, 92–94% of VII being recovered.

Refluxing VII with hydroxylamine and excess sodium hydroxide in ethanol for 10 hours failed to yield the oxime, dimesityl ketone (5%), m.p. 135–136° (reported 136–137°),[18] being the only product isolated.

Conversion of Dimesityl Ketimine (VIII) to Mesitoylmesidide (IX) by Peracetic Acid.—In a small flask fitted with a condenser was placed 5.0 g. (0.019 mole) of dimesityl ketimine (m.p. 128–129°), 25 ml. of glacial acetic acid and 3.0 ml. of 30% hydrogen peroxide.[19] The mixture was heated to 80° for 6 hours, 3.0 ml. more of hydrogen peroxide was then added, and the heating continued for 9 hours longer. After cooling, 15 ml. of water was added. The resulting white precipitate was collected on a funnel and washed with water and sodium bicarbonate solution, and again with water. After drying, the solid weighed 3.5 g. and melted at 190–195°. On recrystallization from 95% ethanol it gave 2.5 g. (50%) of mesitoylmesidide melting at 207°.

Anal. Calcd. for $C_{19}H_{23}NO$: C, 81.10; H, 8.24; N, 4.98. Found: C, 81.05; H, 8.32; N, 5.05.

This substance did not depress the melting point of a sample of mesitoylmesidide prepared by the general Scotten-Baumann procedure from mesidine and mesitoyl chloride.

In another experiment, employing somewhat impure ketimine hydrochloride (m.p. 122–124°), the yield of mesitoylmesidide (VII), m.p. 207°, was only 17%.

In a blank experiment, employing water instead of hydrogen peroxide, dimesityl ketimine failed to react, and 91% of it was recovered.

Treatment of dimesityl ketimine with hydrogen peroxide in methanolic sodium hydroxide or with potassium permanganate in alkaline acetone–water resulted in the recovery of most of the imine. Treatment with potassium periodate and sodium hydroxide in aqueous dioxane produced tars.

(18) E. P. Kohler and R. Baltzly, THIS JOURNAL, **54**, 4023 (1932).

(19) See E. Ochiai, *J. Org. Chem.*, **18**, 534 (1953).

DURHAM, N. C.

[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Hydrolysis of Carbon–Carbon Bonds by α-Chymotrypsin

BY DAVID G. DOHERTY[1,2]

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The enzymic hydrolysis of ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate by α-chymotrypsin to yield *p*-hydroxyphenylpropionic acid has been established. The effect of *p*H, substrate concentration and enzyme concentration have been investigated and K_s and k_3 for the system evaluated. The K_s and k_3 for the system 5-(*p*-hydroxyphenyl)-3-ketovaleric acid–α-chymotrypsin have also been determined. Procedures are given for the synthesis of these substrates.

Introduction

The investigation of the nature of enzyme–substrate or inhibitor complex formation under equilibrium conditions has yielded apparent thermodynamic functions for several substrate and competitive inhibitor systems.[3] Examinations of these functions reveal increasingly negative ΔS and ΔH values for systems catalytically bound at the active center in contrast to the small positive values obtained for those in which no catalytic activity may be demonstrated. The results were explained[3] by the assumption that (1) the active center of the enzyme consisted of an attractive center determining the specificity and orientation and responsible for the high intrinsic binding forces evinced by the large negative ΔH, and (2) a repulsive charge center, activating the labile bond and responsible for the entropy loss that was found experimentally. In the 5-(*p*-hydroxyphenyl)-3-acetaminobutanone-2–α-chymotrypsin system, the above properties are found and the compound is a competitive inhibitor since the carbonyl–methyl bond is relatively unpolarized and not within the activation energy range of the enzyme. However, if the carbonyl–methyl bond could be sufficiently polarized by the addition of electrophilic groups, its activation energy might be lowered to the extent that the enzyme could bring about the scission of the carbonyl to carbon linkage. Polarization of this bond could be effected by the addition of a carbethoxy group to form a β-keto

(1) This work was performed for the Atomic Energy Commission under Contract No. W-4705-eng-26.

(2) Presented in part at the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 12–16 (1954).

(3) D. G. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952); F. Vaslow and D. G. Doherty, *ibid.*, **75**, 928 (1953).

ester. Thus the model compound ethyl 5-(*p*-hydroxyphenyl)-4-acetamino-3-ketovalerate would have all of the necessary requisites for binding on the active center of chymotrypsin plus the labilized carbonyl–carbon bond. Attempts to synthesize this compound have so far been unsuccessful, owing to the multiplicity of functional groups. However, since *p*-hydroxyphenylpropionic acid was catalytically bound to chymotrypsin[3,4] and methyl phenylpropionate was shown by Snoke and Neurath[5] to be hydrolyzed by chymotrypsin, the more accessible β-keto analogs of these compounds should be suitable for testing this assumption.

Substrate Synthesis

The desired keto ester, ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate was prepared from either *p*-hydroxyphenylpropionic acid or *p*-hydroxycinnamic acid as starting materials. The phenolic group was protected by conversion to the benzyl ether which was then transformed to the acid chloride and coupled to ethyl acetoacetate according to the procedure of Viscontini and Merckling.[6] Hydrolysis of the α-acetyl group by sodium methoxide followed by hydrogenolysis of the benzyl group yielded the desired ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate. This ester could be hydrolyzed with alcoholic sodium hydroxide to the free β-keto acid. Ethyl 5-phenyl-3-ketovalerate, was also prepared by this method in better yields than previously reported.[7]

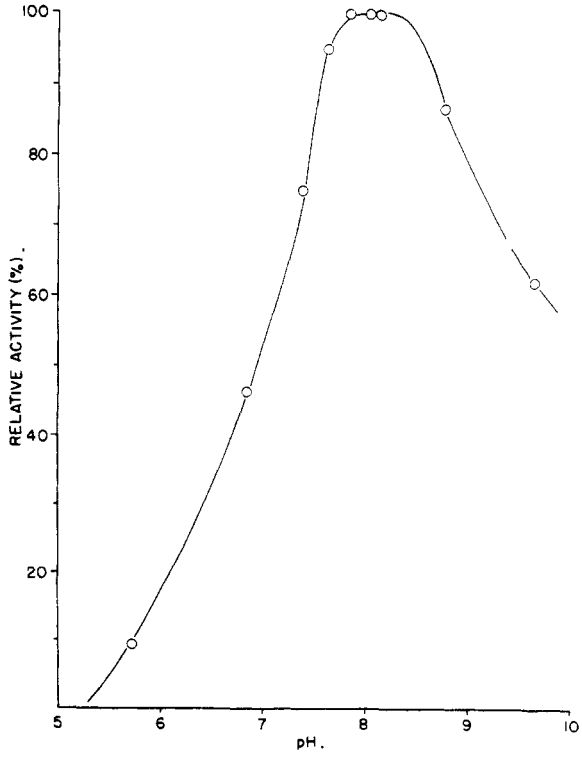

Fig. 1.—Relative activity *vs.* *p*H, 0.05 *M* ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate.

Enzyme Activity Results

Since the substrates, β-keto esters and acids, possess limited solubility in water it was necessary to carry out all enzymic determinations in 30% ethanol. The extent of hydrolysis was followed potentiometrically[8] at 25°, in solutions which were weakly buffered at *p*H 7.9–8.0, for kinetic determinations. The *p*H sensitivity of the major substrate, ethyl hydroxyphenylketovalerate, was determined at one *p*H unit intervals from *p*H 5 to *p*H 11 in an 0.05 *M* solution at 25° in the absence of enzyme. No measurable hydrolysis was observed at *p*H 5–9 in one hour, less than 2% hydrolysis at *p*H 8.0 in 20 hours, 2% in 1 hour at *p*H 10, and 2% in one hour at *p*H 11. Addition of α-chymotrypsin to a weakly buffered solution of the ethyl hydroxyphenylketovalerate brought about an immediate progressive decrease in *p*H which was counteracted by the continual addition of alkali from a microburet. Enzymic action ceased when 1 mole of alkali per mole of substrate had been consumed and *p*-hydroxyphenylpropionic acid could be isolated in good yield as one of the terminal hydrolysis products. Determination of the effect of *p*H on activity for the enzymic hydrolysis of ethyl hydroxyphenylketovalerate at the 0.05 *M* level and 25° gave a curve (Fig. 1), with a maximum from *p*H 7.8–8.2, approximately similar to the *p*H curves for the esterase and amidase activities of chymotrypsin.

The hydrolysis of ethyl hydroxyphenylketovalerate was found to be first order over the range of available substrate concentrations. The enzyme concentration was varied over an eightfold range and the first-order constants obtained were approximately proportional to the enzyme concentration, the greatest deviation being obtained with the most dilute enzyme concentration. The initial velocities over the range of enzyme and substrate concentrations used were estimated from the apparent first-order plot (Fig. 2A, B). These rates were used in $1/v_0$ *versus* $1/S_0$ plots given in Fig. 3 and the values of K_s and k_3 estimated for the system at *p*H 7.95 + 0.05. At enzyme concentrations from $^1/_2$ to 2 mg. of N/ml. the values were $70 \pm 3 \times 10^{-3}$ *M* and $2.5 \pm 0.3 \times 10^{-3}$ *M*/min./mg. of protein N/ml., respectively. If all the protein nitrogen present in the reaction mixture is assumed to be α-chymotrypsin and the molecular weight of the enzyme is 22,000 and its nitrogen content 16%, then k°_3 for this system has the value 0.15 sec.$^{-1}$. The most dilute enzyme concentrations used, $^1/_4$ and $^1/_8$ mg. of protein N/ml., yielded the same *k*, (*i.e.*, $2.5 \pm 0.3 \times 10^{-3}$ *M*/min./mg. of protein N/ml.) but a significantly different K_s of $40 \pm 3 \times 10^{-3}$ *M*. Since, at the three higher enzyme concentrations, ethyl hy-

(4) D. G. Doherty and F. Vaslow, unpublished data.

(5) J. E. Snoke and H. Neurath, *Arch. Biochem. Biophys.*, **21**, 351 (1949).

(6) M. Viscontini and N. Merckling, *Helv. Chim. Acta*, **35**, 2280 (1952).

(7) W. Borsche and M. Lewinsohn, *Ber.*, **66**, 1792 (1933); H. R. Henze and C. B. Holder, THIS JOURNAL, **66**, 1545 (1944); G. W. Anderson, I. F. Halverstadt, W. H. Miller and R. D. Robling, Jr., *ibid.*, **67**, 2197 (1945); S. P. Vila and R. C. Linares, *Anales fis. y quim. (Madrid)*, **41**, 807 (1945); J. P. Vila, *Ion*, **7**, 150 (1947); A. Ya Berlin and Yu V. Markova, *Zhur. Obshchei Khim.*, **18**, 1791 (1948).

(8) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

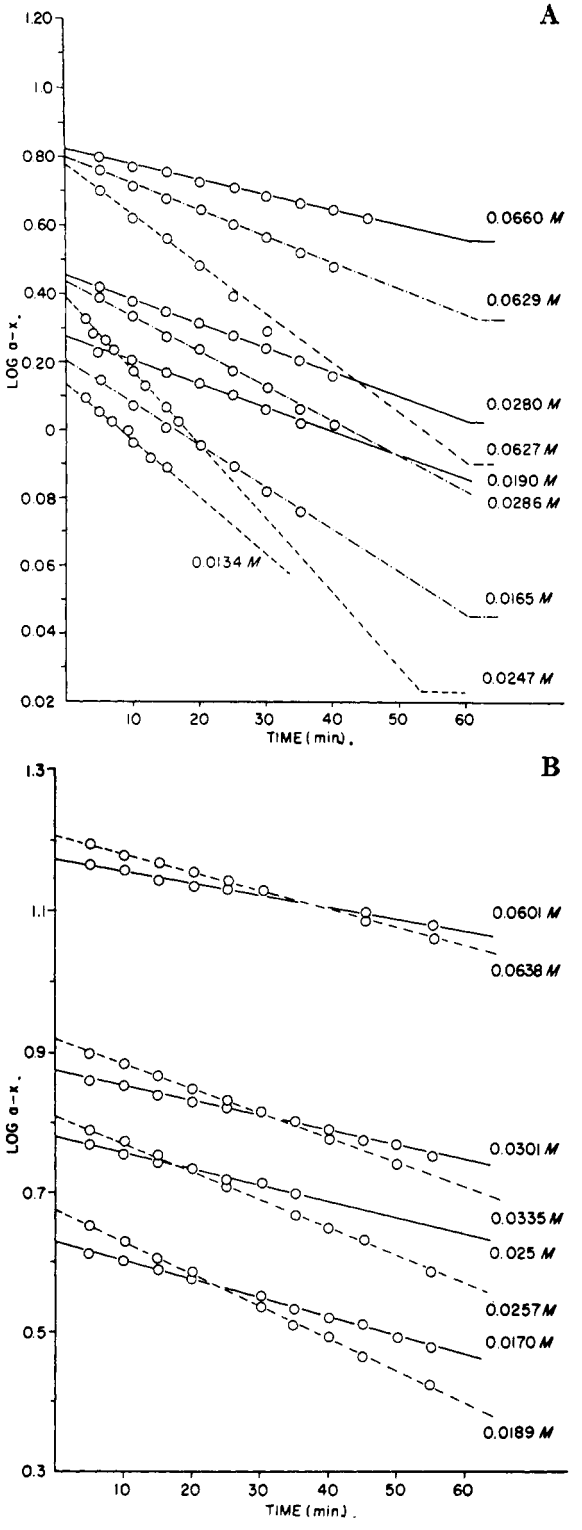

Fig. 2.—Estimation of initial velocities from apparent first-order plots; $[S]_0$ (initial concn.) ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate: (A) $a - x$ = ml. × 10 of 0.5 *N* NaOH per 5 ml. of digest; —, E = 0.5 mg. of N/ml.; —·—·—, 1 mg. of protein N/ml.; ----, 2 mg. of protein N/ml. (B) $a - x$ = ml. × 10 of 0.2 *N* NaOH per 5 ml. of digest; 0.02 *M* tris-(hydroxymethyl)-aminomethane–HCl buffer at 25° and *p*H 7.9: —, E = 1/8 mg. of protein N/ml.; — — — —, 1/4 mg. of protein N/ml.

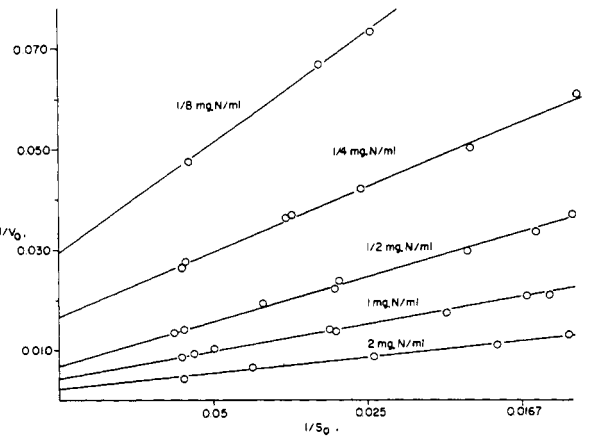

Fig. 3.—α-Chymotrypsin-catalyzed hydrolysis of ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate at 25° and *p*H 7.9; v_0 in units of 10^{-6} *M*/min.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–HCl.

droxyphenylketovalerate was rapidly hydrolyzed (15–30% in 10–15 minutes in contrast to 20% hydrolysis in 35 minutes for 1/4 mg. of protein N digests), competitive inhibition by *p*-hydroxyphenylpropionic acid at the higher enzyme concentrations could easily explain the difference in K_s. Such an effect would be similar to the known inhibition of N-acetyl-L-tyrosine amides, hydroxamides and esters by N-acetyltyrosine. If no such inhibition took place, a plot of $K_s \ln [S]_0/[S] + ([S]_0 - [S])$ *versus* time should give a straight line passing through the origin and having a slope

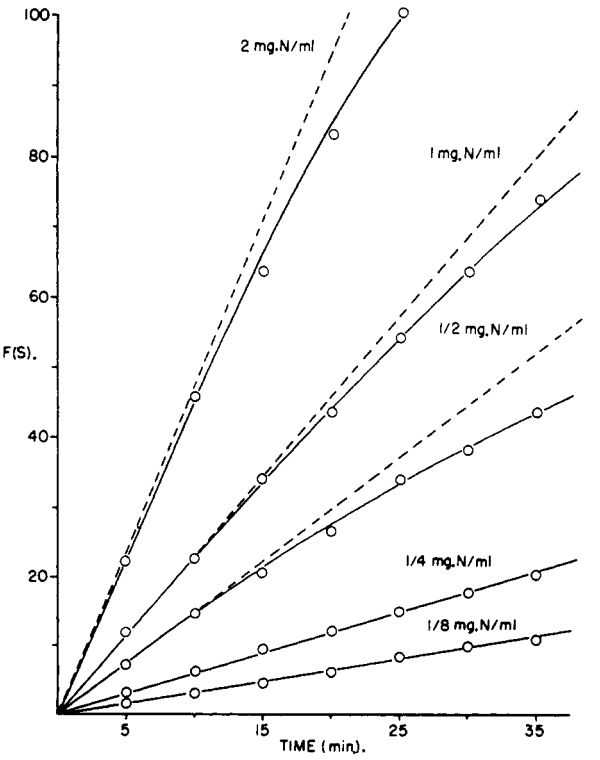

Fig. 4.—Plot of $F(S) = K_s \ln [S]_0/[S] + ([S]_0 - [S])$ in units of 10^3 *M versus* time for α-chymotrypsin and ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate at 25° and *p*H 7.9; $[S]_0 = 60 \times 10^{-3}$ *M*.

of k_3E. The data presented in Fig. 4 show clearly, by the negative deviation from the calculated slope at the three higher enzyme concentrations, that the reaction is competitively inhibited by the split product. In contrast, the experimental values at $^1/_4$ and $^1/_8$ mg. of protein N/ml. are in good agreement with the calculated slope.

Similar determinations at a single enzyme concentration, 1 mg. of protein N/ml., with the free acid, 5-(*p*-hydroxyphenyl)-3-ketovaleric acid, are presented in Figs. 5 and 6. The reaction is again monomolecular, and yields a K_s of $14 \pm 2 \times 10^{-2}$ M and a k_3 of 0.8×10^{-3} M/min./mg. of protein N/ml. Attempted kinetic determinations with ethyl 5-phenyl-3-ketovalerate were unsuccessful owing to the very limited solubility of the ester and the very slow enzymic hydrolysis. However, at the 1 mg. of protein N/ml. level and 0.013 M substrate concentration, approximately 5% hydrolysis was obtained in 1 hour and 30% hydrolysis in 8 hours.

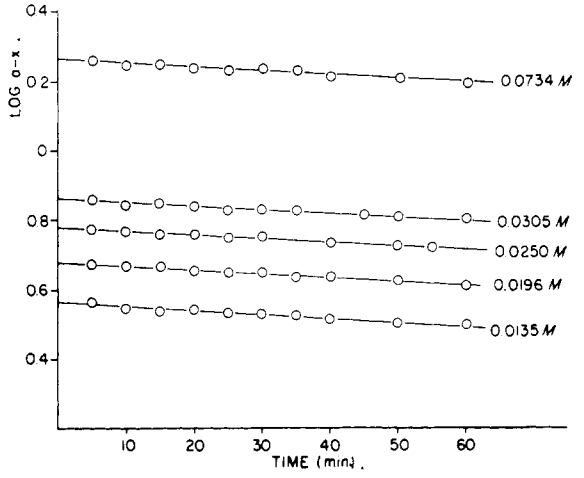

Fig. 5.—Estimation of initial velocities from apparent first-order plots; $[S]_0$ (initial concn.) 5-(*p*-hydroxyphenyl)-3-ketovaleric aicd; $a - x$ = ml. × 10 of 0.2 N NaOH per 5 ml. of digest; 0.02 M tris-(hydroxymethyl)-aminomethane-HCl buffer at 25° and *p*H 7.9; E = 1 mg. of protein N/ml.

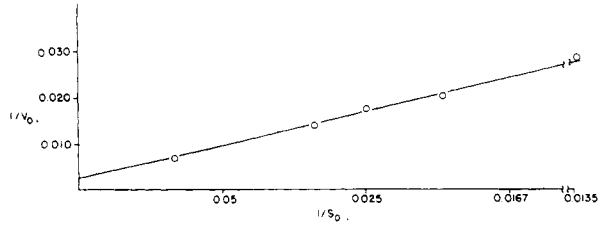

Fig. 6.—α-Chymotrypsin-catalyzed hydrolysis of 5-(*p*-hydroxyphenyl)-3-ketovaleric acid at 25° and *p*H 7.9; v_0 in units of 10^{-6} M/min.; 0.02 M tris-(hydroxymethyl)-aminomethane; E = 1 mg. of protein N/ml.

Discussion

The enzymic nature of the hydrolysis of ethyl hydroxyphenylketovalerate has been well established by the following observations: (1) no appreciable hydrolysis was noted over the *p*H range for enzyme activity in the absence of enzyme; (2) addition of enzyme resulted in an immediate release of titratable carboxyl groups; (3) the reaction rate was proportional to the enzyme concentration and its variation with *p*H resembled the *p*H activity curves for other chymotrypsin substrates. With regard to the site of hydrolysis, it would be unlikely, in view of the extensive studies on the structural requirements for chymotryptic activity, for the scission to occur at the terminal ester group rather than the carbonyl–carbon bond. Hydrolysis at this latter site is indicated by the consumption of 1 mole of alkali per mole of substrate hydrolyzed, the isolation under mild conditions of *p*-hydroxyphenylpropionic acid, and the hydrolysis of the free β-keto acid, hydroxyphenylvaleric acid. The kinetic constants obtained for the keto ester and the keto acid are also in an order that might be expected from the respective electronic configurations of the substrates. The keto group of the keto ester is relatively more polarized than the keto group of the keto acid and this is reflected in its greater affinity for the enzyme (K_s 0.04 *vs.* K_s 0.14) as well as the more rapid decomposition of the ES complex (k_3 2.5×10^{-3} *vs.* k_3 0.8×10^{-3}) owing to its greater susceptibility to anionic attack. The very slow hydrolysis of ethyl phenylketovalerate is also in accord with previous findings that hydrolysis rates are reduced when the phenyl group is substituted for the *p*-hydroxyphenyl group. The question as to whether the active center for carbon–carbon bond hydrolysis is the same as the active center for esterase and amidase activity cannot be unequivocally answered at this time. However, the whole pattern of activity as well as the apparent competitive inhibitions by *p*-hydroxyphenylpropionic acid makes it most likely that the active sites for all the various types of activity are the same. Further studies with competitive inhibitors for both systems are necessary to more firmly establish this point.

The demonstrated hydrolysis of carbon–carbon bonds by α-chymotrypsin extends the range of this versatile hydrolytic enzyme system to a new class of compounds, and is a logical addition to the series that began with the observation of carbon–oxygen bond fission, *i.e.*, esterase activity by Kaufman, Schwert and Neurath,[9] exchange reactions with water by Doherty and Vaslow[3] and by Sprinson and Rittenberg,[9] carbon-N-R bonds (hydrazides and hydroxamides) by MacAllister and Niemann,[10] and, finally, the hydrolysis of the carbon–sulfur bond by Goldenberg, Goldenberg and McLaren.[11] Thus a proteolytic enzyme may exhibit a type of activity formerly associated with oxidative or metabolic enzyme systems, although the biological significance, if any, of carbon–carbon bond hydrolysis remains to be discovered. Apparently, the major factor in the hydrolysis of a bond, provided the specific structural requirements for the enzyme have been fulfilled, is whether the activation energy for bond rupture can be suffi-

(9) S. Kaufman, G. W. Schwert and H. Neurath, *Arch. Biochem. Biophys.*, **17**, 203 (1948); D. B. Sprinson and D. Rittenberg, *Nature*, **167**, 484 (1951).

(10) R. V. MacAllister and C. Niemann, THIS JOURNAL, **71**, 3854 (1949); B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **183**, 403 (1950).

(11) V. Goldenberg, H. Goldenberg and A. D. McLaren, THIS JOURNAL, **72**, 5317 (1950).

ciently lowered by attachment on the active center of the enzyme to bring about an exchange of groups. The enzyme reaction observed here is very similar to the nucleophilic double displacement reactions observed in the hydrolysis of acetoacetic esters by the hydroxyl anion. The β-keto ester system is an ideal one to test this hypothesis and perhaps to provide data on the upper limit of bond strengths susceptible to enzyme action, since the strength of the carbonyl–carbon bond can be readily varied by either substituting other electrophyllic groups for the carboxylate group or by replacing the α-hydrogens with electrophilic or electrophobic groups. Synthetic procedures for the preparation of such substrates are at present under development in this Laboratory.

Acknowledgment.—The author wishes to thank Miss Eloise Eavenson for technical assistance in preparation of the substrates reported in this work.

Experimental

***p*-Benzyloxycinnamic Acid.**—*p*-Hydroxycinnamic acid was prepared from *p*-hydroxybenzaldehyde by a Perkin condensation with acetic anhydride.[12] The recrystallized acid, 32.8 g. (0.2 mole), was dissolved in 600 ml. of ethanol and 400 ml. of *N* NaOH and 34.2 g. (0.2 mole) of benzyl bromide in 50 ml. of ethanol dropped in with stirring over 2 hours. After standing overnight at room temperature, the mixture was poured into 1 l. of ice and water and acidified with concd. HCl to *p*H 2.0 with strong stirring. The crystalline precipitate was filtered, washed with water and dried *in vacuo;* yield 43 g. (85%); m.p. 209–211°. Recrystallization from hot toluene raised the m.p. to 210–213°.

Anal. Calcd. for $C_{16}H_{14}O_3$: C, 75.56; H, 5.55. Found: C, 75.50; H, 5.65.

***p*-Benzyloxyphenylpropionic Acid.**—*p*-Hydroxycinnamic acid was smoothly hydrogenated at low pressure with Pd–C catalyst to *p*-hydroxyphenylpropionic acid. The recrystallized acid, 33.2 g. (0.12 mole), was converted to the benzyl ether according to the above procedure; yield 45 g. (88%); m.p. 118–120°. Recrystallization from benzene plus ligroin raised the m.p. to 123–124°.

Anal. Calcd. for $C_{16}H_{16}O_3$: C, 74.98; H, 6.29. Found: C, 74.84; H, 6.40.

***p*-Benzyloxycinnamoyl Chloride.**—*p*-Benzyloxycinnamic acid, 25.4 g. (0.1 mole), was suspended in 1 l. of toluene and 22.9 g. (0.11 mole) of PCl_5 added in portions with stirring over a period of 1 hour. The resulting clear solution was allowed to stand for 4 hours at room temperature, then was evaporated *in vacuo* at 50°. Ligroin was added and the crystalline product was filtered, washed with ligroin, and dried *in vacuo;* yield 25 g. (92%); m.p. 134°. Recrystallization from a toluene–ligroin mixture raised the m.p. to 136–138°.

Anal. Calcd. for $C_{16}H_{13}O_2Cl$: C, 70.46; H, 4.80; Cl, 13.00. Found: C, 70.32; H, 4.95; Cl, 12.90.

***p*-Benzyloxyphenylpropionyl Chloride.**—*p*-Benzyloxyphenylpropionic acid, 25.6 g. (0.1 mole), was dissolved in 1 l. of benzene and 22.9 g. (0.11 mole) of PCl_5 added in portions with stirring over a period of 1 hour. After 3 hours, the clear solution was evaporated *in vacuo* at 50° to a sirup, ligroin added, and crystallization induced by scratching. The crystalline product was filtered, washed with ligroin, and dried *in vacuo;* yield 26 g. (95%); m.p. 75–78°. The acid chloride was recrystallized from a toluene–ligroin mixture for analysis.

Anal. Calcd. for $C_{16}H_{15}O_2Cl$: C, 69.94; H, 5.50; Cl, 12.91. Found: C, 69.80; H, 5.60; Cl, 12.79.

Ethyl 5-(*p*-Benzyloxyphenyl)-3-keto-2-acetylvalerate.—This compound was prepared by a procedure analogous to that of Viscontini and Merckling.[6] Magnesium turnings, 2.65 g. (0.11 mole), were placed in a 3-necked 500-ml. round-bottom flask fitted with a condenser, stirrer and dropping funnel and 15 ml. of absolute ethanol was added with the exclusion of moisture. Carbon tetrachloride, 0.5 ml., was added to initiate the reaction followed by 100 ml. of dry ether in portions after the initial reaction had moderated. The mixture was stirred for 4 hours at room temperature until the reaction had ceased, cooled in an ice-bath to 0° and 13 g. of acetoacetic ester in 50 ml. of dry ether dropped in slowly with strong stirring. After an additional hour of stirring, the mixture was cooled in an ice–salt bath to −5° and 27.4 g. (0.10 mole) of recrystallized *p*-benzyloxyphenylpropionyl chloride dissolved in 100 ml. of dry ether dropped in slowly with continued stirring. When all the acid chloride had been added, the mixture was allowed to warm up to room temperature, stirred for an additional hour, and allowed to stand overnight. The ether solution was washed with ice-cold dilute sulfuric acid, with water until neutral, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and 31 g. of a light yellow oil obtained. Attempted distillation of a portion at 0.01 mm. and a bath temperature of 230–260° yielded a fraction distilling at 162–165° which crystallized at 0°. An ether solution of the oil shaken with an aqueous saturated copper acetate solution yielded an intense blue ether layer which deposited light blue needles on standing a short time. The insoluble copper complex had a m.p. of 148–151°, which was raised to 152–153.5° by recrystallization from benzene.

Anal. Calcd. for $(C_{22}H_{24}O_5)_2Cu$: C, 66.03; H, 6.04; Cu, 7.93. Found: C, 66.10; H, 6.15; Cu, 7.84.

Ethyl 5-(*p*-Benzyloxyphenyl)-3-ketovalerate.—The oily acetyl keto ester, 11 g. (0.03 mole), was dissolved in 50 ml. of absolute methanol, cooled in ice, and 1.7 g. of sodium methylate in 25 ml. of absolute methanol added. After standing overnight at room temperature the mixture was poured into 150 ml. of ice-water containing 30 ml. of *N* HCl. Crystallization was induced by scratching, yielding 8.1 g. with a m.p. of 48°. Recrystallization from ethanol raised the m.p. to 54°.

Anal. Calcd. for $C_{20}H_{22}O_4$: C, 73.62; H, 6.81. Found: C, 73.47; H, 6.72.

Ethyl 5-(*p*-Hydroxyphenyl)-3-ketovalerate.—Ten grams of the above benzyl ether was dissolved in 50 ml. of absolute ethanol and hydrogenated at low pressure with 3 g. of 5% Pd–C catalyst. One mole of hydrogen was absorbed in 8 hours. After removal of the catalyst, the solution was concentrated *in vacuo* yielding a light yellow oil which was fractionally distilled at 0.01 mm. The pure keto ester distilled at b.p. 97–98° with a bath temperature of 150–160°. The pure ester crystallized at 5° and melts at 20–21°. This compound was also prepared in similar yield by the simultaneous hydrogenation and hydrogenolysis of ethyl 5-(*p*-benzyloxyphenyl)-4-ene-3-ketovalerate with 5% Pd–C catalyst.

Anal. Calcd. for $C_{13}H_{16}O_4$: C, 66.50; H, 7.01. Found: C, 66.34; H, 6.82.

5-(*p*-Hydroxyphenyl)-3-ketovaleric Acid.—The above keto ester, 11.8 g. (0.05 mole), was dissolved in 200 ml. of methanol, and 100 ml. of *N* NaOH added. After 30 minutes, 100 ml. of *N* HCl was added, the mixture evaporated *in vacuo* to a small volume and extracted with benzene. The benzene solution was extracted with bicarbonate, the aqueous layer acidified and re-extracted with benzene. After two such transfers the benzene solution was dried over Na_2SO_4 and evaporated *in vacuo* to a sirup. Attempted distillation at 0.01 mm. resulted in extensive decomposition.

Ethyl 5-(*p*-Benzyloxyphenyl)-4-ene-3-keto-2-acetylvalerate.—*p*-Benzyloxycinnamoyl chloride, 27.2 g. (0.1 mole), was slurried in 100 ml. of absolute ether and added in portions over 1 hour to the ethylmagnesium acetoacetate at −5° according to the previously described procedure. The bright yellow mixture was stirred overnight at room temperature and worked up in the usual manner. Evaporation of the ether solution *in vacuo* yielded 32.5 g. (89%) of a crystalline product; m.p. 90–92°. Recrystallization from absolute methanol raised the melting point to 91–93°.

Anal. Calcd. for $C_{22}H_{22}O_5$: C, 72.11; H, 6.05. Found: C, 71.97; H, 6.12.

Ethyl 5-(*p*-Benzyloxyphenyl)-4-ene-3-ketovalerate.—The above coupling product, 11 g. (0.03 mole), was suspended in methanol and deacetylated as previously described; yield 8.6 g. of amorphous powder. Since efforts at crystallization were unsuccessful, the amorphous material

(12) F. Konek and E. Pacsu, *Ber.*, **51**, 856 (1918).

was directly hydrogenated with Pd-C catalyst to ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate.

Ethyl 5-Phenyl-3-ketovalerate.[7]—Phenylpropionyl chloride was coupled to ethyl acetoacetate as previously described to give an 80% yield of ethyl 5-phenyl-3-keto-2-acetylvalerate, b.p. 88–94° (0.006 mm.).

Deacetylation with sodium methylate gave a 70% yield of the β-keto ester, b.p. 105–110° (1 mm.).

Enzyme Methods.—The enzyme utilized in this study was thrice-recrystallized Worthington α-chymotrypsin. The reaction was carried out in 10-ml. thermostated vessels, stirred magnetically, and the extent of hydrolysis determined by continuous potentiometric titration with 0.2 to 0.5 *N* NaOH essentially according to the procedure of Schwert, *et al.*[8] Insolubility of the substrates made it necessary to run the hydrolyses in 30% ethanol. Control determinations without enzyme at *p*H 7.8 showed less than 2% hydrolysis of the β-keto esters and β-keto acids in 20 hours. *p*-Hydroxyphenylpropionic acid was isolated from an 0.05 *M* terminal enzymic hydrolyzate of the β-keto ester by chilling in ice and acidification to *p*H 2.0. The crystalline precipitate, after filtration and recrystallization, was identified by melting point and mixed melting point with an authentic sample of *p*-hydroxyphenylpropionic acid.

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On Cyclic Intermediates in Substitution Reaction. VI. The Alkaline Solvolysis of N-β-Bromoethylaniline

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The alkaline solvolysis of N-β-bromoethylaniline has been studied in 70% ethanol and it has been established that the reaction product is N-phenylethylenimine. The rate of solvolysis as measured by release of bromide ion satisfies the equation $d(Br^-)/dt = k'(\text{bromoamine}) + k''(OH^-)(\text{bromoamine})$, where k' represents a first-order constant for an internal nucleophilic displacement of the bromine by the anilino group, and k'' represents a second-order constant for the reaction of the base with N-β-bromoethylaniline to form N-phenylethylenimine.

In preceding papers of this series[1] examples of the participation of neighboring groups in displacement reactions as the distance between the seat of substitution and the nucleophilic groups was increased were discussed. It was observed that these nucleophilic groups through the formation of cyclic intermediates exert profound effects on the rate of the displacement reaction and other things being equal, the ease of participation of the neighboring nucleophilic group increases with the strainlessness of the ring formed. The work so far described has been concerned with the carboxylate ion, hydroxyl group and the alkoxide ion.

The present paper reports the results of a kinetic study of the alkaline solvolysis of N-β-bromoethylaniline. The product formed as described in the experimental section is N-phenylethylenimine. In contrast to earlier studies on the alkaline hydrolysis of β-halogenated primary amines[2] the rate is shown to be influenced by the concentration of sodium hydroxide.

Method of Rate Measurement.—The measurements were carried out in a water-bath in which the temperature was thermostatically controlled to ±0.02°. Three to five mmoles of N-β-bromoethylaniline hydrobromide[3] was introduced into a 100-ml. volumetric flask previously immersed in a water-bath and which contained quantities of sodium hydroxide sufficient to make the concentration lie within the range 0.03–0.30 *M* and enough absolute ethanol to always give a 70% ethanol (by volume) solution. Ethanol (70%) preheated to the bath temperature was added to the mark and at convenient time intervals, 10-ml. aliquots were removed with a pipet and immediately delivered into a 125-ml. separatory funnel containing 50 ml. of chloroform and 15 ml. of distilled water. The mixture was shaken thoroughly and the chloroform removed. The water layer was then extracted with an additional 50 ml. of chloroform. Ten ml. of 6 *M* HNO_3 was then added to the water layer and the bromide ion was determined by the Volhard method. Extraction with chloroform was necessary because of the occurrence of undesirable color development during the Volhard titration which completely masked the end-point. Several experiments were carried out at higher ionic strength and added bromide ion to determine the possibility of a salt effect and a common ion effect, respectively.

In calculating the rate constants from the results of the titration, account must be taken of the fact that one-half of the total bromide ion determined in the infinity aliquot was due to the hydrobromide salt of the amine. The concentrations of sodium hydroxide recorded in the tables are after neutralization of the hydrobromide salt.

The rate of release of bromide ion followed a first-order rate law but, as shown in the example of Table I, and as summarized in Table II and Fig. 1, the rate of release of bromide ion is accelerated by increasing the concentration of the base.

TABLE I

RATE OF SOLVOLYSIS OF N-β-BROMOETHYLANILINE IN 70% ETHANOL AT 30.00°

Time (min.)	Vol. of 0.0505 *N* $AgNO_3$ (ml.)		10^2k (min.$^{-1}$)
0.03 *M* N-β-Bromoethylaniline and 0.06 *M* NaOH			
10.20	0.57		0.99
20.36	1.16		1.07
30.23	1.61		1.05
40.23	2.07		1.07
50.28	2.47		1.07
60.53	2.81		1.06
70.28	3.10		1.05
∞	5.93	(Mean)	1.06

N-Phenylethylenimine.—In the present study it is necessary to show that N-phenylethylenimine is the only product formed in the alkaline solvolysis of N-β-bromoethylaniline. Two and one-half liters of a solution 0.05 *M* with N-β-bromoethylaniline and 0.25 *M* with NaOH in 70% ethanol was allowed to react at 30° until all the bromide was displaced. With this concentration of hydroxide ion and at this temperature the half-life of the second-order process would be approximately 140 minutes. The half-life of the first-order process at the same temperature is 74 minutes. Thus, if the second-order process was leading to another

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(2) G. Salomon, *Helv. Chim. Acta*, **19**, 743 (1936).

(3) W. J. Pearlman, THIS JOURNAL, **70**, 871 (1948).