[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

The Acidic, Basic and α-Chymotrypsin-catalyzed Hydrolyses of Some Esters. A Kinetic Comparison[1]

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The kinetics of hydrolysis of a series of ethyl α-substituted-β-phenylpropionates have been determined. Rate constants for alkaline hydrolysis in 85% ethanol, for acid hydrolysis in 70% dioxane, and for α-chymotrypsin-catalyzed hydrolysis in 30% methanol have been determined. A comparison of the relative k_3 values in enzymatic hydrolysis with the relative rate constants for acidic and basic hydrolysis indicates that at least as far as the breakdown of the intermediate enzyme-substrate complex is concerned there is no correlation possible between enzymatic and non-enzymatic hydrolysis. The extraordinarily large k_3 value for benzoyl-L-phenylalanine ethyl ester indicates a possible specific interaction of the substrate at a site somewhat removed from the reactive center. A comparison of the first-order velocity constants of the α-chymotrypsin-catalyzed hydrolysis of benzoyl-L-phenylalanine ethyl ester and its oxygen homomorph suggests the importance of hydrogen bonding of the substrate during enzymatic hydrolysis, probably during the breakdown of the intermediate enzyme-substrate complex. A mechanism is proposed consistent with existing data, for the enzymatic hydrolysis with α-chymotrypsin.

Introduction

The study of the effect of structure on the rates of various reactions has proven effective in helping to elucidate the mechanism of various organic reactions. This has been particularly true in the hydrolyses of esters, in which the effect of substituents often has been striking.[2] In enzymatic reactions, many studies have been made of the specificity of the enzyme for various substrates in an effort to determine the scope of the enzymatic reaction and to provide information concerning the mode of action of the enzyme. Of the many hydrolytic enzymes, α-chymotrypsin has been studied very extensively. This proteolytic enzyme has been shown to hydrolyze many different carboxylic acid derivatives such as esters, amides, hydrazides and hydroxamides.[3]

This research is concerned with a comparison of the rates of hydrolysis of a series of esters under three different kinds of catalysis: acidic, basic and enzymatic in an attempt to carry over the mechanistic ideas of non-enzymatic hydrolysis to enzymatic hydrolysis.

α-Chymotrypsin was chosen as the enzyme because of its structure as a crystalline protein[4] with molecular weight of 22,500[5] or 21,500[6] because it has one catalytic site per molecule[6]; because it is capable of reproducible kinetic results over extended periods of time,[7] and because much is known concerning the specificity and inhibition of its catalytic action.[3] α-Chymotrypsin has exhibited specificity for an extensive series of substrates containing the β-phenylpropionate structure and kinetic investigations have been effected with a number of these esters. The rates of hydrolysis of a group of ethyl α-substituted-β-phenylpropionates are reported here.

(1) This investigation was supported by research grant G-3787 of the National Institutes of Health, Public Health Service.

(2) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 758.

(3) (a) B. M. Iselin, H. T. Huang and C. Niemann, *J. Biol. Chem.*, **183**, 403 (1950); (b) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(4) M. Kunitz, *J. Gen. Physiol.*, **32**, 265 (1948).

(5) E. L. Smith, D. M. Brown and M. L. Laskowski, *J. Biol. Chem.*, **191**, 639 (1951).

(6) G. W. Schwert and S. Kaufman, *ibid.*, **190**, 807 (1951).

(7) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4713 (1952).

Experimental

DL-Phenylalanine Ethyl Ester.—This ester was prepared by allowing a solution of dry hydrogen chloride and phenylalanine in absolute ethanol to stand at room temperature for 4 days. Recrystallization from ethanol–ether gave the ester hydrochloride, m.p. 125–127° (lit. 127°).[8]

N-Benzoyl-DL-phenylalanine Ethyl Ester.—N-Benzoyl-DL-phenylalanine was prepared by the method of Carter.[9] The acid was esterified by refluxing an absolute ethanol solution with hydrogen chloride. Recrystallization from ethanol–water gave the ester, m.p. 94–95° (lit. 95–95.5°).[10]

Ethyl β-Phenylpropionate.—This ester was prepared by refluxing a solution of β-phenylpropionic acid in absolute ethanol with gaseous hydrogen chloride. The product was distilled through a 25-cm. column packed with glass helices, b.p. 90–94° (2–3.5 mm.), $n^{20}D$ 1.4947 (lit. $n^{20}D$ 1.4954).[11]

Ethyl DL-α-Chloro-β-phenylpropionate.—α-Chloro-β-phenylpropionic acid was prepared in 49% yield from phenylalanine by the method of Snoke and Neurath,[12] b.p. 170–174° (2.5–3.5 mm.). The racemic mixture is partially solidified at room temperature although the L-isomer melts at 44°.[10] Esterification was carried out by refluxing the acid (4.5 g., 0.0244 mole) with 100 ml. of absolute ethanol and 5.0 ml. of concd. sulfuric acid for 6 hours. The product was distilled through a 25-cm. column packed with glass helices, b.p. 100–101° (1 mm.), $n^{25}D$ 1.5062.

Anal. Calcd. for $C_{11}H_{13}O_2Cl$: Cl, 16.67; sapon. equiv., 212.7. Found: Cl, 15.95; sapon equiv., 213.4 ± 1.0.

Ethyl DL-α-Hydroxy-β-phenylpropionate.—DL-α-Chloro-β-phenylpropionic acid (4.5 g., 0.0246 mole) was refluxed with 5.5 g. of calcium carbonate in 350 ml. of water for 1 hour.[13] Recrystallization from benzene gave 2.2 g. (53.5%) of colorless crystals, m.p. 94–96.5° (lit. m.p. 97–98°). DL-α-Hydroxy-β-phenylpropionic acid (2.2 g.) was refluxed with 50 ml. of absolute ethanol and 2.5 ml. of concd. sulfuric acid for 6 hours. Distillation gave 1.7 g. of ester, b.p. 94–96° (0.5 mm.), $n^{25.5}D$ 1.5052 (lit. b.p. 152–154° (20 mm.)).[14]

Ethyl DL-α-Benzoxy-β-phenylpropionate.—Benzoyl chloride (2.0 g., 0.0145 mole) was added to a solution of ethyl α-hydroxy-β-phenylpropionate in 15 ml. of pyridine at 0°. The reaction mixture was maintained at 0° for 1 hour and at room temperature for 16 hours. After separation the product was distilled, 2.6 g., b.p. 165° (0.5 mm.), $n^{22}D$ 1.5377 (lit. b.p. 225–226° (15 mm.)).[15]

Anal. Calcd. for $C_{18}H_{18}O_4$: C, 72.50; H, 6.08. Found: C, 72.45; H, 6.11.

(8) T. Curtius and E. Müller, *Ber.*, **37**, 1266 (1904).

(9) H. E. Carter and C. M. Stevens, *J. Biol. Chem.*, **138**, 627 (1941).

(10) J. Max, *Ann.*, **369**, 281 (1909).

(11) Brühl, *ibid.*, **200**, 192 (1880).

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

(13) The method follows that of E. Fischer, *Ber.*, **42**, 489 (1909), for the hydrolysis of the bromo acid.

(14) A. McKenzie and F. Barrow, *J. Chem. Soc.*, **99**, 1922 (1911).

(15) H. Gault and R. Weick, *Bull. soc. chim., France*, [4] **31**, 1001 (1922).

Benzoyl-L-phenylalanine Ethyl Ester.—The preparation of this compound was carried out by the method previously described for the DL compound, m.p. 100–102° (lit. 101.5–102°),[16] $[\alpha]^{25}D$ −40.0° (methanol, *c* 4.3).

Ethyl L-α-Benzoxy-β-phenylpropionate.—The preparation of the necessary precursor, L-α-hydroxy-β-phenylpropionic acid was carried out by the method of Dakin and Dudley[17] in five 2-g. portions. Esterification of the carboxyl and hydroxyl groups by the methods described above gave 0.4 g. of product, b.p. 174–176° (1 mm.), $n^{22}D$ 1.5362, $[\alpha]^{28}D$ −10.5 (benzene, *c* 0.9).

Other Reagents.—α-Chymotrypsin was an Armour and Co. salt-free preparation, Lot. No. 00592. Redistilled dioxane was purified according to the procedure of Fieser,[18] b.p. 99–100°, $n^{25}D$ 1.4199. Absolute ethanol was a Commercial Solvents product. Sodium hydroxide used as titrant was standardized against potassium acid phthalate using phenolphthalein as indicator. Hydrochloric acid used as titrant was standardized against standard hydroxide solution.

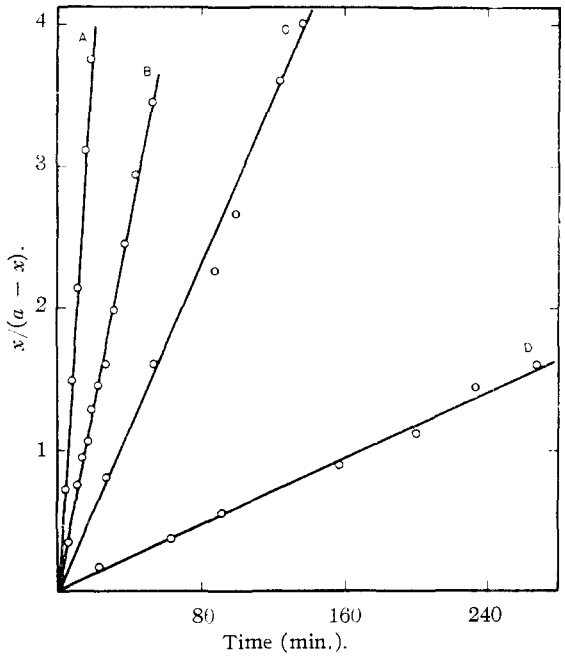

Fig. 1.—Basic hydrolysis of ethyl α-substituted-β-phenylpropionates: A, chloro; B, hydroxy; C, benzoylamino; D, hydrogen.

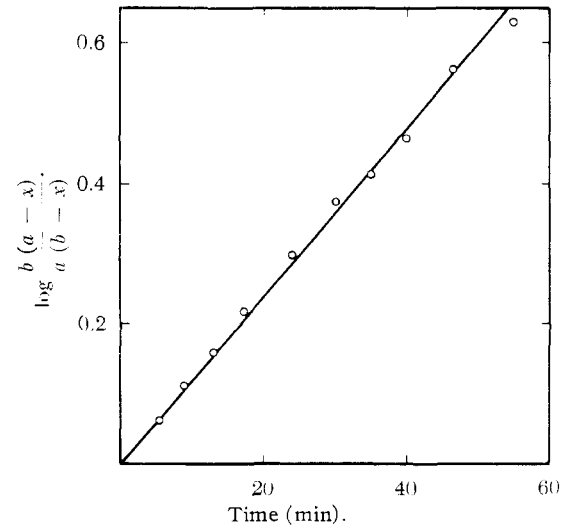

Fig. 2.—Basic hydrolysis of phenylalanine ethyl ester.

Kinetic Methods. Alkaline Hydrolysis.—Equal quantities of approximately 0.1 *N* solutions of ester and sodium hydroxide in 85% ethanol (by weight) which had attained thermal equilibrium in the thermostat (25.04 ± 0.01°) were mixed. Five-ml. samples were removed after appropriate amounts of time and pipetted into a known excess of dilute hydrochloric acid. Titration of this mixture was effected to a phenolphthalein end-point using standard sodium hydroxide solution. For the chloro and hydroxy derivatives, which underwent rapid reaction, a two-cell reaction flask was used for efficient mixing.

There is some question concerning the validity of the determination of the concentration of hydrogen ion in the presence of an amino acid and its corresponding ester,[19] in this case, phenylalanine and phenylalanine ethyl ester. In a blank experiment, it was found that hydrochloric acid could be titrated in the presence of phenylalanine at *p*H 5.4 to the extent of 95–98%, the equivalence point being *p*H 6.5. When phenylalanine ethyl ester was added to such a solution, the titration curve did not exhibit as sharp an end-point but at *p*H 5.4 the quantity of hydrochloric acid titrated was 97–99%. It was thus feasible to determine the kinetics of the alkaline hydrolysis of phenylalanine ethyl ester by titration of samples to *p*H 5.4 using a Beckman Model H *p*H Meter. In order to suppress the equilibrium between phenylalanine and its anion, the initial concentration of sodium hydroxide was 2–3 times that of phenylalanine ethyl ester. See Figs. 1 and 2 for representative experiments.

Acidic Hydrolysis.—The reaction mixtures were made up by taking the appropriate quantity of standard ester solution in 70% (by weight) dioxane, 15.0 ml. of 0.6400 *N* hydrochloric acid (aqueous), 35 g. of dioxane and diluting to 100 ml. with 70% dioxane. Five-ml. samples were removed and titrated with standard sodium hydroxide to the phenolphthalein end-point. In the case of phenylalanine ethyl ester, titration was carried out with 5.0-ml. samples of the reaction mixture to a *p*H of 5.4 using a Beckman Model H *p*H Meter and 0.1 *N* sodium hydroxide as described under alkaline hydrolysis. See Fig. 3 for representative experiments.

Enzymatic Hydrolysis.—Kinetic runs were carried out in a thermostated water-bath at 25.04 ± 0.03°. A Beckman Model G *p*H Meter equipped with external electrodes was employed. All experiments were performed in a solution of 30% methanol in water (by volume). Nitrogen was bubbled through the system for *p*H stability. The low substrate concentrations employed (of the order of 2.5×10^{-4} mole/l. were due to the limited solubility of ethyl L-α-benzoxy-β-phenylpropionate in 30% methanol solution. Enzyme concentrations were determined by measurement of the optical density at 282 mμ in a Beckman DU spectrophotometer and comparison with a curve which was standardized by means of a nitrogen analysis using a semimicro Kjeldahl method. The enzyme solutions were made up in water and stored at 5° until used (never longer than three hours).

The kinetic experiments for benzoyl L-phenylalanine ethyl ester were carried out as follows, using essentially the procedure of Schwert, *et al.*[20] Ten ml. of methanol, 31.0 ml. of water, 5.0 ml. of ester (3×10^{-3} *M*) in methanol and 3.0 ml. of tris-(hydroxymethyl)-aminomethane buffer solution (4.4×10^{-3} *M* in NH_2, 8.8×10^{-3} in NH_3^+) in water were mixed and allowed to attain thermal equilibrium. The *p*H of the solution was then adjusted to some point (7.85–8.0) above that to be operated as the null point (7.75), by the addition of sodium hydroxide solution. One ml. of enzyme solution (0.03 mg.N/ml.) was then added. When the *p*H of the solution fell to 7.75 the timer was started, and an increment of 0.028 *N* sodium hydroxide was added to give a *p*H rise of 0.05–0.10 *p*H unit. When the null point was again reached, the time was recorded.

For ethyl L-α-benzoxy-β-phenylpropionate it was neces-

(16) S. Kaufmann and H. Neurath, *Arch. Biochem.*, **21**, 437 (1949).
(17) H. D. Dakin and H. W. Dudley, *J. Biol. Chem.*, **18**, 46 (1914).
(18) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath and Co., Boston, Mass., 1941, p. 368.
(19) B. Harrow, "Laboratory Manual of Biochemistry," W. B. Saunders Co., Philadelphia, Pa., 1944, p. 24.
(20) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

TABLE I

RATE CONSTANTS FOR THE HYDROLYSIS OF SOME ETHYL β-PHENYLPROPIONATES AT 25.04 + 0.01°

Ethyl α-substituted β-phenylpropionate	Alkaline hydrolysis in 85% ethanol: Initial concn. (mole/l.) (ester = OH^-)	$k_2 \times 10^2$ (l./mole sec.)	Acid hydrolysis in 70% dioxane: Initial concn. (mole/l.) (ester)	(acid)	$k \times 10^7$ (sec.$^{-1}$)
Hydrogen	0.01003	0.503 ± 0.019[a]	0.0602	0.0968	13.1 ± 0.3
	.01504	0.500 ± .007	.0667	.0970	13.9 ± .2
	.01900	0.506 ± .007			
Benzoylamino	.0952	5.03 ± .22	.0529	.0960	3.13 ± .08
	.0952	4.88 ± .23	.0570	.0960	3.15 ± .04
Hydroxy	.00937	11.1 ± .2	.0410	.0965	20.1 ± .4
	.00937	11.4 ± .3	.0414	.0976	20.0 ± .8
Chloro	.01128	29.9 ± 1.2	.0755	.0994	3.11 ± .09
	.01128	29.5 ± 0.9	.0788	.0992	3.09 ± .19
	.01128	28.9 ± .6			
	.00990	29.3 ± .8			
	(ester) (b) ≠ (OH^-) (a)				
Amino	0.01164 0.02720	2.97 ± .08	.0765	.1285	0.759 ± .017
	.01267 .02450	2.82 ± .13	.0705	.1297	.701 ± .024

[a] H. S. Levenson and H. A. Smith, THIS JOURNAL, 62, 2324 (1940), report 0.504 × 10^{-2} liter/mole sec.

sary to add the ester last since it was found that the addition of the concentrated solution of enzyme (0.6 mg. N/ml.) caused a change in the *p*H of the solution from 7.8 to 7.4. The enzyme concentration was generally fifty times as large for the oxygen homomorph as for the nitrogen compound.

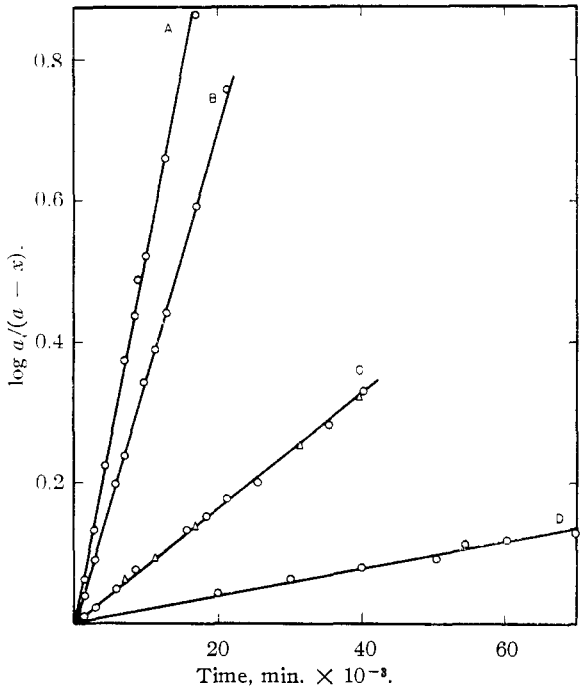

Fig. 3.—Acidic hydrolysis of ethyl α-substituted-β-phenylpropionates: A, hydroxy; B, hydrogen; C, 0 = benzoylamino, Δ = chloro; D, amino.

At such low substrate concentration it is evident from an examination of the Michaelis–Menten equation that first-order kinetics would be expected. When the kinetics of a hydrolytic reaction are followed by measuring the amount of alkali necessary to maintain a constant hydrogen ion concentration as a function of time, there is an inherent uncertainty in the initial substrate concentration. One must then resort to calculation of the initial concentration by graphical means.[20] Figure 4 shows that plots of log $a/(a - x)$ *versus* time give straight lines up to 70% hydrolysis.

Product Analysis.—The product of the alkaline hydrolysis of N-benzoylphenylalanine ethyl ester was isolated in the following manner. One hundred ml. of 0.01627 *M* N-benzoylphenylalanine ethyl ester and of 0.01653 *M* sodium hydroxide were allowed to react for 20 hours at 25.04°. The solvent was removed under reduced pressure at 25°. The residue was dissolved in 25 ml. of water and acidified with dilute hydrochloric acid to *p*H 5. The precipitate was filtered and washed with water, yielding 0.40 g. of N-benzoylphenylalanine, 91%, m.p. 185–187° (lit. m.p. 184°).[9]

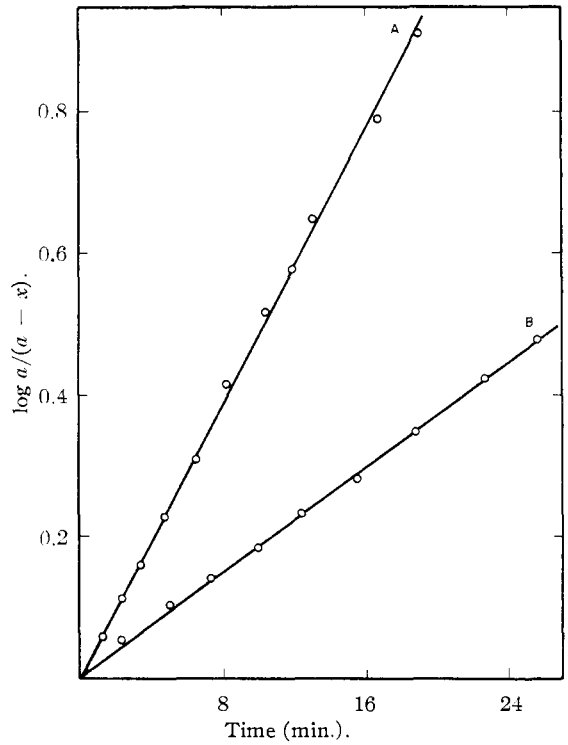

Fig. 4.—α-Chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-phenylalanine ethyl ester (A) and ethyl α-benzoxy-β-phenylpropionate (B).

Results and Discussion

Rate constants have been determined for the acidic and basic hydrolysis of five α-derivatives of ethyl β-phenylpropionate: hydrogen, hydroxyl, chloro, amino and benzoylamino. These values are given in Table I. Representative experiments are plotted in Figs. 1, 2 and 3. Relative rate constants

for the hydrolysis of these compounds are given in Table II together with the kinetic results of Snoke and Neurath[12] on the α-chymotrypsin-catalyzed hydrolysis of the corresponding methyl esters.

TABLE II

RELATIVE RATES OF ACIDIC, BASIC AND α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS

α-Substituent of alkyl β-phenylpropionate	Basic[b]	Acidic[b]	Hydrolysis α-Chymotrypsin[a,c] K_{III} × 10²	k_3 × 10³	Ref. k_3
Hydrogen	1	1	0.39	0.2	1
Amino	6	0.04	..	...	....
1-Benzoylamino	10	0.24	0.46	550	2750
d-Hydroxy	22	1.46	3.5	2.4	12
l-Hydroxy	22	1.46	1.0	14.5	72
dl-Chloro	59	0.22	1.2	2.3	11.5

[a] For the reaction $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$; $K_m = (k_2 + k_3)/k_1$. [b] For the ethyl esters. [c] For the methyl esters.

The relative order obtained in the basic hydrolyses is in agreement with that for the derivatives of ethyl acetate.[21,22] However, there are no rate constants in the literature for α-benzoylamino derivatives of esters. The relative order for the acidic hydrolyses is also in accord with that for the derivatives of ethyl acetate except for the α-amino and α-benzoylamino derivatives which have hitherto not been reported. In comparing the relative rate constants for the acidic and basic hydrolyses with the α-chymotrypsin-catalyzed hydrolysis, it should be noted that since it has never been shown that $k_2 > k_3$ in the enzymatic hydrolysis of these substrates, K_m cannot be used to determine the relative enzyme–substrate affinities. The unwarranted use of K_m for this purpose[12] has been pointed out.[23] Thus one cannot use K_m for any comparative purposes such as those presently under consideration. A comparison of the relative k_3 values of the five substrates with the relative rate constants for acidic or basic hydrolysis shows little if any correlation. At least as far as the breakdown of the intermediate enzyme–substrate complex is concerned, there is no recognizable general connection between enzymatic and non-enzymatic hydrolysis. However in very specific cases such as the hydrolyses of methyl DL-α-chloro-β-phenylpropionate and methyl hydrocinnamate, the higher k_3 value for the former ester can be attributed to an inductive effect of the chlorine atom.[20] This conclusion may be criticized, however, on the basis that the kinetics were determined with a *dl* mixture rather than with the separate isomers. It would not be expected that the formation of the enzyme–substrate complex could be related to the rates obtained in non-enzymatic hydrolysis, since formation of the complex would involve steric and possibly electronic requirements not found in acidic or basic hydrolysis.

One of the striking features of Table II is the extraordinarily large value of k_3 for benzoyl-L-phenylalanine ethyl ester which is several orders of magnitude larger than the other four derivatives. While there are no available constants for phenylalanine ethyl ester, it is certainly of a lower order of magnitude than the benzoyl derivative.[22–26] Neurath and co-workers[3,12,16,27] have presented evidence that the large values of k_3 for acylamino substrates is due to one or more hydrogen bonds involving the acylamino group and the enzyme. Their hypothesis is predicated on the fact that a series of six substrates, analogous to benzoyl-L-phenylalanine ethyl ester, are hydrolyzed extremely slowly or not at all by α-chymotrypsin. Three of these are benzyl derivatives of malonic acid, one of acetoacetic ester. None of these compounds is a homomorph[28] of benzoyl-L-phenylalanine ethyl ester; and thus the use of these substrates introduces added steric complications besides the loss of hydrogen-bonding abilities.

The homomorph chosen to test the hypothesis of hydrogen-bonding at a site removed from the reactive center was ethyl L-α-benzoyl-β-phenylpropionate. The first-order velocity constant found for benzoyl L-phenylalanine ethyl ester was 95.6 min.$^{-1}$/mg. enzyme N/ml.[29] The first order velocity constant for ethyl L-α-benzoxy-β-phenylpropionate was 0.702 min.$^{-1}$/mg. enzyme N/ml. (Fig. 4). The ratio of the first-order rate constants for the benzoylamino and benzoxy esters is 136. The essential difference between the two compounds is that the nitrogen-containing compound has a hydrogen atom attached to nitrogen whereas there is no analogous hydrogen atom in the oxygen homomorph. It is reasonable to assume that the large difference in reactivity of the two compounds toward α-chymotrypsin is attributable to the presence of the hydrogen atom and the most likely effect of such a hydrogen atom would be participation in hydrogen bonding.

Hydrogen bonding may play an important role in the following parts of the enzymatic process: formation of the enzyme–substrate complex, the breakdown of the complex into products or both. Neurath[3,12] has used K_m and k_3 values to substantiate the role of hydrogen bonding in both the formation and breakdown of the enzyme–substrate complex. Niemann[30,31] has rejected the importance of hydrogen bonding in complex formation using a series of D-inhibitors derived from D-tryptophanamide.

The first-order velocity constants observed in the present research are actually k_3/K_m, so that without additional information they cannot be used to determine in which step the hydrogen bonding effect

(21) R. A. Robinson, *Trans. Faraday Soc.*, **26**, 217 (1930).

(22) C. Gustafsson, *Ann. Acad. Sci. Fennicae*, **Ser. A, II**, Chem. No. 15, 11 (1945).

(23) D. S. Hogness and C. Niemann, THIS JOURNAL, **74**, 3183 (1952).

(24) H. Goldenberg, V. Goldenberg and A. D. McLaren, *Biochim. Biophys. Acta*, **7**, 110 (1951).

(25) H. Goldenberg and V. Goldenberg, *Arch. Biochem.*, **29**, 154 (1950).

(26) R. J. Foster, R. R. Jennings and C. Niemann, THIS JOURNAL, **76**, 3142 (1954).

(27) S. Kaufmann and H. Neurath, *J. Biol. Chem.*, **181**, 623 (1949).

(28) H. C. Brown, *et al.*, THIS JOURNAL, **75**, 1 (1953).

(29) The value of $C_{max.}$ for this reaction calculated from the data of Kaufman and Neurath is 29.8, the value of $C_{max.}$ found from the above first order velocity constant is 41.6. The only difference in these two systems is the buffer used, phosphate in the experiments of Kaufman and Neurath and tris-(hydroxymethyl)-aminomethane in the present work.

(30) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 101 (1952).

(31) H. J. Shine and C. Niemann, *ibid.*, **74**, 97 (1952).

occurs. Use may be made of some data obtained by Niemann[32] to answer this question. The Michaelis–Menten constants for L-tryptophanamide and acetyl L-tryptophanamide are 6.3×10^{-3} M and 5.3×10^{-3} M, and the inhibitor constants for the D-enantiomorphs of these compounds are 3.2×10^{-3} and 2.7×10^{-3} M, respectively. In these systems there is evidence that $k_2 >> k_3$, so that K_m may be used as a measure of enzyme–substrate affinity. If this is so, introduction of an acyl group gives a change of only 10% in the formation of the enzyme–substrate complex or the enzyme–inhibitor complex. On the other hand the k_3 values for L-tryptophanamide and acetyl-L-tryptophanamide are 0.006×10^{-3} and 0.50×10^{-3} mole/l./min./mg. enzyme N/ml., a ratio of eighty-three. It is apparent that the large difference in reactivity of the two compounds is in the second step. Introduction of the acyl group would be expected to polarize the nitrogen–hydrogen bond and make the hydrogen atom more able to enter into hydrogen bonding. If an extrapolation can be made from the tryptophan amide system to the benzoyl-L-phenylalanine ethyl ester system ($k_2 >> k_3$), the high first-order velocity constant for this ester over its oxygen homomorph suggests that the hydrogen bonding effect is to be found in the breakdown of the intermediate complex and not in its formation.

(32) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

If hydrogen bonding plays an important role in the breakdown of the enzyme–substrate into products, the following may be suggested: (1) hydrogen bonding to the enzyme "surface" is not important since the complex has already formed by the time hydrogen bonding becomes important; (2) hydrogen bonding to a molecule of water may be important; (3) the water molecule appears in the enzymatic hydrolysis not as a part of the enzyme–substrate complex but as a reactant in the subsequent decomposition of the complex into products. The last conclusion is not only a consequence of the above data but also of the concept of competitive inhibition and is confirmed by the lack of oxygen exchange with carboxylic acids which inhibit enzymatic hydrolysis.[33] The specification of the role of the water molecule in enzymatic hydrolysis seems to have been largely ignored except by several workers who have included the water molecule in the enzyme–substrate complex.[34,35]

Acknowledgment.—The authors wish to express their gratitude to Armour and Co. for a generous gift of α-chymotrypsin.

(33) M. L. Bender and K. C. Kemp, unpublished results.
(34) M. L. Barnard and K. J. Laidler, THIS JOURNAL, **74**, 6099 (1952); K. J. Laidler and J. P. Hoare, *ibid.*, **71**, 2699 (1949).
(35) C. G. Swain and J. F. Brown, Jr., *ibid.*, **74**, 2540 (1952).

CHICAGO, ILLINOIS

[CONTRIBUTION NO. 1982 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Effect of Added Sodium or Potassium Chloride upon the α-Chymotrypsin Catalyzed Hydrolysis of Chloroacetyl-L-tyrosinamide in Aqueous Solutions at 25° and *p*H 7.75[1]

BY HENRY J. SHINE AND CARL NIEMANN[2]

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The initial rate of the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.75 and 0.02 M in the THAM component of a THAM–HCl buffer is increased by the addition of either sodium or potassium chloride. The increase in initial velocities is given by the relation $\log (v_0/v_0^0) = 0.30 \sqrt{M}$ where M is the molarity of the reaction mixture with respect to added sodium or potassium chloride. From a study of the effect of added sodium chloride in systems in which the concentration of the specific substrate was varied it was found that the value of K_S is essentially independent of the concentration of added sodium chloride whereas the value of k_3 is increased by the amount given by the relation $\log (k_3/k_3^0) = 0.30 \sqrt{M}$.

In 1950 Jandorf[3] noted that the addition of magnesium sulfate to systems containing α-chymotrypsin and acetyl-L-tyrosine ethyl ester led to a proportionality between the enzyme concentration and the so-called esterase activity which was not observed in the absence of added magnesium sulfate and that the addition of this salt in a sufficient but otherwise unspecified amount caused a fivefold increase in the apparent esterase activity of the enzyme over that observed in a control experiment where presumably a lesser amount of magnesium sulfate was present. However, this investigator also reported that "no salt which was found to potentiate the esterase activity of α-chymotrypsin had an effect on the proteinase (casein hydrolysis) activity of the enzyme" thus leaving the impression, intended or not, that potentiation by added salt is to be observed only with respect to apparent esterase activity. Two years later Shine and Niemann[4] noted that the rate of the α-chymotrypsin-catalyzed hydrolysis of chloroacetyl-L-tyrosinamide, in aqueous solutions at 25° and *p*H 7.75, increased with increasing concentration of added magnesium sulfate thus demonstrating the generality of an apparent positive salt effect in systems involving α-chymotrypsin and either an amide or an ester type of specific substrate. In the same year Neurath and his coworkers[5] reported that the addition of calcium ion to systems containing α-chymotrypsin and either an ester or an amide type of specific substrate caused an increase in the activity of the enzyme and it was further stated that calcium ion was far more

(1) Supported in part by a grant from Eli Lilly and Co.
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(3) B. J. Jandorf, *Federation Proc.*, **9**, 186 (1950).
(4) H. J. Shine and C. Niemann, THIS JOURNAL, **74**, 97 (1952).
(5) M. M. Green, J. A. Gladner, L. W. Cunningham, Jr., and H. Neurath, *ibid.*, **74**, 2122 (1952).