to the inhibition of the deacylation step.[37,38] Furthermore, the substrate inhibition seen in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine has also been attributed to the same phenomenon.[11,39] Although these phenomena are also theoretically possible with α-chymotrypsin, they will not likely be observed because the acyl–chymotrypsin intermediate derived from a specific substrate preserves essentially all of the original specificity, resulting in blockage of the active site toward a foreign inhibitor.

Activation Parameters.—Since the catalytic rate constant is a complex constant, the activation energy of the catalytic rate constant may not be a constant independent of temperature. If, for example, the temperature dependencies of k_2 and k_3 are different from one another, a change in rate-determining step with temperature may occur. Such a phenomenon has apparently been observed in the acetylcholinesterase-catalyzed hydrolysis of acetylcholine,[11] and in the α-amylase-catalyzed hydrolysis of amylose.[40] No report of such a phenomenon in an α-chymotrypsin-catalyzed hydrolysis is known.

pH Dependence.—If indeed the catalytic rate constant is a complex constant, the pH dependence of this quantity may also be a complex function. This is especially true if the pH dependence of k_2 is different from that of k_3. If this is the case, the pH dependence of the reaction of a specific substrate whose rate-determining step is k_2 will differ from that of a specific substrate whose rate-determining step is k_3. A perusal of the literature indicates this may indeed be the case. For example, the pH–k_{cat} profiles of the specific ester substrates, N-acetyl-L-tryptophan ethyl ester and N-acetyl-L-tyrosine ethyl ester, whose rate-determining steps are postulated above to be deacylation, k_3, are sigmoid curves[41]; on the other hand, the pH-rate profiles (at a S_0/K_s ratio of 2.0 to 3.7 which approaches conditions of maximal velocity and therefore k_{cat}) of the specific amide substrates N-acetyl-L-tryptophan amide and N-nicotinyl-L-tryptophan amide, whose rate-determining steps are postulated above to be acylation, k_2, are bell-shaped curves.[42] Furthermore, if the pH dependencies of the two steps are different from one another, a change in rate-determining step with pH might be found at some point. Finally, if the pH dependencies of k_2 and k_3 are different from one another, and if the catalytic rate constant of specific substrates is a complex function of both steps, the pH dependence of the catalytic rate constant will consist of a family of curves determined by the relative contribution of each step in the over-all catalytic rate constant.[43]

Acknowledgment.—The authors thank Dr. Ferenc J. Kézdy for many helpful suggestions.

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Kinetic Evidence for the Formation of Acyl–Enzyme Intermediates in the α-Chymotrypsin-Catalyzed Hydrolyses of Specific Substrates[1,2]

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The kinetics of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan ethyl, methyl, and *p*-nitrophenyl esters and of N-acetyl-L-phenylalanine ethyl, methyl, and *p*-nitrophenyl esters was determined. The catalytic rate constants of the three tryptophan reactions are practically identical with one another, as are the catalytic rate constants of the three phenylalanine reactions. Ethyl, methyl, and *p*-nitrophenyl esters would be expected to show widely different rates in chymotrypsin reactions which are nucleophilic in character. The identity of each set of catalytic rate constants may therefore only be explained in terms of a rate-determining decomposition of a common intermediate. This intermediate may be most simply identified as N-acetyl-L-tryptophanyl-α-chymotrypsin in the first set of reactions and N-acetyl-L-phenylalanyl-α-chymotrypsin in the second set of reactions. Using the hypothesis that the *p*-nitrophenyl ester reaction proceeds with deacylation completely rate controlling, or using a relationship between the relative K_m(app)'s of these and other compounds, individual rate constants for acylation and deacylation are calculated. The relative k_2's (acylation) are in good agreement with the nucleophilic order of the corresponding carboxylic acid derivative as measured with hydroxide ion.

Introduction

One of the predictions of the kinetic analysis of the previous paper[4] concerns the relative catalytic rate constants of the α-chymotrypsin-catalyzed hydrolysis of a family of compounds having a common acylamino acid moiety but different leaving groups. This prediction is based on the postulate of an acyl–enzyme intermediate in α-chymotrypsin reactions[3,4] (eq. 1) and the nucleophilic character of both the acylation and deacylation reactions.[5,6] The combination of these two hypotheses

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{\underset{P_1}{+}}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

predicts that the catalytic rate constants of those reactions whose rate-determining step is acylation, k_2, will exhibit the full relative nucleophilic reactivity of the various compounds, whereas the catalytic rate constants

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of those reactions whose rate-determining step is deacylation, k_3, will show identical catalytic rate constants since the catalytic rate constants will reflect the decomposition of a common intermediate acyl–enzyme. From a consideration of the known Michaelis constants, K_m(app), it was predicted that the rate-determining step of the hydrolysis of three specific substrates, the ethyl esters of N-acetyl-L-tyrosine, N-acetyl-L-phenylalanine, and N-acetyl-L-tryptophan, is deacylation.[4] Therefore, it would be predicted that the catalytic rate constants of all esters of these compounds of inherent reactivity toward nucleophiles equal to or greater than that of ethyl esters would be essentially equivalent to one another, regardless of the inherent nucleophilic reactivity of these compounds. To this end, the α-chymotrypsin-catalyzed hydrolysis of the ethyl, methyl, and *p*-nitrophenyl esters of N-acetyl-L-tryptophan and N-acetyl-L-phenylalanine have been investigated.

Experimental

Materials.—N-Benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (3 g.), obtained from Mann Biochemical Corp., was dissolved in 15 ml. of pure dry acetone with hand warming. The solution was rapidly filtered through a sintered glass funnel and 200 ml. of anhydrous ether was added. The material was seeded and stored in a freezer. After 48 hr., 2.20 g. of off-white crystals was isolated; m.p. placed on block at 150°, softened slightly 156°, melted with decomposition 158–160°; lit. m.p. 158–159°[7]; 155–157°, 155–156°[8]; $[\alpha]^{28}D$ −8.5 ± 0.5° (*c* 3, acetone); lit. $[\alpha]^{28}D$ 16.3° (*c* 1, acetone), +3.2°(*c* 1, dioxane)[7]; $[\alpha]^{27}D$ −8.3 ± 0.5° (*c* 4, acetone), −8.1 ± 1° (*c* 1, acetone), −12.4 ± 2° (*c* 0.5, acetic acid).[8] The melting point is an excellent criterion of optical as well as chemical purity. The purified material contained 0.15–0.3% excess of *p*-nitrophenol determined spectrophotometrically.

N-Benzyloxycarbonyl-L-tyrosine Ethyl Ester.—L-Tyrosine ethyl ester hydrochloride was prepared in virtually quantitative yield from L-tyrosine.[9] The material was recrystallized from ethanol–ether[10]; m.p. 167–169°, lit. m.p. 165–166°,[10] 166°.[11] N-Benzyloxycarbonyl-L-tyrosine ethyl ester[12] was purified by extraction of the crude solid with large volumes of boiling hexane with stirring. The solution on cooling gave colorless needles, m.p. 78–78.5°. Recrystallization from chloroform–hexane gave material, m.p. 78.5–79°, lit.[7,12–14] m.p. 88.0–88.5°, 78°, 78–78.5°, 75–78°; $[\alpha]^{24}D$ = −2.0 ± 0.1° (5% v./v. ethanol), lit.[12] $[\alpha]^{25}D$ −4.7° (*c* 1.7, ethanol). The optical purity of this material was checked by its alkaline hydrolysis to optically pure N-benzyloxycarbonyl-L-tyrosine.

This compound was recrystallized by dissolving it in the minimum amount of sodium acetate solution followed by the slow addition of 1 *N* hydrochloric acid; m.p. 96.5–97°, lit.[7,12,13] m.p. 94–95°, 101°, 91–93°; $[\alpha]^{24}D$ +10.5 ± 0.3° (*c* 2, acetic acid), lit.[8,12] $[\alpha]^{29}D$ +10.3 ± 0.5° (*c* 3.7, acetic acid), $[\alpha]^{20}D$ +11.1° (acetic acid).

N-Acetyl-L-tryptophan methyl ester (H. M. Chemical Co., Ltd., Santa Monica, Calif., Lot No. 113-100-19) was recrystallized from ethyl acetate; m.p. 154–155°, lit.[15] m.p. 152–155°, $[\alpha]^{24}D$ +13.8 ± 0.3° (*c* 5.6, methanol), lit. $[\alpha]^{23}D$ +11.5 ± 0.5° (*c* 2, methanol).[13]

N-Acetyl-L-tryptophan ethyl ester (Mann Research Laboratories, Lot C2756) was dissolved in the minimum amount of boiling dry ethyl acetate and treated with decolorizing charcoal. After filtering, ether was added to the filtrate (ether–ethyl acetate, 5:1); m.p. 110–111.5°, lit.[16] m.p. 109–109.5°, $[\alpha]^{24}D$ +7.1 ± 0.3° (*c* 6, ethanol), lit.[16] $[\alpha]^{20}D$ +50° (*c* 5, $CHCl_3$). *Anal.* Calcd. for $C_{15}H_{18}N_2O_3$: C, 65.67; H, 6.61; N, 10.21. Found: C, 65.34; H, 6.32; N, 10.17.[17]

N-Acetyl-DL-tryptophan *p*-Nitrophenyl Ester.—N-Acetyl-L-tryptophan (9.62 g., 39 mmoles; Mann Research Laboratories, Lot G1233) and *p*-nitrophenol (5.44 g., 39 mmoles; sublimed at the melting point) were stirred together in 150 ml. of ethyl acetate (redistilled). Dicyclohexylcarbodiimide (8.06 g., 39 mmoles; Aldrich Chemical Co.) was added and the reaction was stirred for 6 hr. at room temperature (30°) and refrigerated overnight. The urea was filtered and the solvent removed *in vacuo* (no heat). The residue on scratching gave a pale yellow crystalline mass. The material was dissolved in the minimum amount of cold acetone, treated with charcoal, and filtered. Addition of ether to the filtrate gave, after addition of a small seed, very pale yellow crystals. Repeated crystallization in this way gave crystals, m.p. 136–137° dec. (sample placed on the block at 125°), $[\alpha]^{24}D$ +0.47 ± 0.1° (*c* 5.7, acetone). The material is almost pure racemate from its behavior with α-chymotrypsin. *Anal.* Calcd. for $C_{19}H_{17}N_3O_5$: C, 62.12; H, 4.67; N, 11.44. Found: C, 62.13; H, 4.87; N, 11.29.

N-Acetyl-L-phenylalanine Methyl Ester.—This ester (Mann Research Laboratories, Inc., Lot G2218) was shown by means of thin layer chromatography on silica gel using 25% ethyl acetate in ether as eluent to be homogeneous; m.p. 88.5–89.5°, lit.[18] m.p 90–91°, $[\alpha]^{25}D$ +21.4° (*c* 1.9, methanol), lit.[18] $[\alpha]^{25}D$ +19 (*c* 2.0, methanol). This compound exhibited a strong singlet n.m.r. peak at 6.4 τ, indicating a methyl group on oxygen.

N-Acetyl-L-phenylalanine Ethyl Ester.—This ester (Mann Research Laboratories, Inc.) was shown by means of thin layer chromatography to exhibit two spots, indicating inhomogeneity. It was, therefore, chromatographed on an alumina column. In benzene and 20% chloroform in benzene, an ethyl ester was eluted which, after two subsequent recrystallizations from methylene chloride–hexane, melted at 90–91.5° and was homogeneous according to the thin layer criterion; $[\alpha]^{25}D$ +19.8° (*c* 2.0, methanol), lit. m.p. 93–94°.[19] This material exhibited peaks centered at 5.9 (quadruplet) and 8.79 τ (triplet) in its n.m.r. spectrum.

N-Acetyl-DL-phenylalanine *p*-nitrophenyl ester (Cyclo Chemical Co.) was used without further purification, m.p. 162–163°, $[\alpha]^{25}D$ ±0° (*c* 1.4, chloroform). The material is pure racemate, from its behavior with α-chymotrypsin.

Anal. Calcd. for $C_{17}H_{16}N_2O_5$; C, 62.3; H, 4.88; N, 8.55. Found: C, 62.61; H, 5.09, N, 8.35.

α-Chymotrypsin.—Worthington α-chymotrypsin (3× crystallized, salt free) was used without further purification. Enzyme solutions were routinely centrifuged at 15,000 r.p.m. to guarantee 100% optically clear solutions. The operational normality of the enzyme solution was determined by titration,[20] using a reverse (method B) titration with 3.0 ml. of enzyme solution.

Phosphate buffers (0.1 *M* total) were prepared from Mallinckrodt reagent grade potassium dihydrogen phosphate and disodium hydrogen phosphate.[21] Acetate buffers were prepared from analytical grade sodium acetate.[19] Acetonitrile was an Eastman Kodak Co. Spectro Grade product which was sometimes purified by the method of Lewis and Smyth.[22] pH measurements were made on a Radiometer 4C pH meter standardized as recommended by Bates, *et al.*[23] At the end of each kinetic run on the spectrophotometer, the pH of the solution in the cuvette was measured.

Kinetic Measurements.—Kinetics of hydrolysis were determined using a Cary 14 PM recording spectrophotometer (0–0.1 and 0–1.0 absorbance slide wires) equipped with a thermostated cell compartment, a stopped-flow mixing device mounted on a Beckman DU spectrophotometer[24] equipped with a thermostated cell compartment, or a Radiometer pH stat equipped

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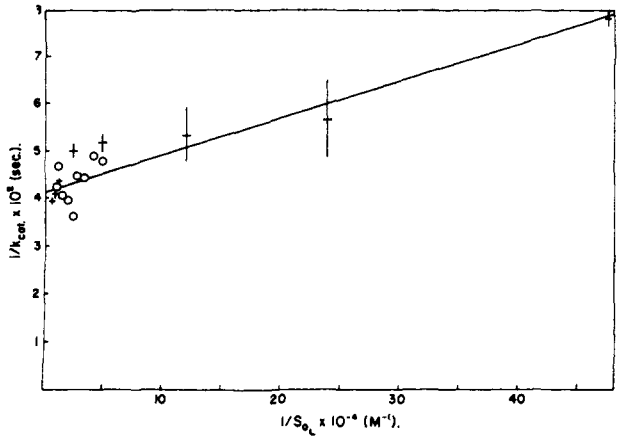

Fig. 1.—The hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester by α-chymotrypsin at 25.0°, pH 6.97, 3.2% (v./v.) acetonitrile–water: O, stopped flow experiments; +, Cary spectrophotometer experiments.

with a Titragraph recorder and a thermostated cell compartment.

The hydrolyses of both N-acetyl-L-tryptophan methyl and ethyl esters were followed at 300 mμ; N-acetyl-DL-tryptophan *p*-nitrophenyl ester at 400 mμ; N-benzyloxycarbonyl-L-tyrosine ethyl and *p*-nitrophenyl esters at 237.5 and 400 mμ, respectively; and N-acetyl-DL-phenylalanine *p*-nitrophenyl ester at 400 mμ. The hydrolyses of N-acetyl-L-phenylalanine methyl and ethyl esters were followed on a Radiometer TTT1 pH Stat.

A typical experiment on the Cary spectrophotometer is described for the hydrolysis of N-acetyl-L-tryptophan methyl ester; 3 ml. of 0.1 *M* phosphate buffer was equilibrated at 25.0 ± 0.1° in the cuvette in the sample compartment of the spectrophotometer. An acetonitrile stock solution of the ester (100 μl.) was added and recording commenced. The molar absorptivity[25] of the ester agreed well with values independently determined volumetrically and Beer's law is accurately followed. After approximately 1 min., 50 μl. of the stock enzyme solution was added and recording commenced after 20 sec. The absorbance data were converted into rate data using the difference in molar absorptivities of the ester and the acid, shown in Table I.

TABLE I

MOLAR ABSORPTIVITIES OF SUBSTRATES AND PRODUCTS[a]

Compound	Wave length, mμ	Molar absorptivity
N-Acetyl-L-tryptophan methyl ester	300	685 ± 3
N-Acetyl-L-tryptophan ethyl ester	300	692 ± 2
N-Acetyl-L-tryptophan	300	926 ± 5[b]
		932 ± 1[c]
p-Nitrophenol	400	8498 ± 5[d]
N-Benzyloxycarbonyl-L-tyrosine ethyl ester[e]	237.5	1320
N-Benzyloxycarbonyl-L-tyrosine[e]	237.5	1000

[a] pH 6.98 ± 0.01, 3.17% (v./v.) acetonitrile–water. [b] From the reaction of the methyl ester. [c] From the reaction of the ethyl ester. [d] In good agreement with the earlier calculated value (8530).[24] [e] pH 7.9, 17.2% acetonitrile–water.

With the *p*-nitrophenyl esters, the enzyme solution was added first to the cuvette. Recording was commenced as rapidly as possible (generally within 10 sec. and sometimes within 4 sec.). The spontaneous hydrolysis of the *p*-nitrophenyl esters at pH 7 is relatively slow ($k_{obsd} \cong 10^{-4}$ sec.$^{-1}$) and within the precision of these experiments may be neglected with respect to the enzymatic reaction.

For hydrolyses of N-acetyl-DL-tryptophan *p*-nitrophenyl ester in the stopped-flow spectrophotometer, approximately 1 ml. of the enzyme solution in a 0.2 *M* phosphate buffer at pH 6.97, and 1 ml. of the substrate solution in a 0.2 *M* potassium chloride solution were mixed. It was necessary to maintain similar refractive indeces of both solutions in order to obviate schlieren effects resulting from incomplete mixing; furthermore, the phosphate buffer could not be mixed prematurely with the *p*-nitrophenyl ester since a rapid reaction ensues in such a system.

A typical experiment with the pH-stat is: 3 ml. of a 0.01 *M* phosphate buffer containing 0.1 *M* sodium chloride (pH 6.95) were pipetted in a 3-ml. capacity reaction cell thermostated to 25.0 ± 0.1°. The electrodes, stirrer, and inlet buret were placed in the solution and the stirrer started. An aliquot of an acetonitrile solution of the substrate (*ca.* 0.3 *M*) was then added. A base line was determined after the solution had been brought to operating pH (6.99). An aliquot of enzyme stock solution (50 μl. of *ca.* 1×10^{-4} *M* also at pH 6.95) was then added and the reaction followed. The sodium hydroxide used was 0.2144 and 0.107 *N*.

Results

The analysis of the data on enzymatic hydrolysis was made in terms of eq. 2 where $k'_{cat} = k_{cat}S_0/(K_m(app) + S_0)$.

$$E + S \underset{}{\overset{K_m(app)}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \qquad (2)$$

The results obtained for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl and ethyl esters are shown in Table II.

Although N-acetyl-DL-tryptophan *p*-nitrophenyl ester is a racemic compound, the L-ester is hydrolyzed by the enzyme several orders of magnitude faster than the D-ester, since 50 ± 1% of the stoichiometric amount of *p*-nitrophenol was released from the DL-ester at sufficiently high enzyme concentration with the formation of an apparent infinity in the reaction. The system is further simplified since $[S_D] \ll K_{sD}$ whereas $[S_L] \gg K_m(app)_L$; thus the measured $K_m(app)$ is $K_m(app)_L$. On the Cary spectrophotometer, only approximate values of the initial rates could be obtained. It was then decided to determine the rate of this reaction using the stopped-flow spectrophotometer where true initial rates at fractions of a second could be easily measured. The results of the experiments using initial rates which were determined both on the stopped-flow spectrophotometer as well as on the Cary spectrophotometer are shown in Fig. 1 which shows a rather poor Lineweaver–Burk plot. From the imprecise intercept and slope of this plot, one may calculate $k_{cat} \cong 24$ sec.$^{-1}$ and $K_m(app) \cong 2 \times 10^{-6}$ *M*. Because of these poor results, we turned to a consideration of the time course of the reaction.

An analysis of the time course of several typical runs using the differential form of the Henri equation[26,27] indicated that the curves did not obey simple enzyme kinetics; in particular, the diminution in the rate of the reaction occurred faster than predicted for an ordinary enzymatic process. Furthermore, the rate at 50% reaction of an experiment where $S_0 = n$ was much slower than the initial rate of reaction of a separate experiment where $S_0 = n/2$. Product inhibition cannot explain this phenomenon since the inhibition constant of N-acetyl-L-tryptophan must be higher than 7×10^{-3} *M* while the substrate concentration used was much less than this value.

Finally, two identical quantities of substrate were added to the same enzyme solution. The first addition was followed to infinity and then the second addition was made. The second identical quantity of substrate was hydrolyzed much more slowly than the first one!

(25) "Spectrometry Nomenclature," *Anal. Chem.*, **33**, 1968 (1961).

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(27) *Cf.* K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Oxford University Press, New York, N. Y., 1958, p. 86.

This experiment clearly shows that the enzyme was inactivated during the first hydrolytic reaction.

Preliminary experiments show that this inactivation is probably a slow acylation of the enzyme by the D-substrate and that this D-acyl–enzyme is reactivated only very slowly.[28] Using very high enzyme concentrations (of the same order of magnitude as the substrate concentration), after (instantaneous) hydrolysis of the L-substrate, the D-substrate slowly reacts with the enzyme with liberation of *p*-nitrophenol, at a rate equal to the rate of disappearance of the enzyme during the hydrolysis of the L-substrate. If the deactivation process is first order with respect to the enzyme, then the rate of the hydrolytic process under conditions in which $[S_0] \gg [E_0]$ will be

$$-dS/dt = k_{cat}ES/(K_m(app) + S) = k_{cat}[S][E_0]e^{-k_d t}/(K_m(app) + S) \quad (3)$$

where k_d is the first-order deactivation constant of the enzyme.[29] If the enzyme is saturated by the substrate (zero-order part of the reaction) then $K_m(app) + S \cong S$, and then

$$V = -dS/dt = k_{cat}E_0e^{-k_d t} \quad (4)$$

Thus by plotting log V *vs.* t, one should obtain a straight line whose intercept at $t = 0$ should yield $k_{cat}E_0$ and hence k_{cat}. Figure 2 indicates that the experimental results do indeed obey eq. 4 over a wide range of concentration, and moreover that the intercepts at $[S_L] = 160.8 \times 10^{-6}$ and 21.07×10^{-6} M yield almost identical k_{cat}'s of 30.5 and 31.0 sec.$^{-1}$, respectively. Thus at these substrate concentrations, which are approximately 80 and 10 times $K_m(app)_L$, saturation of the enzyme is obtained.

The results obtained for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-phenylalanine methyl, ethyl, and *p*-nitrophenyl esters are shown in Table II. The pH-stat experiments with the phenylalanine esters give results more imprecise than the spectrophotometric experiments described above because of enzyme adsorption on the glass at the low enzyme concentrations used (10^{-7} M)[30], because the $K_m(app)$'s of the phenylalanine esters are quite high, necessitating the use of substrate concentrations approaching the solubility limit, because of difficulties with the purity of the ethyl ester, as noted earlier, and finally because the determination of the stoichiometry of the ethyl ester reaction of even the purified sample was consistently in error by about 10%.

At low acetonitrile concentrations, the D-component of N-acetyl-DL-phenylalanine *p*-nitrophenyl ester acylated the enzyme at a rate which interfered with the kinetics of the L-component, in a manner similar to that described for the corresponding tryptophan compound. The technique used with the tryptophan compound to overcome this difficulty was not possible here, because the low solubility of the phenylalanine derivative made it impossible to saturate the enzyme adequately, a necessary requirement of that approach. The difficulty was resolved by using 15% acetonitrile–water as solvent, instead of 3% acetonitrile–water. In the former solvent, the D-ester does not interfere because the K_{sD} is drastically increased, preventing effective acylation of the enzyme. Solvents of different organic content result in slightly different catalytic rate constants, but the difference is quite small. Table II indicates that the catalytic rate constant of the methyl ester in 15% acetonitrile–water is about 6% less than that in 3% acetonitrile–water, a result in agreement with previous studies on the effect of organic solvents on catalytic rate constants.[31] This small effect can thus be taken into account in the comparisons to be made later.

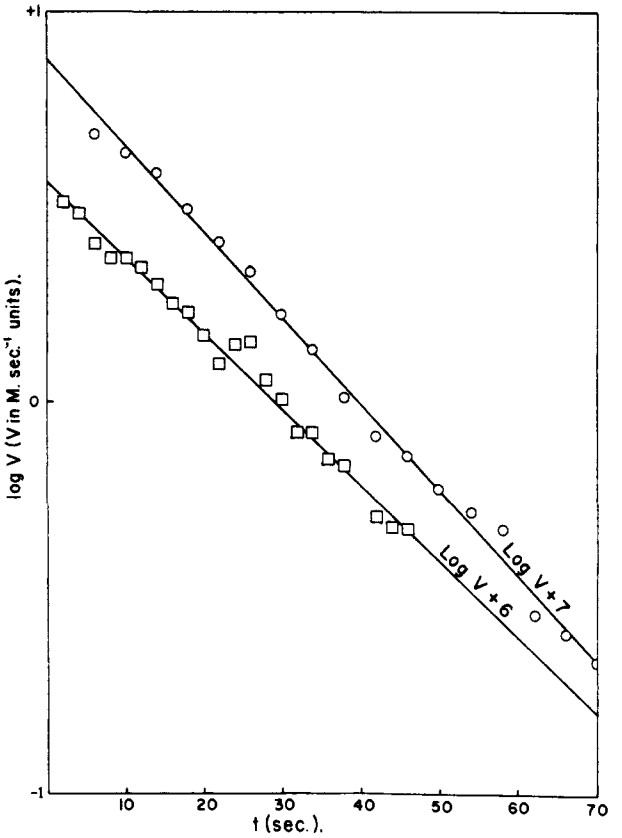

Fig. 2.—The hydrolysis of N-acetyl-L-tryptophan *p*-nitrophenyl ester by α-chymotrypsin at 25.0°, pH 6.97, 3.2% (v./v.) acetonitrile–water: □, $[S_0]_L = 1.608 \times 10^{-4}$ M, $[E_0] = 1.208 \times 10^{-7}$ M; ○, $[S_0]_L = 2.107 \times 10^{-6}$ M, $[E_0] = 2.146 \times 10^{-8}$ M.

Discussion

Table II summarizes the kinetic data for the hydrolysis of the esters determined in this work together with kinetic data from the literature pertinent to the present results. The agreement of the catalytic rate constants and Michaelis constants of the methyl and ethyl esters of N-acetyl-L-tryptophan determined in this investigation with those determined previously is good, if one takes into account the effect of pH on the rate constants. The agreement of the catalytic rate constants and Michaelis constants of the methyl and ethyl esters of N-acetyl-L-phenylalanine is not quite as good as that for the tryptophan derivatives. Considering the fact that the enzyme concentration was determined directly by spectrophotometric titration, whereas previous results were obtained on the basis of an assumed molecular weight and an assumed protein nitrogen content of an assumed pure enzyme preparation, the results are reasonably consistent. The results that will be utilized in

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(29) K. J. Laidler, ref. 27, p. 108.

(30) See J. B. Jones and C. Niemann, *Biochemistry*, **2**, 498 (1963).

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TABLE II

KINETICS OF HYDROLYSIS OF SOME SPECIFIC SUBSTRATES OF α-CHYMOTRYPSIN[a]

Compound	pH	k_{cat}	K_m(app) × 10^{4b}	Ref.
N-Acetyl-L-tryptophan derivatives				
Amide	7.0[c]	0.026	400	35
Amide	7.9[c]	0.036	500	34
Ethyl ester	6.98	26.9 ± 0.5	9.7	[e]
Ethyl ester	8.25[c,d]	51.6	8.9	33
Methyl ester	6.98	27.7 ± 0.5	9.5	[e]
Methyl ester	8.25[c]	54.9	7.8	32
p-Nitrophenyl ester	7.0	30.5 ± 1.2	0.2	[f]
N-Acetyl-L-phenylalanine derivatives				
Amide	7.9[c]	0.046	3000	35
Amide	7.9[c]	.053	3100	34
Ethyl ester	6.99	63.1 ± 1.5	88 ± 19	[g]
Ethyl ester	7.8[c]	160	120	37
Methyl ester	6.99	57.5 ± 1.0	150 ± 20	[g]
Methyl ester	6.99[f]	54.1	729	[g]
Methyl ester	7.80[c]	48.2 ± 12	180 ± 45	36
Methyl ester	7.90[c]	52.5 ± 15.8	125 ± 95	30
p-Nitrophenyl ester	6.99	73 ± 7	11.5 ± 2	[f,h]

[a] 25.0°, 3.17% (v./v.) acetonitrile–water. [b] In the presence of 0.1 *M* $CaCl_2$; calculated from the original V_{max} assuming a molecular weight of 24,800 for α-chymotrypsin and taking protein nitrogen as 16%. [c] Aqueous solution. [d] 0.1 *M* $CaCl_2$. [e] This research; 0.1 *M* phosphate buffer; Lineweaver–Burk least-squares treatment of data obtained at at least 5 substrate concentrations over a 20-fold range, all above K_m(app). [f] This research; see text. [g] This research; using a weighted least-squares treatment,[35] using at least 6 points for each calculation. [h] 15% (v./v.) acetonitrile–water; least-squares treatment of 40 points.

future comparisons are those done in the present investigation, comprising a self-consistent set of data.

The prediction was made earlier that the ethyl, methyl, and *p*-nitrophenyl esters of specific substrates such as acetyl-L-tryptophan derivatives and acetyl-L-phenylalanine derivatives should exhibit the same catalytic rate constants. The catalytic rate constants for the ethyl, methyl, and *p*-nitrophenyl esters of acetyl-L-tryptophan shown in Table III are almost identical, although the rate constant for the corresponding amide is considerably lower. Likewise, the catalytic rate constants for the ethyl, methyl, and *p*-nitrophenyl esters of acetyl-L-phenylalanine shown in Table III are almost identical, although the rate constant for the corresponding amide is considerably lower. Each set of data is internally consistent and also consistent with the large body of other kinetic data on chymotrypsin reactions,[4] assuming all reactions in Table III proceed *via* eq. 1 with the rate-determining step noted in the table.

Chymotrypsin catalyses can be characterized as nucleophilic reactions.[5,6] Toward the nucleophile hydroxide ion, the relative reactivities of *p*-nitrophenyl, methyl, and ethyl esters at 25° are in the ratio 100:2:1.[38] Toward imidazole, the relative reactivities of these three esters is even more disparate.[39] The fact that the three esters of N-acetyl-L-tryptophan and the three esters of N-acetyl-L-phenylalanine possess essentially identical catalytic rate constants (within 10% for the former set and 20% for the latter set) can most readily be explained by postulating a stepwise process involving the rate-determining decomposition of a common intermediate containing the N-acetyl-L-tryptophanyl group or the N-acetyl-L-phenylalanyl group. Of course, α-chymotrypsin is also a common factor to these reactions. Such intermediates may therefore be most simply identified as N-acetyl-L-tryptophanyl-α-chymotrypsin or N-acetyl-L-phenylalanyl-α-chymotrypsin, respectively.

In chymotrypsin reactions, following eq. 1, $(k_2/k_3 + 1) = K_s/K_m(app)$.[4] If acylation is rate controlling ($k_2 \ll k_3$), then $K_s = K_m(app)$; on the other hand, if deacylation is rate controlling ($k_2 \gg k_3$), $K_m(app) = K_s \cdot (k_3/k_2)$. In the latter instance the ratio (k_3/k_2) must necessarily be less than one, and therefore $K_m(app)$ must necessarily be less than K_s. The difference in $K_m(app)$ between the tryptophan or phenylalanine ester and amide substrates cannot be explained on structural grounds since the specificity resides in the tryptophan or phenylalanine residue of these compounds. These differences can be explained readily by postulating that the real K_s's of all these compounds are similar, that the K_s of the amide reaction is the only real K_s (acylation rate controlling), and the K_s's of the ester reactions are apparent K_s's whose values are diminished by the ratio (k_3/k_2) (deacylation rate controlling).[4,40,41] The catalytic rate constants for the α-chymotrypsin-catalyzed hydrolyses of N-benzoyl-L-tyrosine amide, N-benzoyl-L-tyrosine ethyl ester, and N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester have been compared previously.[41] It was found that the catalytic rate constant of the *p*-nitrophenyl ester is only 50% larger than that of the ethyl ester, and the conclusion was drawn that all three reactions proceed according to eq. 1. Unfortunately, the comparison is not valid, for a benzyloxycarbonyl derivative was compared with a benzoyl derivative. This defect was pointed up when we synthesized N-benzyloxycarbonyl-L-tyrosine ethyl ester and compared it to the corresponding *p*-nitrophenyl ester. The catalytic rate constants of these two compounds differ by a factor of *ca.* 19 at pH 7.9 in a 0.1 *M* Tris buffer containing 17.2% (v./v.) acetonitrile, and therefore do not exhibit the identity of rates seen with the compounds of Table III. It is not clear why the *p*-nitrophenyl and ethyl esters of N-benzyloxycarbonyl-L-tyrosine do not show identical rates while the *p*-nitrophenyl and ethyl esters of both N-acetyl-L-tryptophan and of N-acetyl-L-phenylalanine do show this identity. The only reason that can be advanced at this time is that the *p*-nitrophenyl group may pervert the binding in the former compounds but not in the latter compounds.

If one assumes that eq. 1 is operative, it then becomes of interest to calculate the individual rate constants k_2 and k_3 for the various reactions of Table III. This calculation may be done by means of the following

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TABLE III

COMPARISON OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SOME SPECIFIC SUBSTRATES AT pH 7.0[a]

Derivative	k_{cat}, sec.$^{-1}$	K_m(app) × 10^5, M	K_s × 10^5, M	k_2, sec.$^{-1}$	k_3, sec.$^{-1}$	Rel. k_{OH^-} of acetate, M^{-1} sec.$^{-1}$	Rate-determining step
N-Acetyl-L-tryptophan derivatives							
Amide[b]	0.026	730	*730*	0.026	(30)	1	Acylation
Ethyl ester	26.9 ± 0.5	9.7	*252*	*700*	*28*	2750	Deacylation
Methyl ester	27.7 ± 0.5	9.5	*252*	*730*	*29*	5500	Deacylation
p-Nitrophenyl ester	30.5 ± 1.2	0.2	*252*	*38,300*	*30.53*	315,000	Deacylation
N-Acetyl-L-phenylalanine derivatives							
Amide[c]	0.039	3700	*3700*	*0.039*	(72)	1	Acylation
Ethyl ester	63.1	88	*740*	*530*	*72*	2750	Deacylation
Methyl ester	57.5	150	*740*	*280*	*72*	5500	Deacylation
p-Nitrophenyl ester[d]	77	2.4	*740*	*23,700*	*77*	315,000	Deacylation

[a] 25.0°, 3.17% (v./v.) acetonitrile–water. [b] From ref. 35 after correction to 3.17% acetonitrile–water from aqueous solution.[31] This correction is negligible for the k_{cat} and amounts to approximately 85% for K_m(app). The values given here for N-acetyl-L-tryptophan amide are slightly different from the preliminary communication on this subject[40] because of this solvent correction. [c] From ref. 36 after correction to 3.17% (v./v.) acetonitrile–water from aqueous solution.[31] [d] The values of Table III have been corrected to 3.17% (v./v.) from 15% (v./v.) acetonitrile–water according to ref. 31. The correction in k_{cat} is 6% and the correction in K_m(app) is 4.6-fold.

equations derived from eq. 1[4]

$$k_2 = k_{cat}(K_s/K_m(app)) \quad (5)$$

and

$$k_3 = k_{cat}/(1 - (K_m(app)/K_s)) \quad (6)$$

Thus, since k_{cat} and K_m(app) are known quantities, both k_2 and k_3 can be calculated if one can find K_s.

One approach to the estimation of K_s depends on the consideration that the K_m(app)'s given in Table III do not reflect solely relative binding constants, sometimes being considerably perturbed by the ratio of rate constants k_3/k_2. Earlier it was postulated that the ratio K_s N-acetyl-L-tryptophan amide/K_s N-acetyl-L-tryptophan ethyl ester is a constant whose value is not far from one and which may be taken as 2.9 by analogy with the D-inhibitors.[4] Furthermore, for purposes of simplification, the ratio of 2.9 will be taken for the ratio of the K_s's of N-acetyl-L-tryptophan amide/N-acetyl-L-tryptophan ester for all esters of tryptophan. Likewise, the ratio of the K_s N-acetyl-L-phenylalanine amide/K_s N-acetyl-L-phenylalanine ester for all esters of N-acetyl-L-phenylalanine will be taken as 5 since this is the ratio of the K_i's of the corresponding D-inhibitors. Using this approach, the K_s's in Table III were approximated. From these values it was then possible, using eq. 5 and 6, to calculate the k_2 and k_3 values for the hydrolyses shown in Table III.

Another approach to the estimation of the K_s's of the various ester substrates is to assume that the catalytic rate constant of the *p*-nitrophenyl ester of a specific substrate is exactly equivalent to k_3. Then if one further says that k_3 is the same for all derivatives of a given acylamino acid substrate, one can use eq. 5 to calculate k_2 and from this use eq. 6 to calculate K_s. This process leads to values of K_s for acetyl-L-tryptophan ethyl and methyl esters of 82 and 104 × 10^{-5} M, respectively, which are within a factor of two to three of the K_s's of these compounds calculated using the hypothesis of the previous paragraph.

A consideration of the calculated kinetic constants of Table III is of interest. The calculated k_3 values of the three esters of N-acetyl-L-tryptophan are essentially identical, as are the calculated k_3 values of the three esters of N-acetyl-L-phenylalanine. Each set of k_3 values must of course show this identity if k_3 does indeed describe the decomposition of a common intermediate for the set. The calculated k_2 values of each set of four compounds is of more interest since for a given family of acylamino acid derivatives, the calculated relative k_2 values should exhibit the relative rates of these compounds in nucleophilic reactions. The spread in the calculated k_2 values from the amide to the *p*-nitrophenyl ester for the N-acetyl-L-tryptophan series is 1.5 × 10^6, while the spread in the calculated k_2 values for the N-acetyl-L-phenylalanine series is 6 × 10^5. These spreads may be compared with that for the hydroxide ion-catalyzed (nonenzymatic) hydrolysis of the corresponding acetate derivatives; the spread for the latter reactions is 3.15 × 10^5, a value close to the two calculated enzymatic k_2 values. Thus, from the point of view of the two extreme compounds, the relative k_2 values appear to parallel the relative hydroxide ion rates. Likewise, the relative rate ratio of the ethyl ester/amide is 2700 for the N-acetyl-L-tryptophan series, 1400 for the N-acetyl-L-phenylalanine series, and 2750 for the nonenzymatic hydroxide ion series. From these ratios, the relative k_2 values also appear to parallel the relative hydroxide ion rates. The ethyl ester/methyl ester rate ratios in the nonenzymatic hydroxide ion series is 0.5, but in the enzymatic N-acetyl-L-tryptophan series it is 1 and in the enzymatic N-acetyl-L-phenylalanine series it is 2; this lack of parallelism indicates that the present treatment is not able to account for small differences which probably arise from the inapplicability of the assumption of a constant K_s for all esters. However, the over-all parallelism between the calculated k_2 values of the enzymatic reactions and the hydroxide ion-catalyzed (nonenzymatic) rate constants, and the lack of parallelism between k_{cat} values and hydroxide ion-catalyzed reactions, is a striking validation of the approach and assumptions used here.

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