6.2 (w), 6.65 (w), 6.9 (w), 7.2 (m), 7.5–8.4 (m), 9.2 (m), 9.75 (m), and 10.8 (w).

Anal. Found: C, 65.82; H, 6.77.

Hydrolysis of DL-Ethyl α-Benzoyloxypropionate by α-Chymotrypsin.—The ester, 1.80 g. (8.04 mmoles), suspended in 15 ml. of 0.1 *M* sodium chloride under nitrogen at 25°, was brought to pH 7.8 in a pH stat. Hydrolysis in the absence of enzyme was very slow. α-Chymotrypsin (0.201 g., Worthington Biochemical Corp., salt-free, three times recrystallized) was added, the system was brought back to pH 7.8, and the enzymatic hydrolysis was followed. It was slow, and 5 ml. of 0.1 *M* phosphate buffer was added after 3 hr. Uptake of 1 *N* NaOH was 3.97 ml. in 46 hr., 50% reaction, the hydrolysis then proceeding at about one quarter of the initial rate. The suspension was extracted with ether, the extract was washed with water, dried, and concentrated, leading to a residue, 0.79 g., 88% yield. This was distilled, leading to ethyl α-benzoyloxypropionate, 0.48 g. (2.2 mmoles), 53% yield, b.p. 93–95° (<1 mm.), α_{obsd} +0.55°, *c* 5.26 in $CHCl_3$, $[\alpha]^{22}D$ +10.45°. The infrared spectrum in chloroform was identical with that of the starting ester. The water layer, after extraction, was brought to pH 2 with concentrated sulfuric acid and lyophilized. The residue was extracted with acetone and the acetone was evaporated, leaving a crude residue, 1.12 g. This was extracted with benzene, and the benzene was concentrated, leading to α-benzoyloxypropionic acid, m.p. 82–83°, from benzene–petroleum ether, 0.30 g. (1.54 mmoles), 38% yield, α_{obsd} −0.97°, *c* 4.93 in chloroform, $[\alpha]^{22}D$ −19.7°.

Anal. Calcd. for $C_{10}H_{10}O_4$: C, 61.9; H, 5.6. Found: C, 61.8; H, 5.2 (by C. Fitz).

Kinetics of hydrolysis of D-(−)-, L-(+)-, and DL-ethyl α-benzoyloxypropionates by α-chymotrypsin were carried out in 4:1 water–ethanol. The substrate was dissolved in 11 ml. of 0.1 *M* NaCl and 4 ml. of ethanol with magnetic stirring under nitrogen in a pH stat. The rate of nonenzymatic hydrolysis was determined as pH was maintained at 7.8, 0.1 *N* NaOH being delivered from an automatic pipette. α-Chymotrypsin, 0.040 g. in 5 ml. of water, was added, the pH was readjusted, and the initial pseudo-zero-order rates of enzymatic hydrolysis were determined. Corrections were applied in each experiment for the nonenzymatic rates, 4–7% for the D-(−)-ester, 1–3% for the DL-ester. The rates of nonenzymatic hydrolysis of the L-(+)-ester were from one-half to as great as the enzymatic rates.

Hydrolysis of DL-Ethyl α-Acetoxy-β-phenylpropionate by α-Chymotrypsin.—A suspension of 0.516 g. (2.32 mmoles) of the ester in a solution of 0.205 g. of α-chymotrypsin in 20 ml. of 0.1 *M* NaCl was allowed to react at pH 7.8 in a pH stat at 25° under nitrogen. After 1 hr. consumption of alkali was proceeding at about one-third its initial rate, 1.21 ml. of 1 *N* NaOH having been consumed, 52% reaction. The suspension was extracted with ether, and the extract was dried and concentrated, leading to 0.1 g. of the ester, α_{obsd} +0.06, 5% in chloroform. The extracted aqueous layer was brought to pH 2 with hydrochloric acid and lyophilized. The residue was extracted with acetone, leading to 0.3 g. of crude α-acetoxy-β-phenylpropionic acid, α_{obsd} −0.3°, 11% in chloroform. Infrared spectra of the isolated ester and acid were identical with those of the synthesized inactive materials.

Kinetics of hydrolysis of L- and DL-ethyl α-acetoxy-β-phenylpropionate by 0.5 mg./ml. of α-chymotrypsin were studied in 4:1 water–ethanol, as described for the hydrolysis of ethyl α-benzoyloxypropionate. Corrections were applied in each experiment for the nonenzymatic rates, which were 1–3% of the enzymatic rates for L-(−)-compound, 4–7% for the DL-material.

Elemental analyses were by Schwarzkopf Microanalytical Laboratories except where otherwise indicated.

Acknowledgment.—Optical rotatory dispersion measurements were carried out by Miss Carole Lindblow of the Department of Biochemistry.

COMMUNICATIONS TO THE EDITOR

An Identity of the Rates of Deacylation of Nonionic Acyl-α-chymotrypsins and Acyl-trypsins[1]

Sir:

The enzymes α-chymotrypsin and trypsin appear to catalyze the nucleophilic reactions of carboxylic acid derivatives by identical mechanisms. Both reactions follow eq. 1[2] where ES is the enzyme-substrate complex, ES′ is the acyl-enzyme intermediate, P_1 is the

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

leaving group of the substrate, and P_2 is the carboxylic acid. Acyl-enzyme intermediates have been isolated from reactions catalyzed by both enzymes[3] and have been characterized as serine esters for both enzymes.[4] Both acyl-enzymes are partitioned by methanol or water in identical fashion.[5] The effect of deuterium oxide is identical for reactions catalyzed by both enzymes.[5] The individual acylation (k_2) and deacylation (k_3) steps of both enzymes may be characterized as nucleophilic reactions. Both chymotrypsin and trypsin acylations are dependent on two groups, one with a pK_a of 7 and the other with a pK_a of 9 or 10. Both deacylations are dependent on one group with a pK_a of 7 (Fig. 1). The ubiquitous group of pK_a 7 is presumably the imidazole group of a histidine moiety.[6] Thus all major mechanistic criteria point to a parallelism between these enzymes.

α-Chymotrypsin and trypsin show differences in binding constants. α-Chymotrypsin binds substances such as indole and N-acetyl-L- (or D-) tryptophan ethyl ester about 15-fold more effectively than does trypsin. Further, trypsin binds substances containing cationic groups such as benzylammonium ion and benzoyl-L-argininamide 5- to 22-fold more effectively than does α-chymotrypsin. Thus the binding constants define the specificity usually associated with α-chymotrypsin and trypsin.

Although very little data on acylation rate constants exist, a considerable amount of data on deacylation rate

(1) This research was supported by research grants from the National Institutes of Health; presented in part at the Sixth International Congress of Biochemistry, New York, N. Y., July, 1964.

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(5) Unpublished observations from this laboratory.

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TABLE I

RATE CONSTANTS OF THE DEACYLATION OF SOME ACYL-α-CHYMOTRYPSINS AND ACYL-TRYPSINS[a,f,i]

Acyl group	pH	k_3, sec.$^{-1}$ α-Chymotrypsin	k_3, sec.$^{-1}$ Trypsin	k(α-chymotrypsin)/k(trypsin)
Indoleacryloyl-[b]	8.8	0.0019	0.0036	0.53
Furylacryloyl-[b]	8.8	0.0025	0.0019	1.3
Acetyl-[b,g]	8.8	0.0068	0.0099	0.70
Cinnamoyl-[b]	8.8	0.0125	0.0169	0.74
N-Acetyl-L-leucyl-[d]	4.46	0.0918	0.123	0.75
	5.02	0.256	0.192	1.3
	6.10	2.26	1.47	1.5
N-Acetyl-L-tryptophanyl-[d]	3.46	0.0438	0.068[c]	0.55
	7.00	30.5	31	0.99
N-Benzyloxycarbonyl-L-tyrosyl-[h]	3.21	0.0453	0.0470	0.97
	3.21		0.0354[c]	
	5.02	1.47	0.95	1.5
	5.02		0.65	
	6.10	13.32	9.0	1.5
	6.50	23.54	19.4	1.2
	6.50		16.5	1.4
	6.50		16.4[e]	1.4
	7.10	59.6	64.0	0.93
	7.10		28[c]	

[a] 25.0°; 0.8–1.6% (v./v.) acetonitrile–water; citrate, phosphate, Tris, or borate buffers; μ = 0.025 to 0.10. [b] These values correspond to k(lim.), the plateau of the sigmoid curve dependent on a group of pK_a 6.9 to 7.4. [c] Porcine trypsin. [d] The corresponding DL-p-nitrophenyl ester was used as substrate. [e] The trypsin used in this experiment was prepared by Dr. F. H. Carpenter by treatment of trypsin with the α-chymotrypsin inhibitor, L-1-tosylamido-2-phenylethyl chloromethyl ketone,[6] (shown as the square in Fig. 1). [f] Preliminary evidence indicates that a difference exists in the deacylations of the cationic benzoyl-L-arginyl enzymes. [g] Other data on the deacylation of acetyl-α-chymotrypsin and acetyl-trypsin show similar results.[8] [h] The k_{cat} of the α-chymotrypsin- and trypsin-catalyzed hydrolysis of N-acetyl-L-tyrosine ethyl ester is reported to be 7-fold different.[7] [i] The relative order of reactivity of p-nitrophenyl substrates is reported to be similar for thrombin, trypsin, and α-chymotrypsin.[9]

constants, k_3, is shown in Table I.[7–9] The determination of k_3 for the first four acyl-enzymes was carried out by preparing the acyl-enzyme and following the discrete first-order deacylation process. The rate of deacylation of the last three compounds was determined from the steady-state (zero-order) production of p-nitrophenol from the p-nitrophenyl ester. The deacylations of Table I, both slow[3] and fast (Fig. 1), depend on a group with a pK_a very close to 7 in its basic form. On this basis, the data of Table I were extrapolated to limiting rate constants (above pH 8), and it was found that the range of kinetic specificities in both the α-chymotrypsin and trypsin reactions, from an indoleacryloyl- to an N-benzyloxycarbonyl-L-tyrosyl reaction, is 10^5-fold.

The startling conclusion of Table I is that, from the slowest to the fastest deacylation, the rates of deacylation for these (nonionic) α-chymotrypsin and trypsin compounds are essentially identical (within 50%). Certainly, the 10^5-fold range of rate constants means that the enzyme is determining the kinetic specificity of this series of reactions. Electronic differences between the various acyl groups could account for perhaps 10-fold of this 10^5-fold range. But the interaction of the various acyl groups with the two enzymes must explain the other 10^4-fold range. This interaction is obviously the same for both α-chymotrypsin and trypsin.

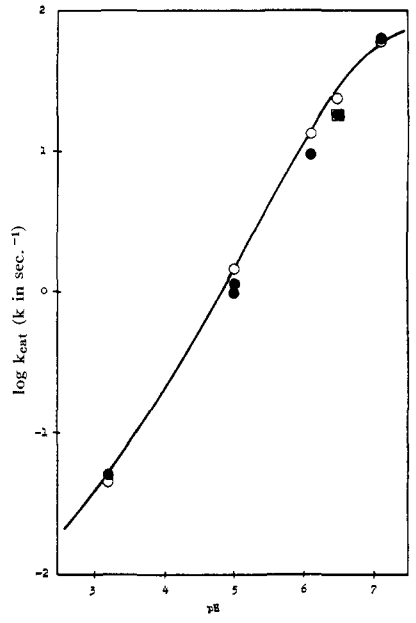

Fig. 1.—The pH dependence of the catalytic rate constant (deacylation) of the α-chymotrypsin-catalyzed hydrolysis of N-benzyloxycarbonyl-L-tyrosine p-nitrophenyl ester, ○, and of the trypsin-catalyzed hydrolysis of this ester, ●. The solid line is the theoretical line for the α-chymotrypsin reaction using pH − log $\alpha/(1-\alpha)$ = pK_{int} − 0.868ωZ.

Acknowledgment.—The authors gratefully acknowledge the assistance of Mr. C. R. Gunter and Dr. J. Feder.

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RECEIVED AUGUST 31, 1964

The Active Site of Chymotrypsin[1]

Sir:

The usual approach to the elucidation of the active site of an enzyme has been to probe the individual groups of the site using specific reagents[2] or to probe the topography of the site by the collection of a large amount of data which can be analyzed as a template reflection of the site.[3] The present report presents a different approach to this problem, based on a comparison between two related enzymes.

The previous communication[4] indicated a very broad identity in deacylation rate constants of nonionic acyl-α-chymotrypsins and acyl-trypsins over a 10^5-fold range in kinetic specificity. These identical deacylation rate constants demand identical gross mechanisms, such as identical serine hydroxyl and imidazole groups. *In addition, this kinetic identity demands an identical interaction of the various acyl groups with identical specificity sites of both enzymes,* since the 10^5-fold range of rates reflects to a large measure the specificity imposed by the

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