anol and 720 ml. of water was adjusted to *p*H 8.0 with 1.0 *N* aqueous sodium hydroxide, 100 mg. of α-chymotrypsin added and the *p*H of the reaction mixture maintained at *p*H 8.0 by the addition of 1.0 *N* aqueous sodium hydroxide. After 30 minutes at 25° the reaction mixture was evaporated *in vacuo* to a volume of 150 ml. whereupon crude IV crystallized from the concentrate. Crude IV was recrystallized twice from aqueous ethanol to give 5.5 g. of IV, m.p. 95–97°, $[\alpha]^{25}D$ −24.8° (*c* 7% in ethanol).

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.6.

Acetyl-D-tyrosinamide (VI).—VI, m.p. 225–226°, $[\alpha]^{25}D$ −49.4° (*c* 0.9% in water) was prepared from IV in the same manner and in approximately the same yield as was II from III.

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222): C, 59.5; H, 6.4; N, 12.6. Found: C, 59.7; H, 6.6; N, 12.5.

Acetyl-DL-tyrosinamide (VII).—VII, optically inactive, m.p. 197–198°, was prepared from V in exactly the same way as was II from III and VI from IV.

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222): C, 59.5; H, 6.4; N, 12.6. Found: C, 59.4; H, 6.4; N, 12.5.

Mechanical Separation of Acetyl-D- and L-Tyrosinamides from Acetyl-DL-Tyrosinamide.—A preparation of VII, m.p. 197–198°, was recrystallized slowly from aqueous ethanol so as to give large well-defined single crystals. The m.p. of any single crystal was found to be 226–228°, which is the m.p. of either II or IV. The mixed m.p. of a single crystal isolated from the DL-mixture with II was found to be either 197–198° or 226–228°, depending upon whether the single crystal selected from the DL-mixture was VI or II.

Enzyme Experiments.—The only departure from the methods described earlier[4,28] was the use of 36–38% Merck and Co., Inc., reagent grade aqueous formaldehyde, adjusted to *p*H 8.0 with aqueous sodium hydroxide instead of with solid magnesium carbonate.

The precision of the measurements reported in this study was identical with that obtained previously.[4] Two enzyme preparations, both obtained from Armour and Company, were used in the present study. Of the constants summarized in Table I, those obtained in experiment no. 3 were with lot no. 70902, and all of the others with lot no. 90402. The initial conditions used for the evaluation of the kinetic constants described in this communication are summarized in Table III. It will be noted that a total of 53 experiments were performed exclusive of those required for the determination of the *p*H-activity curve and those required for the reactions which were allowed to proceed to near completion.

TABLE III

SUMMARY OF EXPERIMENTS USED FOR THE EVALUATION OF KINETIC CONSTANTS

S:I	[E][a]	No. of experiments at $[S]_0 = 10^{-3}$ molar × 5	10	15	20	30	40	*y*
II	0.204[b]	1	1	1	1	1	1	1[c]
II	.150[d]	1	1		1		1	
II	.139[b]		3	2	3	2	3	1,[e] 1[f]
II:I[g]	.139[b]			1	1	1	1	
II:I[h]	.139[b]			1	1	1	1	
II:IV[i]	.139[b]			1	1			1[j]
II:IV[k]	.139[b]			1	1	1	1	
II:IV[l]	.139[b]							1[m]
II:VI[i]	.150[d]		1		1		1	
II:VI[k]	.150[d]		1		1			1[n]
II:VI[o]	.150[d]		1		1		1	
II:VI[p]	.150[d]		1		1		1	

[a] Mg. protein-nitrogen per ml. [b] Lot no. 90402. [c] *y* = 25. [d] Lot no. 70902. [e] *y* = 8. [f] *y* = 13.3. [g] $[I]_0 = [P_1]_0 = 25 \times 10^{-3}$ *M*. [h] $[I]_0 = [P_1]_0 = 40 \times 10^{-3}$ *M*. [i] $[I]_0 = 5 \times 10^{-3}$ *M*. [j] *y* = 35. [k] $[I]_0 = 10 \times 10^{-3}$ *M*. [l] $[I]_0 = 15 \times 10^{-3}$ *M*. [m] *y* = 20.5. [n] *y* = 45. [o] $[I]_0 = 20 \times 10^{-3}$ *M*. [p] $[I]_0 = 40 \times 10^{-3}$ *M*.

(28) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

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[CONTRIBUTION NO. 1468 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin-Catalyzed Competitive Hydrolysis of Acetyl-L-tryptophanamide and Acetyl-L-tyrosinamide in Aqueous Solutions at 25° and *p*H 7.9[1]

BY ROBERT J. FOSTER AND CARL NIEMANN[2]

An investigation of the kinetics of the α-chymotrypsin-catalyzed competitive hydrolysis of acetyl-L-tryptophanamide and acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 has provided independent evidence that these two substrates are hydrolyzed to the corresponding acylated α-amino acids and ammonia *via* combination at the same catalytically active site of the enzyme molecule.

The question as to whether two or more substrates do or do not react at the same catalytically active site of an enzyme molecule can be answered if data are available relative to the reaction kinetics of systems containing the enzyme and substrates, the latter both singly and in competition. In view of the fact that data are now available for the kinetics of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tryptophanamide and of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9[3,4] the kinetics of the α-chymotrypsin-catalyzed competitive hydrolysis of these two substrates, at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, was investigated in order to determine whether the above substrates were hydrolyzed *via* combination at the same reactive site of the enzyme molecule.

For reasons given previously[3,4] the reaction system can be limited to enzyme, substrates and reaction products and the reactions can be formulated in terms of the classical intermediate enzyme–substrate complex theory,[5] *i.e.*

$$E_f + S_{1f} \underset{k_{2.1}}{\overset{k_{1.1}}{\rightleftharpoons}} ES_1 \xrightarrow{k_{3.1}} E_f + P_{1.1f} + P_{2f} \quad (1a)$$

$$E_f + S_{2f} \underset{k_{2.2}}{\overset{k_{1.2}}{\rightleftharpoons}} ES_2 \xrightarrow{k_{3.2}} E_f + P_{1.2f} + P_{2f} \quad (1b)$$

(1) Supported in part by a grant from Eli Lilly and Co.

(2) To whom inquiries regarding this article should be sent.

(3) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1541 (1951).

(4) D. W. Thomas, R. V. MacAllister and C. Niemann, *ibid.*, **73**, 1548 (1951).

(5) J. B. S. Haldane, "Enzymes," Longmans-Green, London, 1930.

where

$[E]$ = molar concentration of total enzyme
$[E_f]$ = molar concentration of free enzyme
$[S_1]$; $[S_2]$ = molar concentration of total respective substrates
$[S_{1f}]$; $[S_{2f}]$ = molar concentration of free respective substrates
$[ES_1]$; $[ES_2]$ = molar concentration of enzyme–substrate complexes
$[P_{1.1f}]$; $[P_{1.2f}]$; $[P_{2f}]$ = molar concentration of free hydrolysis products

Since in all of the experiments reported in this communication the extent of hydrolysis of either of the two substrates was less than 20%, the known inhibition of the hydrolytic reactions by the hydrolysis products[3,4] was justifiably ignored in the development of the rate expressions for the competitive hydrolysis of the two substrates.

For the case where all of the reactants possess unit activity it follows[3-5] that

$$[E_f][S_{1f}]k_{1.1} = [ES_1](k_{2.1} + k_{3.1}) \qquad (2a)$$

and

$$[E_f][S_{2f}]k_{1.2} = [ES_2](k_{2.2} + k_{3.2}) \qquad (2b)$$

With experimental conditions selected so that $[E_f] \doteq ([E] - [ES_1] - [ES_2])$, $[S_1] \doteq [S_{1f}]$ and $[S_2] \doteq [S_{2f}]$ it can be shown that when

$$\frac{d[ES_1]}{dt} \text{ and } \frac{d[ES_2]}{dt} \doteq 0$$

$$-\frac{d[S_1]}{dt} = \frac{V_1}{1 + \frac{K_{S_1}}{[S_1]} + \frac{K_{S_1}[S_2]}{K_{S_2}[S_1]}} \qquad (3a)$$

$$-\frac{d[S_2]}{dt} = \frac{V_2}{1 + \frac{K_{S_2}}{[S_2]} + \frac{K_{S_2}[S_1]}{K_{S_1}[S_2]}} \qquad (3b)$$

where

$$(k_{2.1} + k_{3.1})/(k_{1.1}) = K_{S_1}$$

and

$$(k_{2.2} + k_{3.2})/(k_{1.2}) = K_{S_2}$$

and $V_1 = k_{3.1}[E]$ as $[S_1] \to \infty$ and $V_2 = k_{3.2}[E]$ as $[S_2] \to \infty$. For $[S_T] = [S_1] + [S_2]$ and $-d[S_T]/dt = v_T$

$$\frac{1}{v_T} = \frac{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}}{V_1\frac{[S_1]}{K_{S_1}} + V_2\frac{[S_2]}{K_{S_2}}} + \frac{1}{[S_T]}\frac{[S_1] + [S_2]}{V_1\frac{[S_1]}{K_{S_1}} + V_2\frac{[S_2]}{K_{S_2}}} \qquad (4)$$

Equation 4 is similar in form to the classical equation ordinarily used for the evaluation of K_S,[6] *i.e.*

$$\frac{1}{v_0} = \frac{1}{V} + \frac{1}{[S]_0}\frac{K_S}{V} \qquad (5)$$

Therefore for a plot of $1/v_{T0}$ *versus* $1/[S_T]_0$ *i.e.*, for initial velocities and substrate concentrations it follows that as $[S_T]_0 \to \infty$ the intercept

$$V_T = \frac{V_1\frac{[S_1]}{K_{S_1}} + V_2\frac{[S_2]}{K_{S_2}}}{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}} \qquad (6)$$

the slope

$$\frac{d\left(\frac{1}{v_{T0}}\right)}{d\left(\frac{1}{[S_T]_0}\right)} = \frac{[S_1] + [S_2]}{V_1\frac{[S_1]}{K_{S_1}} + V_2\frac{[S_2]}{K_{S_2}}} \qquad (7)$$

(6) H. Lineweaver and D. Burk. THIS JOURNAL, **56**, 658 (1934).

the apparent Michaelis constant

$$K_{ST} = \frac{[S_1] + [S_2]}{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}} \qquad (8)$$

and the apparent rate constant

$$k_{3T} = \frac{V_T}{[E]} = \frac{k_{3.1}\frac{[S_1]}{K_{S_1}} + k_{3.2}\frac{[S_2]}{K_{S_2}}}{\frac{[S_1]}{K_{S_1}} + \frac{[S_2]}{K_{S_2}}} \qquad (9)$$

From equations 8 and 9 it is seen that the reciprocal of the apparent Michaelis constant K_{ST} is the weighted average of the reciprocals of the individual values of K_{S_1} and K_{S_2} and that the apparent rate constant k_{3T} is dependent not only upon the values of $k_{3.1}$ and $k_{3.2}$ but also upon the values of $[S]_0/K_S$ for the respective substrates. The apparent K_{ST} and k_{3T} values for the α-chymotrypsin-catalyzed competitive hydrolysis of acetyl-L-tryptophanamide and acetyl-L-tyrosinamide at 25° and *p*H 7.9 were determined experimentally for two different ratios of the competing substrates and these data are given in Table I along with the previously determined kinetic constants of each of the above substrates and those calculated for the competitive case from equations 8 and 9. Because of uncertainties in respect to the equivalent weight of α-chymotrypsin the concentration of the enzyme is given in mg. of protein-nitrogen per ml.

TABLE I

α-CHYMOTRYPSIN-CATALYZED COMPETITIVE HYDROLYSIS OF ACETYL-L-TRYPTOPHANAMIDE AND ACETYL-L-TYROSINAMIDE[a]

Mole % acetyl-L-tryptophanamide	Mole % acetyl-L-tyrosinamide	K_S[b] Exptl.	K_S[b] Calcd.	k_3[c] Exptl.	k_3[c] Calcd.
100	0	5.3[d]		0.50[d]	
50	50	9.0	9.1	0.86	0.78
25	75	13.8	13.9	1.3	1.2
0	100	30.5[e]		2.4[e]	

[a] In aqueous solutions at 25° and *p*H 7.9. [b] In units of 10^{-3} *M*. [c] In units of 10^{-3} *M*/min./mg. protein-nitrogen/ml. [d] *Cf.* ref. 3. [e] *Cf.* ref. 4.

It will be noted that the experimental values for k_{3T} are slightly greater than the calculated values. This discrepancy is not unexpected and arises from the difficulty of estimating the initial velocities from either zero or first order plots of the total substrate concentration *versus* time when the ratio of the two substrates is changing with time. From equations 3a and 3b it follows that

$$\frac{v_2}{v_1} = \frac{V}{V_1}\frac{K_{S_1}[S_2]}{K_{S_2}[S_1]} \qquad (10)$$

and for the system under consideration that the ratio of the rate of hydrolysis of acetyl-L-tryptophanamide to that of acetyl-L-tyrosinamide is 1.2 times the ratio of their concentrations. However, for all values of $[S_T]$ encountered in the present investigation the total rate of hydrolysis increased as the ratio of acetyl-L-tryptophanamide to acetyl-L-tyrosinamide decreased as will be seen from the data given in Table II. Therefore, because of the contributions arising from a change in the ratio of the substrate concentrations, the experimental value of k_{3T} will be subject to a positive error and

TABLE II

THE RELATIVE VELOCITIES OF HYDROLYSIS OF ACETYL-L-TRYPTOPHANAMIDE AND ACETYL-L-TYROSINAMIDE[a]

	Acetyl-L-tryptophanamide		Acetyl-L-tyrosinamide		Total relative velocity
$[S_T]_0$[b]	% of $[S_T]_0$	% of v_{T0}	% of $[S_T]_0$	% of v_{T0}	
20	25	29	75	71	1.26
60	25	29	75	71	1.71
20	50	55	50	45	1.00
60	50	55	50	45	1.25

[a] Calculated from equation 16 for the extreme limits of concentration encountered in the competitive case. [b] In units of 10^{-3} M.

will be found to be greater than the true value. It is unfortunate that the limitations of present experimental methods do not permit the use of the ideal procedure wherein k_{3T} is determined under conditions where there is no change in the initial ratio of the concentration of the competing substrates.

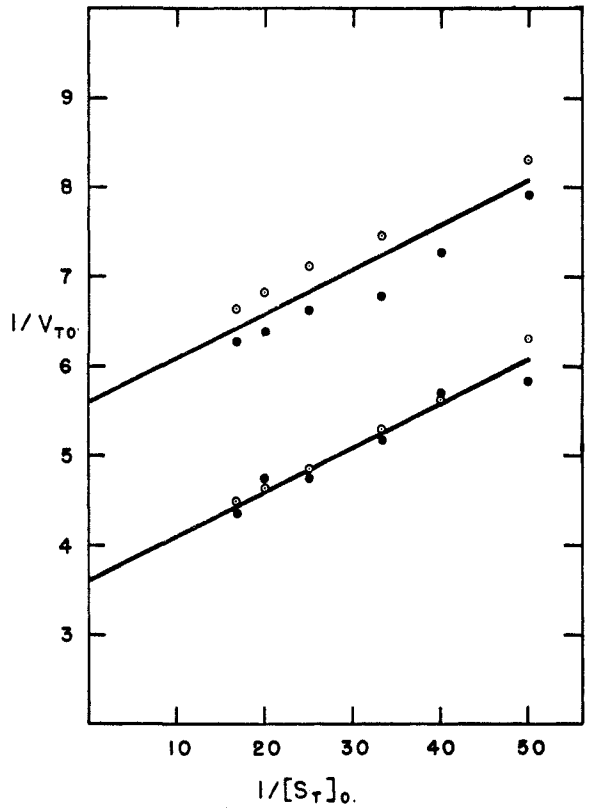

Fig. 1.—$[S_T]_0$ in units of 10^{-3} M; v_{T0} (initial velocities) in units of 10^{-3} M per min.; upper plot 50 mole % of acetyl-L-tryptophanamide and 50 mole % of acetyl-L-tyrosinamide; lower plot 25 mole % of acetyl-L-tryptophanamide and 75 mole % of acetyl-L-tyrosinamide; solid and open circles represent two series of determinations.

From the data presented in this communication there can be little doubt that acetyl-L-tryptophanamide and acetyl-L-tyrosinamide are hydrolyzed *via* combination at the same catalytically active site and it appears reasonable to assume, in the absence of definite proof, that other substrates of the general formula $RCONHCHR_1CONH_2$, where R_1 = a β-indolylmethyl group or a p-hydroxybenzyl group, will behave similarly. It will be noted that the above conclusions derived from kinetic studies are in accord with the independent observation that α-chymotrypsin probably possesses but one catalytically active site per molecule.[7,8]

The authors wish to express their appreciation of the assistance rendered by Dr. H. T. Huang during the course of this investigation.

Experimental[9]

Acetyl-L-tryptophanamide (I).[3]—Ammonolysis of 10 g. of acetyl-L-tryptophan methyl ester gave 8.0 g. of I, m.p. 190–191°, after recrystallization from water and drying *in vacuo* over phosphorus pentoxide. A mixed m.p. of I with an authentic specimen of I[3] showed no depression; $[\alpha]^{25}D$ +20.5 ± 0.5° (c, 2% in methanol).

Acetyl-L-tyrosinamide (II).[4]—Ammonolysis of 10 g. of acetyl-L-tyrosine ethyl ester gave 4.1 g. of II, m.p. 225.5–226° after recrystallization from methanol and drying *in vacuo* over phosphorus pentoxide. The m.p. of II was not depressed when mixed with an authentic specimen of II[4]; $[\alpha]^{25}D$ 50.7 ± 0.5° (c, 0.8% in water).

Procedure.—The methods used have been described previously.[3,10] The α-chymotrypsin was an Armour preparation (lot no. 90402) and in all experiments the enzyme concentration was maintained at 0.208 mg. of protein nitrogen per ml., a concentration leading to a maximum value of E'_8 of 0.91×10^{-2} thus placing all systems in zone A of Straus and Goldstein.[11,12] All experiments were conducted at 25° and pH 7.9 in aqueous solutions 0.02 M in respect to a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. The reduced primary data are summarized in Fig. 1.

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(7) E. Jansen, M. D. Fellows-Nutting, P. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949).

(8) E. Jansen, M. D. Fellows-Nutting and A. K. Balls, *ibid.*, **179**, 201 (1949).

(9) All melting points are corrected.

(10) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

(11) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

(12) A. Goldstein, *ibid.*, **27**, 529 (1944).