Anal. Calcd. for $C_7H_6N_2O$: C, 62.67; H, 4.51; N, 20.89. Found: C, 62.49; H, 4.89; N, 21.02.

1H-Pyrrolo[2,3-b]pyrid-6(7H)-one (XI).—The acid-insoluble diacetyl derivative (1.052 g.) was dehydrogenated by the method used for VI. In this case only polymeric materials were obtained on extraction of the catalyst with ethanol. The Dowtherm–benzene layer was extracted with four 30-ml. portions of *concentrated* hydrochloric acid, in this preparation, and the acid was cooled and neutralized with ammonium hydroxide. Evaporation at low temperature, extraction of the dried residue with acetonitrile and evaporation of this solvent left a sticky solid which, after washing with water and drying, weighed 256 mg. (40%) and melted at 208.5–213° dec. This crude material was purified for analysis by recrystallizations from acetonitrile (Darco) under nitrogen and sublimation at 150° (0.1 mm.). The white filaments had m.p. 226–226.5° dec. This substance, which is less stable than X, gives a dark-purple color with sodium nitroprusside and base, a dark purple color with ferric chloride and a violet color, apparently due to decomposition, with base alone. The ultraviolet maxima (ethanol solution) were found at 227 (log ε 4.17) and 332 mμ (3.93) and the minimum was found at 258 mμ (3.08). In the infrared, the compound absorbed at 3400, 1610 and 1650 cm.$^{-1}$. The last band is attributed to the pyridone carbonyl.

Anal. Calcd. for $C_7H_6N_2O$: C, 62.67; H, 4.51; N, 20.89. Found: C, 62.34; H, 4.54; N, 20.75.

Absorption Spectra.—Ultraviolet spectra were determined with a Beckman model DU quartz spectrophotometer from solutions of 10^{-4} to 5×10^{-5} *M* concentration. The solvent was cyclohexane unless otherwise specified. Infrared spectra were determined on a Baird spectrophotometer (KBr disk) by Dr. S. M. Nagy and associates at the Microchemical Laboratory, Massachusetts Institute of Technology.

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[CONTRIBUTION NO. 2376 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Interaction of α-Chymotrypsin with α-N-Carbethoxy-D- and L-tyrosinmethylamide[1]

BY DAVID T. MANNING AND CARL NIEMANN[2]

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It has been shown that the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide, in aqueous solutions at 25° and *p*H 7.6 and 0.27 *M* in the amine component of a THAM–HCl buffer, is competitively inhibited by α-N-carbethoxy-D- and L-tyrosinmethylamide and that the K_I values parallel the K_I and K_S values previously obtained for α-N-carbethoxy-D- and L-tyrosinamide. These observations have been interpreted as providing support for the contention that $K_S \doteq k_2/k_1$ for the system α-chymotrypsin-α-N-carbethoxy-L-tyrosinamide.

Where the dependence of the initial rate of an enzyme-catalyzed reaction upon the initial specific substrate concentration may be represented by equation 1 and where the constant $K_S = (k_2 + k_3)/k_1$ may be evaluated on the basis of equation

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_f \qquad (1)$$

2 the question frequently arises as to whether the value of K_S obtained for a particular system ap-

$$-d[S]/dt = d[P]/dt = k_3[E][S]/(K_S + [S]) \qquad (2)$$

proximates that of one of the two possible limits, *i.e.*, whether $K_S \doteq k_2/k_1$ or $K_S \doteq k_3/k_1$. When first faced with this question[3] it was realized that a general and unambiguous answer was beyond reach but that one arrived at on the basis of knowledge of the behavior of specific substrates and competitive inhibitors that were structurally similar could provide support for the contention that K_S did or did not approximate the limiting value given by k_2/k_1.[3] In the initial study[3] values of K_S obtained for systems involving α-chymotrypsin and acetyl- or nicotinyl-L-tryptophanamide were compared with K_I values obtained for comparable systems involving the competitive interaction of α-chymotrypsin with the hydrolysis products, *i.e.*, acetyl- or nicotinyl-L-tryptophanate ion, and the enantiomorphs of the above specific substrates, *i.e.*, acetyl-, or nicotinyl-D-tryptophanamide. Subsequently the same procedure was employed with respect to the interpretation of K_S values obtained for systems involving α-chymotrypsin and acetyl- or nicotinyl-L-tyrosinamide,[4,5] chloroacetyl- or trifluoroacetyl-L-tyrosinamide[6,7] and acetyl- or nicotinyl-L-phenylalaninamide.[8] In order to provide additional support for the contention that $K_S \doteq k_2/k_1$ in all of the systems referred to above, and particularly for the first pair, Huang and Niemann[9] turned to a comparison of the K_I values of enantiomorphic pairs of competitive inhibitors in which the L-isomer either did not lead to determinable reaction products with the analytical procedure employed or was hydrolyzed so slowly as to justify its evaluation as a competitive inhibitor rather than as a specific substrate. The pairs considered were acetyl-D- and L-tryptophanate ion, D-and L-tryptophanamide and acetyl-D- and L-tryptophanmethylamide.[9]

In all of the above cases the argument that $K_S \doteq k_2/k_1$ was based upon the supposition that when $K_S \doteq k_2/k_1$ the value of K_S will exhibit approximately the same dependence upon the nature of the specific substrate as is seen in the dependence of the value of K_I upon the nature of related competitive inhibitors, particularly when similar dependencies also are observed for comparable pairs of enantiomorphic competitive inhibitors. It is evident that the reliability of an interpretation of the above

(1) Supported in part by a grant from the National Institutes of Health, Public Health Service.

(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) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).

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

(7) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(8) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).

(9) H. T. Huang and C. Niemann, *ibid.*, **73**, 3223 (1951).

kind is dependent upon the extent to which structural similarity of specific substrate and competitive inhibitor can be achieved without actually producing a pair of specific substrates and the degree to which a given compound can be regarded as a competitive inhibitor rather than as a specific substrate.[10] In view of these considerations it follows that multiple correlations are desirable in order to maximize the possibility of a valid interpretation and to minimize the likelihood of a fortuitous result.

In all of the earlier examples in which the K_S value of an α-N-acylated amino acid amide type of specific substrate[13] was compared with the K_I value of the enantiomorphic competitive inhibitor,[7] it was found[14] that the ratio of K_{S_L}/K_{I_D} was either equal to or greater than unity, *e.g.*, for α-N-acetyl-L- and D-tryptophanamide K_{S_L}/K_{I_D} = 2.3 ± 0.6 and for α-N-acetyl-L- and D-tyrosinamide K_{S_L}/K_{I_D} = 2.8 ± 0.6.[15] Since the ratio of K_{I_L}/K_{I_D} observed for comparable pairs of enantiomorphic competitive inhibitors also was equal to or greater than unity,[14] *e.g.*, for α-N-acetyl-L- and D-tryptophanate ion K_{I_L}/K_{I_D} = 1.5 ± 0.5 and for α-N-acetyl-L- and D-tryptophanmethylamide K_{I_L}/K_{I_D} = 3.9 ± 1.5, it was concluded[9] that support had been provided for the contention that $K_S \doteq k_2/k_1$ for the above specific substrates.

In a recent communication[14] it was noted that the ratio K_{S_L}/K_{I_D} for systems involving α-chymotrypsin and α-N-carbethoxy-L- or D-tyrosinamide had a value of 0.30 ± 0.08, *i.e.*, clearly less than unity. While it was surmised that in this instance $K_S \doteq k_2/k_1$,[14] the fact that the corresponding K_{S_L}/K_{I_D} ratio was less than unity and qualitatively the inverse of the K_{S_L}/K_{I_D} and K_{I_L}/K_{I_D} ratios observed for all of the previously investigated α-N-acylated amino acid amides[14] could not be ignored. Therefore, it became necessary to determine whether or not a similar inversion could be observed with a pair of enantiomorphic competitive inhibitors containing an α-N-carbethoxy group and otherwise structurally related to the first pair. Since secondary amides other than peptides are hydrolyzed much more slowly in the presence of α-chymotrypsin than are the corresponding primary amides[9,16,17] it was decided to adopt the device used by Huang and Niemann[9] and to evaluate α-N-carbethoxy-L- and D-tyrosinmethylamide as competitive inhibitors in a system involving α-chymotrypsin.

In practice the above two compounds were evaluated against acetyl-L-tyrosinhydroxamide in aqueous solutions at 25° and *p*H 7.6 and 0.27 *M* in the amine component of a THAM[18]–HCl buffer. The reactions were followed by a modification of the procedure described by Hogness and Niemann[19] and the corrected initial velocities were evaluated by the method of Jennings and Niemann.[20,21] The values of K_I were obtained by a least squares fit to the linear relationship $([E]/v_0) = 1/k_3 + (K'_S/k_3)(1 + [S]_0)$[22] where $K'_S = K_S(1 + [I]/K_I)$. K_S was assumed to have a value of 43 ± 4 × 10^{-3} *M*.[23] The principal features and results of these experiments are summarized in Table I.

TABLE I

INHIBITION OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINHYDROXAMIDE BY α-N-CARBETHOXY-D- AND L-TYROSINMETHYLAMIDE[a]

Expt. no.	$[S]_0$[b]	v_0[c] Uncor.[d]	v_0[c] Cor.[e]	$1/V$[f]	K_I[b,g]
α-N-Carbethoxy-D-tyrosinmethylamide [I] = 10 × 10^{-3} *M*					
1	5.00	0.071	0.069		
2	6.02	.083	.081		
3	7.50	.100	.098		
4	7.50	.100	.098		
5	10.00	.133	.130		
6	15.00	.174	.170	1.40 ± 0.23	22.2 ± 1.6
α-N-Carbethoxy-D-tyrosinmethylamide [I] = 20 × 10^{-3} *M*					
7	5.00	0.052	0.052		
8	8.00	.082	.082		
9	15.00	.148	.145	1.40 ± 0.27	21.4 ± 0.9
α-N-Carbethoxy-L-tyrosinmethylamide [I] = 10 × 10^{-3} *M*					
10	5.00	0.054	0.053		
11	5.00	.052	.051		
12	6.02	.062	.062		
13	7.50	.077	.076		
14	10.00	.100	.098		
15	15.00	.141	.137	1.27 ± 0.32	10.5 ± 0.5
α-N-Carbethoxy-L-tyrosinmethylamide [I] = 20 × 10^{-3} *M*					
16	10.00	0.064	0.064		
17	15.00	.090	.089		
18	20.00	.120	.118		
19	30.00	.155	.154		
20	40.00	.205	.201	1.39 ± 0.37	9.1 ± 0.5

[a] In aqueous solutions at 25° and *p*H 7.6 and 0.27 *M* in the amine component of a THAM–HCl buffer with [E] = 0.0266 mg. protein-nitrogen per ml. or 0.755 × 10^{-5} *M* in monomeric α-chymotrypsin with an assumed molecular weight of 22,000 and a nitrogen content of 16.0%. [b] In units of 10^{-3} *M*. [c] Apparent first-order initial velocity in units of 10^{-3} *M* per min. [d] Estimated from plots of ln absorbancy *vs.* time. [e] Corrected initial velocity computed by the method of Jennings and Niemann.[20] [f] $1/V = 1/k_3[E]$, *i.e.*, the ordinate intercept of a $1/v_0$ *vs.* $1/[S]_0$ plot in units of 10^3 M^{-1} sec. [g] Evaluated by means of a least squares fit to the relation $([E]/v_0) = 1/k_3 + (K'_S/k_3)(1 + [S]_0)$ where $K'_S = K_S(1 + [I]/K_I)$ and K_S = 43 ± 4 × 10^{-3} *M*.

(10) The evaluation of a single compound both as a specific substrate and as a competitive inhibitor has been considered by Bernhard[11] who, without reference to previous studies[3-8] concluded that $K_S = k_2/k_1$ for the systems trypsin–benzoyl-L-argininamide and α-chymotrypsin–acetyl-L-phenylalaninamide on the basis of the numerical equality of the K_S and K_I values. The ambiguity inherent in Bernhard's argument, which is a restrictive case of the more general argument developed earlier,[3-8] has been pointed out by Ingraham.[12]

(11) S. A. Bernhard, THIS JOURNAL, **77**, 1973 (1955).

(12) L. L. Ingraham, *ibid.*, **79**, 666 (1957).

(13) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

(14) D. T. Manning and C. Niemann, *ibid.*, **80**, 1478 (1958).

(15) Attention is called to a typographical error in ref. 14. The second to the last line of the first paragraph of the text should read "... all other cases ..." rather than "... all cases ..."

(16) Unpublished observations made in these laboratories by Drs. H. T. Huang, B. M. Iselin, D. T. Manning, W. E. M. Lands and T. H. Applewhite.

(17) When examined under conditions where the extent of hydrolysis of the primary amide is extensive, it is difficult to determine whether the corresponding methylamide is or is not being hydrolyzed.

(18) Tris-(hydroxymethyl)-aminomethane.

(19) D. S. Hogness and C. Niemann, THIS JOURNAL, **75**, 884 (1953).

(20) R. R. Jennings and C. Niemann, *ibid.*, **75**, 4687 (1953).

(21) W. E. M. Lands and C. Niemann, *ibid.*, **77**, 6508 (1955).

(22) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).

(23) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

All four values of $1/V = 1/k_3[E]$ given in Table I are essentially identical and the mean value, *i.e.*, 1.35 ± 0.28 is in reasonable agreement with the value of 1.12 ± 0.07 computed on the basis of previously determined constants for the system containing only enzyme and specific substrate.[23] Therefore because of the coincidence, within the limits of experimental error, of all of the above values of $1/V$ we may conclude that the observed inhibition is competitive in nature. Furthermore, the data summarized in Table I leads to the conclusion that all values of K_I were obtained under conditions consistent with all assumptions inherent in the evaluation procedure.[7]

The values of K_S and K_I obtained for α-N-carbethoxy-D- and L-tyrosinamide and for α-N-carbethoxy-D- and L-tyrosinmethylamide are summarized in Table II. It will be seen from these data, and particularly from the ratios corresponding to K_{S_L}/K_{I_D} or K_{I_L}/K_{I_D}, that the pattern observed previously for the α-N-carbethoxytyrosinamide,[14] with the L-antipode functioning as a specific substrate and the D-antipode as a competitive inhibitor, is repeated in the case of the enantiomorphic α-N-carbethoxytyrosinmethylamides, with both antipodes functioning as apparent competitive inhibitors. Since this particular behavior is consistent with the interpretation that $K_S \doteq k_2/k_1$ for the system α-chymotrypsin-α-N-carbethoxy-L-tyrosinamide, we may conclude that support has been provided for the above interpretation of K_S in a case where the ratios K_{S_L}/K_{I_D} and K_{I_L}/K_{I_D} were qualitatively the inverse of those observed for the pairs α-N-acetyl-L- and D-tryptophanamide and α-N-acetyl-L- and D-tryptophanmethylamide, where it was concluded that $K_S \doteq k_2/k_1$ for the system α-chymotrypsin–α-N-acetyl-L-tryptophanamide.[9] Thus the ratio K_{S_L}/K_{I_D} for a particular L-specific substrate and its enantiomorphic D-competitive inhibitor is in itself of limited value in arriving at an estimate of whether $K_S \doteq k_2/k_1$ or $K_S \neq k_2/k_1$, but when it is compared with ratios of K_{I_L}/K_{I_D} observed for comparable enantiomorphic competitive inhibitors a more forceful decision may be reached.

Experimental[24,25]

α-*N*-Carbethoxy-D-tyrosinmethylamide.—A sirupy preparation of N-carbethoxy-D-tyrosine ethyl ester obtained from the enzymatic resolution of 75.0 g. of N-carbethoxy-DL-tyrosine ethyl ester[14] was dissolved in 250 ml. of absolute methanol, the solution cooled to 0–5°, saturated with gaseous methylamine, allowed to stand at room temperature for 70 hr. and evaporated *in vacuo* to give a sirup which rapidly crystallized in the form of fine colorless needles. This product was recrystallized twice from water to give 10.1 g. of α-N-carbethoxy-D-tyrosinmethylamide, m.p. 152.4–154.4°, $[\alpha]^{22}D$ −20.9° (*c* 5.0%, in methanol).

Anal. Calcd. for $C_{13}H_{18}O_4N_2$ (266): C, 58.6; H, 6.8; N, 10.5. Found: C, 58.6; H, 6.8; N, 10.4.

α-N-Carbethoxy-L-tyrosinmethylamide.—A solution of 40.5 g. of L-tyrosine ethyl ester in 300 ml. of absolute methanol was saturated at 0–5° with gaseous methylamine, the solution allowed to stand at room temperature for four days and evaporated *in vacuo* to give a sirup. The sirup was dissolved in 300 ml. of a mixture of acetone and ethyl acetate and the solution placed in a 1000-ml. Florence flask containing 300 ml. of saturated aqueous potassium carbonate. The biphasic system was cooled to 0–5° and 40.6 ml. of ethyl chlorocarbonate added in small portions over a 20 minute period with continuous shaking and cooling. The precipitate which formed on acidification was collected and repeatedly recrystallized from water to give 7.0 g. of α-N-carbethoxy-L-tyrosinmethylamide, colorless needles, m.p. 151.5–153.5° $[\alpha]^{22}D$ 19.2° (*c* 5.0%, in methanol).

Anal. Calcd. for $C_{13}H_{18}O_4N_2$ (266): C, 58.6; H, 6.8; N, 10.5. Found: C, 58.6; H, 6.9; N, 10.5.

Enzyme Experiments.—All experiments were conducted as described previously.[14] The α-chymotrypsin was an Armour preparation, lot no. 10705.

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TABLE II

SUMMARY OF VALUES OF K_S AND K_I FOR SYSTEMS INVOLVING α-CHYMOTRYPSIN AND THE ENANTIOMORPHIC α-N-CARBETHOXYTYROSINAMIDES AND METHYLAMIDES[a]

α-N-Carbethoxy-	K_S[b]	K_I[b]	K_L/K_D
L-Tyrosinamide	6.4 ± 0.5[c]		0.30 ± 0.08[g]
D-Tyrosinamide		21 ± 3[d]	
L-Tyrosinmethylamide		9.8 ± 1.2[e]	0.44 ± 0.08
D-Tyrosinmethylamide		22.7 ± 1.7[f]	

[a] In aqueous solutions at 25°. [b] In units of 10^{-3} *M*. [c] At *p*H 7.9 and 0.02 *M* in the amine component of a THAM–HCl buffer.[14] [d] Mean of value of 20.7 ± 2.9 determined at *p*H 7.9 and value of 17.3 ± 1.2 determined at *p*H 7.6 with former system 0.02 *M* in the amine component of a THAM–HCl buffer and the latter 0.27 *M* in the amine component.[14] [e] Mean of values of 10.5 ± 0.5 and 9.1 ± 0.5 determined for system at *p*H 7.6 and 0.27 *M* in the amine component of a THAM–HCl buffer. [f] Mean of values of 22.2 ± 1.6 and 21.4 ± 0.9 determined for system at *p*H 7.6 and 0.27 *M* in the amine component of a THAM–HCl buffer. [g] If based upon a value of K_{I_D} of 17.3 ± 1.2, *vide ante*, value would be 0.37 ± 0.05.

(24) All melting points are corrected.

(25) Microanalyses by Dr. A. Elek.