dissolved in 60 ml. of water and after gas evolution had ceased 3 ml. of acetic anhydride was added in portions and with shaking to the biphasic reaction mixture. When gas was no longer evolved the ethereal phase was collected, dried and evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml. of anhydrous methanol, the solution saturated at 0° with gaseous methylamine and allowed to stand at room temperature for several days. The reaction mixture then was evaporated to dryness *in vacuo* to give a crystalline residue which was recrystallized repeatedly from a mixture of ethyl acetate and methanol to give 0.68 g. of α-N-acetyl-L-tyrosinmethylamide, m.p. 191–192°.

Anal. Calcd. for $C_{12}H_{16}O_3N_2$ (236): C, 61.0; H, 6.8; N, 11.9. Found: C, 61.1; H, 6.7; N, 11.5.

α-N-Nicotinyl-L-tyrosinmethylamide.—To a solution of 2.5 g. of sirupy L-tyrosinmethylamide in 5 ml. of acetone and 4 ml. of pyridine was added 1.5 g. of crystalline nicotinyl azide, the amber colored solution allowed to stand overnight, evaporated to dryness *in vacuo*, the sirupy residue triturated with water to give a crystalline solid and the latter recrystallized twice from 95% ethanol to give 1.5 g. of α-N-nicotinyl-L-tyrosinmethylamide, m.p. 213–214°.

Anal. Calcd. for $C_{16}H_{17}O_3N_3$ (299): C, 64.2; H, 5.7; N, 14.0. Found: C, 64.0; H, 5.8; N, 13.9.

α-N-Benzoyl-L-tyrosinmethylamide.—A suspension of 2.3 g. of L-tyrosine methyl ester hydrochloride in 75 ml. of diethyl ether was shaken with 4.2 g. of sodium bicarbonate in 60 ml. of water. When the evolution of gas had ceased 2.4 g. of benzoyl chloride was added to the biphasic reaction mixture and it was shaken until no more gas was evolved. Sufficient diethyl ether was added to the reaction mixture to permit collection of the precipitated solid by filtration. The precipitate was washed with aqueous sodium bicarbonate and water and dried to give 1.93 g. of crude ester. An additional 0.24 g. was recovered from the ether phase. The crude ester was dissolved in 20 ml. of anhydrous methanol, the solution saturated at 0° with gaseous methylamine and allowed to stand at room temperature for 70 hours. The solution then was evaporated to dryness *in vacuo*, the solid residue recrystallized first from aqueous methanol and then from a mixture of methanol and ethyl acetate and dried to give 0.9 g. of α-N-benzoyl-L-tyrosinmethylamide, m.p. 211–212°.

Anal. Calcd. for $C_{17}H_{18}O_3N_2$ (298): C, 68.4; H, 6.1; N, 9.4. Found: C, 68.3; H, 6.1; N, 9.3.

α-N-Acetyl-L-tyrosinhydrazide.—This compound, m.p. 232–233° with dec., was prepared from acetyl-L-tyrosine methyl ester and hydrazine hydrate essentially as described previously.[29]

Anal. Calcd. for $C_{11}H_{15}O_3N_3$ (237): C, 55.7; H, 6.4; N, 17.7. Found: C, 55.8; H, 6.4; N, 17.6.

Enzyme Experiments.—The procedure employed has been described previously.[16,17,29] Other pertinent details are given in Table II. The α-chymotrypsin was an Armour preparation lot no. 10705.

(29) R. Lutwack, H. F. Mower and C. Niemann, THIS JOURNAL, **79**, 2179 (1957).

PASADENA, CALIFORNIA

[CONTRIBUTION NO. 2386 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Interaction of α-Chymotrypsin with a Series of α-N-Acetyl-α-amino Acid Methylamides[1]

BY THOMAS H. APPLEWHITE AND CARL NIEMANN[2]

RECEIVED SEPTEMBER 15, 1958

The α-N-acetyl-α-amino acid methylamides derived from glycine, L-alanine, L-isoleucine, L-methionine, L-proline, L-hydroxyproline, L-histidine, D- and L-phenylalanine, D- and L-tyrosine and D- and L-tryptophan have been examined with respect to their ability to inhibit the α-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester. The extent of interaction of the enzyme with the first seven compounds was insufficient to permit evaluation of the enzyme–inhibitor dissociation constants, beyond assignment of a lower limit for the magnitude of these constants, despite the fact that several of the analogous methyl or ethyl esters are known to be specific substrates of the enzyme. In contrast to α-N-acetyl D- and L-phenylalaninmethylamide, α-N-acetyl-D- and L-tyrosinmethylamide and α-N-acetyl-D-tryptophanmethylamide, which function as fully competitive inhibitors of the α-chymotrypsin catalyzed hydrolysis of both methyl hippurate and benzoyl-L-valine methyl ester, α-N-acetyl-L-tryptophanmethylamide does so only in the latter system. In the former, this methylamide appears to participate in the formation of ternary complexes, involving enzyme, specific substrate and inhibitor, that are capable of yielding reaction products, *i.e.*, its action is only partially competitive.

In previous studies conducted in these Laboratories[3–5] it has been shown that the methylamides of certain α-N-acyl-L-α-amino acids can be evaluated as apparent competitive inhibitors of α-chymotrypsin even though the corresponding amides are identifiable as specific substrates of this enzyme. Thus, it was possible for Lands and Niemann[5] to evaluate the dependence of the apparent enzyme–inhibitor dissociation constants of a series of α-N-acyl-L-tyrosinmethylamides upon the nature of the acyl moiety with less ambiguity than that inherent in a comparison of a series of specific substrates, *e.g.*, the corresponding amides. In the present study advantage has been taken of this technique to examine a series of α-N-acetyl-L-α-amino acid methylamides, and the D-enantiomorphs of several members of this series, with respect to their interaction with α-chymotrypsin. It was hoped that such a study, involving the compounds listed in Table I, would supply needed information as to the dependence of the enzyme–inhibitor dissociation constants upon the nature of the α-amino acid side chain and inferentially upon the characteristics of the combination process.

At the time this investigation was initiated the only system involving α-chymotrypsin that had been examined with the aid of a *p*H-stat was one in which methyl hippurate was used as the specific substrate.[6] Therefore, in order to take advantage of this experience and to place the proposed studies in proper perspective it was decided to re-evaluate α-N-acetyl-D-tryptophanamide as a competitive

(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**, 3223 (1951).

(4) D. T. Manning and C. Niemann, *ibid.*, **81**, 747 (1959).

(5) W. E. M. Lands and C. Niemann, *ibid.*, **81**, 2204 (1959).

(6) T. H. Applewhite, R. B. Martin and C. Niemann, *ibid.*, **80**, 1457 (1958).

TABLE I

PROPERTIES OF THE α-N-ACETYL-α-AMINO ACID METHYLAMIDES USED IN THE ENZYMATIC STUDIES

α-Amino acid	M.p.,[a] °C.	$[\alpha]^{25}D$, degree
Glycine	157.5–158.0	.....
L-Alanine	181.2–182.0[b]	−51.1[c]
L-Leucine	165.3–166.8[b]	−33.9[d]
L-Methionine	180.9–181.5[b]	−11.4[e]
L-Proline	104.5–105.0	−87.5[e]
L-Hydroxyproline	167.0–168.5	−60.9[e]
L-Histidine	243.0–244.5[f]	+ 4.5[d]
D-Phenylalanine	205.5–207.0	−21[e]
L-Phenylalanine	207.3–208.6	+19.4[e]
D-Tyrosine	192.0–192.5	−42.4[d]
L-Tyrosine	192.2–193.3	+44.2[d]
D-Tryptophan	185.0–186.0	−20[g]
L-Tryptophan	187.1–189.0	+22.9[e]

[a] Corrected. [b] In a sealed tube. [c] (*c* 2%, in ethanol). [d] (*c* 1%, in water). [e] (*c* 1%, in ethanol). [f] With decomp. [g] (*c* 0.5%, in methanol).

inhibitor of the α-chymotrypsin catalyzed hydrolysis of methyl hippurate using in this instance a *p*H-stat instead of the formol titration which was employed earlier.[7-9] These experiments are summarized in Table II. As before,[6] the initial velocities were determined by the empirical procedure of Booman and Niemann[10] and the kinetic constants evaluated by a least squares fit to the equation $([S]_0[E]/v_0) = (K_S'/k_3) + ([S]_0/k_3)$, where $K_S' = K_S(1 + [I]/K_I)$.

The agreement of the value of k_3 given in Table II with that observed in the absence of α-N-acetyl-D-tryptophanamide, *i.e.*, 2.97 ± 0.12,[6,11] is compatible with the conclusion that this compound can be considered as a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of methyl hippurate.[7] While the experimental uncertainty encountered in the present study is somewhat greater than that associated with the preferred value based upon previous investigations it will be noted that the two values of K_I, *i.e.*, 3.7 ± 1.5 and 2.4 ± 0.4,[8] are in agreement within the limits of experimental error. Therefore, it was concluded that the system α-chymotrypsin–methyl hippurate, when examined with the aid of a *p*H-stat, was suitable for the evaluation of the various α-N-acetyl-L-α-amino acid methylamides as competitive inhibitors.

Since Huang and Niemann[3] previously had evaluated α-N-acetyl-D- and L-tryptophanmethylamide as competitive inhibitors of the α-chymotrypsin catalyzed hydrolysis of α-N-nicotinyl-L-tryptophanamide, it was decided to initiate the present investigation by evaluating α-N-acetyl-L-tryptophanmethylamide as a competitive inhibitor of the α-chymotrypsin catalyzed hydrolysis of methyl hippurate under the conditions used for the evaluation of K_I for α-N-acetyl-D-tryptophanamide, *cf.* Table II. A series of experiments were performed with values of $[S]_0$ varying from 8 to 20 × 10^{-3} *M*, [E] = 0.150 mg. protein-nitrogen per ml. and [I] = 5.0 × 10^{-3} *M*. Proceeding as before a value of $K_I = 33$ was obtained. This was an unexpected result since Huang and Niemann[3] had obtained a value of $K_I = 4.8$ which was revised to 6.5 ± 1.5 by Foster and Niemann.[12] In order to be certain that the above result was not due to an artifact a second series of experiments were conducted as described in Table II. When these experiments were evaluated as before a value of $K_I = 24 \pm 4$ was obtained. Since α-N-acetyl-L-tryptophanmethylamide had been evaluated as an apparent competitive inhibitor it is of interest to note that the value of $k_3 = 3.09 \pm 0.22$ given in Table II is identical, within the limits of experimental error, with the value of $k_3 = 2.97 \pm 0.12$ obtained in the absence of added methylamide.[6] Furthermore, an examination of the parameters *p*, *q*, *r* and *I'*[5] revealed that in an operational sense the evaluation of the above compound as an apparent competitive inhibitor was fully justified, although a value of I' = 0.2 could not lead to a very precise value of K_I.[5] Thus, when faced with the fact that the value of K_I for α-N-acetyl-L-tryptophanmethylamide was 6.5 ± 1.5 when evaluated against α-N-nicotinyl-L-tryptophanamide and *ca.* four times this value when evaluated against methyl hippurate, it was decided to depart from our original plan to examine only the α-N-acetyl-L-α-amino acid methyl amides and to re-evaluate K_I for α-N-acetyl-D-tryptophanmethylamide,[3] this time using methyl hippurate as the specific substrate. It will be seen from the experiments summarized in Table II that the value of $K_I = 2.6 \pm 0.4$ obtained in the present study and evaluated against methyl hippurate is in reasonable agreement with the value of $K_I = 1.8 \pm 0.3$ obtained earlier[3,12] with α-N-nicotinyl-L-tryptophanamide as the specific substrate.

Since the data at hand indicated that the value of K_I for α-N-acetyl-L-tryptophanmethylamide was dependent upon and that of α-N-acetyl-D-tryptophanmethylamide was independent of the nature of the specific substrate present in the reaction system, it was decided to establish this point with greater certainty by evaluating both of these inhibitors against a third specific substrate. The two specific substrates used previously were α-N-nicotinyl-L-tryptophanamide[3] and methyl hippurate. Because the unexpected behavior had been encountered with the L-antipode and methyl hippurate, it was felt that more significant information could be obtained if the third specific substrate were more closely related to methyl hippurate than to α-N-nicotinyl-L-tryptophanamide. Since benzoyl-L-valine methyl ester had become available as a specific substrate,[13] it was decided to employ this compound even though it was antici-

(7) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634 (1952).

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

(9) An additional factor in the choice of α-N-acetyl-D-tryptophanamide was that this compound also had been shown to function as a competitive inhibitor in the α-chymotrypsin catalyzed hydrolysis of α-N-nicotinyl-L-tryptophanamide, α-N-acetyl-L-tyrosinamide, α-N-trifluoroacetyl-L-tyrosinamide, α-N-chloroacetyl-L-tyrosinamide and α-N-nicotinyl-L-tyrosinamide.[8]

(10) K. A. Booman and C. Niemann, THIS JOURNAL, **78**, 3642 (1956).

(11) All values of k_3 are in units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. and those of K_S and K_I in units of 10^{-3} *M*.

(12) R. J. Foster and C. Niemann, THIS JOURNAL, **77**, 3370 (1955).

(13) T. H. Applewhite, H. Waite and C. Niemann, *ibid.*, **80**, 1465 (1958).

TABLE II

PARTIAL SUMMARY OF ENZYMATIC STUDIES[a]

Exptl. series	Specific substrate	Inhibitor	k_3[b]	K'_S[c]	K_I[c]
1[d]	Bz-gly-OCH_3[e]	Ac-D-try-NH_2[f]	3.10 ± 0.20	10.2 ± 1.2	3.7 ± 1.5[g]
3[h]	Bz-gly-OCH_3	Ac-L-try-$NHCH_3$[i]	3.09 ± .22	8.0 ± 1.3	24 ± 4[g]
4[j]	Bz-gly-OCH_3	Ac-D-try-$NHCH_3$	3.06 ± .18	19.1 ± 1.4	2.6 ± 0.4[g]
5[k]	Bz-val-OCH_3[l]	Ac-D-try-$NHCH_3$	0.53 ± .03	5.2 ± 0.4	2.1 ± 1.2[m]
6[n]	Bz-val-OCH_3	Ac-L-try-$NHCH_3$	0.60 ± .08	7.2 ± 1.1	7.1 ± 2.9[m]
7[o]	Bz-gly-OCH_3	Ac-D-phe-$NHCH_3$[p]	3.28 ± .11	11.1 ± 0.6	5.9 ± 1.3[q]
8[q]	Bz-gly-OCH_3	Ac-L-phe-$NHCH_3$	2.95 ± .30	8.2 ± 1.7	25[q,r]
9[s]	Bz-gly-OCH_3	Ac-D-tyr-$NHCH_3$[t]	3.07	10	10[q,r]
10[u]	Bz-gly-OCH_3	Ac-L-tyr-$NHCH_3$	2.98 ± .18	8.6 ± 1.0	66 ± 38[q,r]
11[v]	Bz-val-OCH_3	Ac-D-phe-$NHCH_3$	0.55 ± .04	6.0 ± 0.4	9.6 ± 3.5[m]
12[w]	Bz-val-OCH_3	Ac-L-phe-$NHCH_3$	0.61 ± .15	5.6 ± 1.4	19[m,r]
13[x]	Bz-val-OCH_3	Ac-D-tyr-$NHCH_3$	.....	5.6[r]	15[m,r]
14[y]	Bz-val-OCH_3	Ac-L-tyr-$NHCH_3$	0.54 ± .06	4.6 ± 0.6	105[m,r]

[a] In aqueous solutions at 25.0 ± 0.1° and *p*H 7.90 ± 0.02 and 0.02 *M* in sodium chloride with [E] = 0.150 mg. protein-nitrogen per ml. of Armour preparation no. 90402. [b] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [c] In units of 10^{-3} *M*. [d] Eight experiments, *i.e.*, four duplicates with $[S]_0$ = 8, 12, 16 and 20 × 10^{-3} *M*, [I] = 2.0 × 10^{-3} *M*, extent of reaction of 22 to 46% and v_0, which varied from 2.03 to 3.20 × 10^{-4} *M*/min., evaluated from 2nd to 4th degree polynomials without correction for an enzyme blank.[6] [e] Benzoylglycine methyl ester, *i.e.*, methyl hippurate. [f] α-N-Acetyl-D-tryptophanamide. [g] Based upon a value of K_S = 6.6 ± 0.30 × 10^{-3} *M*.[6] [h] Twelve experiments, *i.e.*, four triplicates with $[S]_0$ = 8, 12, 16 and 20 × 10^{-3} *M*, [I] = 5.0 × 10^{-3} *M*, extent of reaction of 33 to 43% and v_0, which varied from 2.30 to 3.38 × 10^{-4} *M*/min., evaluated from 3rd and 4th degree polynomials without correction for an enzyme blank.[6] [i] α-N-Acetyl-L-tryptophanmethylamide. [j] Eight experiments, *i.e.*, four duplicates with $[S]_0$ = 8, 12, 16 and 20 × 10^{-3} *M*, [I] = 5.0 × 10^{-3} *M*, extent of reaction of 24 to 32% and v_0, which varied from 1.36 to 2.40 × 10^{-4} *M*/min., evaluated from 2nd and 3rd degree polynomials without correction for an enzyme blank.[6] [k] Twelve experiments with $[S]_0$ = 1.45 to 3.61 × 10^{-3} *M*, [I] = 0.50 × 10^{-3} *M*, extent of reaction of 14 to 18% and v_0, which varied from 1.73 to 3.31 × 10^{-5} *M*/min., evaluated from 2nd to 4th degree polynomials with correction for an enzyme blank.[13] [l] Benzoyl-L-valine methyl ester. [m] Based upon a value of K_S = 4.2 ± 0.4 × 10^{-3} *M*.[13] [n] Ten experiments with $[S]_0$ = 0.99 to 3.08 × 10^{-3} *M*, [I] = 5.0 × 10^{-3} *M*, extent of reaction of 14 to 18% and v_0, which varied from 1.08 to 2.65 × 10^{-5} *M*/min., evaluated from 1st to 3rd degree polynomials with correction for an enzyme blank.[13] [o] Six experiments with $[S]_0$ = 8.15 to 20.08 × 10^{-3} *M*, [I] = 4.0 × 10^{-3} *M*, extent of reaction of 22 to 36% and v_0, which varied from 2.11 to 3.25 × 10^{-4} *M*/min., evaluated from 2nd to 4th degree polynomials without correction for an enzyme blank.[6] [p] α-N-Acetyl-D-phenylalaninmethylamide. [q] Four experiments with $[S]_0$ = 7.97 to 20.01 × 10^{-3} *M*, [I] = 6.0 × 10^{-3} *M*, extent of reaction of 22 to 36% and v_0, which varied from 2.24 to 3.18 × 10^{-4} *M*/min., evaluated from 2nd and 3rd degree polynomials without correction for an enzyme blank.[6] [r] Reliable only as to order of magnitude. [s] Four experiments with $[S]_0$ = 7.87 to 19.9 × 10^{-3} *M*, [I] = 5.0 × 10^{-3} *M*, extent of reaction of 22 to 40% and v_0, which varied from 2.07 to 3.11 × 10^{-4} *M*/min., evaluated from 2nd and 3rd degree polynomials without correction for an enzyme blank.[6] [t] α-N-Acetyl-D-tryosinmethylamide. [u] Eight experiments with $[S]_0$ = 7.99 to 20.12 × 10^{-3} *M* [I] = 20 × 10^{-3} *M*, extent of reaction of 22 to 40% and v_0, which varied from 2.18 to 3.10 × 10^{-4} *M*/min., evaluated from 3rd and 4th degree polynomials without correction for an enzyme blank.[6] [v] Five experiments with $[S]_0$ = 1.97 to 2.98 × 10^{-3} *M*, [I] = 4.0 × 10^{-3} *M*, extent of reaction of 14 to 21% and v_0, which varied from 2.04 to 2.74 × 10^{-5} *M*/min., evaluated from 2nd and 3rd degree polynomials with correction for an enzyme blank.[13] [w] Seven experiments with $[S]_0$ = 0.97 to 2.48 × 10^{-3} *M*, [I] = 6.0 × 10^{-3} *M*, extent of reaction of 14 to 21% and v_0, which varied from 1.36 to 2.86 × 10^{-5} *M*/min., evaluated from 2nd and 3rd degree polynomials with correction for an enzyme blank.[13] [x] Three experiments with $[S]_0$ = 2.52 to 3.46 × 10^{-3} *M*, [I] = 5.0 × 10^{-3} *M*, extent of reaction of 14 to 20% and v_0, which varied from 2.51 to 3.09 × 10^{-6} *M*/min., evaluated from 2nd degree polynomials with correction for an enzyme blank.[13] [y] Six experiments with $[S]_0$ = 1.48 to 3.51 × 10^{-3} *M*, [I] = 10 × 10^{-3} *M*, extent of reaction of 14 to 20% and v_0, which varied from 2.03 to 3.63 × 10^{-5} *M*/min., evaluated from 2nd degree polynomials with correction for an enzyme blank.[13]

pated that its use would present difficulties and lead to results of lesser precision than those that might be obtained with another specific substrate. The experiments conducted with α-chymotrypsin and benzoyl-L-valine methyl ester and α-N-acetyl-D- and L-tryptophanmethylamide are summarized in Table II.

The results obtained in all of the experiments with α-N-acetyl-D- and L-tryptophanmethylamide are presented in Table III. It can be shown from the data given in Table II that in every case both antipodes can be considered as apparent competitive inhibitors in the sense used by Lands and Niemann.[5] Furthermore, if consideration were limited to the k_3 values which are listed in Table III, it might be concluded that in every case the inhibition was fully competitive in nature. However, considering all available information, it is clear that whereas α-N-acetyl-D-tryptophanmethylamide appears to function as a fully competitive inhibitor in all three of the systems investigated, this is not true for the L-antipode. The fact that the same K_I value was obtained for the L-antipode when evaluated against two trifunctional specific substrates and a different value was obtained when this inhibitor was evaluated against methyl hippurate, a bifunctional specific substrate, suggests that in the latter system the L-antipode and the bifunctional specific substrate form ternary complexes with the enzyme that are capable of yielding reaction products. Such ternary complexes apparently are not formed with the L-antipode and the trifunctional specific substrates nor with the D-antipode and the bi- and trifunctional specific substrates. A situation comparable to that of the L-antipode and methyl hippurate was encountered earlier in studies with the system α-chymotrypsin–methyl hippurate–indole,[6,14] wherein it was concluded that the latter three components formed ternary complexes capable of giving reaction products. While it is true that for this latter situation one expects the value of k_3, and also that of K_I, to vary with the concentration of the inhibitor,[6,14] it is possible that the magnitudes of these changes are within the limits of experimental error and that the more

(14) H. T. Huang and C. Niemann, THIS JOURNAL, **75**, 1395 (1953).

TABLE III

SUMMARY OF KINETIC CONSTANTS FOR SYSTEMS INVOLVING α-CHYMOTRYPSIN AND α-N-ACETYL-D- AND L-TRYPTOPHAN-METHYLAMIDE[a]

System	k_3[b]	K_S[c]	K_I[c]
α-N-Ni-L-tryNH$_2$[d]	0.45 ± 0.05	2.5 ± 0.2	.....
α-N-Ni-L-tryNH$_2$[d] + D-antipode	.....[g]	.....	1.8 ± 0.3
α-N-Ni-L-tryNH$_2$[d] − L-antipode	.....[g]	.....	6.5 ± 1.5
Bz-glyOCH$_3$[e]	2.97 ± 0.12	6.6 ± 0.3	.....
Bz-glyOCH$_3$[e] + D-antipode	3.06 ± .18	.....	2.6 ± 0.4
Bz-glyOCH$_3$[e] + L-antipode	3.09 ± .22	.....	24, 30
Bz-L-valOCH$_3$[f]	0.55 ± .04	4.2 ± 0.4	.....
Bz-L-valOCH$_3$[f] + D-antipode	.53 ± .03	.....	2.1 ± 1.2
Bz-L-valOCH$_3$[f] + L-antipode	.60 ± .08	.....	7.1 ± 2.9

[a] For aqueous systems at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer for studies with α-N-nicotinyl-L-tryptophanamide and 0.02 *M* in sodium chloride for studies with methyl hippurate and benzoyl-L-valine methyl ester. [b] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [c] In units of 10^{-3} *M*. [d] α-N-Nicotinyl-L-tryptophanamide. [e] Methyl hippurate. [f] Benzoyl-L-valine methyl ester. [g] K_I evaluated by a graphical procedure which indicated that the value of k_3 was identical within the limits of experimental error with that observed in the absence of the inhibitor.

sensitive criterion is the dependence of K_I upon the nature of the specific substrate.[15,16]

The fact that ternary complex formation is inferred for the ternary systems involving α-chymotrypsin, methyl hippurate and indole and α-chymotrypsin, methyl hippurate and α-N-acetyl-L-tryptophanmethylamide and is not apparent for the ternary systems involving the same enzyme and methyl hippurate and α-N-acetyl-D-tryptophanmethylamide, benzoyl-L-valine methyl ester and α-N-acetyl-D-tryptophanmethylamide and benzoyl-L-valine methyl ester and α-N-acetyl-L-tryptophanmethylamide establishes a significant characteristic of the catalytically active site of α-chymotrypsin. Namely, the features of this site are such that concomitant combination of α-N-acetyl-D-tryptophanmethylamide and methyl hippurate is excluded but that of α-N-acetyl-L-tryptophanmethylamide and methyl hippurate is permitted. However, the replacement of one of the two α-hydrogen atoms present in methyl hippurate by an isopropyl group with the production of a specific substrate of the L-configuration no longer permits concomitant combination with either α-N-acetyl-D or L-tryptophanmethylamide.

The results obtained with α-N-acetyl-D- and L-tryptophanmethylamide led us to investigate the behavior of the analogous phenylalanine and tyrosine derivatives. These experiments are described in Table II and a summary of all studies conducted with the three enantiomorphic pairs of α-N-acetyl-α-amino acid methylamides is given in Table IV.

(15) These observations call attention to the possibility that the characterization of a process as one involving fully competitive inhibition solely on the basis of the invariance of k_3 and the dependence of K_S' upon [I] may be deceptive and that the error may become apparent only when the value of K_I is found to be dependent upon the nature of the specific substrate.

(16) The conclusions contained in this paragraph are based upon the assumption that in every case zone A conditions are satisfied.[13] The validity of this assumption is apparent from an examination of the data given in Table II.

TABLE IV

SUMMARY OF THE KINETIC CONSTANTS FOR THE INHIBITION OF THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF METHYL HIPPURATE AND OF BENZOYL-L-VALINE METHYL ESTER BY THREE ENANTIOMORPHIC PAIRS OF COMPETITIVE INHIBITORS[a]

Inhibitor[b]	k_3[c]	K_S[d,e]	K_I[d]	I_1'[f]	*p.q.-r*[g]
Methyl hippurate					
None	2.97 ± 0.12	6.6 ± 0.3			
D-Tryptophan	3.06 ± .18	19.0 ± 1.4	2.6 ± 0.4	1.9	0.01
L-Tryptophan	3.09 ± .22	8.0 ± 1.3	24[h]	0 2	.01
D-Phenylalanine	3.28 ± .11	11.1 ± 0.6	5.9 ± 1.3	.7	.01
L-Phenylalanine	2.95 ± .30	8.2 ± 1.7	25[h]	.2	.01
D-Tyrosine	3.07	10.0	9 7[i]	.5	.01
L-Tyrosine	2.98 ± 0.18	8.6 ± 1.0	66 ± 38	.3	.01
Benzoyl-L-valine methyl ester					
None	0.55 ± 0.04	4.2 ± 0.4			
D-Tryptophan	53 ± .03	5.2 ± 0.4	2.1 ± 1.2	0.2	.01
L-Tryptophan	.60 ± .08	7.2 ± 1.1	7.1 ± 2 9	.7	.05
D-Phenylalanine	.55 ± .04	6.0 ± 0.4	9.6 ± 3.5	4	.02
L-Phenylalanine	.61 ± .15	5.6 ± 1.4	19[h]	.3	.03
D-Tyrosine		5.6	15[i]	.3	.02
L-Tyrosine	.54 ± .06	4.6 ± 0.6	105[h]	.1	.02

[a] In aqueous solutions at 25° and *p*H 7.9. [b] α-N-acetyl-α-amino acid methylamide. [c] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [d] In units of 10^{-3} *M*. [e] Value given is that of K_S' when inhibitor is present. [f] $I_1' = [I]/K_I$. [g] See ref. 5. [h] Reliable only as to order of magnitude. Value uncertain because of low value of I_1' and high value of K_S'. [i] Approximate value due to limited number of experiments.

It will be seen from Table IV that the K_I values for α-N-acetyl-L-phenylalaninmethylamide and α-N-acetyl-L-tyrosinmethylamide do not exhibit the dependence upon the nature of the specific substrate that was noted earlier for α-N-acetyl-L-tryptophanmethylamide. While it is true that several of the K_I values involved in the comparison are reliable only as to order of magnitude, it may be noted that the value for α-N-acetyl-L-tyrosinmethylamide determined against methyl hippurate, *i.e.*, 66 ± 38, is in agreement with the value of 61 ± 7 determined by Lands and Niemann[5] using α-N-acetyl-L-tyrosinhydrazide as the specific substrate. Thus, of the six members of the three enantiomorphic pairs that were examined, it appears that the only one that has the capability of forming ternary complexes with α-chymotrypsin and methyl hippurate is α-N-acetyl-L-tryptophanmethylamide and that the other five members appear to function only as fully competitive inhibitors with both the bi- and tri- functional specific substrates.

With regard to the absolute magnitude of the K_I values of the methylamides of α-N-acetyl-D- and L-tryptophan, D- and L-phenylalanine and D- and L-tyrosine, when all of these compounds function as fully competitive inhibitors, it can be seen from the data summarized in Table V that the K_I values of the D-antipodes appear to be identical, within the limits of experimental error, with those of the corresponding amides. In the case of the L-antipodes, in which the amides function as specific substrates,[17] it will be noted that the K_S and K_I value of α-N-acetyl-L-tryptophanamide and α-N-acetyl-L-tryptophanmethylamide are identical within the limits of experimental error whereas the K_I value of α-N-acetyl-L-tyrosinmethylamide is

(17) R. J. Foster and C. Niemann, THIS JOURNAL, 77, 1886 (1955).

approximately double that of the K_S value of α-N-acetyl-L-tyrosinamide. Because of the lack of precision inherent in the K_I value of α-N-acetyl-L-phenylalaninmethylamide, it is not possible to determine whether the phenylalanine derivatives follow the pattern of the tyrosine or of the tryptophan derivatives.

TABLE V

SUMMARY OF PREFERRED K_S AND K_I VALUES OF A SERIES OF α-N-ACETYL-D- AND L-α-AMINO ACID AMIDES AND METHYLAMIDES[a]

α-N-Acetyl-	K_S[b]	K_I[b]	Ref.
D-Tryptophanamide	.....	2.3 ± 0.4	11
D-Tryptophanmethylamide	.....	2.2 ± 0.5[c]	15
L-Tryptophanamide	5.0 ± 0.5	.....	20
L-Tryptophanmethylamide	.....	6.5 ± 1.5	15
D-Phenylalaninamide	.....	12 ± 3	11
D-Phenylalaninmethylamide	.....	8 ± 3[d]	..
L-Phenylalaninamide	31 ± 3	.....	20
L-Phenylalaninmethylamide	.....	20[e]	..
D-Tyrosinamide	.....	12 ± 2	11
D-Tyrosinmethylamide	.....	10[e]	..
L-Tyrosinamide	34 ± 2	.....	20
L-Tyrosinmethylamide	.....	61 ± 7	5

[a] In aqueous solutions at 25° and *p*H 7.9. [b] In units of 10^{-3} *M*. [c] Mean of values of 1.8 ± 0.3[12] and 2.6 ± 0.4, *cf.*, Table IV. [d] Mean of values of 5.9 ± 1.3 and 9.6 ± 3.5. [e] Order of magnitude only.

The remaining seven methylamides listed in Table I were examined with respect to their ability to inhibit the α-chymotrypsin catalyzed hydrolysis of benzoyl-L-valine methyl ester. However, with systems at 25° and *p*H 7.9 and 0.02 *M* in sodium chloride and with $[S]_0 = 3.5 \times 10^{-3}$ *M*, [I] = 50×10^{-3} *M* and [E] = 0.150 mg. protein-nitrogen per ml. of Armour preparation no. 90492 the values of v_0 that were obtained were identical, within the limits of experimental error, with those observed in the absence of added inhibitor. From these experiments, which were conducted in duplicate, it can be concluded that the K_I values of α-N-acetyl-glycinmethylamide, α-N-acetyl-L-alaninmethylamide, α-N-acetyl-L-leucinmethylamide, α-N-acetyl-L-methionmethylamide, α-N-acetyl-L-prolinmethylamide, α-N-acetyl-L-hydroxyprolinmethylamide and α-N-acetyl-L-histidinmethylamide are probably of the order of 250×10^{-3} *M* or greater and that these compounds are not useful competitive inhibitors of α-chymotrypsin. This conclusion was reinforced when it was noted that the enzyme blank, which invariably was diminished upon the addition of the methylamides of the α-N-acetylated aromatic α-amino acids, was not influenced by the addition of any of the above α-N-acylated aliphatic α-amino acid methylamides. It is noteworthy that whereas the K_I value of α-N-acetyl-L-leucinmethylamide was so large as to be indeterminable, acetyl-L-leucine methyl ester was hydrolyzed so rapidly that the reaction could not be followed with the *p*H-stat.[13]

Experimental[18,19]

α-N-Acetylglycinmethylamide.—Glycine ethyl ester hydrochloride, 22.5 g., was allowed to react with 19 ml. of acetic anhydride in the presence of 450 ml. of 1 *M* aqueous sodium bicarbonate and 200 ml. of ethyl acetate. The aqueous phase was saturated with sodium chloride, the ethyl acetate phase withdrawn, dried over magnesium sulfate and evaporated *in vacuo* to give 10.4 g. (45%) of crude methyl aceturate, m.p. 45.5–47.0°. Ten g. of the crude ester was dissolved in 100 ml. of methanol, the solution maintained at 0° during the addition of 46 g. of gaseous methylamine, the reaction mixture allowed to stand at 25° for 16 hr. and then concentrated *in vacuo* under a stream of nitrogen to give 8.1 g. (90%) of the crude methylamide. The crude product was recrystallized twice from a 16:1 mixture of ethyl acetate and ethanol and once from ethyl acetate to give α-N-acetylglycinmethylamide, m.p. 157.5–158.0°, lit.[20] m.p. 158°.

Anal. Calcd. for $C_5H_{10}O_2N_2$ (130): C, 46.1; H, 7.8; N, 21.5. Found: C, 46.0; H, 7.7; N, 21.5.

α-N-Acetyl-L-alaninmethylamide.—Application of the procedure of Brenner and Huber[21] to the reaction of 5.0 g. of L-alanine with 18 ml. of methanol and 4.5 ml. of redistilled thionyl chloride gave 7.62 g. (97.5%) of L-alanine methyl ester hydrochloride, m.p. 109.0–111.5°. Following an abortive attempt to acetylate the above ester hydrochloride with acetyl chloride by the procedure of Curtiss,[22] the desired acetyl-L-alanine methyl ester was obtained as a low melting solid in 86% yield through use of the procedure which Reihlen and Knöpfle[23] had employed for the acetylation of L-valine methyl ester. The crude ester, 6.6 g., was converted to the crude methylamide in 95% yield in the same manner as for the corresponding glycine derivative. The product so obtained was recrystallized twice from 200-ml. portions of toluene to give 2.3 g. of α-N-acetyl-L-alaninmethylamide, colorless needles, m.p. 181.2–182.0°, in a sealed tube, $[\alpha]^{25}D$ −51.1° (*c* 2%, in ethanol). In an open tube the compound rapidly sublimed at *ca.* 170°.

Anal. Calcd. for $C_6H_{12}O_2N_2$ (144): C, 50.0; H, 8.4; N, 19.4. Found: C, 50.1; H, 8.6; N, 19.4.

α-N-Acetyl-L-leucinmethylamide.—L-Leucine methyl ester hydrochloride, 18 g., prepared from L-leucine in 97% yield by the method of Brenner and Huber,[21] was suspended in benzene and heated with an excess of acetyl chloride for 4 hr. under refluxing conditions.[22] The resulting clear solution was extracted with three 35-ml. portions of 1 *N* aqueous hydrochloric acid and dried over magnesium sulfate. Removal of the solvent *in vacuo* gave a mobile oil which was taken up in methanol and treated with methylamine as described previously to give 12.9 g. of crude methylamide, m.p. 154–160°. The crude product was repeatedly recrystallized, with considerable loss, from ethyl acetate and from a toluene–hexane mixture to give α-N-acetyl-L-leucinmethylamide, m.p. 165.3–166.8° in a sealed tube, $[\alpha]^{25}D$ −33.9° (*c* 1%, in water).

Anal. Calcd. for $C_9H_{18}O_2N_2$ (186): C, 58.0; H, 9.7; N, 15.0. Found: C, 58.1; H, 9.8; N, 15.1.

α-N-Acetyl-L-methioninmethylamide.—A solution of 5.0 g. of acetyl-L-methionine methyl ester[13] and 28.5 g. of methylamine in 100 ml. of absolute methanol was allowed to stand at room temperature for two days, the solvent removed *in vacuo* and the solid residue recrystallized twice from ethyl acetate to give 3.0 g. of α-N-acetyl-L-methioninmethylamide, m.p. 180.9–181.5° in a sealed tube, $[\alpha]^{25}D$ −11.4° (*c* 1%, in ethanol). When heated in an open tube the compound sublimed at temperatures above 160°.

Anal. Calcd. for $C_8H_{16}O_2N_2S$ (204): C, 47.0; H, 7.9; N, 13.7. Found: C, 46.8; H, 8.0; N, 13.5.

α-N-Acetyl-L-prolinmethylamide.—Ten grams of L-proline was acetylated with acetic anhydride in the presence of 2 *N* aqueous sodium hydroxide as directed by du Vigneaud and Meyer[24] and the crude product recrystallized from water to give 7.4 g. of acetyl-L-proline, m.p. 114–116°. This latter material was esterified with methanol and thionyl chloride[21] and the oily product, obtained in 78% yield, dissolved in methanolic methylamine and the crude methylamide prepared as described above. The crude product was recrys-

(18) All melting points are corrected unless otherwise noted.

(19) Microanalyses by Dr. A. Elek.

(20) S. Mizushima, T. Shimanouchi, M. Tsuboi, T. Sugita, E. Kato and E. Kondo, *ibid.*, **73**, 1330 (1951).

(21) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

(22) T. Curtiss, *J. prakt. Chem.*, [2] **94**, 85 (1916).

(23) H. Reihlen and L. Knöpfle, *Ann.*, **523**, 199 (1936).

(24) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295 (1932).

tallized three times from a mixture of toluene and hexane and three times from ethyl acetate to give α-N-acetyl-L-prolinmethylamide, large colorless bipyramids, m.p. 104.5–105.5°, $[\alpha]^{25}D$ −87.5° (*c* 1%, in ethanol).

Anal. Calcd. for $C_8H_{14}O_2N_2$ (170): C, 56.5; H, 8.3; N, 16.5. Found: C, 56.3; H, 8.3; N, 16.4.

α-N-Acetyl-L-hydroxyprolinmethylamide.—L-Hydroxyproline, 10 g., was stirred for 2 hr. at room temperature in 150 ml. of glacial acetic acid containing 10.5 ml. of acetic anhydride as directed by Kolb and Toennies.[25] A trace of undissolved solid was removed by filtration and the filtrate concentrated *in vacuo* to give an oil which solidified when triturated with 75 ml. of anhydrous ethyl ether. The solid was collected, washed with three 25-ml. portions of anhydrous ethyl ether and dried *in vacuo* over calcium chloride and sodium hydroxide. A 3.5-g. portion of the crude acetyl-L-hydroxyproline so obtained was esterified by reaction with diazomethane as described by Neuberger.[26] The oily ester was allowed to react with methanolic methylamine as described previously to give the crude methylamide in *ca.* 95% yield based upon crude acetyl-L-hydroxyproline. The crude methylamide was recrystallized twice from a mixture of 9 ml. of ethanol and 18 ml. of ethyl acetate to give α-N-acetyl-L-hydroxyprolinmethylamide, clusters of hexagonal rods which when air-dried sintered at *ca.* 130°. This product was dried *in vacuo* at 68° to give an anhydrous product, m.p. 167.0–168.5°, $[\alpha]^{25}D$ −60.9° (*c* 1%, in ethanol).

Anal. Calcd. for $C_8H_{14}O_3N_2$ (186): C, 51.6; H, 7.6; N, 15.0. Found: C, 51.7; H, 7.6; N, 15.1.

α-N-Acetyl-L-histidinmethylamide.—Anhydrous L-histidine, m.p. 284–286° with decomp., prepared from the hydrochloride according to Pyman[27] was acetylated by the method of Bergmann and Zervas.[28] From 5 g. of the free amino acid there was obtained 5.8 g. of acetyl-L-histidine monohydrate, m.p. 164.5–167.0° with decomp. The procedure used earlier for the preparation of acetyl-L-methioine methyl ester was adapted to the esterification of 4.2 g. of acetyl-L-histidine monohydrate by adding sufficient additional thionyl chloride to react with the water of hydration. The oily product so obtained was converted into the crude methylamide as described previously except that in this case the solid methylamide which separated from the reaction mixture was collected, repeatedly washed with methanol and air-dried to give 2.48 g. of product. Two recrystallizations from methanol gave α-N-acetyl-L-histidinmethylamide, fine needles, m.p. 243.0–244.5° with decomp., $[\alpha]^{25}D$ 4.5° (*c* 1%, in water).

Anal. Calcd. for $C_9H_{14}O_2N_4$ (210): C, 51.4; H, 6.7; N, 26.7. Found: C, 51.5; H, 6.7; N, 26.5.

α-N-Acetyl-D-phenylalaninmethylamide.—Acetyl-D-phenylalanine methyl ester, 2.2 g., prepared as described previously[29] was treated with methanolic methylamine in the usual manner and the reaction product recrystallized twice from water to give α-N-acetyl-D-phenylalaninmethylamide, long colorless needles, m.p. 205.5–207.0°, $[\alpha]^{25}D$ −21.0° (*c* 1%, in ethanol).

Anal. Calcd. for $C_{12}H_{16}O_2N_2$ (220): C, 65.4; H, 7.3; N, 12.7. Found: C, 65.4; H, 7.5; N, 12.7.

α-N-Acetyl-L-phenylalaninmethylamide.—L-Phenylalanine methyl ester hydrochloride, 5 g., prepared by the method of Brenner and Huber,[21] was acetylated by the procedure of Reihlen and Knöpfle[23] to give 4.8 g. of acetyl-L-phenylalanine methyl ester, m.p. 88.0–90.0°. The preceding compound was converted into the methylamide and the crude product, obtained in 95% yield, was recrystallized once from ethyl acetate and twice from water to give α-N-acetyl-L-phenylalaninmethylamide, colorless needles, m.p. 207.3–208.6°, $[\alpha]^{25}D$ 19.4° (*c* 1%, in ethanol).

Anal. Calcd. for $C_{12}H_{16}O_2N_2$ (220): C, 65.4; H, 7.3; N, 12.7. Found: C, 65.4; H, 7.3; N, 12.8.

α-N-Acetyl-D-tyrosinmethylamide.—α-N-Acetyl-D-tyrosine ethyl ester,[30] 1 g., was treated with a sixfold excess of methanolic methylamine. Removal of the excess methylamine and solvent *in vacuo* after the reaction mixture had stood for two days at room temperature gave a solid residue which was recrystallized from a mixture of 3 ml. of methanol and 10 ml. of ethyl acetate to give 0.45 g. of α-N-acetyl-D-tyrosinmethylamide, fine colorless needles, m.p. 185.5–186.0° which resolidified to give prisms, m.p. 192.0–192.5°. A second recrystallization from the same solvent did not alter the melting behavior nor the optical rotation, *i.e.*, $[\alpha]^{25}D$ −42.4° (*c* 1%, in water).

Anal. Calcd. for $C_{12}H_{16}O_3N_2$ (236): C, 61.0; H, 6.8; N, 11.9. Found: C, 61.0; H, 6.9; N, 11.8.

α-N-Acetyl-L-tyrosinmethylamide.—To a chilled solution of 3.7 ml. of thionyl chloride in 40 ml. of absolute methanol was added 8.2 g. of acetyl-L-tyrosine,[24] m.p. 147.5–150.5°, in *ca.* four equal portions. The reaction mixture was heated at 40° for 1 hr., the clear solution allowed to stand overnight at room temperature, the solvent removed *in vacuo*, the glassy residue triturated with aqueous sodium carbonate, the solid so obtained collected, washed with water, air-dried and recrystallized from 50 ml. of ethyl acetate to give 8.14 g. of α-N-acetyl-L-tyrosine methyl ester, m.p. 135.0–139.0°. A 7.1-g. portion of this product was converted into the methylamide as before and the crude product recrystallized from a mixture of 25 ml. of methanol and 100 ml. of ethyl acetate to give 4.3 g. of α-N-acetyl-L-tyrosinmethylamide, well formed bipyramids, m.p. 192.2–193.3°, $[\alpha]^{25}D$ 44.2° (*c* 1%, in water), lit.[5] m.p. 191–192°.

Anal. Calcd. for $C_{12}H_{16}O_3N_2$ (236): C, 61.0; H, 6.8; N, 11.9. Found: C, 61.0; H, 6.9; N, 12.0.

α-N-Acetyl-D-tryptophanmethylamide.—This compound, m.p. 185–186°, $[\alpha]^{25}D$ −20° (*c* 1%, in methanol) was prepared as before.[3]

Anal. Calcd. for $C_{14}H_{17}O_2N_3$ (259): C, 64.9; H, 6.6; N, 16.2. Found: C, 65.0; H, 6.7; N, 16.1.

α-N-Acetyl-L-tryptophanmethylamide.—This compound was prepared as described previously.[3] The crude methylamide was recrystallized repeatedly from water to give α-N-acetyl-L-tryptophanmethylamide, m.p. 187.1–189.0°, $[\alpha]^{25}D$ 22.9° (*c* 1%, in ethanol), lit.[3] m.p. 183–184°, $[\alpha]^{25}D$ 20° (*c* 1%, in methanol). A redetermination of the m.p. of the original preparation gave 187.0–188.5° and a mixed m.p. of the two preparations was 187.5–189°.

Anal. Calcd. for $C_{14}H_{17}O_2N_3$ (259): C, 64.8; H, 6.6; N, 16.2. Found: C, 65.0; H, 6.7; N, 16.1.

Enzyme Experiments.—The general procedure has been described.[6] Specific details are given in Table II. With the tyrosine and histidine derivatives a buffering action was encountered which resulted in a sluggish response of the *p*H-stat.

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