cold water. The yield of *p*-nitrobenzoate was 95% and the crude product did not require recrystallization. It was identified by a mixed melting point with an authentic sample and by a comparison of infrared spectra of the reaction product and the authentic sample.

Smaller quantities of sodium methoxide or sodium iodide, *i.e.*, 10 mg., are sufficient to cause methanolysis at room temperature.

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The Interaction of α-Chymotrypsin with a Series of α-N-Acylated-L-tyrosinmethylamides[1]

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α-N-Formyl-, α-N-acetyl-, α-N-nicotinyl- and α-N-benzoyl-L-tyrosinmethylamide have been evaluated as apparent competitive inhibitors in a representative α-chymotrypsin catalyzed hydrolysis. Operationally valid K_I' values have been obtained for the first three compounds. A comparison of these K_I' values with the K_S values previously obtained for systems involving α-chymotrypsin and the corresponding amides and hydrazides has provided further evidence regarding the interpretation of the above K_S and K_I' values as apparent dissociation constants.

In 1951 Huang and Niemann[4] evaluated α-N-acetyl-D- and L-tryptophanmethylamide as competitive inhibitors of the α-chymotrypsin catalyzed hydrolysis of α-N-nicotinyl-L-tryptophanamide and more recently Manning and Niemann[5] performed a similar evaluation of α-N-carbethoxy-D- and L-tyrosinmethylamide using acetyl-L-tyrosinhydroxamide as the specific substrate. In both of these studies it was assumed that the methylamides possessing the L-configuration would be hydrolyzed so slowly, relative to the analogous or corresponding amide or hydroxamide, as to permit their evaluation as competitive inhibitors rather than as components of a binary mixture of specific substrates. Since it has been shown[6] that a dissociation constant cannot be evaluated for the less reactive of two competitive specific substrates solely on the basis of the dependence of the rate of reaction upon the concentration of the specific substrates, despite an earlier claim to the contrary,[7] our immediate concern is the development of an argument, based upon operational rather than theoretical considerations, that can be used as a guide to determine when a given compound can be considered as a competitive inhibitor rather than as a competitive specific substrate.

The case of two competitive specific substrates has been considered by Foster and Niemann[8] who showed that the combined rate of disappearance of the two specific substrates is given by

$$1/v_T = \{([S_1]/K_{S_1}) + ([S_2]/K_{S_2})\}/\{(V_1[S_1]/K_{S_1}) + (V_2[S_2]/K_{S_2})\} + 1/[S_T]\{\{([S_1] + [S_2])\}/\{(V_1[S_1]/K_{S_1}) + (V_2[S_2]/K_{S_2})\}\} \quad (1)$$

eq. 1, where $[S_T] = ([S_1] + [S_2])$, $-d[S_T]/dt = v_T$, $V_1 = k_{3.1}[E]$, $V_2 = k_{3.2}[E]$, $K_{S_1} = (k_{2.1} + k_{3.1})/k_{1.1}$ and $K_{S_2} = (k_{2.2} + k_{3.2})/k_{1.2}$. Rearrangement of eq. 1 and substitution of the quantities $p = K_{S_1}/K_{S_2}$, $q = V_2/V_1$ and $r = [S_2]/[S_1]$ leads to equation 2

$$1/v_T = \{K_{S_1}(1 + ([S_2]/K_{S_2}))\}/\{V_1[S_1](1 + p \cdot q \cdot r) + 1/V_1(1 + p \cdot q \cdot r)\} \quad (2)$$

If we limit consideration to the operational situation where the magnitude of $p.q.r$ is less than that associated with experimental error it follows that $V_1 \doteq V_1(1 + p.q.r)$ and eq. 2 may be reduced to eq. 3

$$1/v_T \doteq \{K_{S_1}(1 + [S_2]/K_{S_2})\}/\{V_1[S_1]\} + 1/V_1 \quad (3)$$

It will be recognized that eq. 3 is, in an operational sense, the equivalent of eq. 4, which may be employed for the evaluation of K_I of a competitive

$$1/v = \{K_S(1 + [I]/K_I)\}/\{V[S]\} + 1/V \quad (4)$$

inhibitor. As long as it is realized that the act of replacing $[S_1]$ by $[S]$, $[S_2]$ by $[I]$, V_1 by V, K_{S_1} by K_S and K_{S_2} by K_I' is an approximation that is dependent upon the validity of the assumption that operationally $V_1 \doteq V_1(1 + p.q.r)$ it is not unreasonable to use eq. 4, or its equivalent, for the approximate evaluation of the more slowly reacting component as an apparent competitive inhibitor provided every effort is made to minimize experimental error and to be certain that the magnitude of $(1 + p.q.r)$ approximates unity within the limits of experimental error.[9]

If the quantities p, q and r are considered individually, it is clear that $p = K_{S_1}/K_{S_2} \doteq K_S/K_I'$ can be minimized only to a limited degree by the use of specific substrates with low K_S values relative to the K_I' values of the apparent competitive inhibitors. The quantity $q = V_2/V_1$ is more amenable to minimization since many pairs of potential reactants can be selected as to yield differences in reactivity, with respect to the production of reaction products, of the order of 10^2 to 10^3, or even greater, between the members of a given pair. However, the advantage so gained can be lost if the

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(4) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

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

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

(7) S. A. Bernhard, *ibid.*, **77**, 1973 (1955).

(8) R. J. Foster and C. Niemann, *ibid.*, **73**, 1552 (1951).

(9) This condition implies that S_2 will not be hydrolyzed at a sufficient rate nor be present in a high enough concentration to contribute significantly to the total rate of formation of reaction products. Its function will then be restricted to combination with the active site of the enzyme thus limiting the hydrolysis of S_1.

quantity $r = [S_2]/[S_1] = [I]/[S]$ is not minimized even though the quantity $(1 + [I]/K_I')$ must remain significantly greater than unity.[10] Thus in contrast to the usual case where it is desirable to maximize [I][11,12] the case at hand may require that precision in the evaluation of K_I' be sacrificed in order that the quantity $r = [I]/[S]$ may be minimized.

Since we are to be concerned with the situation where one member of the pair of reactants is a methylamide it was imperative that an estimate of the magnitude of k_3 for a system involving α-chymotrypsin and a representative α-N-acylated-α-amino acid methylamide be made. Although it was realized that only a value of limited precision could be obtained because of the relative inertness of this class of compounds, with respect to the formation of reaction products, a system was established involving α-chymotrypsin and α-N-acetyl-L-tyrosinmethylamide in aqueous solutions at 25° and *p*H 7.95 and 0.10 *M* in the phosphate component of a sodium phosphate buffer with [E] = 0.144 mg. protein-nitrogen per ml., $[S]_0$ = 25.4 and 50.8 × 10^{-3} *M* and a total reaction time of 16 hours. The course of the reaction was followed by a modification of the ninhydrin procedure developed by Bernhard and Niemann[13] for following the comparable hydrolysis of α-N-acylated-α-amino acid amides. The results of these experiments led to a value of k_3 for the system α-chymotrypsin-α-N-acetyl-L-tyrosinmethylamide of the order of 10^{-5} *M*/min./mg. protein-nitrogen per ml., which because of the uncertainties associated with the enzyme blank[14] could be an order of magnitude less, *i.e.*, 10^{-6} *M*/min./mg. protein-nitrogen per ml. However, for the purpose at hand the higher and more conservative value will be used.

The above estimate of the magnitude of k_3 for the system α-chymotrypsin–α-N-acetyl-L-tyrosinmethylamide may be used to assess the validity of the results of Huang and Niemann[4] who employed α-N-nicotinyl-L-tryptophanamide as the specific substrate, under conditions where K_S = 2.5 × 10^{-3} *M*,[14] k_3 = 1.5 × 10^{-3} *M*/min./mg. protein-nitrogen per ml.,[15] [E] = 0.208 mg. protein-nitrogen per ml. and $[S]_0$ = 8 to 20 × 10^{-3} *M*, to evaluate α-N-acetyl-D- and L-tryptophanmethylamide as apparent competitive inhibitors, when [I] = 5 × 10^{-3} *M*. The values of K_I obtained were 1.8 and 6.5 × 10^{-3} *M* for the D- and L-isomers, respectively.[12] We may assume that the value of k_3 for the system α-chymotrypsin-α-N-acetyl-L-tryptophanmethylamide is of the same order of magnitude as that for α-chymotrypsin-α-N-acetyl-L-tyrosinmethylamide, *i.e.*, *ca.* 10^{-5} *M*/min./mg. protein–nitrogen per ml.[15] From this value and those above we may now evaluate *p*, *q* and *r* and their product. The observation that the product *p.q.r* is of the order of 10^{-3}, $(1 + p.q.r)$ is equal to unity within the limits of experimental error and the value of $[I]/K_I = 0.8$ provides ample evidence, from an operational point of view, that the evaluation of α-N-acetyl-L-tryptophanmethylamide as an apparent competitive inhibitor by Huang and Niemann[4] was justified. Since the value of k_3 for the D-antipode is certainly less than that for the L-antipode it follows that the same conclusion also can be drawn with respect to the evaluation of α-N-acetyl-D-tryptophanmethylamide as a competitive inhibitor.[4]

In the second and only other example involving α-N-acylated-α-amino acid methylamides Manning and Niemann[5] evaluated α-N-carbethoxy-D- and L-tyrosinmethylamide as apparent competitive inhibitors of the α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-L-tyrosinhydroxamide by observing the rate of disappearance of the latter species in the presence and absence of the methylamides. Thus, in contrast to the previous example, where the analytical procedure employed was capable of determining the rate of appearance of one of the products of hydrolysis of both the amide and methylamide,[4] in this instance only the rate of disappearance of the more reactive specific substrate, *i.e.*, the hydroxamide, was measurable.[5] Therefore, in this latter case the operational criteria used previously cannot be applied since in this instance eq. 2 necessarily reduces to equation 3 without the application of the condition that $V_1 \doteq V_1(1 + p.q.r)$. However, there may be other reasons for assuming that equation 3 may be interpreted in terms of equation 4, *e.g.*, the parallel behavior of operationally unambigous competitive inhibitors and structurally related specific substrates.[5]

In contrast to the example considered immediately above it is possible to assess the operational validity of the example provided by Bernhard,[7] which is similar to the one examined by Huang and Niemann,[4] and which was concerned with the evaluation of α-N-benzoyl-L-argininamide as a competitive inhibitor of the trypsin catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester. Bernhard's experiments were conducted under conditions which were such as to lead to a value of $p = 1$, $q = 0.0033$, $r = 34$ to 272 and $p.q.r = 0.1$ to 0.9. Since a value of $(1 + p.q.r)$ of 1.1 to 1.9 cannot be considered to approximate unity within the limits of reasonable experimental error it must be concluded that Bernhard's evaluation of α-N-benzoyl-L-argininamide as a competitive inhibitor cannot be justified either on a theoretical[6] or on an operational basis.

An examination of the K_S values of a series of α-N-acylated-L-tyrosinamides[15] and hydrazides,[16,17] *cf.* Table I, revealed that with both amide and hydrazide the magnitude of the K_S values is in the order acetyl > formyl ≥ nicotinyl > benzoyl. Because of the structural similarity of the hydrazides and the methylamides it was decided to evaluate a series of α-N-acylated-L-tyrosinmethylamides as apparent competitive inhibitors of the

(10) It will be seen from eq. 4 that unless the quantity $(1 + [I]/K_I')$ is significantly greater than unity the constant K_I' cannot be evaluated.

(11) R. J. Foster, H. J. Shine and C. Niemann, THIS JOURNAL, **77**, 2378 (1955).

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

(13) R. A. Bernhard and C. Niemann, *ibid.*, **79**, 4085 (1957).

(14) R. B. Martin and C. Niemann, *Biochim. Biophys. Acta*, **26**, 634 (1957).

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

(16) R. Lutwack, H. F. Mower and C. Niemann, *ibid.*, **79**, 5690 (1957).

(17) R. J. Kerr and C. Niemann, *ibid.*, **80**, 1469 (1958).

TABLE I

SUMMARY OF KINETIC CONSTANTS FOR SYSTEMS INVOLVING α-CHYMOTRYPSIN AND A SERIES OF α-N-ACYLATED-L-TYROSINAMIDES, HYDRAZIDES AND METHYLAMIDES[a]

Specific substrate or competitive inhibitor	K_S[b]	K_I'[b]	k_3[c]	Ref.
Acetyl-L-amide	32 ± 4	.....	2.4 ± 0.3	14
Formyl-L-amide	12 ± 3	.....	0.45 ± 0.05	14
Nicotinyl-L-amide	12 ± 3	.....	5.0 ± 1.0	14
Benzoyl-L-amide	2.5 ± 0.3	.....	4.0 ± 0.5	14
Acetyl-L-hydrazide	22 ± 8	.....	0.7 ± .2	15
Formyl-L-hydrazide	9.8 ± 0.5	.....	0.058 ± .002	15
Nicotinyl-L-hydrazide	8.0 ± 1.6	.....	0.91 ± .16	15, 16
Benzoyl-L-hydrazide	*ca.* 1	.....	*ca.* .1	15
Acetyl-L-methylamide	.....	61 ± 7[d,e]	*ca.* .01	...
Formyl-L-methylamide	.....	31 ± 8[d,e,f]	.....	...
Nicotinyl-L-methylamide	.....	8.8 ± 1.1[d,g]	.....	...
Benzoyl-L-methylamide	.....	6.4 ± 1.6[d,h,i]	.....	...

[a] In aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM component of a THAM–HCl buffer. [b] In units of 10^{-3} *M*. [c] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [d] This value, given with a variability of 2σ, is the simple average of those values whose deviation from the simple average of all values listed in Table II is less than 4 σ. [e] Value from experiment 6 has been excluded. [f] Value from experiment 18 has been excluded. [g] Values from experiments 28 and 31 have been excluded. [h] Values from experiments 47 and 51 have been excluded. [i] Value of questionable significance because of the low value of $[I]/K_I$.

α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-L-tyrosinhydrazide in order that their respective K_I' values could be compared with the K_S values observed for the corresponding amides and hydrazides. It was anticipated that such a comparison would be of value with respect to the interpretation of the K_S values of the above two classes of specific substrates, provide additional examples of the use of methylamides as apparent competitive inhibitors and afford an opportunity of examining the interaction of α-chymotrypsin with a homogeneous series of α-N-acylated-α-amino acid amides, hydrazides and methylamides.

The apparent competitive inhibition of the α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-L-tyrosinhydrazide by α-N-acetyl-, α-N-formyl, α-N-nicotinyl- and α-N-benzoyl-L-tyrosinmethylamide was examined in aqueous solutions at 25° and *p*H 7.95 and 0.02 *M* in the THAM[18] component of a THAM–HCl buffer. The details of these experiments, in which the initial velocities were evaluated by the empirical procedure of Booman and Niemann,[19] are summarized in Table II.

Since prior studies with comparable α-N-acylated-α-amino acid methylamides[4,5] had removed the necessity of again demonstrating that the above group of compounds were capable of functioning as apparent competitive inhibitors, at least in an operational sense, the K_I' values were obtained by taking advantage of equation 5,[20] which for the

$$v/v_i = \{1 + (K_S/K_I')([I])\}/\{(K_S + [S])\} \quad (5)$$

purpose at hand was rearranged to give eq. 6.

$$K_I' = ([I])/\{((v_{0_S}/v_{0_I}) - 1)(1 + ([S]_0/K_S))\} \quad (6)$$

It will be noted from the data summarized in Table II that the magnitude of the term $(1 + ([S]_0/K_S))$ present in equation 6 ranged from 1.02 to 1.11 largely because of the relatively high K_S value of α-N-acetyl-L-tyrosinhydrazide and a particularly sensitive analytical procedure which permitted the reactions to be examined at a relatively low initial specific substrate concentration. Since the difference between the values noted above and unity is within the limits of experimental error encountered in the evaluation of K_I', all of the values of this constant which are given in Table II were evaluated on the basis of equation 7

$$K_I' \doteq [I]/((v_{0_S}/v_{0_I}) - 1) \quad (7)$$

It will be seen from the data given in Table II that the $[I]/K_I'$ values for the acetyl derivative vary from 0.11 to 0.73, the formyl derivative from 0.09 to 0.25, the nicotinyl derivative from 0.18 to 0.40 and the benzoyl derivative from 0.04 to 0.07. From these values it follows that the K_I' values for the first three compounds can be considered as being operationally significant but that of the benzoyl derivative cannot be so regarded. Therefore, the value given for this compound in Table I is questionable and is useful only in suggesting that the magnitude of the constant is probably greater than 1×10^{-3} *M*.

A comparison of the K_S and K'_I values summarized in Table I reveals that the dependence of the K_I' values of the α-N-acylated-L-tyrosinmethylamides upon the nature of the α-N-acyl moiety leads to an ordering with respect to the magnitude of the K_I' values, *i.e.*, acetyl > formyl > nicotinyl ≥ benzoyl that is similar to that observed previously for the K_S values of the corresponding α-N-acylated-L-tyrosinamides and hydrazides, *i.e.*, acetyl > formyl ≥ nicotinyl > benzoyl. This behavior coupled with the fact that the magnitude of the K_S and K_I' values are approximately of the same order whereas the values of k_3 vary over more than a 200-fold range suggests that in no case does k_3 contribute significantly to the value of $K_S = (k_2 + k_3)/k_1$ and that both the K_S and K_I' values given in Table I can be interpreted as apparent dissociation constants.[4,21–26]

(18) Tris-(hydroxymethyl)-aminomethane.

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

(20) P. W. Wilson in H. A. Lardy, "Respiratory Enzymes," revised edition, Burgess Publ. Co., Minneapolis, Minn., 1949.

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

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

It will be seen from the data given in Table I that the K_S and K_I' values associated with the interaction of α-chymotrypsin with α-N-nicotinyl-L-tyrosinamide and the corresponding hydrazide and methylamide are of comparable magnitude, *i.e.*, *ca.* 10^{-2} *M*. However, with the analogous α-N-acetyl and α-N-formyl derivatives this situation no longer obtains and it is clear that even with molecules of the type RCONHCHR'CONHR", where the configuration about the asymmetric carbon atom is L and the nature of R' is invariant, the extent to which such molecules can combine with the active site of the enzyme may be determined by the nature of both R and R" rather than by that of R alone, as might have been inferred from the behavior of the α-N-nicotinyl derivatives or of the various α-N-acylated amides or hydrazides. It is noteworthy that when R = CH_3 or H and R" = H or NH_2 the values of K_S for comparable pairs are nearly equivalent whereas when R" = CH_3 the values of K_I' are markedly increased. Since we have no reason to doubt our interpretation of the above K_S and K_I' values as apparent dissociation constants the preceding observation suggests that the lesser extent of interaction of the methylamides with the active site, relative to that of the hydrazides, is not the consequence of a simple steric effect but may be one that is associated with the dielectric properties of those regions of the active site that are in juxtaposition to the -CONHR" group in the ES or EI complex.

TABLE II

INHIBITION OF THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF α-N-ACETYL-L-TYROSINHYDRAZIDE BY α-N-ACETYL-, FORMYL-, NICOTINYL- AND BENZOYL-L-TYROSINMETHYLAMIDE[a]

Expt. no.	$[S]_0$[b]	[I][b]	v_{0S}[c]	v_{0I}[c]	v_{0S}/v_{0I}	K_I'[b]
	α-N-Acetyl-L-tyrosinmethylamide					
1	1.23	..	20.1	..	..	..
2	1.23	24.4	..	14.5	1.39	63
3	1.23	48.8	..	11.6	1.73	67
4	1.84	..	29.3	..	..	..
5	1.84	24.4	..	21.4	1.37	66
6	1.84	48.8	..	18.1	1.62	79
7	2.45	..	44.0	..	..	..
8	2.45	7.10	..	39.5	1.11	65
9	2.45	11.4	..	37.0	1.19	60
10	2.45	12.2	..	35.8	1.23	53
11	2.45	24.4	..	30.1	1.46	53
	α-N-Formyl-L-tyrosinmethylamide					
12	0.61	..	10.8	..	..	..
13	.61	3.11	..	9.3	1.16	19
14	.61	5.18	..	9.5	1.14	37
15	.64	..	10.8	..	..	..
16	.64	3.63	..	9.9	1.09	40
17	1.21	..	20.7	..	..	..
18	1.21	3.11	..	19.7	1.05	62
19	1.21	5.18	..	18.3	1.13	40
20	1.27	..	21.0	..	..	..
21	1.27	9.06	..	16.8	1.25	36
22	1.84	..	33.0	..	..	..
23	1.84	3.11	..	29.0	1.14	22
24	1.84	5.18	..	28.0	1.18	29
25	1.91	..	32.6	..	..	..
26	1.91	3.63	..	28.4	1.15	24
	α-N-Nicotinyl-L-tyrosinmethylamide					
27	0.32	..	5.4	..	..	..
28	.32	1.75	..	4.1	1.32	5.5
29	.64	..	10.5	..	..	..
30	.64	1.75	..	8.8	1.19	9.2
31	.64	3.50	..	8.1	1.30	12
32	.95	..	16.7	..	..	..
33	.95	3.50	..	12.1	1.38	9.5
34	1.27	..	22.8	..	..	..
35	1.27	1.75	..	18.1	1.26	6.7
36	1.27	3.50	..	16.5	1.38	8.8
37	1.27	3.50	..	16.3	1.40	8.8
38	1.91	..	31.8	..	..	..
39	1.91	1.75	..	26.9	1.18	9.7
	α-N-Benzoyl-L-tryrosinmethylamide					
40	0.32	..	5.4	..	..	..
41	.32	0.49	..	5.1	1.06	8.2
42	.64	..	10.5	..	..	..
43	.64	.30	..	9.8	1.07	4.3
44	.64	.49	..	9.8	1.07	7.0
45	.95	..	16.5	..	..	..
46	.95	.30	..	15.6	1.06	5.0
47	.95	.49	..	14.4	1.15	3.3
48	1.27	..	21.2	..	..	..
49	1.27	.30	..	20.3	1.04	7.5
50	1.91	..	30.9	..	..	..
51	1.91	.30	..	30.0	1.03	10

[a] In aqueous solutions at 25° and *p*H 7.95 and 0.02 *M* in the THAM component of a THAM–HCl buffer with [E] = 0.144 mg. protein-nitrogen per ml. or 4.1×10^{-6} *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] In arbitrary units of O.D. per min. and evaluated by the empirical procedure of Booman and Niemann from nine observations of the extent of reaction at 3 min. intervals from 0 to 24 min. where the maximum extent of reaction was *ca.* 10%.

Experimental[27,28]

α-N-Formyl-L-tyrosinmethylamide.—A suspension of 15 g. of L-tyrosine methyl ester hydrochloride in 200 ml. of ether was allowed to react with an excess of saturated aqueous sodium bicarbonate, the crystalline L-tyrosine methyl ester collected by filtration, washed with water, air dried, dissolved in a mixture of methanol and diethyl ether and the solution saturated at 0° with gaseous methylamine. The mixture was allowed to stand at room temperature for several days and then evaporated to dryness *in vacuo* to give *ca.* 10 ml. of a sirupy residue. To 7 ml. of this residue maintained at 60° was added 45 ml. of 80% formic acid and 15 ml. of acetic anhydride. Evaporation of the mixture *in vacuo* gave a semi-crystalline solid which was successively triturated with diethyl ether, saturated aqueous sodium bicarbonate and water to give a crystalline solid which was dried and repeatedly recrystallized from a mixture of methanol and ethyl acetate to give *ca.* 2 g. of α-N-formyl-L-tyrosinmethylamide, m.p. 201–202°.

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

α-N-Acetyl-L-tyrosinmethylamide.—A suspension of 2.3 g. of L-tyrosine methyl ester hydrochloride in 100 ml. of diethyl ether was shaken with 4.2 g. of sodium bicarbonate

(23) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, THIS JOURNAL, **73**, 3231 (1951).
(24) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).
(25) C. Niemann, *Science*, **117**, 469 (1953).
(26) H. J. Shine and C. Niemann, THIS JOURNAL, **77**, 4275 (1955).
(27) All melting points are corrected.
(28) Microanalyses by Dr. A. Elek.

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).

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

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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).