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Re-evaluation of the Inhibition Constants of Previously Investigated Competitive Inhibitors of α-Chymotrypsin. II. Mono-, Bi- and Trifunctional Inhibitors Evaluated under Zone A Conditions[1]

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The enzyme–inhibitor dissociation constants of α-chymotrypsin and thirty-three previously investigated competitive inhibitors of this enzyme have been re-evaluated by methods which are more objective than those used earlier.

Recently we have reported[3] revised values for the enzyme–inhibitor dissociation constants of α-chymotrypsin and fifteen competitive inhibitors which are either hydrolysis products or D-enantiomorphs of previously investigated specific substrates of this enzyme.[4] In this communication we wish to describe the re-evaluation of the enzyme–inhibitor dissociation constants of α-chymotrypsin and thirty-three additional competitive inhibitors which, in common with those mentioned above, were evaluated originally[5–14] by methods which we now regard as lacking in objectivity.[3,4,15,16]

The primary data that were available for the thirty-three competitive inhibitors had been collected during the initial stages of the various reactions and therefore the procedure employed for the reevaluation of all of the respective enzyme–inhibitor dissociation constants was that described by Jennings and Niemann[15] in which the corrected initial velocities were first determined from $([S]_0 - [S]_t)$ *vs.* t and $\ln [S]_0/[S]_t$ *vs.* t plots and the values of K_I then obtained from subsequent v_0 *vs.* $v_0/[S]_0$ plots[17–20] based upon equation 1. As before[3,4] care has been taken to specify as completely as possible

$$-d[S]/dt = k_3[E][S]/(K_S(1 + [I]/K_I) + [S]) \quad (1)$$

the reaction conditions that were employed for each particular set of experiments and in every case attention has been directed to those parameters, *i.e.*, [E], E_S', E_I', S_S' and I_I', which must be maintained within certain limits in order to satisfy the assumptions inherent in the various treatments.[3,4,21,22] For purposes of calculation the molecular weight of monomeric α-chymotrypsin was taken to be 22,000 and its nitrogen content 16.0%.[3,4]

In Table I there are included accounts of several previously unpublished experiments[23] which are pertinent to the topic under discussion. In these as well as in all other experiments reported in this communication a single α-chymotrypsin preparation, *i.e.*, Armour No. 90402, was used. Furthermore, all experiments were conducted in the presence of a THAM–HCl buffer.[24] All of the competitive inhibitors considered in this study are presented in Table I and are ordered in this table in two groups, *i.e.*, those containing an indole nucleus and those containing a benzene nucleus, and are so listed generally in increasing order of complexity within each group.

Examination of Table I reveals that in every instance [E] was of the order of 10^{-5} *M*, E_S' less than 2.4×10^{-2}, E_I' less than 10×10^{-2} and that S_S' was within the limits of 0.1 and 8.0. Thus, it may be concluded that the data which were employed for the re-evaluation of the enzyme–inhibitor dissociation constants given in Table I were obtained under conditions which favored the presence of essentially monomeric α-chymotrypsin and which satisfied the usual zone A criteria with respect to both the specific substrate and the competitive inhibitor.[3,4,21,22] Furthermore, the values of S_S' were such as to justify the use of equation 1, in the form of v_0 *vs.* $v_0/[S]_0$ plots,[17–20] for the evaluation of the various enzyme–inhibitor dissociation constants.[3,4] With respect to values of I_I' in no case was a value of I_I' so low as to preclude the use of a set of data for the re-evaluation of K_I. However, in three instances where a value of K_I was computed previously from data obtained in experiments conducted at a single specific substrate concentration and a single inhibitor concentration, *i.e.*, for β-phenyl propionate *vs.* acetyl-L-tyrosinamide,[12] chloramphenicol *vs.* acetyl-L-tyrosinamide[8] and indole *vs.* nicotinyl-L-tryptophanamide at an inhibitor concentration of 10×10^{-3} *M*,[14] we have not re-evaluated K_I because we now believe that such data are too limited to be of quantitative significance.

The observation that all of the experimental data obtained in the various inhibition studies which are summarized in Table I can be interpreted in terms of v_0 *vs.* $v_0/[S]_0$ plots[17–20] based upon equation 1 permits the conclusion that all of the inhibitors listed in Table I are competitive inhibitors of α-chymotrypsin in so far as can be determined within the

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(24) Tris(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

TABLE I

K_I VALUES OF A GROUP OF THIRTY THREE COMPETITIVE INHIBITORS EVALUATED UNDER ZONE A CONDITIONS[a]

Competitive inhibitor[b,c]	Ref.	$[S]_0 \times 10^3$ M	$[I] \times 10^3$ M	$EI' \times 10^2$	II'	$K_I \times 10^3$ M Orig.[d]	$K_I \times 10^3$ M Re-eval.[e,f,g]
Indole	14	8–20[h,i]	0.7	6.5	0.78	0.71	0.9 ± 0.2[j]
Indole	14	8–20[k,i]	1.6	7.4	2.0	.68	.8 ± .2
Indole	14	8–20[k,i]	2.4	8.4	3.5	.68	.7 ± .2[j]
Indole	14	10[l]	5.0	..	..	.72	.....
Tryptamine	9	5–20[m,n]	3.0	2.6	1.3	2.5	2.3 ± 0.4
Acetyltryptamine	9	5–20[o,n]	2.5	3.3	1.4	1.8	1.8 ± .3
Trifluoroacetyltryptamine	9	5–20[o,n]	0.8	4.9	0.67	0.95	1.2 ± .3
β-Indoleacetate	12	8–20[p,i]	30	0.33	1.7	25	18 ± 3
β-(β-Indole-)propionate	12	8–20[p,i]	15	0.39	1.0	13	15 ± 3
β-(β-Indole)-propionamide	12	5–20[m,n]	2.1	2.6	0.91	1.7	2.3 ± 0.4
γ-(β-Indole)-butyrate	12	8–20[p,i]	20	0.26	0.87	17	23 ± 5
D-Tryptophanamide	6	5–20[o,n]	3.0	1.5	0.75	3.2	4.0 ± 1.0
L-Tryptophanamide	6	5–20[p,n]	6.0	0.74	0.75	6.3	8.0 ± 2.0[q]
L-Tryptophanamide	6	5–15[r,s]	10	.66	1.11		9.0 ± 2.0[q]
Acetyl-D-tryptophanate	6	8.5–20[t,u]	5.0	.79	0.67	4.8	7.5 ± 1.5
Acetyl-D-tryptophanmethylamide	6	8–20[k,i]	5.0	3.3	4.2	1.7	1.8 ± 0.3
Acetyl-L-tryptophanmethylamide	6	8–20[k,i]	5.0	0.91	0.77	4.8	6.5 ± 1.5
Acetyl-D-tryptophanhydrazide	9	8.5–20[t,u]	0.8	7.4	1.0	0.75	0.8 ± 0.2
Acetyl-D-tryptophan-isopropyl ester	9	8.5–20[t,u]	0.5	7.4	0.63	0.58	0.8 ± 0.2
Trifluoroacetyl-D-tryptophanamide	9	8.5–20[t,u]	2.0	1.5	.50	2.5	4.0 ± 1.0
Benzoyl-D-tryptophanamide	9	8.5–20[t,u]	0.5	8.4	.71	0.48	0.7 ± 0.2
p-Methoxybenzoyl-D-tryptophanamide	9	8.5–20[t,u]	0.25	9.9	.42	0.30[v]	0.6 ± 0.2
Benzoate	12	6–12.5[r,w]	80	0.04	.53	200	150 ± 50
Benzamide	12	6–20[m,x]	10	.59	1.0	6.6	10 ± 2
Acetanilide	12	6–20[m,x]	10	.46	0.77	10.4	13 ± 3
Phenylacetate	12	6–12.5[r,w]	70	.03	.35	120	200 ± 50
Phenylacetamide	12	6–20[m,x,y]	10	.39	.67	10.2	15 ± 3
β-Phenylpropionate	12	8–20[k,i]	20	.24	.80	28	25 ± 5
β-Phenylpropionamide	12	6–20[m,x]	7.0	.84	1.0	6.7	7.0 ± 2.0
γ-Phenylbutyrate	12	8–20[p,i]	60	.99	1.0	72	60 ± 10
γ-Phenylbutyramide	12	6–20[m,x]	7.0	.49	0.58	7.2	12 ± 3
Hippurylamide	11	8–20[k,i]	10	.45	.77	11	13 ± 3[z]
Hippurylamide[aa]	..	8–20[ab,ac]	10	.47	.80	.....	12.5 ± 2[ad,z]
Hippurylamide[ae,af]	..	12.5–25[p,ag]	10	.32	.83	.....	12 ± 3[ah,z]
Chloramphenicol	8	5–20[m,n]	10	.24	.40	13.5	25 ± 5
Acetyl-D-phenylalanine methyl ester	10	5–20[ai,n]	3.0	3.0	1.5	2.5	2.0 ± 0.5[aj]
Acetyl-D-phenylalanine methyl ester[aa]	11	8–25[ak,al]	2.0	2.3	0.77	2.8[am]	2.6 ± .4[ad,aj]
Acetyl-D-phenylalanine methyl ester[ae,af]	..	15–25[r,an]	2.0	1.7	0.91	.....	2.2 ± .4[ah,aj]
Acetyl-D-tyrosine ethyl ester[ao,ap]	13	5–35[aq,ar]	5.0	0.17	1.0	4.0[as]	5.0 ± .5[at,au]
Acetyl-D-tyrosine ethyl ester[av,aw]	5	15–35[r,ax]	5.0	.79	1.0	3.5 ± 0.5[ay]	5.0 ± 1.0[az,au]
Acetyl-D-tyrosine ethyl ester	5	15–40[p,ba]	10	.99	2.5		4.0 ± 1.0[az,au]
Acetyl-D-tyrosinhydrazide[ao,ap]	13	5–35[aq,ar]	7.5	.11	1.0	6.8[as]	7.5 ± 1.5[at]
Nicotinyl-D-tyrosine ethyl ester	7	10–20[p,bb]	1.0	7.4	1.3	0.97	0.8 ± 0.2

[a] In aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer and with a single α-chymotrypsin preparation, *i.e.*, Armour No. 90402, unless otherwise noted. [b] *Vs.* nicotinyl-L-tryptophanamide unless otherwise noted. [c] Enzyme concentration of 0.208 mg. protein-nitrogen/ml., *i.e.* $[E] = 5.91 \times 10^{-6}$ *M* on the basis of an assumed molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin. [d] Based upon values of K_S and k_3 for nicotinyl-L-tryptophanamide of 2.7×10^{-3} *M* and 1.6×10^{-3} *M*/min./mg. protein-nitrogen/ml., respectively, unless otherwise noted. [e] Re-evaluated by the method of Jennings and Niemann[15] and a v_0 *vs.* $v_0/[S]_0$ plot.[17-20] [f] Based upon values of K_S and k_3 for nicotinyl-L-tryptophanamide of 2.5×10^{-3} *M* and 1.5×10^{-3} *M*/min./mg. protein-nitrogen/ml., respectively,[4] unless otherwise noted. [g] Re-evaluated value to be taken as the preferred value unless otherwise noted. [h] Eight experiments at four initial specific substrate concentrations within the limits indicated. [i] $E_S' = 2.37 \times 10^{-2}$, $S_S' = 3.2$–8.0. [j] Preferred value $0.8 \pm 0.2 \times 10^{-3}$ *M*. [k] Six experiments at four initial specific substrate concentrations within the limits indicated. [l] One experiment at the initial specific substrate concentration indicated. [m] Five experiments at five initial specific substrate concentrations within the limits indicated. [n] $E_S' = 2.37 \times 10^{-2}$, $S_S' = 2.0$–8.0. [o] Six experiments at six initial specific substrate concentrations within the limits indicated. [p] Four experiments at four initial specific substrate concentrations within the limits indicated. [q] Preferred value $0.85 \pm 0.2 \times 10^{-3}$ *M*. [r] Three experiments at three initial specific substrate concentrations within the limits indicated. [s] $E_S' = 2.37 \times 10^{-2}$, $S_S' = 2.0$–6.0. [t] Six experiments at five initial specific substrate concentrations within the limits indicated. [u] $E_S' = 2.37 \times 10^{-2}$, $S_S' = 3.4$–8.0. [v] K_I originally determined from a *Q vs.* α ($K_S/[S]$) plot[9] based upon an assumed zone B behavior for this competitive inhibitor under the conditions specified. [w] $E_S' = 2.37 \times 10^{-2}$, $S'_S = 2.4$–5.0. [z] $E_S' = 2.37 \times 10^{-2}$, $S_S' = 2.4$–8.0. [y] Original evaluation based upon four experiments at four initial specific substrate concentrations within the limits of 6–12.5 $\times 10^{-3}$ *M*. [z] Preferred value $12.5 \pm 3 \times 10^{-3}$ *M*. [aa] *Vs.* methyl hippurate. [ab] Eleven experiments at four specific substrate concentrations within the limits indicated. [ac] $E_S' = 0.91 \times 10^{-2}$, $S_S' = 1.2$–3.1. [ad] Based upon values of K_S and k_3 for methyl hippurate of 6.5×10^{-3} *M* and 2.2×10^{-3} *M*/min./mg. protein-nitrogen/ml. respectively.[4] [ae] *Vs.* chloroacetyl-L-tyrosinamide at *p*H 7.75. [af] Enzyme concentration 0.135 mg. protein-nitrogen/ml., *i.e.*, $[E] = 3.83 \times$

10^{-5} M. [aq] $E_S' = 0.14 \times 10^{-2}$, $S_S' = 0.5$–0.9. [ah] Based upon values of K_S and k_3 for chloroacetyl-L-tyrosinamide of 27×10^{-3} M and 4.0×10^{-3} M/min./mg. protein-nitrogen/ml., respectively.[4] [ai] Seven experiments at six initial specific substrate concentrations within the limits indicated. [aj] Preferred value $2.4 \pm 0.4 \times 10^{-3}$ M. [ak] Nine experiments at five initial specific substrate concentrations within the limits indicated. [al] $E_S' = 0.91 \times 10^{-2}$, $S_S' = 1.2$–3.9. [am] Based upon values of K_S and k_3 for methyl hippurate of 8.5×10^{-3} M and 2.2×10^{-3} M/min./mg. protein-nitrogen/ml., respectively. [an] $E_S' = 0.14 \times 10^{-2}$, $S_S' = 0.6$–0.9. [ao] *Vs.* acetyl-L-tyrosinhydroxamide at *p*H 7.6 and 0.3 M in the THAM component of a THAM–HCl buffer. [ap] Enzyme concentration 0.0294 mg. protein-nitrogen/ml., *i.e.*, [E] = 0.835×10^{-5} M. [aq] Nine experiments at nine initial specific substrate concentrations within the limits indicated; original evaluation based upon eight experiments at eight initial specific substrate concentrations within the same limits. [ar] $E_S' = 0.02 \times 10^{-2}$; $S_S' = 0.1$–0.8. [as] Based upon values of K_S and k_3 for acetyl-L-tyrosinhydroxamide of 51×10^{-3} M and 34×10^{-3} M/min./mg. protein-nitrogen/ml., respectively. [at] Based upon values of K_S and k_3 for acetyl-L-tyrosinhydroxamide of 43×10^{-3} M and 33×10^{-3} M/min./mg. protein-nitrogen/ml., respectively.[4] [au] Preferred value $5.0 \pm 1.0 \times 10^{-3}$ M. [av] *Vs.* acetyl-L-tyrosinamide. [aw] Enzyme concentration 0.139 mg. protein-nitrogen/ml., *i.e.*, [E] = 3.95×10^{-5} M. [ax] $E_S' = 0.12 \times 10^{-2}$, $S'_S = 0.5$–1.1. [ay] Based upon values of K_S and k_3 for acetyl-L-tyrosinamide of 30.5×10^{-3} M and 2.4×10^{-3} M/min./mg. protein-nitrogen/ml., respectively. [az] Based upon values of K_S and k_3 for acetyl-L-tyrosinamide of 32×10^{-3} M and 2.4×10^{-3} M/min./mg. protein-nitrogen/ml., respectively.[4] [ba] $E_S' = 0.12 \times 10^{-2}$, $S_S' = 0.5$–1.3. [bb] $E_S' = 2.37 \times 10^{-2}$, $S_S' = 4.0$–8.0.

limits of experimental error. The validity of the above conclusion is confirmed by the fact that in three cases, *i.e.*, indole *vs.* nicotinyl-L-tryptophanamide, L-tryptophanamide *vs.* nicotinyl-L-tryptophanamide and acetyl-D-tyrosine ethyl ester *vs.* acetyl-L-tyrosinamide, the value of K_I was found to be independent of (I) over a range of values of [I] of 3.4, 2.0 and 2.0 fold, respectively, and by the added fact that in three cases, *i.e.*, hippurylamide *vs.* methyl hippurate or nicotinyl-L-tryptophanamide or chloroacetyl-L-tyrosinamide, acetyl-D-phenylalanine methyl ester *vs.* methyl hippurate or nicotinyl-L-tryptophanamide or chloroacetyl-L-tyrosinamide, and acetyl-D-tyrosine ethyl ester *vs.* acetyl-L-tyrosinhydroxamide or acetyl-L-tyrosinamide, the value of K_I was found to be independent of the nature of the specific substrate used for its evaluation.

While most of the competitive inhibitors listed in Table I were evaluated in aqueous solutions at 25° and *p*H 7.9 and 0.02 M in the THAM component of a THAM–HCl buffer there were two, *i.e.*, hippurylamide and acetyl-D-phenylalanine methyl ester, which also were evaluated under the same conditions except at *p*H 7.75. With these two uncharged competitive inhibitors the values of K_I at *p*H 7.9 and 7.75 were identical within the limits of experimental error. Two other uncharged competitive inhibitors, *i.e.*, acetyl-D-tyrosine ethyl ester and acetyl-D-tyrosinhydrazide, were evaluated in aqueous solutions at 25° and *p*H 7.6 and 0.3 M in the THAM component of a THAM–HCl buffer and the K_I value of acetyl-D-tyrosine ethyl ester so determined was in good agreement with that evaluated in aqueous solutions at 25° and *p*H 7.9 and 0.02 M in the THAM component of a THAM–HCl buffer. From these limited data there is no suggestion that the value of K_I for an uncharged competitive inhibitor is dependent either upon the *p*H or the ionic strength of the reaction system.

In contrast to the situation discussed immediately above, where the addition of the inhibitor did not cause an increase in the ionic strength of the reaction system, there were a number of cases encountered in this study where this was not so. Eight of the competitive inhibitors listed in Table I are carboxylate ions. In each instance these inhibitors were added to the reaction system in the form of their sodium salts thereby increasing the ionic strength of the reaction system over that obtained for the uninhibited reaction by an amount proportional to the amount of each particular inhibitor added. Under these conditions the initial velocities of the uninhibited reactions are simultaneously increased, by the increase in ionic strength of the system, and decreased, by the normal competitive interaction, leading to an underestimation of the true competitive interaction.[3] Therefore, the K_I values of the anionic competitive inhibitors which are listed in Table I must be regarded as provisional values subject to correction for the above ionic strength effect.

The remaining uncertainty with respect to values of K_I listed in Table I relates to the charge state of several amine types of competitive inhibitors, *i.e.*, tryptamine and D- and L-tryptophanamide. At *p*H 7.9 tryptamine would be expected to be substantially protonated and we may regard the value of K_I $2.3 \pm 0.4 \times 10^{-3}$ M as the enzyme–inhibitor dissociation constant of α-chymotrypsin and monoprotonated tryptamine. However, with D- and L-tryptophanamide, whose respective pK_A' values are approximately 7.5 ± 0.1,[6] it is clear that the K_I values which are given in Table I for these two competitive inhibitors are composite in nature and refer to that mixture of the unprotonated and protonated forms of each of these competitive inhibitors which prevails at *p*H 7.9.

Of those competitive inhibitors of α-chymotrypsin which have been considered in previous publications from these laboratories there are now available revised values of K_I, at some particular set of conditions, for forty-eight competitive inhibitors of this enzyme which have been studied under zone A conditions, *cf.* Table I and ref. 3. Since we are now engaged in a reevaluation of those primary data which have been obtained[9,14,25] from studies of competitive inhibitors of α-chymotrypsin in systems which do not appear at this moment to satisfy the usual zone A criteria[3,4,21,22] we wish to defer any discussion of the relations existing between the above K_I values and the structures of the respective competitive inhibitors until this latter study is completed.

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