pronounced structural alterations in acid media is in accord with this idea since it also possesses a high isoelectric rotation (above $-50°$). The structural rigidity of β-lactoglobulin and insulin, in this study, is also in accord since they have very low rotations in the isoelectric state. However, by the same token, it would be predicted that most of the enzymes should be structurally adaptable, particularly pepsin and ribonuclease which have specific rotations of around $-70°$. Further studies of these proteins in alkaline solution, and perhaps also in urea, would clearly be in order.

Acknowledgment.—We are indebted to Miss Ana Pellecer for her technical assistance in carrying out a large proportion of the experimental measurements. We would also like to express our thanks to Drs. J. N. Ashworth, T. L. McMeekin, W. G. Gordon, J. B. Lesh and E. G. Samsa for supplying protein samples.

AMES, IOWA

[CONTRIBUTION NO. 1938 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Re-evaluation of the Inhibition Constants of Previously Investigated Competitive Inhibitors of α-Chymotrypsin. I. Hydrolysis Products and Enantiomorphs of Previously Investigated Specific Substrates[1]

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RECEIVED SEPTEMBER 9, 1954

The enzyme–inhibitor dissociation constants of α-chymotrypsin and five hydrolysis products and ten enantiomorphs of previously investigated specific substrates of this enzyme have been re-evaluated from primary data by procedures which are more objective than those used previously and more reliable and realistic values for these constants have been obtained.

It is to be expected[3,4] that an α-chymotrypsin-catalyzed hydrolysis of a given specific substrate when conducted under zone A conditions[4-6] may be competitively inhibited by one or more of the hydrolysis products and that such a reaction can be described throughout its course, in so far as it is dependent upon the concentration of enzyme and specific substrate, in terms of equations 1, 2, 3 and 4 where $K_S = (k_2 + k_3)/k_1$, $K_{P_1} = k_5/k_4$ and $K_{P_2} = k_7/k_6$.

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (2)$$

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \quad (3)$$

$$k_3[E]t = K_S\left(1 + [S]_0 \sum_{j=1}^{n} 1/K_{P_j}\right) \ln [S]_0/[S]_t + \left(1 - K_S \sum_{j=1}^{n} 1/K_{P_j}\right)([S]_0 - [S]_t) \quad (4)$$

In previous studies conducted in these laboratories[3,7-11] it was shown that with a number of acylated α-amino acid amides and hydroxamides only one of the hydrolysis products, *i.e.*, the carboxylate ion derived from the acylated α-amino acid, may competitively inhibit the hydrolytic reaction and that the other hydrolysis product, *i.e.*, ammonia or hydroxylamine or the corresponding monoprotonated species, is without effect even when present in concentrations which are considerably greater than those of the specific substrate. Thus, with knowledge of the K_{P_1} value of the carboxylate ion of the acylated α-amino acid, and the K_S and k_3 values of the corresponding specific substrate, it is possible to describe, within the limits of experimental error, the α-chymotrypsin-catalyzed hydrolysis of a number of specific substrates over a substantial portion of the reaction in terms of equation 4.[3,7,8,10,11] To date we have reported[3,7-10] K_{P_1} values for the carboxylate ions derived from six acylated α-amino acids which are hydrolysis products of eight of the sixteen specific substrates of α-chymotrypsin which have been studied in these laboratories and for which revised values of K_S and k_3 are now available.[4] Since the above K_{P_1} values were determined by procedures which can now be questioned[4] we have, in this study, re-evaluated these constants from the original primary data by more objective procedures than those used previously and thus have obtained a set of more reliable and realistic constants.

Most of the primary data available for the re-evaluation of the enzyme–inhibitor dissociation constants of α-chymotrypsin and the carboxylate ions of the various acylated α-amino acids relate to the situation obtaining during the initial stages of hydrolysis of a particular specific substrate in the presence of known amounts of added inhibitor. Therefore, these primary data were re-evaluated through the use of the procedure proposed by Jennings and Niemann[12] and the initial velocities so

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

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TABLE I

K_I VALUES OF HYDROLYSIS PRODUCTS AND OF D-ENANTIOMORPHS OF PREVIOUSLY INVESTIGATED SPECIFIC SUBSTRATES OF α-CHYMOTRYPSIN[a]

Competitive inhibitor	Ref.	pH[b]	$[E] \times 10^5$ M[c,d]	$[S]_0 \times 10^3$ M	$[I] \times 10^3$ M	$E'_S \times 10^2$	$E'_I \times 10^2$	S'_S	I'_I	$K_S \times 10^3$ M[e] Orig.	$K_S \times 10^3$ M[e] Re-eval.	$k_3 \times 10^3$ M/min./mg. P-N/ml.[e] Orig.	$k_3 \times 10^3$ M/min./mg. P-N/ml.[e] Re-eval.	$K_I \times 10^3$ M Orig.	$K_I \times 10^3$ M Re-eval.[f]	Preferred
Acetyl-L-tryptophanate[g]	7	7.9	5.91	5–25[h]	10	1.2	0.59	1.0–5.0	1.0	5.3	5.0	0.50	0.55	17.5 ± 1.5	10 ± 2	
Acetyl-L-tryptophanate[g]	4, 7	7.9	5.91	5–20[i]	..	1.2	.66	1.0–4.0	..	..	5.0	...	0.55	..	9 ± 2[j]	10 ± 2
Nicotinyl-L-tryptophanate[k]	7	7.9	5.91	5–20[i]	9.7	2.4	.39	2.0–8.0	0.65	2.7	2.5	1.6	1.5	8.8 ± 1.0	15 ± 5	15 ± 5
Acetyl-L-tyrosinate[l]	8	7.9	3.95	12.5–40[m]	40	0.12	.04	0.4–1.3	0.36	30.5	32	2.4	2.4	115 ± 15[n]	110 ± 30	110 ± 30
Acetyl-L-tyrosinate[o]	3, 4	7.65[p]	0.57[q]	5–80[r]	..	.01	.01	.1–1.9	..	..	43	..	33	..	80 ± 10	80 ± 10
Trifluoroacetyl-L-tyrosinate[s]	9	7.75	1.91	15–30[t]	80	.07	.01	.6–1.1	0.50	27.6	27	4.2	4.0	119	160 ± 60	
Trifluoroacetyl-L-tyrosinate[s]	9	7.75	3.83	15–30[t]	80	.14	.03	.6–1.1	.67	27.6	27	4.2	4.0		120 ± 30	120 ± 30
Chloroacetyl-L-tyrosinate[s]	9	7.75	1.91	15–30[u]	80	.07	.01	.6–1.1	.53	27.6	27	4.2	4.0	122	150 ± 50	
Chloroacetyl-L-tyrosinate[s]	9	7.75	3.83	15–30[t]	80	..	..	.....	..	27.6	27	4.2	4.0		...[v]	150 ± 50
Nicotinyl-L-tyrosinate[l]	10	7.9	3.95	10–25[w]	12.8	..	..	.....	..	30.5	32	2.4	2.4	60	...[x]	...
D-Tyrosinhydroxamide[y]	24	6.95[z]	2.95	5–20[g]	20	0.07	0.07	0.1–0.5	0.50	41	41	3.6	3.6	40 ± 8	40 ± 8	40 ± 8
Acetyl-D-tyrosinhydroxamide[aa]	11	7.6[aa]	0.83	8–35[r]	7.5	0.02	.11	0.2–0.8	1.0	51	43	34	33	7.7	7.5 ± 1.5	
Acetyl-D-tryosinhydroxamide[k]	25	7.9	5.91	10–12.5[ab]	7.0	2.4	.8	4.0–5.0	1.0	..	2.5	...	1.5	...	7	7.5 ± 1.5
Acetyl-D-tryptophanamide[ac]	20	7.9	5.91	8–20[ad]	2.0	0.91	2.5	1.2–3.1	0.83	8.5	6.5	2.2	2.2	2.9	2.4 ± 0.4	
Acetyl-D-tryptophanamide[d]	21, 22	7.9	5.91	5–20[ad]	2.5	1.2	2.4	1.0–4.0	1.0	5.3	5.0	0.50	0.55	2.7	2.5 ± .3	
Acetyl-D-tryptophanamide[d]	21, 22	7.9	5.91	5–25[i]	5.0	1.2	2.1	1.0–5.0	1.8	5.3	5.0	.50	.55	2.9	2.8 ± .3	
Acetyl-D-tryptophanamide[d]	21, 22	7.9	5.91	5–15[ae]	7.5	1.2	2.4	1.0–3.0	3.0	5.3	5.0	.50	.55	2.4	2.5 ± .3	
Acetyl-D-tryptophanamide[d]	21, 22	7.9	5.91	5–15[ae]	10	1.2	3.0	1.0–3.0	5.0	5.3	5.0	.50	.55	2.3	2.0 ± .3	
Acetyl-D-tryptophanamide[k]	22	7.9	5.91	5–20[af]	2.5	2.4	2.1	2.0–8.0	0.89	2.7	2.5	1.6	1.5	2.5	2.8 ± .5	
Acetyl-D-tryptophanamide[k]	22	7.9	5.91	5–20[i]	5.0	2.4	2.7	2.0–8.0	2.3	2.7	2.5	1.6	1.5	2.9	2.2 ± .3	
Acetyl-D-tryptophanamide[l]	22	7.9	3.95	10–40[ag]	2.5	0.12	1.3	0.3–1.3	0.83	30.5	32	2.4	2.4	2.7	3.0 ± .5	
Acetyl-D-tryptophanamide[l]	25	7.9	3.95	20–30[ah]	2.5	.12	2.0	.6–0.9	1.25	..	32	...	2.4	...	2.0 ± .4	
Acetyl-D-tryptophanamide[ai]	25	7.85	5.91	10–15[ae]	2.0	.23	3.0	.4–0.6	1.0	..	26	...	2.6	...	2.0 ± .4	
Acetyl-D-tryptophanamide[ai]	25	7.85	5.91	10–20[i]	2.5	.23	3.0	.4–0.8	1.25	..	26	...	2.6	...	2.0 ± .4	
Acetyl-D-tryptophanamide[s]	25	7.75	1.91	15–30[t]	2.5	.07	1.0	.6–1.1	1.25	..	27	...	4.0	...	2.0 ± .4	
Acetyl-D-tryptophanamide[s]	25	7.75	3.83	12.5–30[i]	2.0	.14	1.9	.5–1.1	1.0	..	27	...	4.0	...	2.0 ± .4	
Acetyl-D-tryptophanamide[aj]	25	7.9	1.48	12.5–17[ae]	2.5	.12	0.6	1.0–1.4	1.0	..	12	...	5	...	2.5 ± .5	2.3 ± 0.4
Nicotinyl-D-tryptophanamide[k]	21	7.9	5.91	5–20[ag]	2.5	2.4	3.7	2.0–8.0	1.56	2.7	2.5	1.6	1.5	1.4	1.6 ± .3	
Nicotinyl-D-tryptophanamide[s]	25	7.75	1.91	15–25[ae]	1.0	0.07	1.3	0.6–0.9	0.57	..	27	...	4.0	...	1.5 ± .3	
Nicotinyl-D-tryptophanamide[s]	25	7.75	3.83	12.5–30[i]	0.75	.14	2.2	.5–1.1	.44	..	27	...	4.0	...	1.7 ± .3	1.6 ± 0.3
Acetyl-D-tyrosinamide[o]	11	7.6[aa]	0.83	8.8–35[ak]	10	.02	0.06	.2–0.8	.77	51	43	34	33	11.2	13 ± 2	
Acetyl-D-tyrosinamide[l]	8	7.9[al]	4.26[am]	10–40[ae]	5.0	.13	.49	.3–1.3	.45	30.5	32	2.4	2.4	12 ± 1	11 ± 3	
Acetyl-D-tyrosinamide[l]	8	7.9[al]	4.26[am]	10–45[ae]	10	.13	.36	.3–1.4	.83	30.5	32	2.4	2.4		12 ± 2	
Acetyl-D-tyrosinamide[l]	8	7.9[al]	4.26[am]	10–40[ae]	20	.13	.39	.3–1.3	1.82	30.5	32	2.4	2.4		11 ± 2	
Acetyl-D-tyrosinamide[l]	8	7.9[al]	4.26[am]	10–40[ae]	40	.13	.36	.3–1.3	3.33	30.5	32	2.4	2.4		12 ± 2	
Acetyl-D-tyrosinamide[s]	25	7.75	3.83	12.5–30[an]	10	.14	.29	.5–1.1	0.77	..	27	...	4.0	...	13 ± 3	12 ± 2
Trifluoroacetyl-D-tyrosinamide[k,as]	25	7.9	5.91	8–12.5[ae]	12	2.4	.33	3.2–5.0	0.67	..	2.5	...	1.5	...	18 ± 4	

TABLE I (*Continued*)

Competitive Inhibitor	Ref.	pH[b]	E_1 × 10^3 M[c,d]	[S]$_0$ × 10^3 M	[I] × 10^3 M	E'_S × 10^2	E'_I × 10^2	S'_S	I'_I	K_S × 10^3 M[e] Orig.	Re-eval.	k_3 × 10^3 M/min./mg. P-N/ml. Orig.	Re-eval.	K_I × 10^3 M Orig.	Re-eval.[f]	Preferred
Trifluoroacetyl-D-tyrosinamide[ai]	25	7.85	5.91	10-15[ab]	5.0	..	..	.....	..	..	26	...	2.6	...	...[ap]	
Trifluoroacetyl-D-tyrosinamide[ai]	25	7.85	5.91	10–15[ae]	8.0	0.23	0.28	0.4–0.6	0.38	..	26	...	2.6	...	21 ± 5	
Trifluoroacetyl-D-tyrosinamide[ai]	25	7.85	5.91	8–12.5[ae]	10	.23	.30	.3–0.5	.50	..	26	..	2.6	...	20 ± 5	
Trifluoroacetyl-D-tyrosinamide[s]	25	7.75	1.91	15–30[h]	10	.07	.10	.6–1.1	.50	..	27	...	4.0	...	20 ± 5	20 ± 5
Chloroacetyl-D-tyrosinamide[g,aq]	25	7.9	5.91	15–25[ae]	7.0	1.2	.84	3.0-5.0	1.0	..	5.0	...	0.55	...	7 ± 2	
Chloroacetyl-D-tyrosinamide[k]	25	7.9	5.91	8–20[ar]	7.0	2.4	.74	3.2–8.0	0.88	..	2.5	..	1.5	...	8 ± 2	
Chloroacetyl-D-tyrosinamide[l]	25	7.9	3.78	20–25[as]	3.0	0.12	.63	0.6–0.8	.50	..	32	...	2.4	...	6 ± 2	
Chloroacetyl-D-tyrosinamide[l]	25	7.9	3.78	15–30[t]	5.0	.12	.63	.5–0.9	.83	..	32	...	2.4	...	6 ± 2	
Chloroacetyl-D-tyrosinamide[ai]	25	7.85	5.91	10-20[i]	3.0	.23	1.1	.4–0.8	.55	..	26	...	2.6	...	5.5 ± 1	
Chloroacetyl-D-tyrosinamide[ai]	25	7.85	5.91	10-20[i]	5.0	.23	1.2	.4–0.8	1.0	..	26	...	2.6	...	5 ± 1	
Chloroacetyl-D-tyrosinamide[ai]	25	7.85	5.91	12.5–20[i]	10	.23	0.91	.5–0.8	1.54	..	26	...	2.6	...	6.5 ± 1	
Chloroacetyl-D-tyrosinamide[s]	25	7.75	1.91	15–30[at]	5.0	.07	.28	.6–1.1	0.72	..	27	...	4.0	...	7 ± 2	
Chloroacetyl-D-tyrosinamide[s]	25	7.75	3.83	12.6–30[i]	5.0	.14	.59	.5–1.1	.77	..	27	...	4.0	...	6.5 ± 1.5	
Chloroacetyl-D-tyrosinamide[aj]	25	7.9	1.42	12.5–15[ab]	3.0	.12	.19	1.0–1.3	.40	..	12	...	5	...	7.5	6.5 ± 1.5
Nicotinyl-D-tyrosinamide[k]	22	7.9	5.91	8.5–20[av]	5.0	2.4	.74	3.4–8.0	.63	2.7	2.5	1.6	1.5	6.0	8 ± 2	
Nicotinyl-D-tyrosinamide[s]	25	7.75	3.83	12.5–30[i]	5.0	0.14	.38	0.5-1.1	.50	..	27	4.0	4.0	...	10 ± 2	
Nicotinyl-D-tyrosinamide[s]	25	7.75	3.83	15–30[ab]	4.0	.14	.38	.6–1.1	.40	..	27	...	4.0	...	10	
Nicotinyl-D-tyrosinamide[aj]	10, 22	7.9	1.33	10.8-25[an]	5.0	.11	.17	.9–2.1	.63	15	12	6.2	5	6.2	8 ± 2	
Nicotinyl-D-tyrosinamide[aj]	10, 22	7.9	1.33	12.5-20[i]	2.5[au]	..	..	.....	..	15	12	6.2	5	6.4	...[aw]	9 ± 2
Acetyl-D-phenylalaninamide[l]	23	7.9	5.91	10–25[i]	10	0.18	0.49	0.3–0.8	0.83	30.5	32	2.4	2.4	14	12 ± 3	12 ± 3
Nicotinyl-D-phenylalaninamide[k]	22	7.9	5.91	8–20[i]	5.0	2.4	.59	3.2–8.0	.50	2.7	2.5	2.5	1.5	6.8	10 ± 2	
Nicotinyl-D-phenylalaninamide[s]	25	7.75	3.83	12.5–30[t]	6.0	0.14	.43	0.5–1.1	.67	..	27	...	4.0	...	9 ± 2	
Nicotinyl-D-phenylalaninamide[aj]	22	7.9	1.33	12.5-25[i]	5.0	0.11	.17	1.0–2.1	.63	15	12	6.2	5	7.1	8 ± 2	9 ± 2

[a] In aqueous solutions at 25° and under the conditions specified. [b] 0.02 M in the THAM component of a THAM–HCl buffer unless otherwise specified. [c] Based upon a molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin, 0.10 mg. protein-nitrogen/ml. equivalent to 2.84 × 10^{-5} M. [d] Armour preparation No. 90402 unless otherwise specified. [e] *Cf.* ref. 4. [f] Re-evaluated by the method of Jennings and Niemann[12] and a v_0 *vs.* $v_0/[S]_0$ plot[13–16] unless otherwise specified. [g] *Vs.* acetyl-L-tryptophanamide. [h] Eight experiments at five initial specific substrate concentrations within the limits indicated. [i] Four experiments at four initial specific substrate concentrations within the limits indicated. [j] Evaluated by the method of Foster and Niemann.[3] [k] *Vs.* nicotinyl-L-tryptophanamide. [l] *Vs.* acetyl-L-tyrosinamide. [m] Five experiments at five initial specific substrate concentrations within the limits indicated. [n] The mean of two values, one determined for [I] = 25 × 10^{-3} M and the other for [I] = 40 × 10^{-3} M; re-evaluation of the first set of data based upon four experiments at four initial specific substrate concentrations within the limits of 15–40 × 10^{-3} M clearly showed that these data could not be used to evaluate K_I because of the low value of I'_I, *i.e.*, 0.23. [o] *Vs.* acetyl-L-tyrosinhydroxamide. [p] 0.5 M in the THAM component of a THAM–HCl buffer. [q] Armour preparation No. 10705. [r] Seven experiments at seven initial specific substrate concentrations within the limits indicated. [s] *Vs.* chloroacetyl-L-tyrosinamide. [t] Six experiments at four initial specific substrate concentrations within the limits indicated. [u] Eight experiments at five initial specific substrate concentrations within the limits indicated, original evaluation based upon six experiments at four initial specific substrate concentrations within the same limits. [v] Corrected data failed to give a v_0 *vs.* $v_0/[S]_0$ plot that could be used for the re-evaluation of K_I. [w] Seven experiments at five initial specific substrate concentrations within the limits indicated. [x] K_I not re-evaluated because of the low value of I'_I, *i.e.*, 0.14. [y] *Vs.* L-tyrosinhydroxamide. [z] 0.2 M in the THAM component of a THAM–HCl buffer. [aa] 0.3 M in the THAM component of a THAM–HCl buffer. [ab] Two experiments at two initial specific substrate concentrations within the limits indicated. [ac] *Vs.* methyl hippurate. [ad] Eight experiments at four initial specific substrate concentrations within the limits indicated. [ae] Three experiments at three initial specific substrate concentrations within the limits indicated. [af] Five experiments at four initial specific substrate concentrations within the limits indicated. [ag] Six experiments at six initial specific substrate concentrations within the limits indicated, original evaluation based upon four experiments at four initial specific substrate concentrations within the limits of 12.5–40 × 10^{-3} M. [ah] Four experiments at three initial specific substrate concentrations within the limits indicated. [ai] *Vs.* trifluoroacetyl-L-tyrosinamide. [aj] *Vs.* nicotinyl-L-tyrosinamide. [ak] Eight experiments at eight initial specific substrate concentrations within the limits indicated. [al] 0.02 M in the EDA component of an EDA–HCl buffer. [am] Armour preparation No. 70902. [an] Seven experiments at five initial specific substrate concentrations within the limits indicated. [ao] This compound, m.p. 228–229°, was prepared from D-tyrosinamide as described for the L-isomer.[11]

[ap] K_I not evaluated because of the low value of I'_I, *i.e.*, 0.20. [aq] This compound, m.p. 202–203°, was prepared from D-tyrosinamide as described for the L-isomer.[9] [ar] Six experiments at six initial specific substrate concentrations within the limits indicated. [as] Six experiments at two initial specific substrate concentrations within the limits indicated. [at] Ten experiments at five initial specific substrate concentrations with the limits indicated. [au] Added as the DL-mixture. [av] K_I not re-evaluated because of an anticipated low value of I'_I, *i.e.*, <0.2.

obtained, from both zero- and first-order plots of the primary data, were then presented in the form of v_0 *vs.* $v_0/[S]_0$ plots[13–16] whose slopes in the presence of added competitive inhibitor are equal to $-K_S(1 + [I]/K_I)$. Thus, with knowledge of this latter quantity and of K_S and k_3 for the uninhibited reaction it is possible to arrive at a value of $K_I = K_{P_i}$ and the conclusion that the inhibition is or is not competitive in nature. In view of the evidence presented earlier[3,4,7–12] there appears to be little need for further comment upon the validity and adequacy of the above treatment and to again attest that in every case the observed inhibitions are competitive in nature in so far as can be determined within the limits of experimental error.

In three cases, *cf.* Table I, sufficient data were available to permit the use of the procedure of Foster and Niemann[3] and to arrive at a simultaneous evaluation of K_S, k_3 and K_{P_i} for the specific substrate and its only competitive hydrolysis product from a study of the extended hydrolysis of the specific substrate in the absence of added hydrolysis product.[3,4] As will be pointed out later, values of K_{P_i} so determined are in a sense more reliable than those evaluated by the other procedure described above.

As before[4] care was taken to specify, as completely as possible, the reaction conditions which 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, that must be maintained within certain limits[4–6] in order to satisfy the assumptions inherent in the various treatments. For purposes of calculation it was assumed that the molecular weight of monomeric α-chymotrypsin is 22,000 and that its nitrogen content is 16.0%.[4]

The revised values of $K_I = K_{P_i}$ for the five acylated α-amino acid anions for which satisfactory data are available[3,7–10] are summarized in Table I. It will be noted that in every case [E] was of the order of 10^{-5} *M*, $E'_S = [E]/K_S$ less than 2.4×10^{-2}, $E'_I = [E]/K_I$ less than 0.7×10^{-2} and $S'_S = [S]/K_S$ within the limits of 0.1 and 8.0. Thus, in all of the experiments with these acylated α-amino acid anions the reaction conditions were such as to ensure the presence of substantially monomeric α-chymotrypsin, the maintenance of zone A conditions with respect to both the specific substrate and the competitive inhibitor,[4–6] and the attainment of values of S'_S between the limits necessary for the application of equation 5, in the form of

$$-d[S]/dt = k_3[E][S]/K_S(1 + [I]/K_I) + [S] \quad (5)$$

(13) G. S. Eadie, *J. Biol. Chem.*, **146**, 85 (1942).
(14) K. B. Augustinssen, *Acta Physiol. Scand.*, **15**, suppl. 52 (1948).
(15) H. J. Hofstee, *Science*, **116**, 329 (1952).
(16) G. S. Eadie, *ibid.*, **116**, 688 (1952).

v_0 *vs.* $v_0/[S]_0$ plots.[13–17] However, the values of $I'_I = [I]/K_I$ in most cases were too low to permit the re-evaluation of K_I with more than modest accuracy and in two instances, *i.e.*, acetyl-L-tyrosinate, $[I] = 25 \times 10^{-3}$ *M*, *vs.* acetyl-L-tyrosinamide and nicotinyl-L-tyrosinate *vs.* acetyl-L-tyrosinamide the values of I'_I were so low as to preclude a re-evaluation of K_I.[18]

Of the hydrolysis products listed in Table I the values of $K_I = K_{P_i}$ for acetyl-L-tryptophanate and for acetyl-L-tyrosinate at *p*H 7.65 are the most reliable and those for nicotinyl-L-tryptophanate, acetyl-L-tyrosinate at *p*H 7.9, trifluoroacetyl-L-tyrosinate and chloroacetyl-L-tyrosinate are of sufficient reliability to be useful in most cases. However, it should be appreciated that all but two of these K_I values were evaluated in systems containing added inhibitor and that in adding the inhibitor, in the form of its sodium salt, the ionic strengths of the systems were increased. It has been noted earlier[4,9] that an increase in the ionic strength of the reaction system causes an increase in the rate of the reaction and therefore in addition to the anticipated decrease in rate associated with normal competitive action one must allow for an increase in rate when ionizable competitive inhibitors are used in relatively high concentrations. When allowance is not made for the increase in the ionic strength of the reaction system it follows that the effectiveness of the inhibitor in the competitive process is underestimated. Studies on the effect of ionic strength on the over-all rate of the reaction and upon values of K_S and k_3 and K_{P_i} are now in progress and until these studies are completed almost all of the $K_I = K_{P_i}$ values given in Table I must be accepted as provisional values subject to correction for ionic strength effects. However these provisional values may be used as constants in equation 4 when K_{P_j} is large relative to K_S since under these circumstances a rather large error in K_{P_j} can be tolerated without serious consequences. If the K_I values in Table I are so used it must be remembered that values of K_I for the acylated α-amino acid anions are *p*H dependent[19] and that even when they have been determined under conditions where no added inhibitor is present they also may be dependent upon the ionic strength of the reaction system. Thus extensive extrapolations with respect to these latter two parameters are not justifiable at the present time.

In previous communications from these laboratories[8,10,11,20–24] we have reported values for the en-

(17) For an experimental error of ±5% the permissible limits of S's are 0.05 to 20 and for an error of ±10%, 0.1 to 10.
(18) It will be seen from equation 5 that for a value of I'_I of 0.1 and an experimental error of ±10% the term $K_S(1 + [I]/K_I)$ is equal to K_S within the limits of error. Since, in the evaluation of K_I the probable experimental error is likely to be at least twice this value it is obvious that values of I'_I must exceed 0.2 for K_I values to be of significance and if they are to be estimated with any reasonable degree of accuracy the value of I'_I should be of the order of 1.0 or greater.
(19) Unpublished observations of R. J. Foster and R. R. Jennings.
(20) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634 (1952).
(21) H. T. Huang and C. Niemann, *ibid.*, **73**, 1541 (1951).
(22) H. T. Huang and C. Niemann, *ibid.*, **73**, 1555 (1951).
(23) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, **74**, 105 (1952).
(24) R. J. Foster, R. R. Jennings and C. Niemann, *ibid.*, **76**, 3142 (1954).

zyme–inhibitor dissociation constants of α-chymotrypsin and the D-enantiomorphs of eight of the previously investigated specific substrates of this enzyme. Since these K_I values, with but one exception,[24] were obtained from primary data by methods which involved a more or less subjective estimation of initial velocities obtaining in the initial stages of the reactions in question, we have in this communication re-evaluated all of these data by the method of Jennings and Niemann[12] and from subsequent v_0 *vs.* $v_0/[S]_0$ plots,[13-16] based upon equation 5, have obtained a set of revised values of K_I. In addition we have collected all unpublished data which is in our possession[25] which relates to the competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of L-specific substrates by their D-enantiomorphs and have obtained, by the same evaluation procedures noted immediately above, additional values of K_I including those for two D-enantiomorphs, *i.e.*, trifluoroacetyl- and chloroacetyl-D-tyrosinamide, which have not been described in our earlier publications. The same precautions observed in the re-evaluation of the K_I values for the hydrolysis products also have been observed in the corresponding operation with the D-enantiomorphs.

The data relating to the D-enantiomorphs are summarized in Table I and it will be seen 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 3.7×10^{-2} and S's within the limits of 0.1 and 8.0. Thus, as for the hydrolysis products noted above, it may be concluded that the experimental data employed for the re-evaluation of the enzyme–inhibitor dissociation constants of α-chymotrypsin and the D-competitive inhibitors considered in this study were obtained under conditions which ensured the presence of essentially monomeric α-chymotrypsin, which satisfied the usual zone A criteria with respect to both the specific substrate and the competitive inhibitor[4-6] and which were compatible with the use of equation 5, in the form of v_0 *vs.* $v_0/[S]_0$ plots,[13-16] for the evaluation of the various enzyme–inhibitor dissociation constants. With respect to values of I'_I it was found in a few instances that the values were so low, *e.g.*, a value of 0.20 in one set of experiments with trifluoroacetyl-D-tyrosinamide *vs.* trifluoroacetyl-L-tyrosinamide, and a value of 0.28 in a set of experiments with nicotinyl-D-tyrosinamide *vs.* nicotinyl-L-tyrosinamide, where the inhibitor was added as the DL-mixture,[10] to preclude the use of the data in question for the re-evaluation of K_I. However, in general, the magnitude of I'_I was such as to lead to significant values of K_I.

The fact that the experimental data obtained in the various inhibition studies could be interpreted without exception in terms of v_0 *vs.* $v_0/[S]_0$ plots[13-16] based upon equation 5 is in itself an argument that in every case the observed inhibition was competitive in nature in so far as could be determined within the limits of experimental error. A corollary of the above criterion of competitive inhibition under zone A conditions,[3-6] *i.e.*, that in a v_0 *vs.* $v_0/[S]_0$ plot[13-16] the ordinate intercept remains constant while the slope increases from $-K_S$ in the absence of the inhibitor to $-K_S(1 + [I]/K_I)$ in the presence of the inhibitor and that the abscissa intercept increases similarly from $k_3[E]/K_S$ to $k_3[E]/K_S(1 + [I]/K_I)$, demands that K_I for a given system be independent of [I]. There are in Table I several sets of experiments which bear on this point. For example K_I was found to be independent of [I], within the limits of experimental error, for the system α-chymotrypsin *vs.* acetyl-D-tryptophanamide *vs.* acetyl-L-tryptophanamide over a fourfold range of values of [I] and for the system α-chymotrypsin *vs.* acetyl-D-tyrosinamide *vs.* acetyl-L-tyrosinamide over an eightfold range of values of [I].

Although it is usually assumed that adherence to the above criterion and its corollary is sufficient to establish competitive inhibition, it has been noted[22] that a second criterion, which demands that the value of K_I for a given competitive inhibitor be independent of the nature of the specific substrate used in its evaluation, *i.e.*, that K_S be an independent variable in the evaluation of K_I, also must be satisfied. If adherence to both of these criteria is observed one can be doubly assured that the inhibition is competitive in nature. In addition, if the value of K_I for a given competitive inhibitor is found to be independent of the value of K_S used in its evaluation, useful information relative to the accuracy of the respective values of K_S and K_I is obtained as is knowledge of those structures which are capable of interacting with the enzyme at a common site.

Of the ten D-enantiomorphs considered in Table I two were studied in competition with but a single specific substrate, two in competition with two different specific substrates, four in competition with three different specific substrates, one in competition with six different specific substrates and one in competition with seven different specific substrates. The results of these experiments, particularly those with acetyl-D-tryptophanamide and chloroacetyl-D-tyrosinamide, provided convincing evidence that the inhibition being studied is competitive in nature, and that the values of K_S for the various specific substrates which were used for the evaluation of the enzyme–inhibitor dissociation constants of these competitive inhibitors were reasonably reliable.

It will be seen from data presented in Table I that a change from a 0.02 *M* THAM–HCl buffer[26] to a 0.02 *M* EDA–HCl buffer[27] was without demonstrable effect upon the K_I value of acetyl-D-tyrosinamide and also that there was no indication that the K_I value of an uncharged competitive inhibitor is affected, within the limits of experimental error, by modest changes in the *p*H of the reaction system. The few data that are available with respect to the effect of changes in the ionic strength of the reaction system also suggest that with uncharged inhibitors the value of K_I is essentially independent of the ionic strength of the reaction system.

The preferred values of K_I which are given in

(25) From unpublished experiments of H. T. Huang, H. J. Shine and D. W. Thomas.

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

(27) Ethylenediamine-hydrochloric acid buffer.

Table I are of sufficient reliability to be used in equation 6 to describe the course, over an extended

$$k_3[E]t = K_S(1 + [S]_0/K_{P_1} + [I]/K_I) \ln [S]_0/[S]_t + (1 - K_S/K_{P_1})([S]_0 - [S]_t) \quad (6)$$

range, of the α-chymotrypsin-catalyzed hydrolysis of a number of representative specific substrates of this enzyme in the presence of their D-enantiomorphs. With but one exception we wish to defer discussion of the relationships existing between the K_I values and the structures of the various competitive inhibitors listed in Table I until we have had an opportunity to present revised values for the other competitive inhibitors which have been studied in these laboratories.

From a comparison of the K_I and K_S values of the pairs acetyl-D- and L-tryptophanamide, nicotinyl-D- and L-tryptophanamide, acetyl-D- and L-tyrosinamide, nicotinyl-D- and L-tyrosinamide, acetyl-D- and L-phenylalaninamide and nicotinyl-D- and L-phenylalaninamide and a consideration of molecular models of these compounds and of surfaces which were complementary to certain aspects of these models we were led to the hypothesis that the modes of combination of the D- and L-enantiomorphs with the catalytically active site of the enzyme were substantially the same, and because of the parallel behavior of K_I and K_S in the above series it was believed that K_S could be taken as being approximately equal to k_2/k_1.[7,10,23] Substituting the revised values of K_S and K_I for the original values of the above series does not require us to alter the above conclusion. However, with data now available for the first time for trifluoroacetyl-D- and L-tyrosinamide and chloroacetyl-D- and L-tyrosinamide it is clear that our original conclusion is in error in at least one respect. For the series acetyl-, trifluoroacetyl-, chloroacetyl- and nicotinyl-L-tyrosinamide the values of K_S are in the ratio of 32:26:27:12 whereas the corresponding values of K_I for the D-enantiomorphs are in the ratio of 12:20:6.5:9. Thus, with no tendency for parallel behavior of the K_S and K_I values in this series it is clear that unless one resorts to the undesirable practice of introducing additional *ad hoc* hypotheses it must be concluded that (a) the modes of combination of the D- and L-enantiomorphs are significantly different and that the variation of the K_S and K_I values with the structures of the above specific substrates and competitive inhibitors cannot be used to determine whether K_S does or does not approximate k_2/k_1 or, (b) that the modes of combination of the enantiomorphs are substantially the same and that K_S does not approximate k_2/k_1. Because we have at hand a considerable amount of unpublished data which supports the conclusion that K_S does approximate k_2/k_1 for the specific substrates which have been considered in earlier studies conducted in these laboratories[4] we suggest that the first explanation given above is the correct one.

The authors are indebted to Drs. H. T. Huang and D. W. Thomas for the use of previously unpublished data.

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Reactivation of Human Serum Esterase Inhibited by Alkylphosphates[1]

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RECEIVED NOVEMBER 1, 1954

Human serum esterase inhibited by tetraethyl pyrophosphate or diisopropyl fluorophosphate may be reactivated 50% in 15 minutes in the former case and in 3 hours in the latter instance by suitable hydroxamic acids. Hydroxylamine and pyridine also were active. Quaternary ammonium hydroxamic acids are much better reactivators than the tertiary amino or primary ammonium acids. Thus the distinction between quaternary, tertiary and primary nitrogen groups observed in the enzyme still persists in the inhibited enzyme, indicating that the anionic site of the enzyme still functions to bind and orient the reactivating molecules.

Introduction

Certain phosphate esters such as tetraalkyl pyrophosphates, dialkyl *p*-nitrophenyl phosphates, and dialkyl fluorophosphates are potent irreversible inhibitors of the cholinesterases and esterases in general. The development of the theory of enzymic hydrolysis clarified the mechanism of irreversible inhibition and suggested the means whereby reactivation might be achieved. During the course of the enzymatic hydrolysis of carboxylic esters a basic group in what has been termed the esteratic site of the enzyme makes a nucleophilic attack upon the carbonyl carbon of acetylcholine. An acylated enzyme is formed as an intermediate which reacts rapidly with water to yield carboxylic acid and free enzyme.[2] In the case of the irreversible inhibitors the basic group of the esteratic site is phosphorylated, involving the same nucleophilic substitution mechanism,[3,4] but unlike the acylated enzyme this compound reacts only very slowly with water. As a result the enzyme is converted to a dialkylphosphoryl enzyme which in view of the theory is expected to be without activity. It is the slowness with which the phosphoryl enzyme reacts with water which makes these compounds inhibitors rather than substrates.

The theory suggests that esterase might be

(1) This work was supported in part by the Medical Research and Development Board, Department of the Army, Office of the Surgeon General, Contract No. DA-49-007-MD-37, and in part by the Division of Research Grants and Fellowships of the National Institutes of Health, Grant No. B-573, United States Public Health Service.

(2) (a) I. B. Wilson, F. Bergmann and D. Nachmansohn, *J. Biol. Chem.*, **186**, 781 (1950); (b) I. B. Wilson, "The Mechanism of Enzyme Action," ed. by W. D. McElroy and Bentley Glass, The Johns Hopkins Press, Baltimore, Md., 1954, p. 642.

(3) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950).

(4) I. B. Wilson, *ibid.*, **190**, 111 (1951).