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Re-evaluation of the Kinetic Constants of Previously Investigated Specific Substrates of α-Chymotrypsin[1]

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The constants K_S and k_3 of sixteen representative specific substrates of α-chymotrypsin have been re-evaluated from primary data by procedures which are more objective than those employed previously and more reliable and realistic values for these constants have been obtained. Attention has been called to a relationship existing between the refractivity of the R′ group in specific substrates of the general formula $R'CONHCH(CH_2C_6H_4OH)CONH_2$ and the corresponding values of k_3.

For an enzyme-catalyzed reaction whose rate, in so far as it is dependent upon the concentration of enzyme and specific substrate, can be described within the limits of experimental error and for the initial stages of the reaction by equations 1 and 2,

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

$$k_3[E]t = K_S \ln [S]_0/[S]_t + ([S]_0 - [S]_t) \quad (2)$$

where $K_S = (k_2 + k_3)/k_1$, and throughout its course by equations 1, 3, 4 and 5,

$$E_f + P_{1f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 \quad (3)$$

$$E_f + P_{2f} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP_2 \quad (4)$$

$$k_3[E]t = K_S(1 + [S]_0 \sum_{j=1}^{n} 1/K_{P_j}) \ln [S]_0/[S]_t + (1 - K_S \sum_{j=1}^{n} 1/K_{P_j})([S]_0 - [S]_t) \quad (5)$$

where $K_{P_1} = k_5/k_4$ and $K_{P_2} = k_7/k_6$, it has been customary to evaluate the constants K_S and k_3 by graphical procedures based upon the differential form of equation 2; *i.e.*, equation 6.[3-7] However, as has been noted previously,[8,9] all of these graphical procedures usually require prior evaluation of the

$$-d[S]/dt = k_3[E][S]/(K_S + [S]) \quad (6)$$

initial velocities associated with the various initial specific substrate concentrations and since this requirement ordinarily has been satisfied by extrapolations to $t = 0$ from arbitrarily selected non-linear zero or first-order plots of $([S]_0 - [S]_t)$ *vs.* t or of $\ln[S]_0/[S]_t$ *vs.* t it is possible that the values of the initial velocities so obtained, and the values of K_S and k_3 derived from them, may be in error because of the subjective nature of the above operations. Therefore, with the availability of two new procedures,[8,9] one[8] primarily intended for the evaluation of the data obtained during the initial stages of the reaction, *i.e.*, where equations 1 and 2 are a valid representation of the system under consideration, and the other[9,10] which is most useful when the first becomes very cumbersome,[8] *i.e.*, where the reaction has been allowed to proceed to its terminal stages so that its course is most accurately described by equations 1, 3, 4 and 5, which permit a far more objective evaluation of the initial velocities, and in turn the constants K_S and k_3, than can be achieved by the older methods which we had used previously in the evaluation of the kinetic constants of α-chymotrypsin and sixteen specific substrates of this enzyme[8,9,11-26] we have in this communication re-evaluated all of these constants by one or both of the newer and more objective procedures and thus have obtained a set of more reliable and realistic values of K_S and k_3 for α-chymotrypsin and these specific substrates. In several instances where previously unpublished data were available[27] these data were employed along with the original primary data in the re-evaluation process.

In view of the fact that both of the procedures used for the re-evaluation of the constants K_S and k_3 are based, at least in part, on equation 1 it is appropriate to inquire as to whether the assumptions inherent in a steady-state formulation[28] can

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(10) Attention is called to three typographical errors in ref. 9. on pp. 1000 and 1002 the equation describing the slopes of the $([S]_0 - [S]_t)/t$ *vs.* $(\ln[S]_0/[S]_t)/t$ plots should read "$-K_S(K_P + [S]_0)/(K_P - K_S)$" instead of "$-K_S(K_P + [S]_0)/(K_S - K_P)$" and in Fig. 1 the slopes of the lines drawn through the hypothetical experimental points should read "$-K_S(K_P + [S]_0)/(K_P - K_S)$" instead of "$K_S(K_P + [S]_0)/(K_P - K_S)$."

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be satisfied for the particular cases of interest. The assumption that the concentration of ES be low, *i.e.*, that ES be more reactive than either E or S,[28] has been satisfied for the cases at hand by the repeated observation that individually neither E nor S gives rise to products under the conditions employed in our studies.[11-26] The second assumption that $d[ES]/dt \doteq 0$, *i.e.*, that the induction period has been passed prior to the beginning of observations,[28] also appears to be a valid one since at no time has it been possible to observe such an induction period in any of the systems which have been studied to date.[11-26] While it is implied in equation 1 that an equilibrium precedes the rate-determining step it is appreciated that this will be true only if k_2 is substantially greater than k_3. However, since, for the present, we are concerned only with the redetermination of values of K_S and k_3, and not with their interpretation,[29-31] we can accept equation 1 as a satisfactory formulation of the initial stages of the reactions under consideration and reserve for the future the interpretation of K_S for each particular situation, *i.e.*, whether $K_S = (k_2 + k_3)/k_1$, or $\doteq k_2/k_1$, or $\doteq k_3/k_1$.

Equation 2, or equation 6, may be taken as the rate equation for the reaction depicted in equation 1 provided $[S] \doteq [S_f] >> [ES]$, *i.e.*, that the reaction be allowed to proceed under zone A condition.[32,33] In order to be certain that this requirement was satisfied in all cases encountered in this study $E'_S = [E]/K_S$ was evaluated in every instance and in no case was the value of E'_S found to exceed 2.5×10^{-2}, a value far below the maximum values of 0.1 and 0.6 ordinarily associated with observations which may be in error by ±1 and ±5%, respectively.[32,33]

A second condition that is important for the simultaneous evaluation of K_S and k_3 by methods based upon equations 2 and 6 relates to the relative magnitudes of [S] and K_S. If [S] is very small relative to K_S equation 6 may be approximated, within the limits of experimental error, by equation 7 and if [S] is very large relative to K_S equation 6 may be

$$-d[S]/dt = k_3[E][S]/K_S \qquad (7)$$

similarly approximated by equation 8. Since solutions of equation 7 can lead to values of only K_S/k_3

$$-d[S]/dt = k_3[E] \qquad (8)$$

or its reciprocal and solutions of equation 8 to values of only k_3 it is obvious that if both K_S and k_3 are to be simultaneously evaluated care must be taken to select values of [S] with reference to expected values of K_S.

For an experimental error of ±5% it follows that for values of $[S]/K_S = S'_S$ of less than 0.05 the experimental observations will be satisfactorily described by equation 7 and for values of S'_S of greater than 20 by equation 8. Only if values of S'_S are greater than 0.05 and less than 20 will the use of methods based upon equations 2 or 6 lead to the simultaneous evaluation of both K_S and k_3. In this study we have taken ±5% as an optimistic, and in a sense as a conservative, estimate of the probable experimental error and have computed values of S'_S for each system investigated. Since in no case was a value of S'_S found to be less than 0.05 or greater than 10 it is clear that in so far as the relation between [S] and K_S was concerned the use of equations 2 and 6 for the evaluation of K_S and k_3 of these systems was completely justified. In only one instance, *i.e.*, of L-tyrosinhydroxamide, was the lower limit of the S'_S values identical with the lower limit of S'_S specified above.

While knowledge of the molecular weight of α-chymotrypsin is not needed for the calculation of values of K_S and k_3 for the various specific substrates, under the conditions specified, the lack of such knowledge results in the necessity of expressing values of k_3 in very awkward units, *i.e.*, *M*/min./mg. protein-nitrogen/ml. Furthermore, without knowledge of the molecular weight, or more properly the combining weight, of the enzyme values of E'_S cannot be computed. Therefore, on the basis of values which have been reported previously[34-43] we have chosen, for purposes of calculation in this investigation, a value of 22,000 for the molecular weight of monomeric α-chymotrypsin and in addition have assumed that the nitrogen content of the enzyme is 16.0%.[35,44] Since there is reason to believe[40-42] that at concentrations of the order of 10^{-5} *M* or less α-chymotrypsin is present in aqueous solutions essentially in the form of the monomer we have assumed that a solution which contains 0.1 mg. of protein-nitrogen/ml. is, on the basis of a molecular weight of 22,000 and a nitrogen content of 16.0%, 2.84×10^{-5} *M* in this enzyme. It will be seen from the accounts of the various experiments reported in this communication that α-chymotrypsin concentrations of the order of 10^{-5} *M* were used in almost every instance.

In the interpretation of values of E'_S, *i.e.*, the specific enzyme concentration, it is appropriate to recall that the permissible upper limit of E'_S, of 0.1 or 0.6 for assumed experimental errors of ±1 or ±5%, respectively, for the practical fulfillment of zone A conditions for the cases at hand is based upon the supposition that the enzyme in question contains but one catalytically active site per molecule.[32,33] That this latter condition is satisfied for α-chymotrypsin is evident from previous investigations[38,39,43,45] which provide substantial evidence

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that this enzyme contains but one catalytically active site per molecule of *ca.* 25,000.

Equation 1 contains the tacit assumptions that both the enzyme and the specific substrate are monomeric and that both of these species are monofunctional in the sense employed in previous communications from these laboratories.[19,22,31,46] While it can be concluded from the previous discussion that the enzyme, *i.e.*, α-chymotrypsin, will be monomeric in aqueous solutions at 25°, provided that its concentration is of the order of 10^{-5} *M* or less, it must be admitted that there is very little direct evidence, other than that provided by MacAllister, Harmon and Niemann,[11] to support the contention that the specific substrate will be monomeric in aqueous solutions at 25° for all specific substrates and for all concentrations employed. However, in view of the nature of the various specific substrates that have been used in the experiments under consideration it is not unreasonable to assume that these compounds will be monomeric under the conditions specified.

For operations under zone A conditions[32,33] and where the specific substrate concentrations are never sufficiently high so as to permit the formation of significant amounts of enzyme–substrate complexes of the type $E(S)_2$ and $E(S)_3$ there still remains the possibility that a variety of enzyme–substrate complexes of the type ES may be formed, by alternative modes of combination, if both the catalytically active site of the enzyme and the specific substrate are polyfunctional.[19,22,31,46] Since competitive inhibition by an excess of specific substrate has not been encountered in any of the reactions considered in this communication, probably because the solubilities of the various specific substrates, in aqueous solutions at 25°, were so low as to preclude the attainment of sufficiently high specific substrate concentrations for the systems in question, it is clear that we need not concern ourselves at this time with the consequences arising from the formation of enzyme–substrate complexes of the type $E(S)_2$ and $E(S)_3$. However, for the remaining case, *i.e.*, that involving the possible formation of several enzyme–substrate complexes of the type ES by alternative modes of combination, it must be recalled[19,22,31,46] that while this situation will not influence the actual numerical values obtained through the use of equations 2, 5 or 6 it does afford the possibility that these values do not correspond to K_S and k_3 values as defined by equations 1, or 1, 3 and 4, and thus are in this sense apparent rather than true values.

The *p*H optima for the α-chymotrypsin-catalyzed hydrolysis, in aqueous solutions at 25°, of twelve of the sixteen specific substrates considered in this investigation are given in Table I.[47] In the re-evaluation of the kinetic constants of the sixteen specific substrates we have used the method of Jennings and Niemann[8] for the determination of the initial velocities from both $([S]_0 - [S]_t)$ *vs.* t and $\ln([S]_0/[S]_t)$ *vs.* t plots coupled with subsequent v_0 *vs.* $v_0/[S]_0$ plots[4-7] for all of those data which were derived from observations made during the initial stages of the various reactions under consideration.[48] For those cases where the primary data were derived from observations made beyond the initial stages of the reaction in question the method of Foster and Niemann[9] was used for the re-evaluation of such data. In addition to its pertinence and convenience this latter procedure also provides for the simultaneous evaluation of K_{P_1} in the cases under consideration.[9] Care has been taken to specify as completely as possible the reaction conditions that were employed for each particular set of experiments and in the course of re-evaluation the primary data available for a particular specific substrate were grouped as to disclose any variation that might arise from a change in experimental conditions. The indicated limits of error for the re-evaluated constants represent the extremes of error, as determined graphically from v_0 *vs.* $v_0/[S]_0$ plots, for each particular set of data.

TABLE I

*p*H OPTIMA FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF TWELVE SPECIFIC SUBSTRATES[a]

Specific substrates	*p*H Opt.	Buffer system
Methyl hippurate[22]	7.2–8.3	0.02 *M* THAM[b]
L-Tyrosinhydroxamide[20]	6.95 ± 0.05	.1 *M* THAM[c,d]
Acetyl-L-tyrosinhydroxamide[24]	7.6 ± 0.05	.3 *M* THAM[e]
Acetyl-L-tryptophanamide[14]	7.2–8.5	.02 *M* THAM[b]
Nicotinyl-L-tryptophanamide[14]	7.2–8.5	.02 *M* THAM[b]
Acetyl-L-tyrosinamide[15]	7.9 ± 0.1	.02 *M* THAM[b,f]
Trifluoroacetyl-L-tyrosinamide[19]	7.85 ± 0.05	.02 *M* THAM[b]
Chloroacetyl-L-tyrosinamide[19]	7.75 ± 0.05	.02 *M* THAM[b]
Nicotinyl-L-tyrosinamide[18]	7.9 ± 0.1	.02 *M* THAM[b]
Acetyl-L-phenylalaninamide[20]	7.75 ± 0.15	.02 *M* THAM[b]
Nicotinyl-L-phenylalaninamide[20]	7.75 ± 0.15	.02 *M* THAM[b]
Acetyl-L-hexahydrophenylalaninamide[8]	7.9 ± 0.1	.02 *M* THAM[b]

[a] In aqueous solutions at 25°. [b] 0.02 *M* in the THAM component of a THAM–HCl buffer. [c] 0.1 *M* in the THAM component of a THAM–HCl buffer. [d] Several determinations were also made with a cacodylic acid–sodium cacodylate buffer, which was 0.1 *M* in arsenic added as cacodylic acid. [e] 0.3 *M* in the THAM component of a THAM–HCl buffer. [f] Several determinations were also made with an EDA–HCl buffer which was 0.02 *M* in the EDA component.

The preferred values of K_S and k_3 which are given in Table II, and which are based upon the results of our re-evaluation of all of the primary data which have been obtained in these laboratories, are not of equal reliability and some of them are no more than provisional values. The constants for L-tyrosinhydroxamide, acetyl-L-tyrosinhydroxamide, nicotinyl-L-tryptophanamide, acetyl-L-tyrosinamide, chloroacetyl-L-tyrosinamide and acetyl-L-hexahydrophenylalaninamide are probably the most reliable and those for formyl-L-tyrosinamide, benzoyl-L-tyrosinamide, isonicotinyl-L-tyrosinamide and acetyl-L-tyrosylglycinamide the least reliable. It also must be emphasized that with no system considered in this study was the initial specific substrate concentration varied over more than a twenty-fold range and the enzyme concentration over more than a three-fold range. Thus, in accepting equation 2 as the integrated rate equation for the initial stages of the α-chymotrypsin-catalyzed hydrolysis, under the conditions

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(47) In this table and elsewhere THAM is used as an abbreviation for tris-(hydroxymethyl)-aminomethane and EDA for ethylenediamine.

(48) In general, those reactions which were allowed to proceed to an extent of *ca.* 30% were considered to be in their initial stages.

TABLE II

RE-EVALUATION OF THE KINETIC CONSTANTS OF SIXTEEN SPECIFIC SUBSTRATES OF α-CHYMOTRYPSIN[a]

Specific substrate	Ref.	pH	[E] × 10^5, M[b]	$[S]_0$ × 10^3, M	E's × 10^2	S's	K_S × 10^3, M Orig.	K_S × 10^3, M Re-eval.	K_S × 10^3, M Preferred	k_2 × 10^3, M/min./mg. P-N/ml Orig.	k_2 × 10^3, M/min./mg. P-N/ml Re-eval.	k_2 × 10^3, M/min./mg. P-N/ml Preferred	k_2, M/sec./M[b] Re-eval.	k_2, M/sec./M[b] Preferred
Methyl hippurate	22	7.9[c]	5.91[d]	5–25[e]	0.9	0.8–3.9	8.5	6.5 ± 0.5[f]	6.5 ± 0.5	2.2	2.2 ± 0.2[f]	2.2 ± 0.2	$0.12_9 \pm 0.01_2$	$0.12_9 \pm 0.01_2$
L-Tyrosinhydroxamide	23, 26	6.9[g]	2.95[d]	2–40[h]	0.07	0.05–1.0	32 ± 2	41 ± 2[f]	41 ± 2	3.0 ± 0.3	3.6 ± 0.2[f]	3.6 ± 0.2	$0.21_2 \pm .01_2$	$0.21_2 \pm .01_2$
Acetyl-L-tyrosinhydroxamide	24, 25	7.6[i]	0.59[d]	5–40[j]	0.01	0.1–0.8	51	50 ± 5[f]		34	32 ± 3[f]		$1.8_8 \pm 0.1_8$	
	24, 25	7.6[i]	0.84[d]	5–35[k]	0.02	0.15–0.9		40 ± 5[f]			32 ± 3[f]		$1.8_8 \pm .1_8$	
	9	7.65[l]	0.57[m]	5–80[n]	0.01	0.1–1.9	42 ± 2	42 ± 2[o,p]	43 ± 4	34 ± 2	34 ± 2[o]	33 ± 3	$1.9_9 \pm .1_1$	$1.9_4 \pm .1_8$
Acetyl-L-tryptophanamide	14	7.9[c]	2.95[d]	5–25[q]	0.65	1.1–5.6	5.3 ± 0.2	4.5 ± 1[f]		0.5	0.5 ± 0.1[f]		$0.02_9 \pm .00_6$	
	14	7.9[c]	5.91[d]	5–25[r]	1.2	1.0–5.0		5.0 ± 0.5[f]			$0.55 \pm .1$[f]		$.03_2 \pm .00_6$	
	14	7.9[c]	5.91[d]	5–20[s]	1.2	1.0–4.0		5.0 ± 0.5[o,t]	5.0 ± 0.5		$0.55 \pm .1$[o]	0.55 ± 0.1	$.03_2 \pm .00_6$	$0.03_2 \pm .00_6$
Nicotinyl-L-tryptophanamide	14	7.9[c]	1.96[d]	5–20[u]	0.75	1.9–7.4	2.7 ± 0.2	2.7 ± 0.3[o]		1.60	$1.6 \pm .2$[o]		$.09_4 \pm .01_2$	
	14	7.9[c]	4.12[d]	5–20[u]	1.85	2.3–9.1		2.2 ± 0.3[o]			$1.5 \pm .2$[o]		$.08_8 \pm .01_2$	
	14	7.9[c]	5.91[d]	5–20[v]	2.45	2.1–8.3		2.4 ± 0.2[o,w]			$1.5 \pm .2$[o]		$.08_8 \pm .01_2$	
	21	7.9[c]	4.09[x]	5–20[y]	1.65	2.0–8.0	2.7	2.5 ± 0.2[f]		1.5	$1.5 \pm .2$[f]		$.08_8 \pm .01_2$	
	27	7.9[c]	5.91[d]	5–20[z]	2.35	2.0–8.0	.....	2.5 ± 0.2[f]		.....	$1.5 \pm .2$[f]		$.08_8 \pm .01_2$	
	27	7.9[c]	2.44[x]	5–20[s]	0.85	1.8–7.1	.....	2.8 ± 0.3[f]	2.5 ± 0.2	.....	$1.6 \pm .2$[f]	1.5 ± 0.2	$.09_4 \pm .01_2$	$0.08_8 \pm .01_2$
Formyl-L-tyrosinamide	12	8.0[aa]	4.26[ab]	10–40[ac]	0.35	0.8–3.3	11.2	12 ± 3[f]	12 ± 3	0.39	$0.45 \pm .05$[f]	0.45 ± 0.05	$.02_6 \pm .00_3$	$0.02_6 \pm .00_3$
Acetyl-L-tyrosinamide	12, 15, 16	7.8[aa]	3.95[d]	8–40[q]	0.10	0.3–1.3	30.5 ± 1.0	32 ± 4[f]		2.4 ± 0.1	$2.3 \pm .03$[f]		$.13_5 \pm .01_8$	
	12, 15, 16	7.9[c]	3.95[d]	10–30[ad]	.10	.3–1.2		33 ± 4[f]			$2.5 \pm .3$[f]		$.14_7 \pm .01_8$	
	12, 15, 16	8.0[aa]	4.26[ab]	5–40[s]	.15	.2–1.3		30 ± 5[f]			$2.5 \pm .5$[f]		$.14_7 \pm .03_0$	
	12, 15, 16	7.8[c]	5.91[d]	5–40[n]	.20	.2–1.3		32 ± 4[f]	32 ± 4		$2.4 \pm .3$[f]	$2.4 \pm .3$	$.14_1 \pm .01_8$	$.14_1 \pm .01_8$
Trifluoroacetyl-L-tyrosinamide	19	7.85[c]	4.43[d]	8–17.5[ae]	.15	.3–0.6	30.0	30 ± 5[f]		2.8	$2.7 \pm .5$[f]		$.15_8 \pm .03_0$	
	19	7.85[c]	5.91[d]	5–17.5[af]	.25	.2–0.7	30.2	25 ± 5[f]	26 ± 5	2.9	$2.5 \pm .5$	$2.6 \pm .5$	$.14_7 \pm .03_0$	$.15_3 \pm .03_0$
Chloroacetyl-L-tyrosinamide	19	7.75[c]	1.92[d]	10–30[ag]	.05	.4–1.2	26.0	26 ± 3[f]		4.3	$4.0 \pm .2$[f]		$.23_5 \pm .01_2$	
	19	7.75[c]	3.85[d]	5–30[ah]	.15	.2–1.1	27.6	27 ± 2[f]		4.2	$4.0 \pm .2$[f]		$.23_5 \pm .01_2$	
		7.75[c]	3.85[d]	10–30[ai]	.15	.4–1.1	.....	27 ± 2[f]	27 ± 2	.....	$4.1 \pm .2$[f]	$4.0 \pm .2$	$.24_0 \pm .01_2$	$.23_5 \pm .01_2$
Benzoyl-L-tyrosinamide	12	8.0[aa]	2.13[ab]	2.5–10[ac]	.85	1.0–4.0	1.9	2.5 ± 0.3[f]	2.5 ± 0.3	4.0	$4.0 \pm .5$[f]	$4.0 \pm .5$	$.2_4 \pm .0_3$	$.2_4 \pm .0_3$
Nicotinyl-L-tyrosinamide	16, 18	7.8[c]	1.33[d]	5–20[aj]	.10	0.4–1.7	15.0 ± 1.0	12 ± 3[f]		6.2	5.0 ± 1.0[f]		$.29_4 \pm .05_9$	
	12, 18	8.0[aa]	4.26[ab]	5–20[ac]	.	...		.....[ak]	12 ± 3		.....[ak]	5.0 ± 1.0	.....	$.29_4 \pm .05_9$
Isonicotinyl-L-tyrosinamide	12	8.0[aa]	2.13[ab]	2.5–20[ac]	.25	0.3–2.2	8.4	9 ± 2[f]	9 ± 2	6.2	6.4 ± 0.6[f]	6.4 ± 0.6	$.37_6 \pm .03_5$	$.37_6 \pm .03_5$
Acetyl-L-phenylalaninamide	20, 23	7.9[c]	5.91[d]	10–40[al]	.20	0.3–1.3	34	31 ± 3[f]	31 ± 3	0.7	0.8 ± 0.2[f]	0.8 ± 0.2	$.04_7 \pm .01_2$	$.04_7 \pm .01_2$
Nicotinyl-L-phenylalaninamide	20, 23	7.9[c]	5.91[d]	5–12.5[am]	.30	0.3–0.7	18	19 ± 4[f]	19 ± 4	2.1	2.0 ± 0.3[f]	2.0 ± 0.3	$.11_8 \pm .01_8$	$.11_8 \pm .01_8$
Acetyl-L-hexahydrophenylalaninamide	8	7.9[c]	2.95–5.91[d]	10–40[an]	0.10–0.20	0.4–1.5	27 ± 4[f]	27 ± 4[f]		0.65 ± 0.05[f]	0.65 ± 0.05[f]		$.03_8 \pm .00_3$	
	8	7.9[c]	2.78–5.57[d]	12.5–40[ao]	..	.....	30 ± 3[f]	.....	27 ± 4	0.67 ± 0.05[f]	.....	0.65 ± 0.05	.....	$.03_8 \pm .00_3$
Acetyl-L-tyrosylglycinamide	11–13	7.8[ap]	4.26[aq]	5–50[q]	0.20	0.2–2.2	30	23 ± 3[f]	23 ± 3	8.9	7.5 ± 1.0[f]	7.5 ± 1.0	$.44_0 \pm .0_6$	$.44_0 \pm .0_6$

[a] In aqueous solutions at 25° and under the conditions specified. [b] Based upon a molecular weight of 22,000 and a nitrogen content of 16.0% for monomeric α-chymotrypsin, 0.1 mg. protein-nitrogen/ml. equiv. to 2.84×10^{-5} M. [c] 0.02 M in the THAM component of a THAM–HCl buffer. [d] Armour preparation no. 90402. [e] Sixteen experiments at seven initial specific substrate concentrations within the limits indicated. [f] Re-evaluated by the method of Jennings and Niemann[8] and a v_0 *vs.* $v_0/[S]_0$ plot[4-7]. [g] 0.2 M in the THAM component of a THAM–HCl buffer. [h] Thirteen experiments at twelve initial specific substrate concentrations within the limits indicated, original evaluation based upon ten experiments at ten initial specific substrate concentrations within the limits of $2–20 \times 10^{-3}$ M. [i] 0.3 M in the THAM component of a THAM–HCl buffer. [j] Eight experiments at five initial specific substrate concentrations within the limits indicated. [k] Ten experiments at nine initial specific substrate concentrations within the limits indicated. [l] 0.5 M in the THAM component of a THAM–HCl buffer. [m] Armour preparation no. 10705. [n] Seven experiments at seven initial specific substrate concentrations within the limits indicated. [o] Re-evaluated by the method of Foster and Niemann.[9] [p] Simultaneous evaluation of $K_{P_1} = 80 \pm 10 \times 10^{-3}$ M. [q] Five experiments at five initial specific substrate concentrations within the limits indicated. [r] Eight experiments at five initial specific substrate concentrations within the limits indicated. [s] Four experiments at four

initial specific substrate concentrations within the limits indicated. [t] Simultaneous evaluation of $K_{P_1} = 9 \pm 2 \times 10^{-3}$ M. [u] Eight experiments at four initial specific substrate concentrations within the limits indicated. [v] Twelve experiments at four initial specific substrate concentrations within the limits indicated. [w] Simultaneous evaluation of $K_{P_1} = 15 \pm 3 \times 10^{-3}$ M. [x] Jansen preparation.[21] [y] Eight experiments at seven initial specific substrate concentrations within the limits indicated. [z] Twelve experiments at six initial specific substrate concentrations within the limits indicated. [aa] 0.02 M in the EDA component of an EDA–HCl buffer. [ab] Armour preparation no. 70902. [ac] Three experiments at three initial specific substrate concentrations within the limits indicated. [ad] Eleven experiments at five initial specific substrate concentrations within the limits indicated, original evaluation based upon ten experiments at five initial specific substrate concentrations within the same limits. [ae] Eight experiments at five initial specific substrate concentrations within the limits indicated, original evaluation based upon six experiments at five initial specific substrate concentrations within the same limits. [af] Twenty-one experiments at six initial specific substrate concentrations within the limits indicated, original evaluation based upon ten experiments at five initial specific substrate concentrations within the limits of 8–17.5 $\times 10^{-3}$ M. [ag] Eleven experiments at five initial specific substrate concentrations within the limits indicated, original evaluation based upon nine experiments at five initial specific substrate concentrations within the same limits. [ah] Eighteen experiments at nine initial specific substrate concentrations within the limits indicated, original evaluation based upon ten experiments at five initial specific substrate concentrations within the limits of 10–30 $\times 10^{-3}$ M. [ai] Nineteen experiments at eight initial specific substrate concentrations within the limits indicated. [aj] Thirteen experiments at seven initial specific substrate concentrations within the limits indicated, original evaluation based upon ten experiments at seven initial specific substrate concentrations within the same limits. [ak] K_S and k_3 were not re-evaluated in this instance because of the discordance of the corrected data. [al] Seven experiments at seven initial specific substrate concentrations within the limits indicated, original evaluation based upon six experiments at six initial specific substrate concentrations within the same limits. [am] Ten experiments at four initial specific substrate concentrations within the limits indicated. [an] Thirty-eight experiments at essentially thirteen initial specific substrate concentrations within the limits indicated. [ao] Eight experiments at three initial specific substrate concentrations within the limits specified but with an enzyme preparation of uncertain history. [ap] 0.015 M in the phosphate component of a potassium dihydrogen phosphate–disodium hydrogen phosphate buffer. [aq] Lehn and Fink preparation previously shown to be equivalent to Armour preparation no. 70902.

specified, of any one of the specific substrates listed in Table II, and the appropriate values of K_S and k_3 as the relevant constants of this equation it must be appreciated that in no case was equation 2 tested over a wide range of enzyme and initial specific substrate concentrations.

Although all of the values of K_S and k_3 which are presented in Table II are based upon experiments which in every instance were conducted in aqueous solutions at 25° and at a specified pH, which for twelve of the specific substrates was known to be the optimum pH of the particular system, there were a number of other variables which were not uniformly controlled and which must be considered. Since four different preparations of crystalline α-chymotrypsin were employed at various times it is fortunate that the experiments with nicotinyl-L-tryptophanamide and either an Armour preparation or the highly purified Jansen preparation and with acetyl-L-tyrosinamide and two different Armour preparations, one of which was identical with the Armour preparation used above, have led to revised values of K_S and k_3 which do not require us to alter our earlier conclusion[21] that it is possible to obtain preparations of crystalline α-chymotrypsin which are kinetically indistinguishable and that these preparations can be used interchangeably to provide a characteristic and reproducible catalytic species.

For twelve of the specific substrates listed in Table II values of K_S and k_3 were derived from experiments conducted with THAM–HCl buffers. With three additional specific substrates EDA–HCl buffers were employed and with the sixteenth a sodium potassium phosphate buffer was used. However, with one specific substrate, *i.e.*, acetyl-L-tyrosinamide, both 0.02 M THAM–HCl and 0.02 M EDA–HCl buffers were employed.[15,49] From the data obtained with these latter systems revised values of K_S and k_3 have been computed which support our earlier conclusion[15] that with THAM–HCl and EDA–HCl buffers of the same pH and molarity the buffer components appear to have no function other than to control the pH of the reaction systems. Therefore, for fifteen of the sixteen sets of values of K_S and k_3 presented in Table II we can consider the systems to which these values refer to be equivalent in so far as the nature of the buffer components are concerned.

The situation with respect to phosphate buffers is not clear at the present time since no direct comparison has been made of a system containing a phosphate buffer with for example one containing a THAM–HCl buffer of equivalent ionic strength. This problem is currently under investigation and until the results of this study are available it appears desirable to suspend judgment as to whether or not it is permissible to compare values of K_S and k_3 obtained from experiments conducted in the presence of a phosphate buffer with values derived from experiments in which THAM–HCl or EDA–HCl buffers were employed, all other factors being constant.

Among the values of K_S and k_3 that are given in

(49) Attention is called to the fact that in ref. 15, p. 1550, Table I, for expt. 1 under the heading of buffer system the line should read 0.02 M THAM–HCl instead of 0.02 M EDA–HCl.

TABLE III

RE-EVALUATION OF SEVERAL α-CHYMOTRYPSIN CATALYZED COMPETITIVE HYDROLYSES[a]

Specific structures	Ref.	Mole %[b]	E'_{S_T} × 10²	$[S_T]_0$ × 10³, M	S'_{S_T}	K_{S_T} × 10³, M Orig. Found	Orig. Calcd.[c]	Re-eval. Found[d]	Re-eval. Calcd.[c]	k_{3_T} × 10⁴, M/min./mg. P-N/ml. Orig. Found	Orig. Calcd.[c]	Re-eval. Found[d]	Re-eval. Calcd.[c]
Acetyl-L-tryptophanamide	17, 23	50	0.4	20–60[e]	1.4–4.3	9.0	9.1[f]	14 ± 3	10 ± 2[g]	0.86	0.78[f]	0.9 ± 0.2	0.8 ± 0.2[g]
vs. acetyl-L-tyrosinamide	17, 23	25	0.4	20–60[h]	1.3–3.8	13.8	13.9[f]	16 ± 3	12 ± 2[g]	1.3	1.2[f]	1.3 ± 0.2	1.2 ± 0.2[g]
Acetyl-L-phenylalaninamide *vs.* acetyl-L-tyrosinamide	20	50	0.2	10–50[i]	0.3–1.6	..[i]	...	32 ± 3	32 ± 3[k]	..[i]	...	1.6 ± 0.2	1.6 ± 0.3[k]

[a] In aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the THAM component of a THAM–HCl buffer with [E] = 0.208 mg. protein-nitrogen/ml. = 5.91 × 10⁻⁵ *M* of the Armour preparation no. 90402. [b] Of the first named specific substrate. [c] As described by Foster and Niemann.[17] [d] Re-evaluated by the method of Jennings and Niemann[8] and a v_0 *vs.* $v_0/[S]_0$ plot.[4-7] [e] Eleven experiments at six total initial specific substrate concentrations within the limits indicated. [f] On the basis of K_{S_1} and K_{S_2} = 5.3 and 30.5 × 10⁻³ *M*, respectively and k_{3_1} and k_{3_2} = 0.50 and 2.4 × 10⁻³ *M*/min./mg. protein-nitrogen/ml., respectively. [g] On the basis of K_{S1} and K_{S2} = 5.0 ± 0.5 and 32 ± 4 × 10⁻³ *M*, respectively and k_{3_1} and k_{3_2} = 0.55 ± 0.1 and 2.4 ± 0.3 × 10⁻³ *M*/min./mg. protein-nitrogen/ml., respectively. [h] Twelve experiments at six total initial specific substrate concentrations within the limits indicated. [i] Five experiments at five total initial specific substrate concentrations within the limits indicated. [j] Data originally used with a value of K_S = 30.5 × 10⁻³ *M* and k_3 = 2.4 × 10⁻³ *M*/min./mg. protein-nitrogen/ml. for acetyl-L-tyrosinhydroxamide to obtain the following values of K_S and k_3 for acetyl-L-phenylalaninamide, K_S = 35 × 10⁻³ *M*, k_3 = 0.9 × 10⁻³ *M*/min./mg. protein-nitrogen/ml., re-evaluation of these data[4-8] with K_S = 32 ± 4 × 10⁻³ *M* and k_3 = 2.4 ± 0.3 × 10⁻³ *M*/min./mg. protein-nitrogen/ml. for acetyl-L-tyrosinamide gave the following values of K_S and k_3 for acetyl-L-phenylalaninamide, K_S = 32 ± 3 × 10⁻³ *M*, k_3 = 0.8 ± 0.2 × 10⁻³ *M*/min./mg. protein-nitrogen/ml. [k] On the basis of K_{S_1} and K_{S_2} = 31 ± 3 and 32 ± 4 × 10⁻³ *M*, respectively, and k_{3_1} and k_{3_2} = 0.8 ± 0.2 and 2.4 ± 0.3 × 10⁻³ *M*/min./mg. protein-nitrogen/ml., respectively.

Table II there are two sets of constants, *i.e.*, those for L-tyrosinhydroxamide and acetyl-L-tyrosinhydroxamide, which are based upon experiments in which THAM–HCl buffers were employed but at concentrations of 0.2–0.5 *M* instead of at the usual concentration of 0.02 *M*. Since we have at hand information[50] which indicates that the value of the constant k_3 may be dependent upon the ionic strength of the reaction system, *i.e.*, k_3 increasing with increasing ionic strength, it is clear that caution should be exercised in comparing the k_3 values of two specific substrates when such values were obtained from experiments in which the ionic strengths of the reaction systems were substantially different. While it is true that the revised values of K_S and k_3 for acetyl-L-tyrosinhydroxamide in 0.3 and 0.5 *M* THAM–HCl buffers are identical within the limits of experimental error it is also possible that the above k_3 values are significantly greater than the k_3 value that would be derived from the observation of a reaction system that was 0.02 *M* in a THAM–HCl buffer if this were experimentally feasible. Thus in comparing the k_3 value of acetyl-L-tyrosinhydroxamide with say that of acetyl-L-tyrosinamide it should be remembered that the difference between these two values may be exaggerated by the substantial difference in the ionic strengths of the buffer systems employed with these two specific substrates.

In order to demonstrate that certain of the above specific substrates react at one and the same catalytically active site the kinetics of several α-chymotrypsin catalyzed competitive hydrolyses were investigated.[17,20,23] The primary data obtained from these experiments have been re-evaluated, in a manner comparable to that employed for the systems containing but one specific substrate, and the results of this operation have led to revised values of K_{S_T} and k_{3_T} for each of these pairs of specific substrates which are in satisfactory agreement with those calculated on the basis of the respective K_S and k_3 values of the individual specific substrates, *cf.*, Table III. Therefore, we can reaffirm our previous conclusion[17,20,23] that the three specific substrates listed in Table III are hydrolyzed in the presence of α-chymotrypsin *via* combination with the same catalytically active site.

While many of the values of K_S and k_3 that were reported previously[8,9,11-26] lie within the limits of error of the corresponding revised values presented in this communication there can be no doubt that the elimination of subjective methods for the estimation of initial velocities has resulted not only in the attainment of more reliable values of K_S and k_3 for sixteen specific substrates of α-chymotrypsin but also in a more realistic appreciation of the limits of error that are inherent in these values. Although we now have at hand a set of values of K_S and k_3 for a number of characteristic specific substrates of α-chymotrypsin that we can view with some confidence we wish, with but one exception, to defer discussion of these values and their relation to the structure of the respective specific substrates until some of the points mentioned in the previous discussion have been clarified and until revised values of K_I are available for the relatively large number of competitive inhibitors of α-chymotrypsin that have been previously examined in these laboratories. It will be realized by some that this caution stems in large part from the realization that knowledge of the values of K_S and k_3 for a family of specific substrates does not permit one to draw conclusions which require an unambiguous interpretation of the constant K_S.[29-31] However, since a satisfactory description of all of the primary data has been obtained in terms of equations 2 or 5 it is clear that our choice of these equations as a basis for the re-evaluation of the constants K_S and k_3 is a reasonable one.

Among the specific substrates that are listed in Table II there are seven for which we may write the general formula $R'CONHCH(CH_2C_6H_4OH)CONH_2$ where R′ = H, CH_3, CF_3, CH_2Cl, C_6H_5, β-C_5H_4N and γ-C_5H_4N. If the refractivities of these groups are estimated from the component atomic refractivities and these values are then plotted against the appropriate value of k_3 for the corresponding specific substrate, a plot such as that given in Fig. 1 is obtained. For four of the specific sub-

(50) Unpublished experiments of H. J. Shine and R. A. Bernhard.

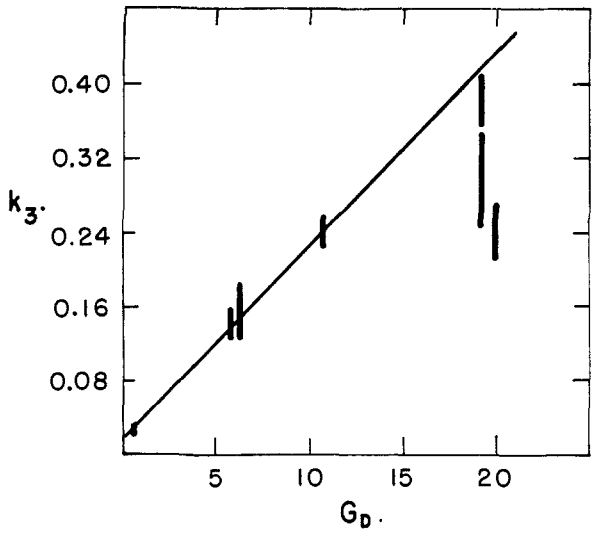

Fig. 1.—Relation between group refractivities, estimated on the basis of H = 1.1, C = 2.4, F = 1.3, Cl = 6.0, N = 2.8, of the group R′ present in R′CONHCH($CH_2C_6H_4OH$)-$CONH_2$ and the corresponding values of k_3 in units of M/sec./M and subject to error to the extent indicated in Table II.

strates, *i.e.*, formyl-, acetyl-, trifluoroacetyl- and chloroacetyl-L-tyrosinamide, the values of k_3 increase in apparent linear manner with increasing values of the refractivities of the corresponding R′ groups present in these specific substrates but for the three remaining specific substrates, *i.e.*, benzoyl-, nicotinyl- and isonicotinyl-L-tyrosinamide the values of k_3 are significantly less than those expected on the basis of the above relationship. It is possible to reconcile the discrepancy that appears to exist between these two sets of data if it is assumed, in the case at hand, that an increase in the value of k_3 is associated only with an increased probability of the specific substrate being oriented in the enzyme–substrate complex in such a manner as to facilitate subsequent reaction. In the situation where k_3 appears to increase in a linear manner with increasing refractivity of the R′ group it is probable that when R′ = H the specific substrate combines with the catalytically active site of the enzyme in a variety of modes only a few of which possess the requisite orientation for subsequent reaction. As the refractivity of the R′ group is increased two things or a combination of these, may happen. One, an increasing number of the unproductive modes of combination may be excluded through steric repulsion arising from the increased volume of the R′ group thereby increasing the probability of occurrence of those modes of combination which can lead to subsequent reaction, or two, combination in a mode suitable for subsequent reaction may be increasingly favored through continued enhancement of a van der Waals interaction, arising from the increased polarizability of the R′ group, with a structural component of the enzyme present at or in the immediate neighborhood of the catalytically active site. Either phenomenon, or a combination of both, would lead to a regular increase in the value of k_3 with increasing refractivity of the R′ group although it is not obvious at the present time as to why the relationship should be a linear one if this is indeed the case for more than a tenfold increase of either value.

For the four specific substrates where an apparent linear increase in the values of k_3 with increasing refractivity of the R′ group was observed it is noteworthy that in no case was the structure of the R′ group even remotely related to the structure of the α-amino acid side-chain which was common to all of the specific substrates. In contrast to formyl-, acetyl-, trifluoroacetyl- and chloroacetyl-L-tyrosinamide, benzoyl-L-tyrosinamide possesses two groups which are structurally very similar and with this latter specific substrate there is a clear possibility that in the course of combination with the catalytically active site of the enzyme the phenyl R′ group is frequently mistaken by the enzyme for the *p*-hydroxyphenyl group present in the α-amino acid side-chain thus increasing the number of unproductive combinations. Therefore the effect of the substantial refractivity of the phenyl group in increasing the value of k_3 is opposed by a probability factor which causes a decrease in the value of k_3. On the basis of the data which are now available it appears that this probability factor has a value in the neighborhood of 0.5 since the value of k_3 for benzoyl-L-tyrosinamide is identical, within the limits of experimental error, with that of chloroacetyl-L-tyrosinamide whereas the refractivity of the phenyl group is approximately twice that of the chloromethyl group. Although the refractivities of the R′ groups present in nicotinyl-, isonicotinyl- and benzoyl-L-tyrosinamide are approximately the same it is not surprising that the k_3 values of the first two specific substrates are greater than that of the latter when it is recalled that the affinity of the catalytically active site for benzamide is substantially greater than that for the more heavily hydrated nicotinamide.[51,52] Thus, if the R′ group is uncharged and is relatively compact and is structurally dissimilar to the α-amino acid side-chain it appears that one can anticipate an increase in the value of k_3 with increasing refractivity of the R′ group all other factors being constant.

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