ture was filtered to remove the precipitated L-valine methyl ester hydrochloride and the filtrate evaporated *in vacuo*. The residue so obtained was recrystallized from pentane using the procedure described for the recrystallization of acetyl-L-valine methyl ester to give 4.8 g. (52.5%) of chloroacetyl-L-valine methyl ester, long colorless needles, m.p. 45.8–46.6°, $[\alpha]^{25}D$ −37.8° (*c* 2.75% in water).

Anal. Calcd. for $C_8H_{14}O_3NCl$ (207.5): C, 46.2; H, 6.8. Found: C, 46.3; H, 6.9.

Benzoyl-L-valine Methyl Ester.—This compound, described by Reihlen and Knöpfle,[42] was prepared from the ester hydrochloride by treatment with benzoyl chloride in a manner similar to that described for the acetylation of L-tryptophan methyl ester.[35] Two g. (0.012 mole) of the ester hydrochloride was dissolved in 20 ml. of water containing 0.08 mole of potassium carbonate. Ethyl acetate, 40 ml., was introduced and with rapid stirring 9.5 ml. (0.039 mole) of benzoyl chloride was added slowly from a dropping funnel. The reaction mixture was stirred for 1 hr. at room temperature, 2 ml. of pyridine introduced and the mixture stirred for an additional 20 minutes. The phases were separated, the organic phase extracted with 25 ml. of 1 *N* aqueous hydrochloric acid, then with 15 ml. of water and dried over magnesium sulfate. The crude, oily solid obtained by evaporation of the solvent was twice recrystallized from hexane to give 1.1 g. (39%) of benzoyl-L-valine methyl ester, colorless silky needles, m.p. 110.5–111.0°, $[\alpha]^{25}D$ +46.0° (*c* 0.4% in chloroform); lit.[42] m.p. 110.5°; $[\alpha]D$ +44.6° (*c* 0.4% in chloroform).

Anal. Calcd. for $C_{13}H_{17}O_3N$ (235.3): C, 66.4; H, 7.3. Found: C, 66.5; H, 7.4.

Enzyme Experiments.—The general procedure has been described in a previous communication.[28] Other details are summarized in Tables I–III. For the determination of the *p*H optima, *cf.*, Fig. 1, the following conditions were employed: acetyl-L-valine methyl ester, [E] = 0.994 mg. protein-nitrogen per ml. of Armour preparation No. 283, $[S]_0 = 23.33 \times 10^{-3}$ *M*, [NaCl] = 0.1 *M*; chloroacetyl-L-valine methyl ester, [E] = 0.994 mg. protein-nitrogen per ml. of Armour preparation No. 283, $[S]_0 = 14.71 \times 10^{-3}$ *M*, [NaCl] = 0.1 *M*; benzoyl-L-valine methyl ester, [E] = 0.150 mg. protein-nitrogen per ml. of Armour preparation No. 90492, $[S]_0 = 4.0 \times 10^{-3}$ *M*, [NaCl] = 0.02 *M*. The initial velocities, corrected for enzyme and specific substrate blanks,[28] were evaluated by the orthogonal polynomial procedure of Booman and Niemann[43] except in those cases where the recorder traces of extent of reaction *vs.* time were essentially linear throughout their course. In these cases the extent of reaction was determined, to the nearest 0.1, at 1-min. intervals beginning with $t = 1$ min., each value corrected for the appropriate enzyme and specific substrate blank and the mean value of extent of reaction *vs.* time for each of the 1 min. intervals used in arriving at a value of v_0. The average deviation in most cases was within the accuracy of observation of extent of reaction for each point, *i.e.*, ±0.1.

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

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[CONTRIBUTION NO. 2249 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Dependence of the α-Chymotrypsin-catalyzed Hydrolysis of α-N-Nicotinyl-L-tyrosinhydrazide upon the Concentration of the Buffer[1]

BY RICHARD J. KERR AND CARL NIEMANN[2]

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The kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-N-nicotinyl-L-tyrosinhydrazide in aqueous solutions at 25° and *p*H 7.9 have been determined in the presence of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer whose concentration has been varied from 0.01 to 0.90 *M* in the amine component. From these studies, and those with a tris-(hydroxymethyl)-aminomethane–sulfuric acid buffer, it has been concluded that the value of K_S is independent of the buffer concentration and that of k_3 is described by the relation $\log (k_3/k_3^0) = 0.46 \pm 0.03 \sqrt{\mu}$ where $k_3^0 = 0.94 \pm 0.16 \times 10^{-3}$ *M*/min./mg. protein-nitrogen per ml.

The advantages of a THAM[3]–HCl buffer for the poising of a reaction system in the region of *p*H 8 were first recognized by Gomori.[4] Its use in studies involving α-chymotrypsin was initiated by Iselin and Niemann.[5] The concentration of buffer required to maintain a given *p*H depends upon the nature of the specific substrate. Most frequently employed is the one 0.02 *M* in the amine component.[6] However, with the hydroxamides concentrations as high as 0.5 *M* have been used.[6–8] Since it is reasonable to expect that a variation in the concentration of the buffer could influence the values of one or more of the kinetic constants, it was decided to examine the α-chymotrypsin-catalyzed hydrolysis of α-N-nicotinyl-L-tyrosinhydrazide over an extended range of buffer concentrations. This substrate was first examined in 1949[9] and more recently[10] a subjective estimate of the magnitude of K_S and k_3 for the aqueous system at 25° and *p*H 7.8 and 0.02 *M* in the THAM component of a THAM–HCl buffer has been made.

Five sets of experiments were conducted under the conditions summarized in Table I. The extent of hydrolysis, which varied from 12.0 to 66.3% was determined as described previously.[11] Plots of $\ln [S]_0/[S]$ *vs.* *t* and $([S]_0 - [S])$ *vs.* *t*, led to preliminary values of v_0 which were used in the equation, $1/v_0 = (K_S + [S]_0)/k_3[E][S]_0 = (K_S/k_3[E]) \cdot (1/[S]_0) + 1/k_3[E]$ to obtain tentative values of K_S and k_3. From these values of K_S and k_3 values of the corrected time, *i.e.*, *t'*, were obtained by the method of Jennings and Niemann.[12] With these

(1) Supported in part by a grant from the National Institutes of Health, Public Health Service.

(2) To whom inquiries regarding this article should be sent.

(3) Tris-(hydroxymethyl)-aminomethane.

(4) G. Gomori, *Proc. Soc. Exptl. Biol. Med.*, **62**, 33 (1946).

(5) B. M. Iselin and C. Niemann, *J. Biol. Chem.*, **182**, 821 (1950).

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

(7) D. S. Hogness and C. Niemann, *ibid.*, **75**, 884 (1953).

(8) R. J. Foster and C. Niemann, *Proc. Nat. Acad. Sci.*, **39**, 999 (1953).

(9) R. V. MacAllister and C. Niemann, THIS JOURNAL, **71**, 3854 (1949).

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

(11) R. Lutwack, H. F. Mower and C. Niemann, *ibid.*, **79**, 2179 (1957).

(12) R. R. Jennings and C. Niemann, *ibid.*, **75**, 4687 (1953).

TABLE I

INITIAL VELOCITIES AND KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF NICOTINYL-L-TYROSINHYDRAZIDE WITH VARIOUS CONCENTRATIONS OF THE AMINE COMPONENT OF A THAM–THAM·HCl BUFFER[a]

[THAM], M	$[S]_0$[b]	v_0[c]	v'_0[d]	$\sigma v'_0$[e]	P_m[f]
0.01	1.01	0.24	0.22	±0.03	2
	2.52	0.44	0.43	± .05	3
	5.03	1.04	1.05	± .13	2
	10.06	1.95	1.85	± .33	2
	20.12	3.54	3.24	± .27	3
	20.12	3.56	3.34	± .10	2
	30.10	4.62	4.20	± .63	3
	40.24	5.95	5.67	± .55	2
K_S = 7.35 ± 1.65[g]			K'_S = 6.97 ± 2.50		
k_3 = 0.91 ± 0.16[g]			k'_3 = 0.84 ± 0.13		
0.10	1.01	0.30	0.34	±0.02	3
	2.52	0.70	0.67	± .02	2
	5.03	1.39	1.35	± .13	2
	10.06	2.71	2.72	± .09	3
	20.12	4.91	4.98	± .14	3
	30.08	6.36	6.00	± .27	2
	40.24	8.08	8.59	± .14	3
K_S = 8.25 ± 1.18			K'_S = 8.36 ± 2.68		
k_3 = 1.37 ± 0.30			k'_3 = 1.39 ± 0.23		
0.50	20.12	5.20	5.27	±0.03	2
	30.00	7.98	7.08	± .75	2
	40.10	9.15	10.02	± .24	2
	50.10	10.70	10.41	± .50	2
	60.10	11.57	11.41	± .93	4
K_S = 8.66 ± 1.78			K'_S = 8.48 ± 2.71		
k_3 = 1.57 ± 0.29			k'_3 = 1.52 ± 0.34		
0.75	5.01	1.40	1.41	±0.04	2
	20.00	5.74	6.17	± .09	3
	30.00	8.16	8.42	± .16	3
	50.10	10.75	11.05	± .46	3
	60.10	12.16	12.05	± .14	2
K_S = 7.52 ± 1.53			K'_S = 7.66 ± 1.72		
k_3 = 1.96 ± 0.30			k'_3 = 1.92 ± 0.39		
0.90	1.02	0.43	0.42	±0.09	2
	2.56	1.16	1.15	± .06	2
	5.03	2.33	2.12	± .03	2
	10.22	4.28	4.36	± .07	2
	20.36	7.29	7.55	± .25	3
	40.72	12.60	12.60	± .09	2
K_S = 8.31 ± 1.79			K'_S = 8.57 ± 2.62		
k_3 = 2.15 ± 0.32			k'_3 = 2.30 ± 0.40		

[a] In aqueous solutions at 25.0 ± 0.1° and *p*H 7.9 ± 0.1 with [E] = 0.1785 mg. protein-nitrogen per ml. of Armour preparation no. 10705. [b] In units of 10^{-4} M. [c] In units of 10^{-5} M/min. and determined by the method of Jennings and Niemann.[12] [d] In units of 10^{-5} M/min. and determined by the orthogonal polynomial procedure.[13] [e] Probable error in units of 10^{-5} M/min. [f] Order of polynomial used in calculation. [g] All values of K_S in units of 10^{-3} M and those of k_3 in units of 10^{-3} M/min./mg. protein-nitrogen per ml.

values of t', values of v_0 for each specific substrate concentration were again computed and used in a least squares fit to the preceding equation to obtain the values of K_S and k_3 summarized in Table I. Each experiment of this series was evaluated a second time using the orthogonal polynomial procedure of Booman and Niemann.[13] These initial velocities, signified by v_0', are given in Table I, along with the probable errors, $\sigma v_0'$, the order of the polynomial, P_m, employed in the calculation and the corresponding values of K_S' and k_3' obtained by a least squares fit to the equation $[E]/v_0 = (K_S/k_3) \cdot (1/[S]_0) + 1/k_3$. The agreement between the values of v_0 and v_0' and between K_S and k_3 and K_S' and k_3' is generally satisfactory and confirms the validity of the first method used for the evaluation of these constants.[13] Since the experiments were not designed for optimal use of the orthogonal polynomial procedure,[13] the constants evaluated by the method of Jennings and Niemann[12] were selected as being the most reliable.

It is seen from Table I that the constant, K_S, evaluated at five concentrations of the THAM–THAM·HCl buffer appears to be essentially constant within the limits of experimental error. The arithmetic mean of the five values corresponds to a value of K_S = 8.0 ± 1.6,[14] which is in agreement with the earlier subjective estimate of 9.[10] While K_S remains constant, the value of k_3 is seen to increase from 0.91 ± 0.16[14] at 0.01 M THAM to 2.15 ± 0.32 at 0.9 M THAM. This is an increase of approximately 130%.

If it is assumed that K_S remains constant at a value of 8.0 ± 1.6, values of v_0, based upon an arbitrarily chosen initial specific substrate concentration, can be calculated for each of the THAM–THAM·HCl concentrations. These are given in Table II. These velocities were used in plots of various relationships which might exist between ionic strength or buffer concentration and the logarithm of the velocities or the velocities themselves. While it is difficult to determine which plots gave the best linear relationships, it appears that those observed between log v_0 and $\sqrt{\mu}$ or M are the most satisfactory. The values of least squares fits of all the reasonable relationships are listed in Table II. The initial velocities calculated from the relation $\log v_0 = 0.423 + 0.467\sqrt{\mu}$ are compared to those found experimentally in Table III.

In order to determine the consequences of employing a THAM–THAM·H_2SO_4 instead of a THAM–THAM·HCl buffer, a series of experiments were conducted under the conditions summarized in Table IV. The general procedure was identical with that used previously.

From the least squares fits that are summarized in Table V, it again appears that the best linear relationships are those observed between log v_0 and $\sqrt{\mu}$ or M. The initial velocities calculated from the relation $\log v_0 = 0.373 + 0.461\sqrt{\mu}$ are compared with the experimentally determined values in Table III.

Since different initial specific substrate concentrations were employed in the two series of experiments, the intercepts cannot be compared. However, it is interesting to compare the various slopes. While the values of the slopes in the case of the log v_0 *vs.* M relation for the two types of THAM buffers are coincident, within the limits of experimental error, the agreement for the log v_0 *vs.* $\sqrt{\mu}$ rela-

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

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

TABLE II

LEAST SQUARES CONSTANTS FOR VARIOUS RELATIONSHIPS BETWEN INITIAL VELOCITIES, IONIC STRENGTH AND MOLALITY OF THAM–THAM·HCl BUFFER SYSTEMS

v_0[a]	log v_0[b]	[THAM][c]	$\sqrt{[\text{THAM}]}$	μ[d]	$\sqrt{\mu}$
2.56	0.4082	0.01	0.100	0.006	0.025
3.85	.5855	.10	.316	.061	.247
4.42	.6454	.50	.707	.305	.552
5.51	.7412	.75	.866	.458	.677
6.05	.7812	.90	.949	.549	.741

A least squares fit of the relationship $Y = a + bX$ where Y and X are the indicated functions gave, within the limits indicated, the values of slope and intercept below

Y	X	Slope[b]	Intercept[a,e]
log v_0	$\sqrt{\mu}$	0.467 ± 0.032	0.423 ± 0.022
log v_0	μ	.578 ± .085	.473 ± .029
log v_0	$\sqrt{M}$	.390 ± .032	.404 ± .026
log v_0	M	.353 ± .052	.473 ± .030
v_0	μ	.557 ± .064	.294 ± .041[f]

[a] In units of 10^{-5} M/min. and calculated by using values of k_3 determined at each concentration of THAM, assuming a value of $K_S = 8.0 \times 10^{-3}$ M and $[S]_0 = 1.0 \times 10^{-3}$ M. [b] For purposes of calculation of the least squares fit the mantissa corresponding to 10^{-5} has been dropped. [c] Molar concentration of the amine component of the buffer includes both protonated and unprotonated species. [d] Ionic strength calculated on the basis of the concentration of the buffer species and all other ions present in the reaction system. [e] Characteristic of the logarithm of the velocity in the absence of added buffer at $[S]_0 = 1.0 \times 10^{-3}$ M, the antilog must be multiplied by 10^{-5} M/min. [f] In units of 10^{-4} M/min.

TABLE III

COMPARISON OF EXPERIMENTALLY DETERMINED INITIAL VELOCITIES WITH THOSE CALCULATED

THAM–THAM·HCl			THAM–THAM·H_2SO_4		
[THAM]	v_0 (exp.)[a]	v_0 (calcd.)[b]	[THAM]	v_0 (exp.)[c]	v_0 (calcd.)[d]
0.01	2.56	2.66	0.02	2.43	2.66
.10	3.85	3.56	.05	2.89	2.96
.50	4.42	4.79	.10	3.16	3.26
.75	5.51	5.48	.20	3.86	3.72
.90	6.05	5.88	.30	4.36	4.12
			.40	4.63	4.49
			.50	5.05	4.85
			.75	5.43	5.69
			1.00	6.61	6.53

[a] *Cf.*, Table II. [b] From the relation log $v_0 = 0.467\sqrt{\mu} + \log v_0^0$ where log $v_0^0 = 0.423$. [c] *Cf.*, Table IV. [d] From the relation log $v_0 = 0.461\sqrt{\mu} + \log v_0^0 = 0.373$.

tionship appears to be better. It is recognized that ionic theory would not predict such a linear relationship at high ionic strengths. However, in the absence of a better relationship, it appears that the initial velocities observed in the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tyrosinhydrazide in the presence of buffer species considered in this communication can be described in terms of the relation $\log (v_0/v_0^0) = (0.46 \pm 0.03)\sqrt{\mu}$, at least over the concentration range studied.

In an earlier investigation, Shine and Niemann[15] noted that the addition of sodium or potassium chloride to the system α-chymotrypsin–chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.75 and 0.02 M in the THAM component of a THAM–HCl buffer caused an increase in the initial velocities which was described by the relation $\log (v_0/v_0^0) = (0.30 \pm 0.01)\sqrt{M}$ where M, the molality of the added sodium or potassium chloride was within the limits of 0 and 1.5 M. It also was found that with the above system K_S was invariant and only k_3 varied with the concentration of the added salt. A similar situation with respect to the behavior of K_S and k_3 also has been observed in the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 in the presence of sodium or potassium phosphate buffers.[16]

TABLE IV

INITIAL VELOCITIES FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF NICOTINYL-L-TYROSINHYDRAZIDE WITH VARIOUS CONCENTRATIONS OF THE AMINE COMPONENT OF THAM–THAM·H_2SO_4 BUFFER[a]

[THAM], M	v_0[b]	v_0'[c]	σv_0[d]	P_m[e]
0.02	2.43			
.05	2.89			
.10	3.16	3.16	±0.05	2
.20	3.86	3.60	± .06	3
.30	4.36	4.35	± .14	2
.40	4.63	4.94	± .14	2
.50	5.05	5.00	± .12	3
.75	5.43	5.10	± .13	3
1.00	6.61	6.39	± .07	4

[a] In aqueous solutions at 25.0° and *p*H 7.9 ± 0.1 with [E] = 0.1785 mg. protein-nitrogen per ml. of Armour preparation no. 10705 and $[S]_0 = 3.324 \times 10^{-4}$ M. [b] In units of 10^{-5} M/min. and determined by the method of Jennings and Niemann.[12] [c] In units of 10^{-5} M/min. and determined by the orthogonal polynomial procedure.[13] [d] Probable error in units of 10^{-5} M/min. [e] Order of polynomial used in calculation.

TABLE V

LEAST SQUARES CONSTANTS FOR VARIOUS RELATIONSHIPS BETWEEN INITIAL VELOCITIES, IONIC STRENGTH AND MOLALITY OF THAM–THAM·H_2SO_4 BUFFER SYSTEMS

v_0[a]	log v_0[b]	[THAM][c]	$\sqrt{[\text{THAM}]}$	μ[d]	$\sqrt{\mu}$
2.43	0.4031	0.02	0.1414	0.0183	0.1353
2.89	.4609	.05	.2236	.0458	.2143
3.16	.4997	.10	.3162	.0915	.3025
3.86	.5866	.20	.4472	.1830	.4280
4.36	.6395	.30	.5477	.2745	.5240
4.63	.6656	.40	.6325	.3660	.6050
5.05	.7033	.50	.7071	.4575	.6764
5.43	.7348	.75	.8660	.6863	.8288
6.61	.8202	1.00	1.000	.9150	.9566

A least squares fit of the relationship $Y = a + bX$ where Y and X are the indicated functions, gave, within the limits indicated, the values of slope and intercept below

Y	X	Slope[b]	Intercept[a,e]
log v_0	$\sqrt{\mu}$	0.461 ± 0.018	0.373 ± 0.017
log v_0	μ	.424 ± .062	.470 ± .029
log v_0	$\sqrt{M}$	.468 ± .016	.359 ± .010
log v_0	M	.387 ± .030	.470 ± .016
v_0	μ	.427 ± .048	.283 ± .029[f]

[a] In units of 10^{-5} M/min., *cf.*, Table IV. [b] For purposes of calculation of the least squares fit the mantissa corresponding to 10^{-5} has been dropped. [c] Molar concentration of the amine component, includes both protonated and unprotonated species. [d] Ionic strength calculated on basis of the concentration of the buffer species and all other ions present in the reaction system. [e] Characteristic of the logarithm of the velocity in the absence of added buffer at $[S]_0 = 3.324 \times 10^{-4}$ M, the antilog must be multiplied by 10^{-5} M/min. [f] In units of 10^{-4} M/min.

(15) H. J. Shine and C. Niemann, THIS JOURNAL, **77**, 4275 (1955).

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

If v_0^0 is defined as v_0 in the absence of added buffer and if K_S remains invariant we may, in the case at hand, expect the relationship log $(k_3/k_3^0) = 0.46 \pm 0.03\sqrt{\mu}$ where k_3^0 is the value in the absence of buffer. A least squares fit of the data given in Table I to this relationship leads to a slope of 0.468 ± 0.064 and an intercept of 0.974 ± 0.088. The intercept, *i.e.*, log k_3^0, corresponds to a value of $k_3^0 = 0.94 \pm 0.16$, which is in good agreement with that obtained for the system 0.01 *M* in THAM–THAM·HCl.

The preceding results suggest that k_3 may be overestimated when buffer concentrations greater than 0.02 *M* THAM–THAM·HCl are employed. While it is tempting to use the relation log $(k_3/k_3^0) = 0.46 \pm 0.03\sqrt{\mu}$ to correct values of k_3 obtained from studies employing higher concentrations of the above buffer,[6-8] it must be noted that there is no evidence to indicate that the preceding relationship or its coefficient is independent of the nature of the specific substrate or the concentration of the enzyme since these two reaction parameters, along with the temperature, hydrogen ion concentration and the solvent system, were held constant in the present study.

Any explanation of the effects associated with the presence of electrolytes must recognize that with chloroacetyl-L-tyrosinamide and sodium chloride,[15] acetyl-L-tyrosinamide and sodium phosphate[16] and α-N-nicotinyl-L-tyrosinhydrazide and THAM–THAM·HCl K_S has been found to be independent of, and k_3 to increase with increasing concentration of electrolyte at all concentrations employed whereas with methyl hippurate and acetyl-L-valine methyl ester K_S decreases and k_3 increases with increasing concentrations of sodium chloride below *ca.* 1.0 *M*, and only at concentrations above *ca.* 1.0 does K_S appear to approach constancy.[17] The differing behavior of the acylated α-amino acid esters and the acylated α-amino acid amides and hydrazide introduces another factor into a situation that is already complex. Thus, any general theory must be deferred until sufficient information is available relative to the interpretation of K_S for the former class of specific substrates and to the effects observed upon the addition of electrolytes to systems in which the enzyme and hydrogen ion concentrations have been varied over wide limits.

In a recent communication[18] Tinoco noted that α-chymotrypsin may undergo appreciable polymerization in aqueous solutions at 25° and *p*H 7.7 and 0.02 *M* in THAM component of a THAM–HCl buffer at the enzyme concentrations employed in this and similar studies.[6] Tinoco advocates the use of solutions with an ionic strength of 0.1 or higher on the grounds that in such solutions α-chymotrypsin will be essentially monomeric, and this will simplify the interpretation of kinetic experiments. This advice is both premature and questionable because it is not known whether polymeric and monomeric α-chymotrypsin possess different or identical kinetic characteristics. Furthermore, k_3 reaches no known upper limit with increasing concentration of sodium chloride or THAM–THAM·HCl with a representative acylated α-amino acid amide or hydrazide and for both, K_S is independent of the electrolyte concentration. The independence of K_I upon the concentration of THAM–THAM·HCl may be inferred from previous studies.[19] Finally the indicated preference for the ester type of specific substrate[18] was made in the absence of knowledge of the consequences noted above.[17]

(17) R. B. Martin and C. Niemann, THIS JOURNAL, **79**, 4814 (1957).
(18) I. Tinoco, Jr., *Arch. Biochem. Biophys.*, **68**, 367 (1957).

Experimental[20,21]

Nicotinyl-L-tyrosinhydrazide.—Esterification of 25 g. of L-tyrosine (0.014 mole) with ethanolic hydrogen chloride gave 24.1 g. of L-tyrosine ethyl ester hydrochloride (80%). Liberation of the ester by slurrying the hydrochloride in chloroform followed by the addition of chloroform saturated with ammonia, removal of the ammonium chloride by filtration and evaporation of the chloroform gave 19.4 g. of L-tyrosine ethyl ester (67%), m.p. 106–108°.

Acylation of 19.4 g. of L-tyrosine ethyl ester (0.0108 mole) with nicotinyl azide gave 15.8 g. of nicotinyl-L-tyrosine ethyl ester (52%), m.p. 145.4–147.5°. The acylated ester was dissolved in ethanol and slowly added to an excess of hydrazine. The solution was refluxed for 3 hr. and the colorless solid collected and dried. Two crystallizations from one liter of water gave 14.5 g. of nicotinyl-L-tyrosinhydrazide (95%), m.p. 245.5–246.1°, $[\alpha]^{25.3}D$ +24.8 ± 0.6 (*c* 2.3% in methyl cellosolve).

Anal. Calcd. for $C_{15}H_{16}O_3N_4$ (300): C, 60.0; H, 5.4; N, 18.7. Found: C, 60.1, 60.2; H, 5.4, 5.5; N, 18.8, 18.6.

Buffer Solutions.—The tris-(hydroxymethyl)-aminomethane used was Matheson and Company practical grade decolorized with Norite and recrystallized three times from water, m.p. 169.3–169.6°. In each case, the stock solution was made up with 10 times the desired concentration by dissolving the required amount of the amine in water and bringing to *p*H 7.93 ± 0.05 with concentrated hydrochloric acid or concentrated sulfuric acid. The addition of one milliliter of these solutions to the ten milliliter reaction flasks gave the desired final concentration.

Enzyme Solutions.—Solutions of 75 mg. of enzyme preparation in five ml. of water were prepared. One ml. of this solution in a 10-ml. reaction flask gave the required final concentration of 1.5 mg./ml. This corresponds to 0.1785 mg. of protein-nitrogen per ml. for Armour preparation no. 10705. No aliquots were taken from stock solutions which had been kept for more than 2 hr. at 25.0 ± 0.1°. No decrease in activity could be noted in this time.

Reaction and Analysis.[11]—Solutions containing the desired amount of substrate in 8 ml. of water and 1.0 ml. of the buffer system were equilibrated in a Sargent constant temperature bath for 15–20 minutes at 25.0 ± 0.1°. Fifteen seconds before zero time, 1.0 ml. of the enzyme solution was withdrawn from the stock solution pipetted into the reaction flask at zero time. The flasks were swirled gently and replaced in the bath. At equal time intervals of one, two or three minutes, depending upon rate of hydrolysis, 1.0-ml. aliquots were withdrawn from the reaction flask and pipetted usually into 10-ml. volumetric flasks containing the acid and aldehyde solution described under the next heading and made up to volume. The color was allowed to develop for 20 minutes and the optical density determined in 1-cm. quartz cells in a Beckman Model B spectrophotometer at 455 mμ with a water blank. Flasks larger than 10 ml. were employed for the more extended reactions which in no case exceeded 66.3% and were never less than 12.0%.

Acid and Aldehyde Solutions.—One hundred and forty-four ml. of J. T. Baker Analyzed hydrochloric acid (spec. grav. 1.186, 36.8%) was diluted to one liter with distilled water. One milliliter of this solution in a 10-ml. flask gave a final acid concentration of 0.172 *N*.

(19) R. J. Foster, H. J. Shine and C. Niemann, THIS JOURNAL, **77**, 2378 (1955).
(20) All melting points are corrected.
(21) Microanalyses by Dr. A. Elek.

A solution of one g. of *p*-dimethylaminobenzaldehyde in 100 ml. of absolute ethanol was prepared. The aldehyde was Matheson and Co. reagent grade, recrystallized from aqueous methanol, m.p. 75.5–75.9°. One milliliter of this solution in a 10-ml. flask corresponds to a final aldehyde concentration of $0.671 \times 10^{-2} M$.

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[CONTRIBUTION NO. 2250 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Catalytic Activity of Dimeric α-Chymotrypsin[1]

BY R. BRUCE MARTIN AND CARL NIEMANN[2]

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The kinetic consequences of the dimerization of α-chymotrypsin in aqueous solutions at 25° and *p*H 7.0 and 1.0 *M* and 0.30 *M* in sodium chloride have been investigated. Of the seven possible representations that were examined, one was definitely disproved, an additional three were rejected with considerable confidence and of the remaining three, preference has been indicated for two based upon the supposition that ES can combine with E, and E_2 with S, to give E_2S and that E_2S can combine with S to give E_2S_2. However, of the three intermediate enzyme-substrate complexes, *i.e.*, ES, E_2S and E_2S_2 only the first appears to be able to decompose to give reaction products at a substantial rate.

The anomalous behavior observed in sedimentation and light scattering studies with α-chymotrypsin in aqueous media have been interpreted in terms of an equilibrium between monomeric and dimeric species.[3–7] In particular, it has been suggested that the equilibrium is reversible and that above *p*H 5 the association of monomer increases with decreasing *p*H and increasing ionic strength. It is believed that polymeric species larger than dimer[8,9] are not likely to occur except at very low ionic strengths.

Since it has been shown[10,11] that the association of α-chymotrypsin may have a significant influence on the rates of certain reactions catalyzed by this enzyme it appeared desirable to determine the catalytic properties of the dimeric species insofar as they could be inferred from the kinetic behavior of systems involving α-chymotrypsin in aqueous solutions at 25° and *p*H 7.0 and 1.0 *M* in sodium chloride.

In principle it should be possible to distinguish between various alternative formulations involving monomeric and dimeric enzyme and monomeric specific substrate. In this study we have considered the situations summarized in Fig. 1 and Table I. The corresponding equations for K_D, the dissociation constant for the dimer, are given in Table II.

Case 1 is the simplest mechanism that can account for dimerization of the enzyme. In this case, which is free of special simplifying assumptions, it is postulated that the dimer is incapable of combining with the specific substrate.

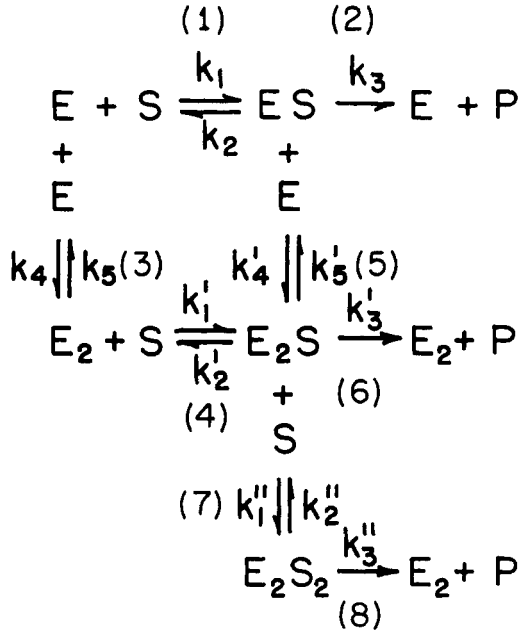

Fig. 1.—Probable equilibria involving enzyme and specific substrate.

In case 2, the dimer is considered capable of combining with the specific substrate to give the ternary complex E_2S, which also can arise by combination of the monomeric enzyme with the complex ES

TABLE I

SITUATIONS CONSIDERED[a]

Case no.	Reactions	Assumptions
1	1–3	
2	1–5	$K_S^{\circ} = K_S^{c}$; $K_D^{d} = K_D'^{e}$
3	1–6	$K_S = K_S'$; $K_D = K_D'$; $k_3 = k'_3$
3a	1–6	$K_S = {}^1/_2K_S'$; $K_D = {}^1/_2K_D'$; $k_3 = k_3'$
4	1–5,7	$K_S = K_S' = K_S''^{f}$; $K_D = K_D'$
4a	1–5,7	$K_S = K_S' = {}^1/_2K_S''$; $K_D = K_D'$
5	1–8	$K_S = K_S' = K_S''$; $K_D = K_D'$; $k_3 = k_3' = k_3''$

[a] Based upon equilibria given in Fig. 1. [b] $K_S = [E][S]/[ES]$. [c] $K_S' = [E_2][S]/[E_2S]$. [d] $K_D = [E]^2/[E_2]$. [e] $K_D' = [ES][E]/[E_2S]$. [f] $K_S'' = [E_2S][S]/[E_2S_2]$.

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(2) To whom inquiries regarding this article should be sent.

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