stituting groups in place of the $(CH_3)_2N-$ substituent on the azobenzene nucleus of I. A corresponding dye with a *para* O–H group, for example, would allow one to study ionization behavior in the region near *p*H 7–8, an *ortho* O–H group, higher *p*H ranges. One is also not limited to attachments at mercaptan side chains of proteins. The use of a sulfonyl chloride substituent in place of –HgAc in I, for example, would permit one to make adducts to lysine residues. There is thus a wide range of covalent complexes which can be prepared. These should offer suitable examples for establishing how much emphasis should be put on the role of "frozen" water in determining the chemical and physical behavior of dissolved protein molecules.

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Further Studies on the Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Acetyl-L-tyrosinamide and of Acetyl-L-tryptophanamide in Aqueous Solutions at 25° and *p*H 7.9 ± 0.1[1]

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It has been found that the values of K_S, k_3 and K_P for the system α-chymotrypsin-acetyl-L-tyrosinamide, in aqueous solutions at 25° and *p*H 7.9 ± 0.1, obtained by observing the rate of formation of either one of the two reaction products, are identical within the limits of experimental error, that the values of K_S and K_P are independent of the nature and concentration of the buffer components of the four buffer systems investigated and that the values of k_3 are dependent upon the concentration of the buffer components and indirectly upon the nature of the buffer species. More limited observations with the system α-chymotrypsin–acetyl-L-tryptophanamide suggest that the behavior of this system is identical with that observed for the system α-chymotrypsin–acetyl-L-tyrosinamide. An experimental criterion for the recognition of bi- and trifunctionality in anionic competitive inhibitors of the type $RCH_2CO_2^-$ and $R'CONHCHRCO_2^-$ has been noted.

In a previous study Thomas, MacAllister and Niemann[3] examined the kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM[4] component of a THAM–HCl buffer, or 0.02 *M* in the EDA[5] component of an EDA–HCl buffer, by determining the rate of formation of acetyl-L-tyrosinate ion. The results obtained by these investigators were re-evaluated by Foster and Niemann[6] and by Foster, Shine and Niemann[7] and it was concluded that the above reaction systems could be represented throughout their course by equations 1 and 2, where P_{1f} is acetyl-L-tyrosinate ion and P_{2f} either ammonia or ammonium ion, and that their rates could be described, within the limits of experimental error, by eq. 3, where $K_S = (k_2 + k_3)/k_1 = 32 \pm 4 \times 10^{-3}$ *M*[6],

$$E_f + S_1 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \quad (1)$$

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

$$k_3[E]t = K_S(1 + [S]_0/K_P)\ln([S]_0/[S]_t) + (1 - K_S/K_P)([S]_0 - [S]_t) \quad (3)$$

$K_P = k_5/k_4 = 110 \pm 30 \times 10^{-3}$ *M*[7] and $k_3 = 2.4 \pm 0.3 \times 10^{-3}$ *M*/min./mg. protein nitrogen per ml.[6],

When the above values of K_S and k_3 are compared with those reported earlier,[8-11] and which were obtained by determining the rate of formation of ammonia and ammonium ion in aqueous solutions at 25° and *p*H 7.8 and 0.1 *M* in an unspecified phosphate buffer containing sufficient lithium chloride to bring the initial ionic strength to 0.292, it is seen, *cf.* Table I, that such a comparison leaves unanswered a number of questions whose answers are required for a more complete understanding of the reaction under consideration. It is the purpose of

TABLE I

KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1

Ref.	K_S[a]	k_3[b]	K_P[a]
3, 6, 7	32 ± 4[c,d]	2.44 ± 0.3[c,d]	110 ± 30[c,d]
8	23[e,f]	2.7[e,f]	.....
9	32.6[e,f]	2.7[e,f]	.....
10	27[e,f,g]	3.0[e,f,g]	.....
11	29[e,f]	3.1[e,f]	.....

[a] In units of 10^{-3} *M*. [b] In units of 10^{-3} *M*/min./mg. protein-nitrogen per ml. [c] Reaction system 0.02 *M* in the THAM component of a THAM–HCl buffer or 0.02 *M* in the EDA component of an EDA–HCl buffer. [d] Based upon the determination of the rate of formation of acetyl-L-tyrosinate ion. [e] Reaction system 0.1 *M* in an unspecified phosphate buffer containing sufficient lithium chloride to bring the initial ionic strength to 0.292. [f] Based upon the determination of the rate of formation of ammonia and ammonium ion. [g] Said to be preferred to the two sets of values immediately above.

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

(4) Tris-(hydroxymethyl)-aminomethane.

(5) Ethylenediamine.

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

(7) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).

(8) S. Kaufman and H. Neurath, *Arch. Biochem.*, **21**, 245 (1949).

(9) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **180**, 181 (1949).

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this communication to provide answers to several of these questions, *i.e.*, are the same values of K_S, k_3 and K_P obtained when the reaction is examined by observing the rate of formation of both P_{1f} and P_{2f}, are the values of K_S, k_3 and K_P derived from studies of the reaction in THAM–HCl and EDA–HCl buffers identical with those observed for systems containing sodium or potassium phosphate buffers and is there any dependence of the values of K_S, k_3 and K_P upon the concentration of the buffer components?

A colorimetric procedure for the determination of ammonia, based upon the earlier work of Moore and Stein,[12] was developed with due consideration being given to its applicability in systems containing α-chymotrypsin, acetyl-L-tyrosinamide, acetyl-L-tyrosine and THAM hydrochloride as well as ammonium chloride. This procedure was capable of determining quantities of ammonia, present as ammonium chloride, of from 0 to 0.6×10^{-3} *M*.

With the above analytical procedure it became possible to examine the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 *M* in the THAM component of a THAM–HCl buffer with respect to the rate of formation of ammonia and ammonium ion. Thirty-two experiments were conducted, *cf.* Table II, and it was found that with 18 the extent of reaction was sufficient to permit the use of the graphical procedure of Foster and Niemann[13–15] for the evaluation of the experimental data. The above graphical procedure could not be used for the evaluation of the remaining fourteen experiments because of a more limited extent of reaction. Therefore, in these latter cases the corrected initial velocities were evaluated by the procedure of Jennings and Niemann[16–18] and these values of v_0 were then used in an Eadie plot[15] to obtain values of K_S and k_3 but not of K_P.

Two separate evaluations were performed. In the first the experimental data obtained from the ten experiments conducted at [E] = 0.200 mg. protein-nitrogen per ml., *cf.* Table II, were presented in a $([S]_0 - [S]_t)/t$ *vs.* $(\ln ([S]_0/[S]_t))/t$ plot[13–15] and from this plot the following values were obtained: $K_S = 34 \pm 2 \times 10^{-3}$ *M*, $k_3 = 2.5 \pm 0.1 \times 10^{-3}$ *M*/min./mg. protein-nitrogen per ml. and $K_P = 80 \pm 20 \times 10^{-3}$ *M*. As the preceding values were derived from experimental data obtained under conditions where $[E] = 5.7 \times 10^{-5}$ *M*,[19] $E'_S = [E]/K_S = 0.2 \times 10^{-2}$ and $S'_S = [S]/K_S = 0.15$ to 1.28 it follows that all assumptions inherent in the evaluation procedure have been satisfied.[6,7]

In the second evaluation, based upon the results obtained in all 32 experiments, *cf.* Table II, the initial velocities, obtained from either a $([S]_0 - [S]_t)/t$ *vs.* $(\ln ([S]_0/[S]_t))/t$ plot or by the procedure of Jennings and Niemann,[16–18] were normalized to an enzyme concentration of 0.200 mg. protein-nitrogen per ml. assuming that the initial velocities were directly proportional to the enzyme concentrations. A line was then fitted to the 32 values of v_0 and of $v_0/[S]_0$ and from the slope and intercepts of this line and the slopes of the 18 lines of slope $-K_S(K_P + [S]_0)/(K_P - K_S)$ the following values were obtained; $K_S = 34 \pm 4 \times 10^{-3}$ *M*, $k_3 = 2.5 \pm 0.3 \times 10^{-3}$ *M*/min./mg. protein-nitrogen per ml. and $K_P = 80 \pm 20 \times 10^{-3}$ *M*. With [E] between the limits of 0.76 to 6.3×10^{-5} *M*,[19] E'_S between 0.02 and 0.2×10^{-2} and S'_S between 0.09 and 1.28 it is clear that the conditions required for a proper evaluation of K_S, k_3 and K_P were satisfied.[6,7]

A comparison of the values of K_S, k_3 and K_P for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H

TABLE II

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1 AND 0.02 *M* IN THE THAM COMPONENT OF A THAM–HCl BUFFER

[E][a]	[S][b]	t_{max}[c]	% Hydrol.[d]
0.222[e]	10	110	72.0
	10	110	72.0
.200[e]	5	122	86.4
	5	110	71.6
	10	110	70.2
	15	150	84.2
	20	150	78.6
	25	150	79.7
	30	165	77.5
	35	150	69.6
	40	180	76.8
	45	150	65.4
.179[f]	5	110	72.4
	5	110	67.2
	10	110	66.9
	10	110	62.5
	30	150	67.5
	40	150	65.1
.133[f]	3	60	40.0
	3	60	40.7
	3	60	39.7
	5	60	38.0
	5	60	39.0
	5	60	44.0
	10	60	37.2
	10	60	38.2
	10	60	36.9
	10	60	37.6
	10	60	36.0
.0266[f]	5	60	8.3
	10	60	7.2
	10	60	7.6

[a] In units of mg. protein-nitrogen per ml. [b] In units of 10^{-3} *M*. [c] Maximum time of observation in minutes with a total of seven observations taken within the time indicated. [d] Armour preparation no. 00592. [e] Armour preparation no. 10705. [f] The first 18 experiments were evaluated by the procedure of Foster and Niemann,[13–15] the remainder by the procedure of Jennings and Niemann.[16–18]

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(18) Based upon a value of $K_S = 32 \times 10^{-3}$ *M*.
(19) Value of [E] is based upon an assumed molecular weight of monomeric α-chymotrypsin of 22,000 and a nitrogen content of 16.0%.[6,7]

7.9 ± 0.1 and 0.02 M in the THAM component of a THAM–HCl buffer with respect to the rate of formation of acetyl-L-tyrosinate ion, *i.e.*, $K_S = 32 \pm 4 \times 10^{-3}$ M, $k_3 = 2.4 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml. and $K_P = 110 \pm 30 \times 10^{-3}$ M,[3,6,7] and with respect to the rate of formation of ammonia and ammonium ion, *i.e.*, $K_S = 34 \pm 2 \times 10^{-3}$ M, $k_3 = 2.5 \pm 0.1 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml. and $K_P = 80 \pm 20 \times 10^{-3}$ M[20] shows that within the limits of experimental error the same values of K_S, k_3 and K_P are obtained irrespective of whether the reaction is studied by determining the rate of formation of P_{1f} or P_{2f}.

From a series of experiments based upon the determination of the amount of ammonia and ammonium ion liberated in aqueous solutions at 25° with [E] = 0.200 mg. protein-nitrogen per ml., $[S]_0 = 5 \times 10^{-3}$ M and $t = 110$ minutes it was found that the *p*H optimum for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in a system 0.02 M in the phosphate component of a sodium phosphate buffer is *ca.* 7.9.

With the knowledge that the *p*H optimum for the above reaction system is identical, within the limits of experimental error, with the *p*H optima for the comparable systems 0.02 M in the THAM or EDA component of a THAM– or EDA–HCl buffer[3] it was decided to evaluate K_S, k_3 and K_P for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.1 M in the phosphate component of a sodium or potassium phosphate buffer[21] by determining the rate of formation of ammonia and ammonium ion. The experimental conditions employed in this study are summarized in Table III.

TABLE III

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1 AND 0.1 M IN THE PHOSPHATE COMPONENT OF A SODIUM OR POTASSIUM PHOSPHATE BUFFER[a]

$[S]_0$[b]	t_{max}[c]	% Hydrol.	$[S]_0$[b]	t_{max}[c]	% Hydrol.
Sodium phosphate buffer			Potassium phosphate buffer		
5	110	88.0	5	110	87.0
10	110	81.6	10	110	81.2
30	150	85.8	20	150	88.7
40	150	80.3	30	150	83.7
			35	120	74.1
			40	150	80.0

[a] With [E] = 0.200 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [b] In units of 10^{-3} M. [c] Maximum time of observation in minutes with a total of seven observations taken within the time indicated.

Since data were available for the extended reaction each set was evaluated by the procedure of Foster and Niemann.[13–15] From the two $([S]_0 - [S]_t)/t$ *vs.* $(\ln ([S]_0/[S]_t))/t$ plots the following values were obtained. For the 0.1 M sodium phosphate buffer; $K_S = 32 \pm 2 \times 10^{-3}$ M, $k_3 = 3.3 \pm 0.2 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml., $K_P = 70 \pm 20 \times 10^{-3}$ M, [E] = 5.7×10^{-5} M,[19] E's = 0.2×10^{-2} and S's = 0.16 to 1.25. For the 0.1 M potassium phosphate buffer; $K_S = 33 \pm 2 \times 10^{-3}$ M, $k_3 = 3.25 \pm 0.15 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml., $K_P = 80 \pm 20 \times 10^{-3}$ M, [E] = 5.7×10^{-5} M,[19] E's = 0.2×10^{-2} and S's = 0.15 to 1.21.

The identity, within the limits of experimental error, of the above two sets of values of K_S, k_3 and K_P demonstrates that in the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 there is no detectable difference in the behavior of systems containing either 0.1 M sodium or potassium phosphate buffers. However, it will be noted that whereas the values of K_S and K_P evaluated in these latter systems, *i.e.*, 32 ± 2 and 33 ± 2 × 10^{-3} M and 70 ± 20 and 80 ± 20 × 10^{-3} M, are identical, within the limits of experimental error, with the values of K_S and K_P obtained in comparable systems 0.02 M in the THAM component of a THAM–HCl buffer, *i.e.*, 32 ± 4[3,6,7] and 34 ± 2 + 10^{-3} M and 110 ± 30[3,6,7] and 80 ± 20 × 10^{-3} M, the corresponding values of k_3 in systems 0.1 M in a sodium or potassium phosphate buffer, *i.e.*, 3.3 ± 0.2 and 3.25 ± 0.15 × 10^{-3} M/min./mg. protein-nitrogen per ml., are significantly greater than those observed in systems 0.02 M in the THAM component of a THAM–HCl buffer, *i.e.*, 2.4 ± 0.3[3,6,7] and 2.5 ± 0.1 × 10^{-3} M/min./mg. protein-nitrogen per ml.

Since the situation noted immediately above could be due to a difference in the nature of the buffer component and/or their concentrations it was decided to examine the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.2 M and 0.4 M in the phosphate component of a sodium phosphate buffer. As before data were obtained with respect to the rate of formation of ammonia and ammonium ion for the extended reaction, *cf.* Table IV, and K_S, k_3 and K_P were evaluated from $([S]_0 - [S]_t)/t$ *vs.* $(\ln ([S]_0/[S]_t)/t$ plots.[13–15] For the system 0.2 M in phosphate $K_S = 30 \pm 2 \times 10^{-3}$ M, $k_3 = 3.85 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml., $K_P = 80 \pm 20 \times 10^{-3}$ M,

TABLE IV

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1 AND 0.2 M AND 0.4 M IN THE PHOSPHATE COMPONENT OF A SODIUM PHOSPHATE BUFFER[a]

$[S]_0$[b]	t_{max}[c]	% Hydrol.	$[S]_0$[b]	t_{max}[c]	% Hydrol.
0.2 M sodium phosphate buffer			0.4 M sodium phosphate buffer		
5	110	87.2	5	90	96.8
5	110	93.2	10	90	92.4
10	110	90.0	30	90	82.2
10	110	88.8	40	90	73.3
30	150	90.0			
40	150	87.1			

[a] With [E] = 0.200 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [b] In units of 10^{-3} M. [c] Maximum time of observation with a total of seven observations taken within the time indicated.

(20) Of the two sets of values given earlier the one presented here is preferred because it was obtained by a more certain evaluation procedure.

(21) While it would have been desirable to study systems which were 0.02 M in the phosphate component such systems could not be poised at *p*H 7.9 for extended reactions at relatively high specific substrate concentrations. The minimum practical concentration is 0.1 M.

$[E] = 5.7 \times 10^{-5}$ M,[19] $E'_S = 0.2 \times 10^{-2}$ and $S'_S =$ 0.17 to 1.33. For the system 0.4 M in phosphate, $K_S = 31 \pm 3 \times 10^{-3}$ M, $k_3 = 5.0 \pm 0.3 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml., $K_P = 75 \pm 20 \times 10^{-3}$ M, $[E] = 5.7 \times 10^{-5}$ M,[19] $E'_S = 0.2 \times 10^{-2}$ and $S'_S = 0.16$ to 1.29.

The information that is now available with respect to the dependence or independence of values of K_S, k_3 and K_P for the system α-chymotrypsin–acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.9 ± 0.1 upon components of the reaction system that are not represented in eq. 1 and 2 is summarized in Table V. Having established for the case at hand that the same values of K_S, k_3 and K_P are obtained irrespective of whether the reaction is followed by determining the rate of formation of P_{1f} or P_{2f}, *vide ante*, it is seen from the information given in Table V that the values of K_S and K_P are independent, within the limits of experimental error, of the nature or concentration of any of the components of the reaction system which may have been added to maintain the system at a constant pH but that the values of k_3 are clearly dependent upon either the nature and/or concentration of such components.

TABLE V

SUMMARY OF KINETIC CONSTANTS FOR THE α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TYROSINAMIDE IN AQUEOUS SOLUTIONS AT 25° AND pH 7.9 ± 0.1

Buffer system	K_S[a]	k_3[b]	K_P[a]	Ref.
0.02 M THAM or EDA[c]	32 ± 4	2.4 ± 0.3	110 ± 30	3,6,7
.02 M THAM[d]	34 ± 2	2.5 ± 0.1	80 ± 20	..
.1 M PO_4[e]	32 ± 2	3.3 ± 0.2	70 ± 20	..
.1 M PO_4[f]	33 ± 2	3.25 ± 0.15	80 ± 20	..
.1 M PO_4[g]	27	3.0	.....	10
.1 M PO_4[g]	29	3.1	.....	11
.2 M PO_4[h]	30 ± 2	3.85 ± 0.3	80 ± 20	..
.4 M PO_4[i]	31 ± 3	5.0 ± 0.3	75 ± 20	..
Any of above	32 ± 2[j]	.....	80 ± 20[j]	..

[a] In units of 10^{-3} M. [b] In units of 10^{-3} M/min./mg. protein-nitrogen per ml. [c] System 0.02 M in the THAM component of a THAM–HCl buffer or 0.02 M in the EDA component of an EDA–HCl buffer and followed by determining the rate of formation of acetyl-L-tyrosinate ion. [d] System 0.02 M in the THAM component of a THAM–HCl buffer and followed by determining the rate of formation of ammonia and ammonium ion. [e] System 0.1 M in the phosphate component of a sodium phosphate buffer and followed by determining the rate of formation of ammonia and ammonium ion. [f] Same as *e* except system 0.1 M in the phosphate component of a potassium phosphate buffer. [g] System 0.1 M in an unspecified phosphate buffer containing sufficient lithium chloride to bring the ionic strength to 0.292 and followed by determing the rate of formation of ammonia and ammonium ion. [h] Same as *e* except system 0.2 M in the phosphate component. [i] Same as *e* except system 0.4 M in the phosphate component. [j] Preferred values.

The observation that the value of K_S for the system α-chymotrypsin–acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.9 ± 0.1 appears to be independent of added THAM and its hydrochloride, EDA and its hydrochloride or sodium or potassium phosphate recalls the experience of Shine and Niemann[22,23] who found that the value of K_S for the system α-chymotrypsin–chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component of a THAM–HCl buffer is essentially independent of added sodium or potassium chloride[22] or of added sucrose.[23] In view of the relatively high concentrations of sodium and potassium phosphate employed in the present studies we can now regard these two salts along with sodium and potassium chloride as members of that class of electrolytes which appear to have no demonstrable effect upon the K_S value of an amide type trifunctional specific substrate.

It is known that the K_I values, *i.e.*, the enzyme-inhibitor dissociation constants, of certain bifunctional anionic competitive inhibitors of α-chymotrypsin, when evaluated in aqueous solutions at 25° and pH 7.9 ± 0.1, are strikingly dependent upon whether the value was obtained by examination of a reaction system 0.02 M in the THAM component of a THAM–HCl buffer or one 0.1 M in an unspecified phosphate buffer.[24–26] Since it was noted previously[26] that no information was available with respect to the behavior of a single trifunctional anionic competitive inhibitor of the type $R'CONHCHRCO_2^-$ in 0.02 M THAM–HCl and 0.1 M phosphate buffers it was of particular interest to find that the K_P value, *i.e.*, the K_I value, of acetyl-L-tyrosinate ion determined in this study was essentially independent of the nature of the buffer systems which included not only 0.02 M THAM–HCl but also 0.1, 0.2 and 0.4 M sodium phosphate, *cf.* Table V. With the knowledge that under certain conditions the K_I values of a trifunctional anionic competitive inhibitor of α-chymotrypsin is independent of the presence of added phosphate and the K_I value of a comparable bifunctional anionic competitive inhibitor of this enzyme appears to be dependent upon the presence of added phosphate it is clear that we now possess an experimental basis for recognizing bi- and trifunctionality in anionic competitive inhibitors of the type $RCH_2CO_2^-$ and $R'CONHCHRCO_2^-$.

In contrast to the essential independence of values of K_S and K_P upon the concentration of the sodium phosphate buffer it is seen from the information summarized in Table V that the value of k_3 for the system α-chymotrypsin–acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.9 ± 0.1 increases with increasing concentration of the sodium phosphate buffer. It was noted previously[22] that with added sodium or potassium chloride the value of k_3 for the α-chymotrypsin catalyzed hydrolysis of chloroacetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.75 and 0.02 M in the THAM component of a THAM–HCl buffer increased linearly in accordance with the relation $\log (k_3/k_3^0) = 0.30 \pm 0.01\sqrt{M}$ where k_3^0 is the value observed in the absence of added sodium or potassium chloride and M the molality of the added salt. When the limited data summarized in Table V were examined with respect to possible relationships between values of k_3 and sodium phosphate concentration, or some function thereof, it was noted that with plots of k_3 *vs.* M, $\log k_3$ *vs.* M, log

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(25) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 5963 (1952).
(26) R. J. Foster and C. Niemann, *ibid.*, **77**, 3365 (1955).

k_3 *vs.* $\sqrt{M}$ and log k_3 *vs.* $\sqrt{\mu}$[27] only the first and third gave reasonably linear plots although all could have been considered to be linear within the limits of experimental error. The log k_3 *vs.* $\sqrt{M}$ plot was selected as the most suitable for an empirical extrapolation and from the relation log $(k_3/k_3^0) = 0.575\sqrt{M}$ the following values of k_3^0, *i.e.*, the value of k_3 in the absence of sodium phosphate, were computed: 2.2, 2.1 and 2.2 $\times 10^{-3}$ M/min./mg. protein-nitrogen per ml. With log k_3^0 = 0.333 $\times 10^{-3}$ M/min./mg. protein-nitrogen per ml. the above relation gave an expected value of $k_3 = 2.6 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml. for a system 0.02 M in the phosphate component of a sodium phosphate buffer of *p*H 7.9 ± 0.1. The agreement of this value of k_3 with that obtained for the comparable system 0.02 M in the THAM component of a THAM–HCl buffer, *i.e.*, 2.5 ± 0.1 $\times 10^{-3}$ M/min./mg. protein-nitrogen per ml, suggests that values of k_3 are basically independent of the nature of the buffers considered in this study but that the dependency of the value of k_3 for a particular specific substrate upon the concentration of the buffer components may vary with the nature of the buffer.

It has been shown[6,7,28] that for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tryptophanamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.02 M in the THAM component of a THAM–HCl buffer $K_S = 5.0 \pm 0.5 \times 10^{-3}$ M, $k_3 = 0.55 \pm 0.1 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml. and $K_P = 10 \pm 2 \times 10^{-3}$ M when the reaction is followed by determining the rate of formation of acetyl-L-tryptophanate ion. In order to determine whether several of the results noted previously could be observed with a second specific substrate the above system was re-examined under conditions where the 0.02 M THAM–HCl buffer was replaced by a potassium phosphate buffer which was 0.1 M in the phosphate component and where the reaction was followed by determining the rate of formation of ammonia and ammonium ion. The reaction conditions employed in this latter study are summarized in Table VI and when the primary data were evaluated through the use of a $([S]_0 - [S]_t)/t$ *vs.* $(\ln ([S]_0/[S])_t)/t$ plot[13-15] the following values were obtained: $K_S = 5.7 \pm 0.3 \times 10^{-3}$ M, $k_3 = 0.68 \pm 0.02 \times 10^{-3}$ M/min./mg. protein-nitrogen per ml., $K_P = 7.3 \pm 0.6 \times 10^{-3}$ M, [E] = 6.3 $\times 10^{-5}$ M,[19] $E'S$ = 0.1 $\times 10^{-2}$ and $S'S$ = 1.75 to 7.05.

TABLE VI

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TRYPTOPHANAMIDE IN AQUEOUS SOLUTIONS AT 25° AND *p*H 7.9 ± 0.1 AND 0.1 M IN THE PHOSPHATE COMPONENT OF A POTASSIUM PHOSPHATE BUFFER[a]

$[S]_0$[b]	t_{max}[c]	% Hydrol.
10	150	80.5
15	150	70.5
15	150	70.5
20	150	61.3
20	150	59.4
25	150	55.1
30	150	49.8
40	150	41.9

[a] With [E] = 0.222 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [b] In units of 10^{-3} M. [c] Maximum time of observation in minutes with a total of seven observations taken within the time indicated.

(27) Based upon the following dissociation constants for phosphoric acid: $k_1 = 1.1 \times 10^{-2}$, $k_2 = 7.5 \times 10^{-8}$ and $k_3 = 4.8 \times 10^{-13}$.

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

A comparison of the above values of K_S, *i.e.*, 5.0 ± 0.5 and 5.7 ± 0.3 $\times 10^{-3}$ M, k_3, *i.e.*, 0.55 ± 0.1 and 0.68 ± 0.02 $\times 10^{-3}$ M/min./mg. protein-nitrogen per ml. and $K_P = 10 \pm 2$ and 7.3 ± 0.6 $\times 10^{-3}$ M reveals a common pattern in the behavior of acetyl-L-tyrosinamide and acetyl-L-tryptophanamide. As with the former specific substrate the value of K_S for the latter appears to be independent of the nature and/or concentration of the buffer components and whether the reaction is followed by determining the rate of formation of P_{1f} or P_{2f}. In contrast, the value of k_3 again appears to be dependent upon the concentration of the buffer components and it is noteworthy that the percentage increase in the value of k_3 in passing from a 0.02 M THAM–HCl buffer to a 0.1 M potassium phosphate buffer is *ca.* 30% for acetyl-L-tyrosinamide and *ca.* 20% for acetyl-L-tryptophanamide. Finally, it may be noted that the value of K_P for the system α-chymotrypsin–acetyl-L-tryptophanate ion in aqueous solutions at 25° and *p*H 7.9 ± 0.1 also appears to be independent of the nature and/or concentration of the buffer components. In this connection it is of interest to recall that Cunningham and Brown[29] have reported that the K_I value of acetyl-L-tryptophanate ion in aqueous solutions at 25° and *p*H 8 and 0.0033 M in the THAM component of a THAM–HCl buffer and 0.1 M in calcium chloride is 8.7 ± 2 $\times 10^{-3}$ M, a value which may be compared with the values of 10 ± 2 and 7.3 ± 0.6 $\times 10^{-3}$ M given above.

Experimental[30]

Analytical Procedure.—The procedure used in this study was based upon one described by Moore and Stein.[12] Because of the necessity, in some cases, of determining small amounts of ammonia in the presence of substantial amounts of THAM many precautions had to be taken beyond those required for a more conventional application of the original procedure. The procedure to be described was the result of a large number of experiments designed to establish optimum conditions for the determination of ammonia in the presence of THAM and phosphate buffers. While these experiments[31] will not be described here it should be realized that the directions given below are based upon a large number of observations and are in no sense arbitrary. Strict adherence to the described procedure is required if satisfactory results are to be obtained.

A. Reagents.—Commercial ninhydrin was recrystallized from hot water, after decolorization with Norite A, the product dried *in vacuo* and stored in a sealed amber colored container. Anhydrous stannous chloride, prepared from the crystalline dihydrate by reaction with acetic anhydride,[32] was dried *in vacuo* and stored in a sealed container. Reagent grade citric acid monohydrate was further purified by recrystallization from hot water. Peroxide-free Methyl Cellosolve was prepared by refluxing the commercial product with anhydrous stannous chloride until a negative test for peroxides was obtained. The reaction mixture was then

(29) L. N. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

(30) All melting points are corrected.

(31) R. A. Bernhard, Ph.D. Thesis, Calif. Inst. Tech., Pasadena, Calif., 1955.

(32) H. Stephen, *J. Chem. Soc.*, 2786 (1930).

fractionally distilled and the fraction b.p. 123–125° stored in a sealed amber colored container. Peroxide-free isopropyl alcohol is also required.

B. **Reagent Solutions.**—A 0.2 *M* sodium citrate buffer of *p*H 4.8 ± 0.1 was prepared from 42 g. of recrystallized citric acid monohydrate and 16 g. of reagent grade sodium hydroxide pellets in 1000 ml. of distilled water. A few crystals of thymol were added and the solution stored at 4° prior to use. This solution was prepared weekly. The ninhydrin reagent was prepared by first dissolving 0.0240 g. of C.P. stannous chloride dihydrate in 15.0 ml. of the citrate buffer and passing dry nitrogen through the solution for three minutes. A solution of 0.600 g. of recrystallized ninhydrin in 15.0 ml. of peroxide-free Methyl Cellosolve was then prepared and dry nitrogen passed through the solution for three minutes. The first solution now was added to the second and the resulting ninhydrin reagent stored in a polyethylene container under an atmosphere of nitrogen. This reagent was prepared immediately prior to use. A diluent solution was prepared daily from equal volumes of distilled water and peroxide-free isopropyl alcohol.

C. **Procedure.**—Prior to the starting of a given kinetic experiment a series of G. S. volumetric flasks were assembled, the capacities being determined by the amount of ammonia expected in a 1.0-ml. aliquot of the reaction mixture, *i.e.*, for 0.0–3.0 × 10^{-3} *M* a 5-ml. flask, for 3.0–7.0 × 10^{-3} *M* a 10-ml. flask, for 7.0–17.5 × 10^{-3} *M* a 25-ml. flask and for 17.5–35.0 × 10^{-3} *M* a 50-ml. flask. To each of the 10-ml. flasks was added *ca.* 8.5 ml. of the 0.2 *M* citrate buffer and when 5-, 25- and 50-ml. flasks were employed the amount of buffer added was *ca.* 3.5, 23.5 and 47.5 ml., respectively. At selected time intervals 1.0-ml. aliquots were withdrawn from the reaction mixture and added to the contents of the above flasks and the solutions made up to volume with the citrate buffer. After the completion of the experiment 1.0-ml. aliquots of each of the above solutions were added to 1.0-ml. aliquots of the ninhydrin reagent contained in a series of 13 cm. rimless Pyrex test-tubes, the tubes capped with close fitting cylindrical aluminum caps and heated in a water-bath maintained at 99–100° for exactly 20 minutes. The tubes were then withdrawn from the bath, allowed to cool for five minutes and a 50-ml. aliquot of the diluent solution added to each of the tubes. The optical density of the resulting solutions, for a 1 cm. path in a Corex cell at 565 mμ, was then promptly determined in a model B Beckman spectrophotometer. A sample of the diluent solution was used as a control.

D. **Calibration Curves and Blanks.**—The examination of solutions of the 0.2 *M* citrate buffer of *p*H 4.8 ± 0.1 containing aliquots of simulated reaction mixtures composed of varying amounts of acetyl-L-tyrosinamide, acetyl-L-tyrosine and ammonium chloride and fixed amounts of α-chymotrypsin and the buffer system employed in the original reaction mixture revealed that for concentrations of ammonia up to 0.04 *M* in the original reaction mixture the optical density was proportional to the ammonia concentration within the limits of experimental error. However, the proportionality constant was found to depend upon the particular ninhydrin reagent employed and upon the concentration of the THAM-HCl buffer when this buffer system was used in the original reaction mixture. Therefore, for each particular reaction system a standard calibration curve was prepared and coincidently with each series of determinations two solutions, one containing all of the components of the original reaction mixture except the specific substrate and the other containing all components present in the first and in addition a known amount of a standard ammonium chloride solution, were prepared and analyzed along with the other solutions of the series.

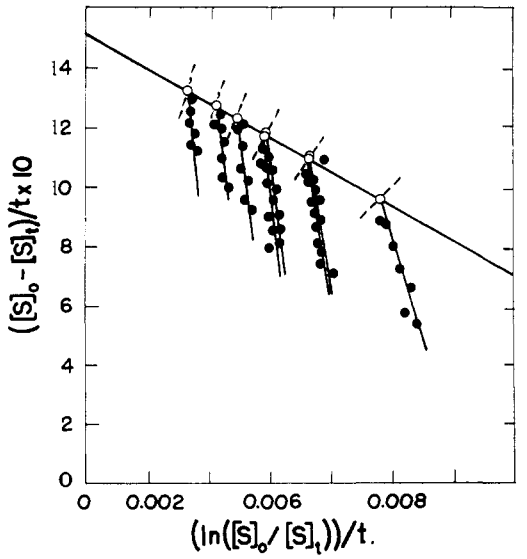

Fig. 1.—Foster-Niemann plot of data obtained for the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tryptophanamide in aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.1 *M* in the phosphate component of a potassium phosphate buffer.

Specific Substrates.—Acetyl-L-tyrosinamide, m.p. 226–228°, $[\alpha]^{24}D$ +52 ± 1° (*c* 0.8% in water) was prepared as directed by Thomas, MacAllister and Niemann.[3] Acetyl-L-tryptophanamide, m.p. 192–193°, $[\alpha]^{25}D$ +20 ± 1° (*c* 2% in methanol) was prepared as described by Huang and Niemann.[28]

Buffer Systems.—A stock solution 0.20 *M* in the thrice recrystallized THAM component of a THAM-HCl buffer of *p*H 8.0 at 25° was prepared. A 1:10 dilution of this stock solution in the reaction mixture gave a solution of *p*H 7.90 ± 0.05 at 25°. Sodium phosphate stock solutions 1.0, 2.0 and 4.0 *M* in the phosphate component were prepared by the addition of sufficient 5 *N* aqueous sodium hydroxide to aqueous solutions of reagent grade sodium dihydrogen phosphate to give solutions of *p*H 8.05 at 25° after the solutions were made up to volume. One to ten dilutions of these stock solutions in the reaction mixture gave solutions of *p*H 7.9 ± 0.05 at 25°. A 1.0 *M* potassium phosphate stock solution was prepared similarly from potassium dihydrogen phosphate and 5 *N* aqueous potassium hydroxide.

Enzyme Solutions.—Crystalline bovine α-chymotrypsin containing magnesium sulfate (Armour lot no. 10705) and crystalline bovine α-chymotrypsin salt-free (Armour lot no. 00592) were employed in these studies. Stock solutions of these preparations were prepared daily.

TABLE VII

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-L-TRYPTOPHANAMIDE[a]

t (min.)	O.D.[b]	$([S]_0 - [S]_t)$[c]	$([S]_0 - [S]_t)/t$	$(\ln([S]_0/[S]_t))/t$
20	0.462	2.175	0.109	0.00784
25	.504	2.550	.102	.00746
50	.748	4.775	.096	.00767
75	.930	6.450	.086	.00749
100	1.105	8.050	.081	.00768
125	1.237	9.225	.074	.00763
150	1.383	10.575	.071	.00814

[a] In aqueous solutions at 25° and *p*H 7.9 ± 0.1 and 0.1 *M* in the phosphate component of a potassium phosphate buffer with [E] = 0.222 mg. protein-nitrogen per ml. of Armour preparation no. 00592 and $[S]_0$ = 15 × 10^{-3} *M*. [b] Optical density based upon a 1:25 dilution of a 1-ml. aliquot of the reaction mixture and a blank value of 0.227. [c] Based upon a value of O.D. = 1.976 for 16 × 10^{-3} *M* ammonium chloride.

Enzyme Experiments.—The substrate was dissolved in 3 to 7 ml. of distilled water contained in a 10-ml. G. S. volumetric flask, 1.0 ml. of the appropriate buffer stock solution added and the solution placed in a 25.0 ± 0.05° bath for 20 to 25 minutes. At minus 20 seconds from time zero the volumetric flask was withdrawn from the bath and at time zero 1.0 ml. of the enzyme stock solution was added and the solution made up to volume with distilled water. The contents of the flask were mixed by gently inverting the flask 12 to 14 times and the flask then returned to the bath. 1.0-

ml. aliquots were withdrawn at convenient time intervals, delivered into a series of volumetric flasks containing the 0.2 M citrate buffer of pH 4.8 ± 0.1, the solution immediately made up to volume with the citrate buffer and then analyzed as directed above.

Analysis of Data.—Data representative of a single kinetic experiment are given in Table VII. Sets of these data were graphically evaluated by the method of Foster and Niemann[13-15] as illustrated in Fig. 1.

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[CONTRIBUTION NO. 2172 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Dependence of the Enzyme-Inhibitor Dissociation Constants of Several Bifunctional Anionic Competitive Inhibitors of α-Chymotrypsin upon Added Potassium Phosphate[1]

BY RICHARD A. BERNHARD AND CARL NIEMANN[2]

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It has been observed that the enzyme-inhibitor dissociation constants of two representative bifunctional anionic competitive inhibitors of α-chymotrypsin, *i.e.*, β-(β-indole)-propionate and phenyl acetate, when evaluated in aqueous solutions at 25° and pH 7.9 ± 0.1 may be decreased by increasing the concentration of the uni-univalent buffer system, or by the addition of sodium chloride or potassium phosphate. A detailed analysis has indicated that the effect produced by added potassium phosphate is to be associated with a general increase in ionic strength and a specific ion effect.

It has been recognized that the enzyme-inhibitor dissociation constants of a number of bifunctional anionic competitive inhibitors of α-chymotrypsin, when evaluated in aqueous solutions at 25° and pH 7.9 and 0.02 M in the THAM[3] component of a THAM–HCl buffer,[4-6] are substantially greater than the constants obtained for the same inhibitors in aqueous solutions at 25° and pH 7.8 and 0.1 M in an unspecified phosphate buffer.[7,8] However, the lack of a suitable analytical procedure has prevented, until recently, a more direct comparison of the behavior of certain bifunctional anionic competitive inhibitors of α-chymotrypsin in the presence of THAM–HCl and phosphate containing buffers. With the development of a procedure which was suitable for following the rate of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.9 ± 0.1 in the presence of THAM–HCl or sodium or potassium phosphate buffers[9] it was thought desirable to re-examine several of the experiments reported earlier[4-8] and if possible to clarify and explain the behavior noted above.

In the first series of experiments the α-chymotrypsin catalyzed hydrolysis of acetyl-L-tyrosinamide in aqueous solutions at 25° and pH 7.0 ± 0.1 and 0.02 M in the THAM component of a THAM–HCl buffer was examined in the presence of added potassium β-(β-indole)-propionate, β-(β-indole)-propionamide, potassium phenylacetate, phenylacetamide or tryptamine hydrochloride. As before[9] the reaction was followed by determining the rate of formation of ammonia and ammonium ion and the primary data so obtained were graphically evaluated by the method of Foster and Niemann.[10-12] The experimental conditions employed in this study are summarized in the footnotes to Table I. It will be seen from these data that the extent of reaction was sufficient to justify the use of the above evaluation procedure.[13] From the evaluated data given in Table I it is evident that all values of K_I were obtained under conditions that were compatible with the assumptions inherent in the procedures used for their evaluation.[6,10-12,14,15]

TABLE I

K_I VALUES OF FIVE COMPETITIVE INHIBITORS OF α-CHYMOTRYPSIN EVALUATED IN AQUEOUS SOLUTIONS AT 25° AND pH 7.9 ± 0.1 AND 0.02 M IN THE THAM COMPONENT OF A THAM–HCl BUFFER[a]

Competitive inhibitor	K_I[b]	E_I'[c]	I_I
Potassium β-(β-indole)-propionate	11 ± 1.5[e]	0.52	3.6
β-(β-Indole)-propionamide	1.7 ± 0.3[f]	3.4	0.88
Potassium phenylacetate	170 ± 15[g]	0.03	0.88
Phenylacetamide	10 ± 1[h]	0.57	1.0
Tryptamine hydrochloride	1.5 ± 0.3[i]	3.8	6.7

[a] Evaluated against acetyl-L-tyrosinamide with K_S = 32 × 10^{-3} M, [E] = 5.7 × 10^{-5} M, [d] E_S' = 0.17 × 10^{-2} and S_S' = 0.625 to 1.25. [b] In units of 10^{-3} M. [c] In units of 10^{-2}. [d] Based upon an assumed molecular weight of monomeric α-chymotrypsin of 22,000 and a nitrogen content of 16.0%. [e] Based upon 4 sets of experiments with $[S]_0$ = 20, 25, 30 and 40 × 10^{-3} M, [I] = 40 × 10^{-3} M, t = 150 minutes with a total of 7 observations of extent of reaction within this time interval, with a total extent of reaction of 36.5, 35.4, 32.2 and 30.0%, respectively, and with [E] = 0.200 mg. protein-nitrogen per ml. of Armour preparation no. 00592. [f] Same as *e* but with [I] = 1.5 × 10^{-3} M and total extent of reaction of 66.3, 64.0, 61.8 and 58.0% respectively. [g] Same as *e* but with [I] = 150 × 10^{-3} M and total extent of reaction of 61.3, 59.0, 56.8 and 52.8%, respectively. [h] Same as *e* but with [I] = 10 × 10^{-3} M and total extent of reaction of 58.5, 57.3, 55.2 and 53.4%, respectively. [i] Same as *h* but with total extent of reaction of 24.3, 23.8, 22.7 and 22.4%, respectively.

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

(3) Tris-(hydroxymethyl)-aminomethane.

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(14) R. J. Foster and C. Niemann, *ibid.*, **77**, 1886 (1955).

(15) R. J. Foster, H. J. Shine and C. Niemann, *ibid.*, **77**, 2378 (1955).