(10) is less ambiguous. Applications of equation (10) will be illustrated by the evaluation of the K_I values of various competitive inhibitors in a subsequent communication.

We wish to express our thanks to Dr. Verner Schomaker for valuable suggestions and discussions in relation to this work.

Experimental

The preparation of acetyl-D-tryptophan methyl ester,[3] nicotinyl-L-tryptophanamide[3] and nicotinyl-L-tyrosinamide[11] has been described previously. All enzymatic experiments are conducted at 25° and *p*H 7.9 ± 0.02 in aqueous solution 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. The methods used are identical with those described previously.[3] The α-chymotrypsin was an Armour preparation lot no. 90402 of bovine origin.

(11) B. M. Iselin, H. T. Huang, R. V. MacAllister and C. Niemann, THIS JOURNAL, **72**, 1729 (1950).

PASADENA 4, CALIF. RECEIVED JANUARY 16, 1951

[CONTRIBUTION NO. 1520 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Kinetics of the α-Chymotrypsin Catalyzed Hydrolysis of Nicotinyl-L-tyrosinamide in Aqueous Solutions at 25° and *p*H 7.9[1]

BY H. T. HUANG, R. V. MACALLISTER, D. W. THOMAS AND CARL NIEMANN[2]

The kinetics of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tyrosinamide in aqueous solutions at 25° and *p*H 7.9 have been found to be similar to those reported previously for this enzyme and other specific substrates of the acylated α-amino acid amide type. For the system α-chymotrypsin–nicotinyl-L-tyrosinamide at 25° and *p*H 7.9 K_S was found to have a value of $15.0 \pm 1.0 \times 10^{-3}$ *M* and k_3 a value of 6.2×10^{-3} *M*/mg. protein nitrogen/ml./min.

It has been noted previously that whereas the *p*H–activity curves for α-chymotrypsin and acetyl- and nicotinyl-L-tryptophanamide are practically identical[3] there is a significant difference between the above curves and that observed for α-chymotrypsin and acetyl-L-tyrosinamide.[4] From the data given in Fig. 1 it will be seen that the *p*H–activity curve for α-chymotrypsin and nicotinyl-L-tyrosinamide is identical, within the limits of experimental error, with that observed for this enzyme and acetyl-L-tyrosinamide. The fact that substantially different *p*H–activity relationships have now been observed for α-chymotrypsin and acetyl- and nicotinyl-L-tryptophanamide on one hand, and α-chymotrypsin and acetyl- and nicotinyl-L-tyrosinamide on the other, clearly indicates that for this enzyme and specific substrates of the general formula $RCONHCHR_1CONH_2$, the nature of the amino acid side chain, *i.e.*, R_1, may have a profound influence upon the *p*H–activity relationship. In contrast, it appears, from the two cases at hand, that the role of the acyl group, *i.e.*, of R, in the above process is relatively unimportant.[5] Experiments designed to provide data relative to effect of *p*H upon the K_S and k_3 values of the above specific substrates are now in progress.

The kinetics of the α-chymotrypsin catalyzed hydrolysis of nicotinyl-L-tyrosinamide, at 25° and *p*H 7.9, are similar to those noted previously for this enzyme and other specific substrates[3,4] in that the course of the hydrolytic reaction is described by equations (1) and (2)

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

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

and, when E's < 0.1, by the rate equation (3)

$$k_3[E]t = 2.3K_S\left(1 + \frac{[S]_0}{K_{P_1}}\right)\log\frac{[S]_0}{[S]} + \left(1 - \frac{K_S}{K_{P_1}}\right)([S]_0 - [S]) \qquad (3)$$

However, with the system α-chymotrypsin–nicotinyl-L-tyrosinamide at 25° and *p*H 7.9, the relation between the values of K_S, K_{P_1} and k_3 is such that the experimental data can also be described, within the limits of experimental error, by rate equation (4)

$$k_3[E]t = 2.3K_S\log\frac{[S]_0}{[S]} + ([S]_0 - [S]) \qquad (4)$$

wherein inhibition of the hydrolytic reaction by the liberated nicotinyl-L-tyrosine is ignored. Therefore, in this instance, and when E's < 0.1, equation (4) may be used with confidence for the evaluation of k_3, even though the extent of hydrolysis may be of the order of 80% (*cf.* Fig. 2). K_S was determined in the usual manner[6] from a plot of $1/v_0$ *versus* $1/[S]_0$, and three independent determinations at two different enzyme concentrations and in two different buffer systems, *i.e.*, tris-(hydroxymethyl)-aminomethane–hydrochloric acid and ethylenediamine–hydrochloric acid, gave a mean value of $K_S = 15.0 \pm 1.0 \times 10^{-3}$ *M*. A typical plot is given in Fig. 3. The value of k_3 was found to be 6.2×10^{-3} *M*/mg. protein nitrogen/ml./min.

The fact that nicotinyl-L-tyrosine must compete, at least to some degree, with nicotinyl-L-tyrosinamide for the catalytically active site on the enzyme molecule was established indirectly when it was shown that the α-chymotrypsin catalyzed hydroly-

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

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

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

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

(5) It should be understood that this generalization relates only to specific substrates derived from acylated α-amino acids. There is evidence to show that replacement of an acylamino group by an amino or ammonium group is accompanied by a change in the position of the maximum of the *p*H-activity curve; *cf.* E. F. Jansen, M. D. Fellows-Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **185**, 209 (1950); H. Goldenberg and V. Goldenberg, *Arch. Biochem.*, **29**, 154 (1950).

(6) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

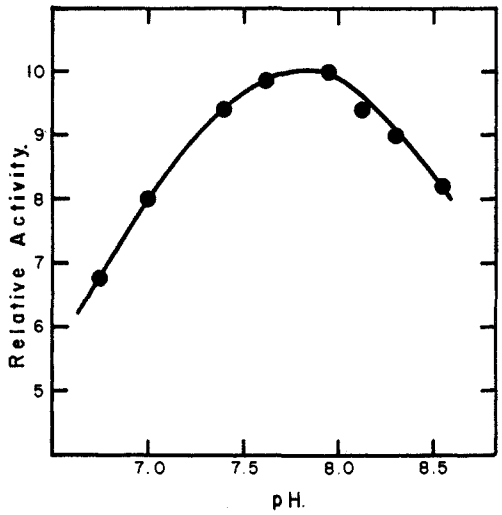

Fig. 1.—Nicotinyl-L-tyrosinamide, $[S]_0 = 12.5 \times 10^{-3}$ *M*; [E] = 0.047 mg. protein nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

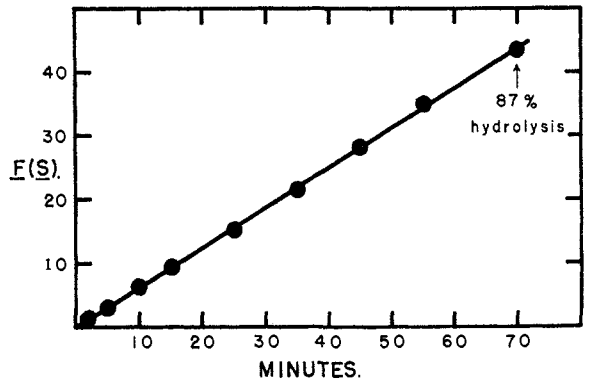

Fig. 2.—F(S) = 2.3 K_S log $[S]_0/[S] + ([S]_0 - [S])$ in units of 10^{-3} *M*; $[S]_0 = 15 \times 10^{-3}$ *M* of nicotinyl-L-tyrosinamide; [E] = 0.104 mg. protein nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

sis of acetyl-L-tyrosinamide is competitively inhibited by nicotinyl-L-tyrosine and that in aqueous solutions at 25° and *p*H 7.9, K_I, or K_{P_i}, = 60 ± 10 × 10^{-3} *M* for the latter compound (*cf.* Fig. 4). The fact that the K_I value of a competitive inhibitor of the above type is independent of the nature of the specific substrate used for its evaluation was demonstrated previously.[7]

The K_I value of nicotinyl-D-tyrosinamide has been redetermined using nicotinyl-DL-tyrosinamide as a source of the D-isomer in systems where the L-isomer was the specific substrate (*cf.* Fig. 3). The value obtained for K_I, *i.e.*, 6.4 ± 0.5 × 10^{-3} *M*, is in good agreement with the values obtained previously,[7] *i.e.*, 6.0 and 6.3 × 10^{-3} *M*, wherein the added inhibitor was the pure D-isomer and the specific substrates were, respectively, nicotinyl-L-tryptophanamide and nicotinyl-L-tyrosinamide. It may be inferred from the above observations that nicotinyl-DL-tyrosinamide behaves as a simple mixture in aqueous solutions and that, under these conditions, there is no significant interaction be-

(7) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1555 (1951).

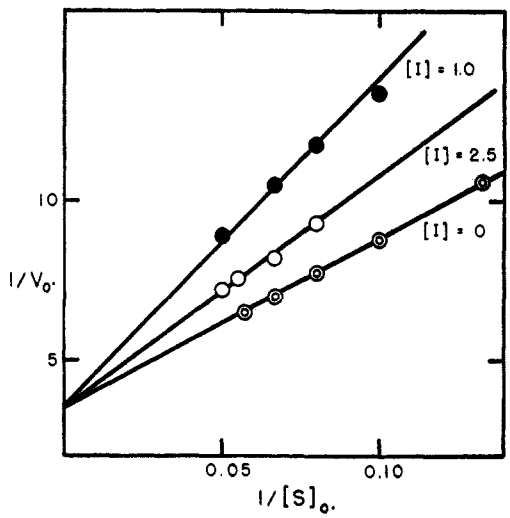

Fig. 3.—v_0 in units of 10^{-3} *M* per min.: ⊙, $[S]_0$ in units of 10^{-3} *M* of nicotinyl-L-tyrosinamide; O, [I] = 2.5 × 10^{-3} *M* of nicotinyl-D-tyrosinamide; ●, [I] = 1.0 × 10^{-3} *M* of nicotinyl-D-tyrosine ethyl ester; [E] = 0.47 mg. protein nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

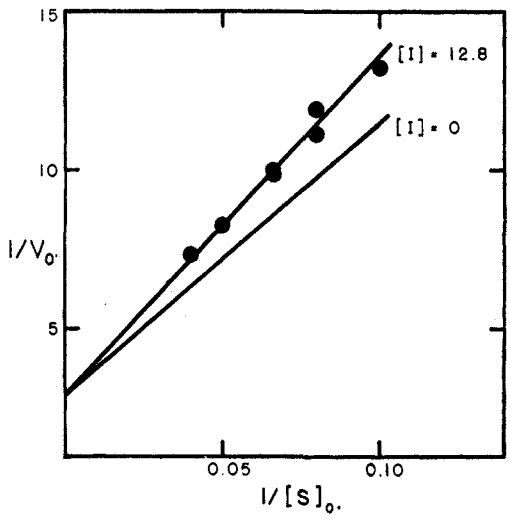

Fig. 4.—Inhibition of hydrolysis of acetyl-L-tyrosinamide by nicotinyl-L-tyrosine; v_0 in units of 10^{-3} *M* per min.; $[S]_0$ and [I] in units of 10^{-3} *M*; [E] = 0.139 mg. protein nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

tween the two enantiomorphs. From the data given in Figs. 3 and 5 it will be seen that nicotinyl-D-tyrosine ethyl ester is a competitive inhibitor in systems containing α-chymotrypsin and a specific substrate and that at 25° and *p*H 7.9, K_I = 0.97 ± 0.05 × 10^{-3} *M*.

In order to facilitate the subsequent discussion, all of the kinetic data obtained in this and in previous studies[3,4,7-9] are summarized in Table I. Since there is reason to believe that the K_S values for the specific substrates listed in Table I may be taken as being approximately equal to k_2/k_1,[8] apparent $-\Delta F^\circ$ values for the formation of the

(8) H. T. Huang and C. Niemann, *ibid.*, **73**, 3223 (1951).
(9) *Ibid.*, **73**, 3228 (1951).

TABLE I

KINETIC CONSTANTS FOR SPECIFIC SUBSTRATES AND COMPETITIVE INHIBITORS OF α-CHYMOTRYPSIN[a]

Compound	K_{S,I,P_1}[b]	k_3[c]	$-\Delta F°$[d]	Ref.
L-Tryptophanamide	6.3[e]		3000	10
D-Tryptophanamide	3.2[e]		3400	10
Acetyl-L-tryptophan	17.5[f]		2390	5
Acetyl-D-tryptophan	4.8[e]		3160	10
Acetyl-L-tryptophanamide	5.3[g]	0.50	3110[h]	5
Acetyl-D-tryptophanamide	2.7[e]		3500	5, 9
Acetyl-L-tryptophanmethylamide	4.8[e]		3160	10
Acetyl-D-tryptophanmethylamide	1.7[e]		3780	10
Acetyl-D-tryptophan methyl ester	0.089[e]		5520	11
Nicotinyl-L-tryptophan	8.8[f]		2810	5
Nicotinyl-L-tryptophanamide	2.7[g]	1.60	3500[h]	5
Nicotinyl-D-tryptophanamide	1.4[e]		3890	5
Acetyl-L-tyrosine	115[f]		1280	6
Acetyl-L-tyrosinamide	30.5[g]	2.4	2080[h]	6
Acetyl-D-tyrosinamide	12.0[e]		2620	6
Acetyl-D-tyrosine ethyl ester	3.5[e]		3350	6
Nicotinyl-L-tyrosine	60[f]		1660	
Nicotinyl-L-tyrosinamide	15.0[g]	6.2	2490[h]	
Nicotinyl-D-tyrosinamide	6.2[e]		3010	9
Nicotinyl-D-tyrosine ethyl ester	0.97[e]		4100	
Nicotinyl-D-phenylalaninamide	7.0[e]		2930	9

[a] In aqueous solutions at 25° and *p*H 7.9. [b] In units of 10^{-3} *M*. [c] In units of 10^{-3} *M*/mg. protein nitrogen/ml./min. [d] In calories per mole at 25° to the nearest 10 calories. [e] K_I value. [f] K_I or K_{P_1} value. [g] K_S value. [h] An apparent value based upon the premise that $K_S \doteq k_2/k_1$.

respective enzyme-substrate complexes are included in the table. It will be noted that irrespective of the nature of the constant, *i.e.*, it may be either K_S or K_I, that with six pairs of specific substrates or competitive inhibitors, characterized by the general formula $R_1CHR_2R_3$, where R_1 = acetamido or nicotinamido, R_2 = β-indolylmethyl or *p*-hydroxybenzyl, and R_3 = carbamido or carboxylate, the replacement of R_1 = acetamido by R_1 = nicotinamido, in either the D- or L-enantiomorphs, is accompanied by an increase in $-\Delta F°$ of approximately 400 calories. In contrast, when R_2 = *p*-hydroxybenzyl and R_3 = carbethoxy, replacement of R_1 = acetamido by R_1 = nicotinamido causes an increase in $-\Delta F°$ of 750 calories for the D-isomers. Where R_1 = amino[10] or acetamido, R_2 = β-indolylmethyl, and R_3 = carbamido, the replacement of R_1 = amino[10] by R_1 = acetamido is accompanied by an increase in $-\Delta F°$ of approximately 100 calories for both the D- and L-enantiomorphs. The fact that the acid strength of an ammonium group in an α-amino acid amide is substantially greater than that of the corresponding α-amino acid[11] precludes the use of the

(10) As indicated previously,[8] it is not known whether this group is an amino group or an ammonium group.

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943.

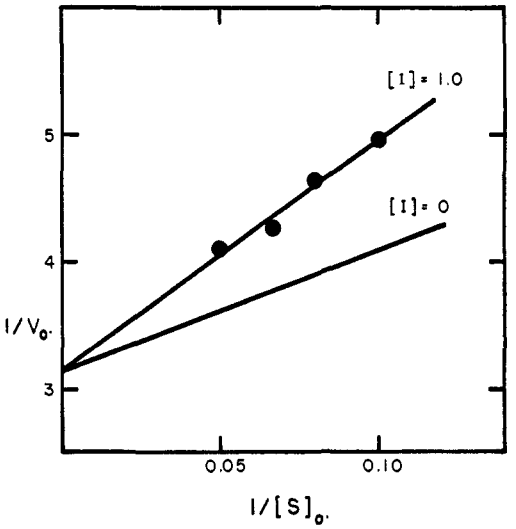

Fig. 5.—Inhibition of hydrolysis of nicotinyl-L-tryptophanamide by nicotinyl-D-tyrosine ethyl ester; v_0 in units of 10^{-3} *M* per min.; $[S]_0$ and [I] in units of 10^{-3} *M*; [E] = 0.208 mg. protein nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

above data for predicting the K_I values for α-chymotrypsin and D- and L-tryptophan and D- and L-tyrosine at *p*H 7.9.

It was noted previously[7] that with competitive inhibitors of the D-configuration and characterized by the general formula $R_1CHR_2R_3$, where R_1 = acetamido or nicotinamido, R_2 = β-indolylmethyl, *p*-hydroxybenzyl or benzyl, and R_3 = carbamido, replacement of R_2 = β-indolylmethyl by R_2 = *p*-hydroxybenzyl caused a decrease in $-\Delta F°$ of approximately 880 calories and replacement of R_2 = *p*-hydroxybenzyl by R_2 = benzyl a decrease in $-\Delta F°$ of 80 calories. It is clear from the data given in Table I that with specific substrates or competitive inhibitors of the L-configuration and where R_1 = acetamido or nicotinamido, R_2 = β-indolylmethyl or *p*-hydroxybenzyl and R_3 = carbamido or carboxylate, the replacement of R_2 = β-indolylmethyl by R_2 = *p*-hydroxybenzyl is accompanied by a decrease in $-\Delta F°$ of 1020 calories when R_3 = carbamido and 1130 calories when R_3 = carboxylate. Thus, where R_1 = acetamido or nicotinamido and R_3 = carbamido, it appears that replacement of R_2 = β-indolylmethyl by R_2 = *p*-hydroxybenzyl causes a greater decrease in $-\Delta F°$ for the L-isomer than for the D-isomer by approximately 150 calories and that the same replacement causes a greater decrease in $-\Delta F°$, by approximately 100 calories for the acids than for the amides.

In respect to changes in the nature of R_3, it is seen that when R_1 = acetamido or nicotinamido, replacement of R_3 = carboxylate by R_3 = carbamido in the L-isomer is accompanied by an increase in $-\Delta F°$ of approximately 700 calories when R_2 = β-indolylmethyl and of 820 calories when R_2 = *p*-hydroxybenzyl. In contrast, when R_1 = acetamido and R_2 = *p*-hydroxybenzyl, replacement of R_3 = carboxylate by R_3 = carbamido in the D-isomer causes an increase in $-\Delta F°$ of only 340 calories. Similarly, when R_1 = acetamido and

TABLE II

SUMMARY OF EXPERIMENTS USED FOR THE EVALUATION OF KINETIC CONSTANTS[a]

S:I[b]	[E][c]	E'_S[h]	E'_I[h]	No. of experiments at $[S]_0 = 10^{-3}$ M x 5	7.5	10	12.5	15	17.5	18.5	20	25
I	0.047	0.72		1	2	2	1	2	1		1	
I	.150[g]	.23		1		1					1	
I:II[d]	.047	.072	0.17				1	1		1	1	
I:III[e]	.047	.072	1.11			1	1	1			1	
IV:V[f]	.138	.11	0.053			1	2	2	1			1
VI:III[e]	.208	1.78	4.95			1	1	1			1	

[a] All experiments at 25° and *p*H 7.9 in an aqueous tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer, 0.02 M with respect to the amine component, unless otherwise noted. [b] I = nicotinyl-L-tyrosinamide, II = nicotinyl-D-tyrosinamide, III = nicotinyl-D-tyrosine ethyl ester, IV = acetyl-L-tyrosinamide, V = nicotinyl-L-tyrosine, and VI = nicotinyl-L-tryptophanamide. [c] Mg. protein nitrogen per ml; on basis of 27,000 for the molecular weight of α-chymotrypsin and one catalytically active site per molecule 0.208 mg. protein nitrogen = 0.048×10^{-3} M. [d] $[I] = 2.5 \times 10^{-3}$ M. [e] $[I] = 1.0 \times 10^{-3}$ M. [f] $[I] = [P_1] = 12.8 \times 10^{-3}$ M. [g] Ethylenediamine-hydrochloric acid buffer, 0.02 M with respect to the amine component. [h] E'_S and $E'_I \times 10^2$.

R_2 = β-indolylmethyl, replacement of R_3 = carboxylate by R_3 = N-methylcarbamido causes an increase in $-\Delta F^\circ$ of 770 calories for the L-isomer and 620 calories for the D-isomer. It is possible that the very large increase in $-\Delta F^\circ$, *i.e.*, 2360 calories, observed when R_3 = carboxylate is replaced by R_3 = carbomethoxy in a compound of the D-configuration, where R_1 = acetamido and R_2 = β-indolylmethyl, may provide an explanation for the difference in the $-\Delta F^\circ$ values noted for acetyl-D-tyrosine ethyl ester and nicotinyl-D-tyrosine ethyl ester, *i.e.*, 750 calories.

The K_S and the k_3 values for the four specific substrates given in Table I demonstrate that, with specific substrates derived from β-indolylpropionamide and from β-(*p*-hydroxyphenyl)-propionamide, the affinity of the enzyme is greater for the α-nicotinamido derivative than for the α-acetamido derivative. If it is true that $K_S \doteq k_2/k_1$ then it appears from the four cases at hand that when R_2 and R_3 are held constant and R_1 is varied, an increase in affinity is accompanied by an increase in susceptibility to hydrolysis, but that when R_1 and R_3 are held constant and R_2 is varied an increase in affinity is accompanied by a decrease in susceptibility to hydrolysis. It is clear that considerably more information will have to be provided before the above observations can be considered as valid generalizations.

Experimental[12,13]

The preparation of nicotinyl-L-tyrosinamide,[14] nicotinyl-D-tyrosine ethyl ester,[7] nicotinyl-L-tryptophanamide[3] and acetyl-L-tyrosinamide[4] has been reported previously.

Nicotinyl-DL-tyrosinamide.—Acylation of 2 g. of DL-tyrosinamide with 1.5 g. of nicotinyl azide in pyridine gave nicotinyl-DL-tyrosinamide, m.p. 224–226°.

Anal. Calcd. for $C_{15}H_{15}O_3N_3$ (285): C, 63.2; H, 5.3; N, 14.7. Found: C, 63.4; H, 5.6; N, 14.5.

Nicotinyl-L-tyrosine.—Hydrolysis of 0.60 g. of nicotinyl-L-tyrosine ethyl ester[15] with chymotrypsin at *p*H 8 gave 200 mg. of nicotinyl-L-tyrosine, m.p. 192°, undepressed upon admixture with an authentic sample.

Enzyme Experiments.—The methods used were identical with those reported previously.[5] The enzyme preparations were obtained from Armour and Company. The experiments in the presence of the ethylenediamine–hydrochloric acid buffer were performed with Lot No. 70902; all other experiments were with Lot No. 90402. The experiments, exclusive of those required for the determination of the *p*H–activity curve, wherein a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer 0.02 M with respect to the amine component was employed, are summarized in Table II. It should be noted that the specific enzyme concentrations, E'_S and E'_I, used for each series of experiments were such as to afford essentially zone A conditions[16,17] for all experiments.

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(12) All microanalyses by Dr. A. Elek.

(13) All melting points are corrected.

(14) B. M. Iselin, H. T. Huang, R. V. MacAllister and C. Niemann, THIS JOURNAL, **72**, 1729 (1950).

(15) The authors are indebted to Mr. R. Foster for this preparation.

(16) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

(17) A. Goldstein, *ibid.*, **27**, 529 (1944).