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The α-Chymotrypsin-catalyzed Hydrolysis of Acetyl-, Chloroacetyl- and Benzoyl-L-valine Methyl Ester[1]

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A search for neutral ester type specific substrates of α-chymotrypsin usable with enzyme concentrations of the order of 10^{-5} M has resulted in the selection of several acylated-L-valine methyl esters. The constants K_S and k_3 have been evaluated for the α-chymotrypsin-catalyzed hydrolysis of acetyl-, chloroacetyl- and benzoyl-L-valine methyl ester in aqueous solutions at 25.0° and pH 7.90 ± 0.01 and 0.1 M in sodium chloride and for the latter specific substrate in 0.02 M sodium chloride.

The discovery of the esterase activity of crystalline α-chymotrypsin, by Schwert, Neurath, Kaufman and Snoke,[3] has resulted in the use of a number of α-amino acid and acylated α-amino acid esters as specific substrates in studies with this enzyme or its cognates.[3-20] Since the ester type of specific substrate is ideally suited for investigations conducted with the recently developed pH-Stat,[21,22] we became interested in identifying those acylated α-amino acid esters that were suitable for such use.

The limited water solubility of many acylated α-amino acid esters led us to employ, in our initial studies, acetyl-L-phenylalanine glycolamide ester, a specific substrate that is very soluble in water.[23] However, with $[S]_0 = 1 \times 10^{-3}$ M, this specific substrate was hydrolyzed so rapidly in aqueous solutions at 25° and pH 7.90 ± 0.02 and 0.01 M in sodium chloride, that the pH-Stat[22] was unable to follow the reaction unless the enzyme concentration was reduced to *ca.* 10^{-4}.[24] While "wall effects" are not observable at enzyme concentrations of *ca.* 10^{-2},[25] it became evident that this was not the case at concentrations of *ca.* 10^{-4} and lower. At these latter concentrations, a significant proportion of the total amount of enzyme is adsorbed on the surfaces of the assembled titration cell and the volumetric equipment used in dilution of enzyme stock solutions. Several pertinent experiments are summarized in Table I where it is shown that reproducible but not necessarily accurate results can be obtained if a rather involved procedure is employed. However, this procedure does not solve the problems encountered in the volumetric manipulation of very dilute enzyme solutions and, more important, calls attention to the probability that under these conditions the reaction system will contain a significant proportion of surface adsorbed enzyme along with that present in solution. In view of the results of McLaren[26,27] on the properties

TABLE I

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF ACETYL-L-PHENYLALANINE GLYCOLAMIDE ESTER[a]

Expt. no.	Vol. titrant[b]	v_0[c]
1	305	13.7
	42[d]	1.9[d]
	26[d]	1.2[d]
	21[d]	1.0[d]
	37[d,e]	1.7[d,e]
2	210[f]	9.4[f]
	21[d,f]	1.0[d,f]
3	352[f,g,h]	15.8[f,g,h]
4	340[f,g,h]	15.3[f,g,h]
5	348[f,g,h]	15.7[f,g,h]

[a] In aqueous solutions at 25° and pH 7.90 ± 0.02 and 0.01 M in sodium chloride with $[S]_0 = 1 \times 10^{-3}$ M and $[E] = 7.0 \times 10^{-5}$ mg. protein-nitrogen per ml. of Armour preparation no. 00592. [b] In units of 10^{-3} ml. of 0.009014 N aqueous sodium hydroxide consumed in the interval from $t = 0$ to 10 min. [c] Approximate initial velocity in units of 10^{-6} M/min. determined by visual inspection of recorder traces of extent of reaction *vs.* time. [d] Consecutive to previous run with all components of the titration cell thoroughly washed with distilled water prior to the addition of 20 ml. of a reaction system containing all components except added enzyme. [e] Electrodes and stirrer pretreated with enzyme solution containing 145×10^{-5} mg. protein-nitrogen per ml. and thoroughly washed with distilled water. [f] All components of the titration cell Dessicoted. [g] Electrodes and stirrer pretreated with enzyme solution containing 145×10^{-5} mg. protein-nitrogen per ml., immersed in 3 N aqueous hydrochloric acid and thoroughly washed with distilled water. [h] Enzyme samples taken from same stock solution containing 145×10^{-5} mg. protein-nitrogen per ml., delivered with pipet prewashed with this stock solution and adjusted to pH 7.90 ± 0.02 immediately prior to use.

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(3) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).
(4) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).
(5) H. Goldenberg and V. Goldenberg, *Arch. Biochem.*, **29**, 154 (1950).
(6) H. Goldenberg, V. Goldenberg and A. D. McLaren, *Biochim. Biophys. Acta*, **7**, 110 (1951).
(7) A. K. Balls and E. F. Jansen, *Adv. Enzymol.*, **13**, 321 (1952).
(8) H. T. Huang and C. Niemann, THIS JOURNAL, **74**, 4634 (1952).
(9) M. L. Barnard and K. J. Laidler, *ibid.*, **74**, 6099 (1952).
(10) R. E. Parks, Jr., and G. W. E. Plaut, *J. Biol. Chem.*, **203**, 755 (1953).
(11) J. Burch, *Biochem. J.*, **58**, 415 (1954).
(12) M. L. Bender, R. D. Ginger and K. C. Kemp, THIS JOURNAL, **76**, 3350 (1954).
(13) M. L. Bender and B. W. Turnquest, *ibid.*, **77**, 4271 (1955).
(14) S. A. Bernhard, *Biochem. J.*, **59**, 506 (1955).
(15) B. R. Hammond and H. Gutfreund, *ibid.*, **61**, 187 (1955).
(16) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).
(17) G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).
(18) B. S. Hartley and V. Massey, *ibid.*, **21**, 58 (1956).
(19) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).
(20) C. E. McDonald and A. K. Balls, *ibid.*, **221**, (1956).
(21) C. F. Jacobsen and L. Leonis, *Compt. rend. trav. lab. Carlsberg, Ser. Chim.*, **27**, 333 (1951).
(22) J. B. Neilands and M. D. Cannon, *Anal. Chem.*, **27**, 29 (1955).
(23) R. J. Kerr and C. Niemann, *J. Org. Chem.*, in press.

(24) Unless otherwise noted, all enzyme concentrations are in units of mg. protein-nitrogen per ml..

(25) R. A. Bernhard and C. Niemann, THIS JOURNAL, **77**, 480 (1955).
(26) A. D. McLaren, *J. Phys. Chem.*, **58**, 129 (1954).
(27) A. D. McLaren and E. F. Estermann, *Arch. Biochem. Biophys.*, **61**, 158 (1956); **68**, 157 (1957).

of α-chymotrypsin adsorbed on kaolin particles, it appeared unwise to commit oneself, at least initially, to a situation where part of the enzyme was adsorbed on a surface and the remainder was in solution and where volumetric surface effects were certain to be troublesome. The alternative solution was to find a set of specific substrates that would be hydrolyzed sufficiently slowly, in aqueous solutions at 25°, so that their rates of hydrolysis could be determined with enzyme concentrations of *ca.* 10^{-1}, where the amount of surface adsorbed enzyme would be negligible relative to the total amount present. One such substrate is methyl hippurate.[8,28] However, others were required.

Previous experience with acylated L-amino acid esters derived from the common aromatic α-amino acids[4,14–16,19] suggested that no specific substrates satisfying the above requirement could be found in this group. Since it was known that acetyl-D-tryptophan methyl ester was hydrolyzed slowly in the presence of large amounts of α-chymotrypsin,[29] this compound was examined under the conditions used for the glycolamide ester but with $[S]_0 = 5 \times 10^{-3}$ *M* and [E] = 0.03 and 0.06. Essentially no hydrolysis was observed in 20 minutes. In contrast to these results, the rates of hydrolysis, with [E] = 0.075, of 5×10^{-3} *M* acetyl-L-methionine ethyl ester, 16×10^{-3} *M* acetyl-L-leucine methyl ester and 4×10^{-3} *M* acetyl-S-benzyl-L-cysteine methyl ester were so rapid that the *p*H-Stat[22] again was unable to follow the reaction.

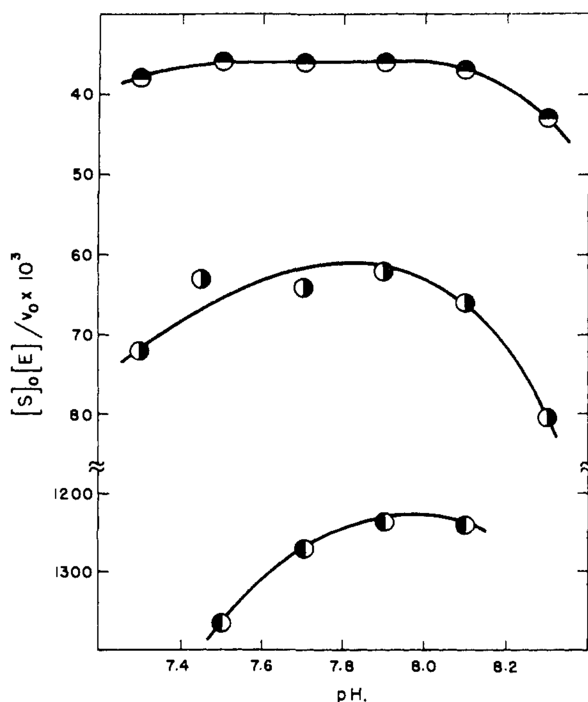

Fig. 1.—*p*H dependence of α-chymotrypsin-catalyzed hydrolysis of: ⊖, chloroacetyl-L-valine methyl ester; ◑, acetyl-L-valine methyl ester; ◐, benzoyl-L-valine methyl ester in aqueous solutions at 25°.

(28) T. H. Applewhite, R. B. Martin and C. Niemann, This Journal, **80**, 1457 (1958).

(29) Unpublished experiments conducted in these laboratories by Dr. R. M. Bock.

Consideration of the known influence of the nature of the acyl moiety on the rates of hydrolysis of acylated α-amino acid amides[30] and hydrazides[31] and of Newman's "Rule of Six,"[32] suggested the use of formyl-L-valine isopropyl ester. The behavior of this ester with α-chymotrypsin, *cf.*, Table II, led

TABLE II

THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SEVERAL ACYLATED α-AMINO ACID ESTERS[a]

Specific substrate	$[S]_0$[b]	[E][c]	v_0[d]
Formyl-L-valine isopropyl ester	24	0.15	<1
Acetyl-L-valine methyl ester	14	.15	3.7
	21	.15	4.4
	28	.15	5.9
Acetyl-L-isoleucine methyl ester	17	.15	5.1
	21	.15	5.9
Acetyl-L-leucine methyl ester	18	.0015	10[e]
	18	.0030	16[e]
Benzoyl-L-valine methyl ester	1.0	.15	2.2
	2.0	.15	3.2
	3.0	.15	4.0

[a] In aqueous solutions at 25.0° and *p*H 7.90 ± 0.02 and 0.02 *M* in sodium chloride. [b] In units of 10^{-3} *M*. [c] In units of mg. protein-nitrogen per ml. of Armour preparation no. 90492. [d] In units of 10^{-6} *M*/min., not corrected for an enzyme blank and based upon an assumed linearity of extent of reaction *vs.* time in the interval from 2 to 4 min. [e] While value is reproducible its accuracy is questionable because of the low enzyme concentration.

to the selection of acetyl-, chloroacetyl- and benzoyl-L-valine methyl ester as a set of specific substrates whose rates of hydrolysis, with an enzyme concentration of *ca.* 10^{-1}, were such as to be suitable for studies involving the use of a *p*H-Stat.[22]

The data summarized in Table II illustrate the apparent dependence of the rates of hydrolysis of a limited series of acylated α-amino acid esters upon their so-called "Six Numbers." For the series acetyl-L-valine, acetyl-L-isoleucine and acetyl-L-leucine methyl ester, whose "Six Numbers" are 8, 8 and 5, respectively, the initial velocities are in the approximate ratio of 4:5:800, if it is assumed that the determined rates are directly proportional to the respective enzyme concentrations. These data not only suggest that a series of specific substrates derived from L-isoleucine would be useful in studies with enzyme concentrations of *ca.* 10^{-1} but also indicate the desirability of attempting to relate the "Six Numbers" of an extensive series of specific substrates with their respective values of K_S and k_3. Such studies are in progress.

The *p*H dependence of the α-chymotrypsin-catalyzed hydrolysis of acetyl-, chloroacetyl- and benzoyl-L-valine methyl ester is illustrated by the curves given in Fig. 1. The first two specific substrates were examined in aqueous solutions at 25.0° and 0.1 *M* in sodium chloride and the latter in solutions 0.02 *M* in the same salt. The features of all three curves suggested the desirability of evaluating K_S and k_3 for all three specific substrates at *p*H 7.9.

The α-chymotrypsin-catalyzed hydrolysis of acetyl-, chloroacetyl- and benzoyl-L-valine methyl

(30) R. J. Foster and C. Niemann, This Journal, **77**, 1886 (1955).

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

(32) M. S. Newman, *ibid.*, **72**, 4783 (1950).

ester was examined in aqueous solutions at 25.0° and pH 7.90 ± 0.01 and 0.10 M in sodium chloride. The latter specific substrate also was studied under the same conditions but at a concentration of sodium chloride of 0.02 M. A summary of the pertinent experimental conditions and of the values of K_S and k_3 that were obtained is given in Table III.

It is clear that the values of K_S and k_3 summarized in Table III were evaluated under conditions consentient with those required if equation 1

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

is to have validity.[30] The experiments with acetyl- and chloroacetyl-L-valine methyl ester presented no unusual difficulties. However, the limited solubility of benzoyl-L-valine methyl ester did cause trouble particularly at the higher specific substrate concentrations where it was necessary to employ stock solutions that were supersaturated at 25° and which had to be prepared by dissolving the compound in boiling water. Fortunately the ester appeared to be reasonably resistant to non-enzymatic hydrolysis prior to adjustment of the stock solution to pH 7.9. However, as the specific substrate began to precipitate from 5×10^{-3} M solutions maintained at 25° for more than 1 hr., all stock solutions of this concentration were discarded after the removal of a single aliquot. It should also be noted that at the lower specific substrate concentrations the correction for the enzyme blank[28] was substantial. Thus, with relatively unfavorable situations being encountered at both limits of permissible specific substrate concentrations, the precision obtained in the studies with benzoyl-L-valine methyl ester was substantially less than that achieved with the other two specific substrates.

The data summarized in Table III demonstrate the usefulness of acetyl-, chloroacetyl- and benzoyl-L-valine methyl ester as specific substrates of α-chymotrypsin in studies where it is desirable to maintain enzyme concentrations at *ca.* 10^{-1}, *i.e.*, *ca.* 10^{-5} M. They also suggest that the values of K_S given in Table III may approximate those of the corresponding enzyme–specific substrate dissociation constants because of the relatively low values of k_3. However, in the absence of information relative to the characteristics of interaction of α-chymotrypsin with the corresponding D-enantiomorphs and with other enantiomorphic pairs, including those where the member possessing the L-configuration is incapable of functioning as a specific substrate, it appears wise to defer final interpretation of the above values of K_S. It is noteworthy that with all three specific substrates considered in this study a decrease in the value of K_S is accompanied by a concomitant decrease in the value of k_3 and that a comparison of the constants observed for methyl hippurate[28] with those for benzoyl-L-valine methyl ester reveals that replacement of an α-hydrogen atom in the former compound by an isopropyl group leads to less than a twofold reduction in the value of K_S but more than a fivefold reduction in the value of k_3.

Experimental[33,34]

Acetyl-L-phenylalanine Glycolamide Ester.—The prepa-

(33) All melting points are corrected.

(34) Microanalyses by Dr. A. Elek.

TABLE III

α-CHYMOTRYPSIN CATALYZED HYDROLYSIS OF ACETYL-, CHLOROACETYL- AND BENZOYL-L-VALINE METHYL ESTER[a]

Acyl moiety	NaCl, M	[E] b	[E] c	$[S]_0$ [d]	t [e]	Extent of reaction, % [f]	v_0 [g]	K_S [d,h]	k_3 [h,i]	k_3/K_S [j]	E'_S [k]	S_S'
Acetyl-	0.10	0.1464[l]	4.16	15.00–66.07[m]	0–8[n]	1.4– 1.9	3.584–11.73[o,p]	125.6 ± 3.4	2.33 ± 0.06	0.0185 ± 0.0009	0.03	0.12–0.53
Chloroacetyl-	.10	.1464[l]	4.16	6.85–42.22[q]	0–8[n]	2.3– 3.8	3.230–12.06[o,n]	47.1 ± 0.8	1.755 ± .028	.0373 ± .0007	.09	.15– .90
Benzoyl-	.10	.1467[l]	4.17	0.93– 3.01[s]	0–8[n]	10.4–13.9	1.694– 3.903[o,t]	4.9 ± .3	0.695 ± .036	.143 ± .016	.86	.20– .62
Benzoyl-	.02	.1545[u]	4.39	1.09– 4.08[v]	0–16[w]	16.0–24.0	1.580– 4.140[o,x]	4.2 ± .4	0.55 ± .04	.133 ± .022	1.0	.23– .97

[a] In aqueous solutions at 25.0° and pH 7.90 ± 0.01. [b] In units of mg. protein-nitrogen per ml. [c] In units of 10^{-5} M based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0%. [d] In units of 10^{-3} M. [e] Time of reaction in min. [f] Max. extent of reaction. [g] In units of 10^{-5} M/min. [h] Evaluated by a least squares fit to the equation $([S]_0[E]/v_0) = (K_S/k_3) + ([S]_0/k_3)$. [i] In units of 10^{-3} M/min./mg. protein-nitrogen per ml. [j] In units of min./mg. protein-nitrogen per ml. [k] In units of 10^{-2}. [l] Armour preparation no. 283. [m] 21 values including replicates within these limits. [n] 9 observations at uniform 1 min. intervals between these limits. [o] Corrected for enzyme and specific substrate blank. [p] Calculated on basis of assumed linear kinetics with a mean value of σv_0 of ±0.9%. [q] 15 values including replicates within these limits. [r] Same as p but with a mean value of σv_0 of ± 1.0%. [s] 14 values including replicates within these limits. [t] Same as p but with a mean value of σv_0 of ±1.5%. [u] Armour preparation no. 90492. [v] 16 values including replicates within these limits. [w] Same as n but with uniform 2 min. intervals. [x] Calculated by the method of orthogonal polynomials[43] with a mean value of σv_0 of ±1.4%.

ration of this compound has been described in another communication.[23]

Acetyl-D-tryptophan Methyl Ester.—The preparation of this compound has been described previously.[35]

Acetyl-L-methionine Methyl Ester.—This compound was prepared from acetyl-L-methionine[36] by a modification of the method of Brenner and Huber.[37] A similar method for the esterification of acylated amino acids has been described by Olechnowitz and Zimmerman.[38] To a 3-necked, 100-ml. flask equipped with a sealed stirrer, dropping funnel and calcium chloride protected outlet to a gas trap and containing 20 ml. of absolute methanol was added in a dropwise manner 4.3 ml. (0.06 mole) of redistilled thionyl chloride accompanied by stirring and cooling in an ice-salt bath. When the addition was complete, 9.5 g. (0.05 mole) of acetyl-L-methionine was added in several portions. A clear solution resulted. The cooling bath was removed, the reaction mixture heated at *ca.* 40° for 1 hr. and allowed to stand overnight. Removal of the solvent *in vacuo* gave a mobile oil that resisted crystallization. The oil was taken up in 100 ml. of chloroform, the solution extracted with 10 ml. of 1 *N* aqueous hydrochloric acid, 20 ml. of 1 *M* aqueous sodium carbonate, 20 ml. of water and the organic phase dried over magnesium sulfate. Removal of the solvent gave a clear oil that rapidly solidified on trituration with *n*-pentane. The solid was recrystallized from a mixture of 100 ml. ether and 20 ml. of pentane to give 6.17 g. (60%) of acetyl-L-methionine methyl ester, clusters of colorless needles, m.p. 43.5–44.5°, $[\alpha]^{25}D$ −21.2° (*c* 1% in ethanol).

Anal. Calcd. for $C_8H_{15}O_4NS$ (205.3): C, 46.8; H, 7.4. Found: C, 47.0; H, 7.2.

Acetyl-L-leucine Methyl Ester.—L-Leucine was converted to the methyl ester hydrochloride as described by Brenner and Huber.[37] The crude ester hydrochloride was acetylated with acetic anhydride in the presence of potassium carbonate as described by Huang and Niemann for the preparation of acetyl-L-tryptophan methyl ester.[35] From 5 g. (0.0275 mole) of crude ester hydrochloride in 40 ml. of 2.75 *M* aqueous potassium carbonate, 50 ml. of ethyl acetate and 6 ml. (*ca.* 0.06 mole) of acetic anhydride, there was obtained 4.2 g. of crude product contaminated with acetic anhydride. This substance was purified by treatment with moist chloroform–pyridine followed by extraction with 1 *N* aqueous hydrochloric acid, 1 *M* aqueous sodium carbonate and water to give 3.9 g. of an oily solid after drying and removal of solvents. The careful addition of pentane to a solution of this solid in ether gave colorless crystals, m.p. 43.0–44.5°. Karrer, *et al.*,[39] give a m.p. 74–75°.

N-Acetyl-S-benzyl-L-cysteine Methyl Ester.—S-Benzyl-L-cysteine, prepared in 50% yield by the reduction of L-cysteine with sodium in liquid ammonia and subsequent reaction with benzyl chloride as described by Wood and du Vigneaud,[40] was converted to the methyl ester hydrochloride in 84% yield by reaction with thionyl chloride and methanol.[37] Acetylation gave a 76% yield of colorless needles, m.p. 76.7–77.5°, after one recrystallization from absolute ether.

L-Valine Isopropyl Ester Hydrochloride.—An attempt to apply the method of Brenner and Huber[37] was unsuccessful. The compound was obtained by repeatedly saturating an ice-cold solution of 2 g. of L-valine in 50 ml. of isopropyl alcohol with hydrogen chloride, heating under refluxing conditions and removing the solvent under reduced pressure until an oil remained. The crude product could be recrystallized from ether but was used as obtained in the following preparation.

Formyl-L-valine Isopropyl Ester.—The method of du Vigneaud, *et al.*,[41] for the formylation of DL-cystine was modified for the preparation of this compound. The ester (0.017 mole), liberated from the hydrochloride with ammonia in chloroform, was allowed to react with 25 ml. of 98–100% formic acid and 21 ml. of acetic anhydride at 60°. Immediate removal of excess reactants *in vacuo* gave a mobile oil containing acidic impurities. The compound was purified by extraction of a chloroform solution with 1 *M* aqueous sodium carbonate and water. Removal of the solvent gave a residue which was crystallized from isopropyl ether. The crystalline solid reverted to an oil at room temperature.

Acetyl-L-isoleucine Methyl Ester.—The method of Reihlen and Knöpfle[42] was employed for the preparation of this compound. From 6.9 g. (0.038 mole) of the crude ester hydrochloride, prepared from L-isoleucine by the thionyl chloride–methanol procedure,[37] 6.6 g. (0.08 mole) of anhydrous sodium acetate, 25 ml. (*ca.* 0.26 mole) of acetic anhydride and three drops of pyridine, there was obtained 4.0 g. (56%) of solid product. Repeated recrystallization of this product from ether–hexane or ether–pentane resulted in the formation of well-formed crystals (melting from 44–56°) contaminated with a yellow oil. Treatment of 1 g. of the twice-recrystallized product with boiling solvent composed of 80 ml. of pentane and 20 ml. of ether caused the solid to dissolve, and the clear solution was decanted from the yellow oil. The solution was cooled whereupon colorless prisms began to separate. The solvent was decanted and the crystals washed with three 10-ml. portions of cold pentane to give acetyl-L-isoleucine methyl ester, m.p. 54.0–55.0°, after drying *in vacuo* over paraffin.

Acetyl-L-valine Methyl Ester.—To 95 ml. of anhydrous methanol contained in a 500-ml. three-necked flask fitted with a mercury sealed stirrer, dropping funnel and calcium chloride protected outlet to a gas trap, and cooled in an ice-salt bath, was added 17 ml. (0.235 mole) of redistilled thionyl chloride in the course of 2 hr. L-Valine, 25.0 g. (0.215 mole), was then added and the mixture held at 50° for 2 hr. or until solution was complete. The solvent and excess reagents were removed by distillation *in vacuo* and the residue dried *in vacuo* for several hours. The resulting solid was taken up in 60 ml. of anhydrous methanol, 600 ml. of anhydrous ether added and the ester hydrochloride allowed to crystallize at 4°. The solid was collected, washed with cold anhydrous ether and dried to give 28.0 g. (78%) of L-valine methyl ester hydrochloride, m.p. 150.0–154.5°, with decomp. A mixture of 7.5 g. (0.0455 mole) of the above ester hydrochloride, 7 g. of sodium acetate and 37.5 ml. of acetic anhydride was stirred for 13 hr. at room temperature after the reaction mixture had been cooled in a water-bath for the first 30 minutes. The slurry was neutralized with solid sodium bicarbonate, diluted with sufficient water to facilitate handling, filtered and the filtrate and inorganic residue exhaustively extracted with chloroform. The chloroform extract was washed with 1 *N* aqueous hydrochloric acid and 1 *M* aqueous sodium bicarbonate and dried over magnesium sulfate. The solvent was removed *in vacuo;* the residue, which was partly crystalline, was taken up in 100 ml. of boiling hexane and decolorized with Norit-A. The hot decolorized solution was allowed to cool to room temperature and sufficient hot hexane added to cause the product to begin to separate as an oil at *ca.* 40°. The mixture was then seeded and stored at 4°. The crystalline product was collected, washed with cold hexane and recrystallized two more times to give 3.6 g. (46%) of acetyl-L-valine methyl ester, m.p. 61.3–62.5°, $[\alpha]^{25}D$ −47.6 ± 0.4° (*c* 7.8% in water), after drying *in vacuo* for several days over solid sodium hydroxide; lit.[42] m.p. 61.5°.

Anal. Calcd. for $C_8H_{15}O_3N$ (173): C, 55.5; H, 8.7. Found: C, 55.7; H, 8.8.

Chloroacetyl-L-valine Methyl Ester.—To 15.0 g. (0.088 mole) of L-valine methyl ester hydrochloride, in 10 ml. of water and 25 ml. of ethyl ether, was added slowly at 0° 4.0 g. (0.10 mole) of sodium hydroxide dissolved in the minimum amount of water. The ethereal phase was separated, the aqueous phase extracted three times with 25-ml. portions of ether, the ethereal extracts combined and dried at 4° over magnesium sulfate. The dried and filtered solution was introduced into a 500-ml. three-necked flask, equipped with stirrer, dropping funnel and calcium chloride drying tube, and 3.5 ml. of chloroacetyl chloride in 20 ml. of anhydrous ether was introduced dropwise, and with stirring at −10°, in the course of 30 minutes. The reaction mix-

(35) H. T. Huang and C. Niemann, This Journal, **73**, 1541 (1951).

(36) V. du Vigneaud and C. E. Meyer, *J. Biol. Chem.*, **98**, 295 (1932).

(37) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1109 (1953).

(38) A. F. Olechnowitz and G. Zimmerman, *Angew. Chem.*, **67**, 209 (1955).

(39) P. Karrer, K. Escher and R. Widner, *Helv. Chim. Acta*, **9**, 301 (1926).

(40) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 109 (1939).

(41) V. du Vigneaud, R. Dorfman and H. S. Loring, *ibid.*, **98**, 577 (1932).

(42) H. Reihlen and L. Knöpfle, *Ann.*, **523**, 199 (1936).

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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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, $1/v_0 = (K_S + [S]_0)/k_3[E][S]_0 = (K_S/k_3[E]).(1/[S]_0) + 1/k_3[E]$ 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).