(VII), m.p. 108–109°, large dense prisms; $[\alpha]^{25}D$ −7.8° (*c* 1.64% in methanol).

Anal. Calcd. for $C_{15}H_{18}O_3N_2$ (274): C, 65.7; H, 6.6; N, 10.2. Found: C, 65.8; H, 6.6; N, 10.2.

Acetyl-D-tryptophan Isopropyl Ester (VIII).[21]—Acetylation of 5.1 g. of crude D-tryptophan isopropyl ester obtained from a resolution of DL-tryptophan isopropyl ester with "Viobin" pancreatic extract, with 3 g. of acetic anhydride in ethyl acetate solution, in the presence of aqueous sodium bicarbonate, gave 3.8 g. of (VIII), m.p. 133°, small stunted needles, after three recrystallizations from ethyl acetate; $[\alpha]^{25}D$ −2.9° (*c* 5% in methanol). No hydrolysis of the ester occurred when tested with α-chymotrypsin at *p*H 7.9.

Anal. Calcd. for $C_{16}H_{20}O_3N_2$ (288): C, 66.7; H, 7.0; N, 9.7. Found: C, 66.7; H, 7.0; N, 9.7.

Tryptamine Hydrochloride (IX).—The Eastman Kodak Co. white label product was recrystallized twice from 5 *N* hydrochloric acid to give dense prisms, m.p. 250–251°.

Acetyltryptamine (X).—One ml. of acetic anhydride was added to a suspension of 2 g. of tryptamine hydrochloride in 10 ml. of aqueous concentrated potassium carbonate, and the mixture shaken vigorously for 10 minutes. The oily product was extracted with ethyl acetate, washed with 1 *N* hydrochloric acid, water and aqueous sodium bicarbonate, the organic phase dried over calcium sulfate and evaporated to dryness in a stream of air. The sirupy residue crystallized on standing for 2 days *in vacuo* at 4°. The product was soluble in ether, chloroform and ethyl acetate and tended to come down as an oil from mixed solvents. Recrystallization was finally effected from hot water by allowing the solution to cool very slowly. Two recrystallizations from this solvent gave 0.8 g. of X, minute prisms, m.p. 75–76°.

Anal. Calcd. for $C_{12}H_{14}ON_2$ (202): C, 71.3; H, 7.0; N, 13.9. Found: C, 71.4; H, 7.0; N; 13.9.

Trifluoroacetyltryptamine (XI).—Trifluoroacetic anhydride (3 ml.) was added to a suspension of 1 g. of tryptamine in 50 ml. of ethyl acetate containing 5 ml. of acetone. The mixture was shaken for 5 minutes and the clear solution washed with aqueous sodium bicarbonate, dried over calcium sulfate and evaporated to dryness *in vacuo*. The solid residue was recrystallized twice from aqueous methanol to give 0.5 g. of XI, fine needles, m.p. 99–100°.

Anal. Calcd. for $C_{12}H_{11}ON_2F_3$ (256): C, 56.2; H, 4.3; N, 10.9. Found: C, 55.6; H, 4.3; N, 10.5.

Benzoyltryptamine (XII).—Benzoyl chloride (0.5 g.) was added to a solution of 1 g. of tryptamine hydrochloride in 10 ml. of anhydrous pyridine. The solution was warmed until it cleared, and allowed to stand at room temperature for two days. Evaporation *in vacuo* gave a gummy residue which was crystallized by rubbing with water. Two recrystallizations from aqueous methanol gave 0.5 g. of XII, fine needles, m.p. 141–142°; m.p. reported,[22] 137–138°.

Anal. Calcd. for $C_{17}H_{16}ON_2$ (264): C, 77.3; H, 6.1; N, 10.6. Found: C, 77.6; H, 6.3; N, 10.6.

Enzyme Experiments.—The methods used in this study were identical with those described previously.[4] All experiments were conducted at 25° and *p*H 7.9 ± 0.02 in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. Tryptamine was used in the form of its hydrochloride and it was found that in the concentration used the final *p*H of the system remained in the region desired without further adjustment. Considerable care had to be exercised in the preparation of the standard stock solutions of the three benzoyl-D-tryptophanamides and of benzoyltryptamine because of the sparing solubility of these compounds. It was found that the appropriate stock solutions of these inhibitors could be prepared at elevated temperatures, allowed to cool slowly at 25°, made up to volume, and aliquots taken immediately before crystallization set in. For each inhibitor a preliminary experiment was performed in order to ascertain what inhibitor concentration was required for the main series of measurements. It will be noted that the concentration chosen for each inhibitor, if the solubility was sufficiently great, is about equal in magnitude to the corresponding K_I value. From equation (3) it follows that the slope of a $1/v_0$ *versus* $1/[S]_0$ plot is $K_S/V(1 + [I]/K_I)$; hence the fact that $[I] = K_I$ will have the effect of increasing the slope of the plot by about 100% over the case where $[I] = 0$. It is believed that this situation represents the optimum conditions for the determination of K_I with our experimental procedures. The advantage of a further increase in the slope would be largely nullified by the greater uncertainty in the value of $1/v_0$ when v_0 is small, since the absolute error in v_0, which is about the same for all experiments, increases in importance as v_0 decreases. From the K_S value of nicotinyl-L-tryptophanamide[4] and the data given in Figs. 1–4, it will be appreciated that the conditions of our experiments were such as to permit the reactions to proceed under essentially zone A conditions,[10,11] except for the four cases where the systems were considered to be in zone B with respect to the inhibitor. The α-chymotrypsin used in this study was an Armour preparation lot no. 90402, of bovine origin.

(21) The authors are indebted to Mr. Richard Bernhard for this preparation.

(22) A. J. Ewins, *J. Chem. Soc.*, **99**, 270 (1911).

PASADENA 4, CALIFORNIA RECEIVED MAY 29, 1951

[CONTRIBUTION NO. 1562 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

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

BY H. T. HUANG, ROBERT J. FOSTER AND CARL NIEMANN[2]

The kinetics of the α-chymotrypsin catalyzed hydrolysis of acetyl- and nicotinyl-L-phenylalaninamide in aqueous solutions at 25° and *p*H 7.9 have been found to be similar to those noted previously for this enzyme and other specific substrates of the acylated α-amino acid amide type. The enzyme-inhibitor dissociation constants of α-chymotrypsin and two new competitive inhibitors derived from D-phenylalanine have also been determined under the above conditions.

The information that has accrued from systematic studies of the α-chymotrypsin-catalyzed hydrolysis of a number of acylated α-amino acid amides[3–7] has prompted us to extend these studies to two additional specific substrates, *viz.*, acetyl- and nicotinyl-L-phenylalaninamide. The *p*H-activity relationships for α-chymotrypsin and these two specific substrates are given in Fig. 1 and as expected[3,4,6,7] the two curves were found to be of the same general character.

In order to facilitate comparisons of the kinetic constants K_S and k_3[8] obtained in this study with those reported previously[3,4,6] all kinetic measurements were made at 25° and *p*H 7.9 in aqueous media 0.02 *M* with respect to the amine component

(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) R. J. Foster and C. Niemann, *ibid.*, **73**, 1552 (1951).

(6) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, **73**, 3231 (1951).

(7) H. J. Shine and C. Niemann, *ibid.*, **74**, 97 (1952).

(8) For definition of symbols *cf.* ref. 3.

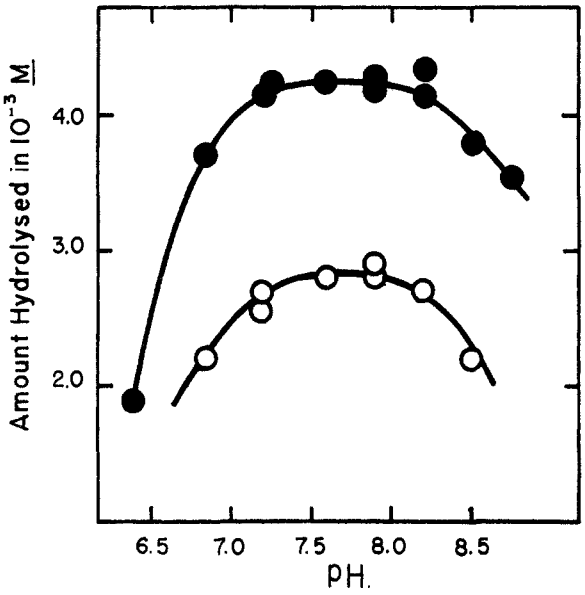

Fig. 1.—*p*H-activity relationships of α-chymotrypsin and acetyl- and nicotinyl-L-phenylalaninamide: ●, $[S]_0 = 10 \times 10^{-3}$ *M* nicotinyl-L-phenylalaninamide, time of hydrolysis = 35 min.; ○, $[S]_0 = 20 \times 10^{-3}$ *M* acetyl-L-phenylalaninamide, time of hydrolysis = 60 min. All experiments at 25° in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer; [E] = 0.208 mg. protein-nitrogen per ml.

of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. For all experiments the enzyme concentration was equivalent to 0.208 mg. of protein nitrogen per ml. of reaction mixture. Because of the slow rate of hydrolysis of acetyl-L-phenylalaninamide and the relatively limited solubility of nicotinyl-L-phenylalaninamide in aqueous solutions neither specific substrate permitted as extensive an investigation as was possible with the corresponding acylated L-tryptophanamides[3] and L-tyrosinamides.[4,6] Thus with acetyl-L-phenylalaninamide the limiting time factor made it inadvisable to study the reaction beyond 30% hydrolysis, and with nicotinyl-L-phenylalaninamide the highest substrate concentration at which successful measurements could be made was 12.5×10^{-3} *M*. The values of K_S and k_3 so determined cannot, therefore, be expected to have the same degree of precision as those determined previously[3-7] under more favorable conditions and are probably accurate to no better than ± 10–15%.

It was expected[3,4,6] that the α-chymotrypsin-catalyzed hydrolysis of acetyl- and nicotinyl-L-phenylalaninamide, under the conditions specified, would be described by equations (1) and (2), and

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

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

provided that E'_S and $E'_{P_1} < 0.1$ that the rate equation would be equation (3),

$$k_3[E]t = 2.3K_S(1 + [S]_0/K_{P_1}) \log [S]_0/[S] + (1 - K_S/K_{P_1})([S]_0 - [S]) \quad (3)$$

However, as has been observed for the system α-chymotrypsin–nicotinyl-L-tyrosinamide,[6] it appears that the relation between the values of K_S, K_{P_1} and k_3 for the system α-chymotrypsin–nicotinyl-L-phenylalaninamide–nicotinyl-L-phenylalanine are such that no direct evidence in respect to the inhibitory action of the nicotinyl-L-phenylalanine formed during the course of the reaction can be obtained by a plot of the kinetic data based upon equation (4) wherein reaction (2) is ignored. This is illustrated in Fig. 2 wherein a typical $F(S)$ *versus*

$$k_3[3]t = 2.3K_S \log [S]_0/[S] + ([S]_0 - [S]) \quad (4)$$

t plot, based upon equation (4), is seen to maintain a satisfactory linear relationship up to 80% hydrolysis.

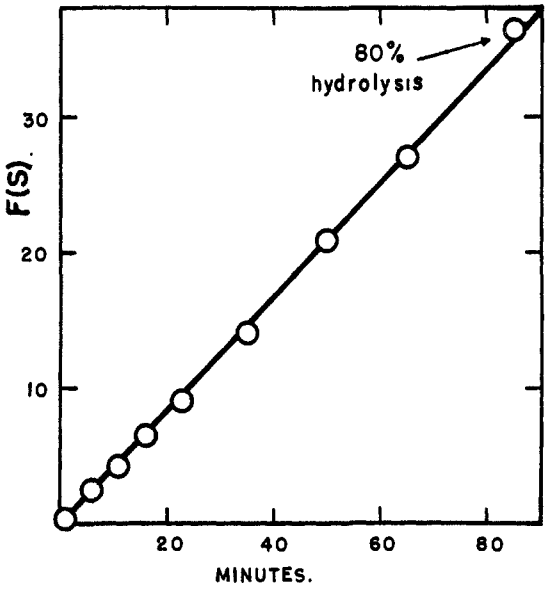

Fig. 2.—Plot of $F(S) = 2.3\ K_s \log [S]_0/[S] + ([S]_0 - [S])$ *versus* t in minutes; $F(S)$ in units of 10^{-3} *M*; $[S]_0 = 10 \times 10^{-3}$ *M* nicotinyl-L-phenylalaninamide, [E] = 0.208 mg. protein-nitrogen per ml., 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

It may be inferred from the above observations and from previous knowledge of the K_S values of acetyl- and nicotinyl-L-tryptophanamide[3] and acetyl- and nicotinyl-L-tyrosinamide,[4,6] and the K_{P_1}, *i.e.*, K_I values of the corresponding acylated-α-amino acids that the K_{P_1} values of acetyl- and nicotinyl-L-phenylalanine are large relative to the K_S values of the corresponding specific substrates, and are of the order of 0.1 *M*. Since it has been shown previously[7] that the K_{P_1}, or K_I, values of the acylated-α-amino acids cannot be accurately evaluated, at least with present techniques, when they are of the order of 0.1 *M*, no attempt has been made in the present study to determine the K_{P_1} values of acetyl- and nicotinyl-L-phenylalanine by independent inhibition experiments.

The K_S values of acetyl- and nicotinyl-L-phenylalaninamide were determined by the customary plot of $1/v_0$ *versus* $1/[S]_0$.[9] A plot based upon ten separate experiments with nicotinyl-L-phenylalaninamide (*cf.* Fig. 3) gave a value of K_S for this specific substrate of 18×10^{-3} *M*. The

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

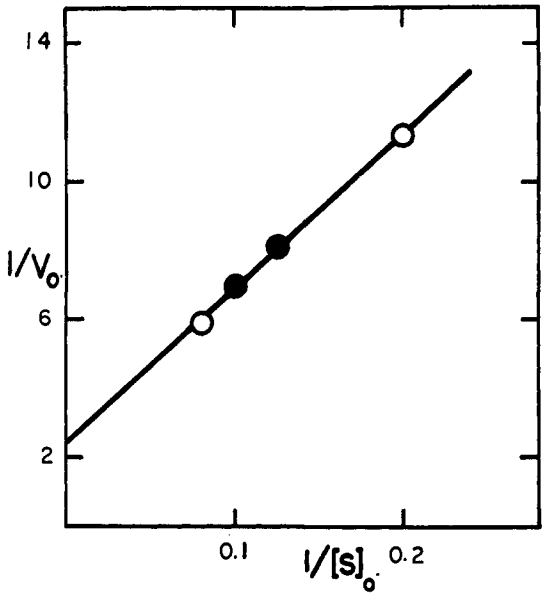

Fig. 3.—Evaluation of K_S and k_3 for α-chymotrypsin and nicotinyl-L-phenylalaninamide; v_0 in units of 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M; [E] = 0.208 mg. protein-nitrogen per ml.; 0.02 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer; O, mean of duplicate determinations; ●, mean of triplicate determinations.

corresponding value of k_3 (*cf.* Figs. 2 and 3) was found to be 2.1 × 10^{-3} M/min./mg. protein-nitrogen/ml. A similar plot for acetyl-L-phenylalaninamide, and based upon six separate experiments (*cf.* Fig. 4), gave for this specific substrate a K_S value of 34 × 10^{-3} M and a k_3 value of 0.7 × 10^{-3} M/min./mg. protein-nitrogen/ml. In a separate series of experiments the kinetics of the α-chymotrypsin-catalyzed competitive hydrolysis of equimolar quantities of acetyl-L-phenylalaninamide and acetyl-L-tyrosinamide were investigated. From the data summarized in Fig. 5 and the known K_S and k_3 values of acetyl-L-tyrosinamide,[4] the method of analysis described by Foster and Niemann[5] gave for acetyl-L-phenylalaninamide a K_S value of 35 × 10^{-3} M and a k_3 value of 0.9 × 10^{-3} M/min./mg. protein-nitrogen/ml. These experiments and those reported previously[5] provide direct proof that acetyl-L-tryptophanamide, acetyl-L-tyrosinamide and acetyl-L-phenylalaninamide are all hydrolyzed at the same catalytically active site.

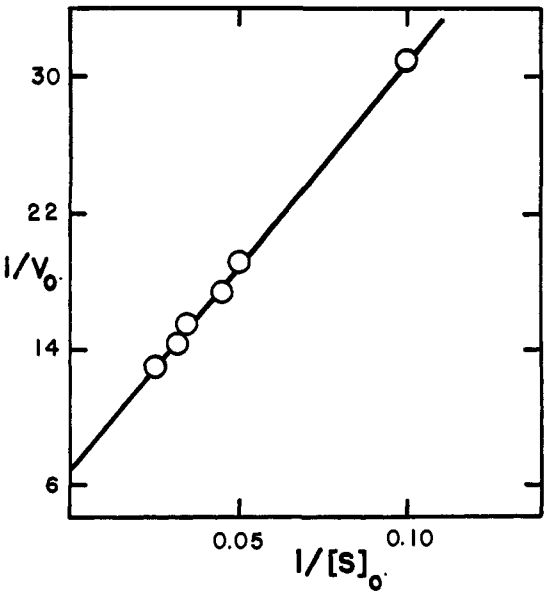

Fig. 4.—Evaluation of K_S and k_3 for α-chymotrypsin and acetyl-L-phenylalaninamide; v_0 in units of 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M; [E] = 0.208 mg. protein-nitrogen per ml.; 0.02 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

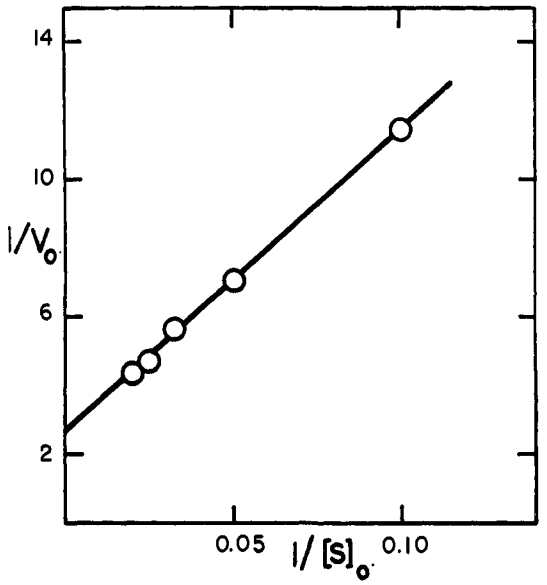

Fig. 5.—Competitive hydrolysis of equimolar quantities of acetyl-L-phenylalaninamide and acetyl-L-tyrosinamide; v_0 in units of 10^{-3} M per min.; $[S]_0 = [S_1]_0 + [S_2]_0$ and $[S_1]_0 = [S_2]_0$ all in units of 10^{-3} M; [E] = 0.208 mg. protein-nitrogen per ml.; 0.02 M tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer.

Data relative to the inhibition of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide by acetyl-D-phenylalaninamide are summarized in Fig. 6. From these data and those given in Fig. 7 in respect to the inhibition of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide by acetyl-D-phenylalanine methyl ester, it follows that in each case the inhibition is competitive in nature and that for acetyl-D-phenylalaninamide K_I = 14 × 10^{-3} M and for acetyl-D-phenylalanine methyl ester K_I = 2.5 × 10^{-3} M. Since the K_S values of acetyl-L-tyrosinamide and nicotinyl-L-tryptophanamide are probably accurate to within ±10%[3,4] it is believed that the two K_I values given immediately above are also of this order of accuracy.

The kinetic constants evaluated in the present study and those determined previously,[3,4,6,10,11] to which reference will be made, are given in Table I. It will be seen that constants are now available for six enantiomorphic pairs wherein the L-enantiomorphs are specific substrates and the D-enantiomorphs are competitive inhibitors of α-chymotrypsin. If, as before,[6,12] these compounds are

(10) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 1555 (1951).
(11) H. T. Huang and C. Niemann, *ibid.*, **73**, 3228 (1951).
(12) H. T. Huang and C. Niemann, *ibid.*, **73**, 3223 (1951).

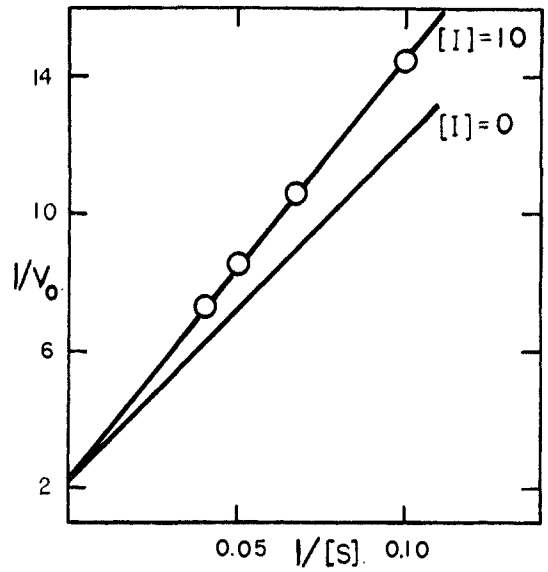

Fig. 6.—Evaluation of K_I for α-chymotrypsin and acetyl-D-phenylalaninamide; v_0 in units of 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M acetyl-L-tyrosinamide; [I] = 10 × 10^{-3} M acetyl-D-phenylalaninamide; [E] = 0.208 mg. protein-nitrogen per ml.; 0.02 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

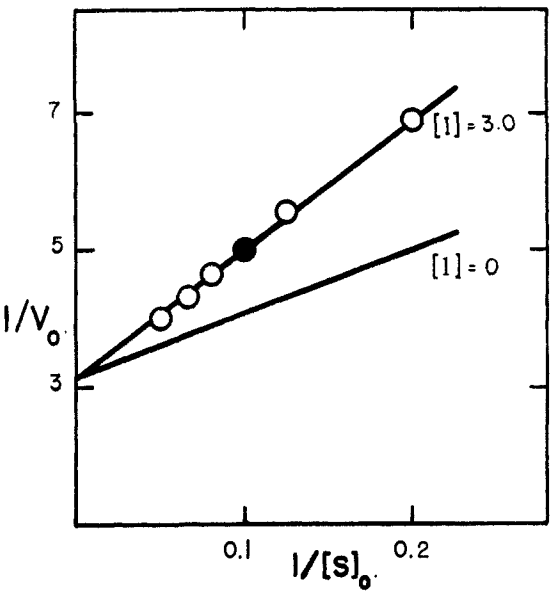

Fig. 7.—Evaluation of K_I for α-chymotrypsin and acetyl-D-phenylalanine methyl ester; v_0 in units of 10^{-3} M per min.; $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide; [I] = 3.0 × 10^{-3} M acetyl-D-phenylalanine methyl ester; [E] = 0.208 mg. protein-nitrogen per ml.; 0.02 M tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer; ●, mean of duplicate determinations.

described 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, it follows (*cf.* Table I) that in both the D- and L-series the replacement of R_1 = acetamido by R_1 = nicotinamido is invariably accompanied by an increase in $-\Delta F^\circ$ of approximately 400 calories per mole. Since the above observation is a reflection of the identity of the

TABLE I

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

Compound	$K_{S,1}$[b]	k_3[c]	$-\Delta F^{\circ d}$	Ref.
Acetyl-L-tryptophanamide	5.3	0.50	3110[e]	3
Acetyl-D-tryptophanamide	2.7		3500	3
Acetyl-D-tryptophan methyl ester	0.089		5520	11
Nicotinyl-L-tryptophanamide	2.7	1.6	3500[e]	3
Nicotinyl-D-tryptophanamide	1.4		3890	3
Acetyl-L-tyrosinamide	30.5	2.4	2080[e]	4
Acetyl-D-tyrosinamide	12.0		2620	4
Nicotinyl-L-tyrosinamide	15.0	6.2	2490[e]	6
Nicotinyl-D-tyrosinamide	6.2		3010	6
Acetyl-L-phenylalaninamide	34	0.8[f]	2000[e]	
Acetyl-D-phenylalaninamide	14		2530	
Acetyl-D-phenylalanine methyl ester	2.5		3550	
Nicotinyl-L-phenylalaninamide	18	2.1	2380[e]	
Nicotinyl-D-phenylalaninamide	7.0		2930	10

[a] At 25° and pH 7.9. [b] In units of 10^{-3} M. [c] In units of 10^{-3} M/min./mg. protein nitrogen/ml. [d] In calories per mole to the nearest 10 calories. [e] An apparent value based upon the premise that $K_S \doteq k_2/k_1$. [f] Average of the two values given in the text.

ratios of the K_S values of the acetyl- and nicotinyl-pairs of the L-series with the ratios of the K_I values of the corresponding pairs of the D-series it is clear that the assumption that $K_S \doteq k_2/k_1$ for the acylated-L-α-amino acid amides[6] has received additional support from the results of the present investigation.

There are now available K_S and k_3 values for three pairs of acylated-L-α-amino acid amides which differ only in respect to the α-amino acid side chains. When R_1 = acetamido or nicotinamido and R_3 = carbamido the affinity of the enzyme for specific substrates, in respect to the nature of the R_2 groups, is in the order β-indolylmethyl≫ p-hydroxybenzyl > benzyl. In contrast, when the susceptibility to hydrolysis of the intermediate enzyme–substrate complex is considered, the order, in respect to the nature of R_2, is p-hydroxybenzyl > benzyl > β-indolylmethyl. It should be appreciated that a change in the nature of R_2 which in one case may cause an increase in affinity and an increase in susceptibility to hydrolysis, *i.e.*, benzyl by p-hydroxybenzyl, may in another, cause an increase in affinity and a decrease in susceptibility to hydrolysis, *i.e.*, benzyl by β-indolylmethyl, and in a third, cause a decrease in affinity and an increase in susceptibility to hydrolysis, *i.e.*, β-indolylmethyl by p-hydroxybenzyl or benzyl. It is clear that the above conclusions (which are based upon the validity of the assumption that $K_S \doteq k_2/k_1$) appear to reinforce the arguments given previously that there is no simple relationship between the affinity of the enzyme for a given specific substrate and the susceptibility to hydrolysis of the corresponding enzyme–substrate complex.[7,12]

Experimental[13,14]

Nicotinyl-L-phenylalaninamide (I).—I, m.p. 185°, $[\alpha]^{21}$

(13) Microanalyses by Dr. A. Elek.

(14) All melting points are corrected.

$-36.5°$ (c 2% in methanol), was prepared as described previously.[15]

Acetyl-L-phenylalaninamide (II).—Esterification of 4.7 g. of acetyl-L-phenylalanine, prepared by the acetylation of L-phenylalanine,[16] with methanolic hydrogen chloride, gave 4 g. of acetyl-L-phenylalanine methyl ester, m.p. 89–90°, large prisms, after three recrystallizations from ether; $[\alpha]^{25}D$ $+19.5°$ (c 2% in methanol).

Anal. Calcd. for $C_{12}H_{15}O_3N$ (221): C, 65.2; H, 6.8; N, 6.3. Found: C, 65.0; H, 6.8; N, 6.0.

Ammonolysis of the above ester gave II, fine needles, m.p. 176–177°, after two recrystallizations from water, $[\alpha]^{25}D$ $+27°$ (c 1% in methanol).

Anal. Calcd. for $C_{11}H_{14}O_2N_2$ (206): C, 64.1; H, 6.8; N, 13.6. Found: C, 64.2; H, 6.8; N, 13.6.

Acetyl-D-phenylalanine Methyl Ester (IV).—To a solution of 22.1 g. of acetyl-DL-phenylalanine methyl ester in 75 ml. of methanol and 500 ml. of water, contained in a beaker thermostated at 30° was added 50 mg. of α-chymotrypsin and the pH of the solution maintained at approximately 7.8 by the addition of 1 N aqueous sodium hydroxide. Although the reaction appeared to be completed in two hours, the solution was stirred for another half-hour and then evaporated, at room temperature in a current of air, to about 200 ml. The reaction mixture was stored at 0° overnight, the precipitate recovered, washed with a small quantity of cold water and dried in air to give 9.7 g. of crude IV. The crude IV was recrystallized twice from ether to give IV, dense prisms, m.p. 90–91°, $[\alpha]^{25}D$ $-19°$ (c 2% in methanol).

Anal. Calcd. for $C_{12}H_{15}O_3N$ (221): C, 65.2; H, 6.8; N, 6.3. Found: C, 65.2; H, 6.8; N, 6.4.

Acetyl-D-phenylalaninamide (V).—Ammonolysis of 1 g. of IV gave 0.6 g. of V, fine needles, m.p. 176–177°, after two recrystallizations from water; $[\alpha]^{25}D$ -27 (c 1% in methanol).

Anal. Calcd. for $C_{11}H_{14}O_2N_2$ (206): C, 64.1; H, 6.9; N, 13.6. Found: C, 64.2; H, 6.8; N, 13.6.

Enzyme Experiments.—The methods used were identical with those reported previously.[3] The enzyme preparation, lot no. 90402, was obtained from Armour and Company. The K_S values for the two substrates employed in the inhibition experiments are nicotinyl-L-tryptophanamide, 2.7×10^{-3} M,[3] acetyl-L-tyrosinamide, 30.5×10^{-3} M.[4] It will be noted that the specific enzyme concentrations, E'_S and E'_I, for each series of experiments were such as to provide essentially zone A conditions for all experiments.[17,18] For both of the specific substrates, at the concentrations experimentally feasible, the course of hydrolysis approximated a first-order reaction, and initial velocities were calculated from the respective apparent first order rate constants.

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

(16) H. T. Huang and C. Niemann, *ibid.*, **73**, 475 (1951).

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

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

PASADENA, CALIFORNIA RECEIVED MAY 29, 1951

[CONTRIBUTION FROM THE U. S. PUBLIC HEALTH SERVICE, TUBERCULOSIS RESEARCH LABORATORY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Glutamic γ-Semialdehyde and Δ^1-Pyrroline-5-carboxylic Acid, Intermediates in the Biosynthesis of Proline[1,2]

BY HENRY J. VOGEL[3] AND BERNARD D. DAVIS

Δ^1-Pyrroline-5-carboxylic acid (V) has been synthesized from γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde with intermediate formation of glutamic γ-semialdehyde. The structure of V has been confirmed by its catalytic reduction to proline and its reaction with o-aminobenzaldehyde. V satisfies the proline requirement of one *Escherichia coli* mutant and appears to be structurally identical with a proline precursor (A) accumulated by another; a third mutant responds to proline, A or glutamic acid. The accumulation of A is enhanced by addition of its precursor, glutamic acid, or of o-aminobenzaldehyde, which functions as an effective "trapping agent." A microbiological assay method for proline or V has been described. The present results support the following scheme of proline biosynthesis: glutamic acid → glutamic γ-semialdehyde → Δ^1-pyrroline-5-carboxylic acid → proline.

The route of biosynthesis of proline in certain microörganisms has been reported to proceed *via* glutamic acid[4,5]; a relationship between the two amino acids has also been shown in mammalian metabolism.[6-14] The possibility of throwing additional light on proline biosynthesis arose when a proline-requiring mutant of *Escherichia coli* (55-1) was found to accumulate in its culture filtrate a substance (A), presumably a proline precursor, which supported growth of another proline auxotroph (55-25).[15] A third mutant (22-64) responded alternatively to proline, A or glutamic acid. These growth responses together with the accumulation of A indicate that glutamic acid, A, and proline form a biosynthetic sequence. The proline requirement of strain 22-64 is much larger than that of the other two strains and is of the order of magnitude of its glutamic acid requirement; it therefore appears that in strain 22-64 glutamic acid is formed from proline by a reversal of the above sequence.

The studies in mammals or with mammalian enzymes have primarily been concerned with the conversion of proline to glutamic acid; among the compounds that have been considered as possible intermediates in this process are α-amino-δ-hydroxyvaleric acid (I),[7] glutamic γ-semialdehyde (II)[11] and 2-pyrrolidone-5-carboxylic acid (III),[7] all of which may be regarded as derived from glutamic acid by some modification of its γ-carboxyl group. While in mammals I and III have been shown to be unlikely as intermediates,[7] compounds

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