**Determination of Dissociation Constants.**—One hundredth molar solutions of acetyl-, chloroacetyl- and trifluoroacetyl-L-tyrosine in 0.1 *M* sodium chloride were titrated potentiometrically at 25° with 0.01 *M* sodium hydroxide. The half-neutralization points were taken as the respective $pK_A'$ values. Each determination was repeated three times.

PASADENA 4, CALIFORNIA RECEIVED MAY 29, 1951

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[CONTRIBUTION NO. 1560 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

# The Enzyme-Inhibitor Dissociation Constants of α-Chymotrypsin and Three Series of Competitive Inhibitors Derived from D-Tryptophan[1]

BY H. T. HUANG AND CARL NIEMANN[2]

The enzyme-inhibitor dissociation constants of α-chymotrypsin and three series of competitive inhibitors derived from D-tryptophan, *i.e.*, a series of acylated-D-tryptophanamides, a series of derivatives of acetyl-D-tryptophan, and a series of acylated tryptamines, have been evaluated for aqueous solutions of these substances at 25° and *p*H 7.9.

It was pointed out in a previous communication[3] that the elucidation of the mode of action of α-chymotrypsin by studies on the structural requirements of synthetic specific substrates for this enzyme is predicated on the recognition of two distinct series of relationships, *i.e.*, one, that between the structure of the specific substrate and the affinity of the enzyme for that substrate and two, that between the structure of the specific substrate and the susceptibility to hydrolysis of the corresponding intermediate enzyme–substrate complex. While the syntheses of numerous structurally desirable specific substrates can be achieved by known methods, the sparing solubility of many of these compounds in aqueous media, or their slow rates of hydrolysis, has severely limited the number that can be used in detailed kinetic studies with present analytical techniques. Therefore, increasing attention has been paid in recent communications from these laboratories[3,4] to the more modest objective of securing data on relation one above, *viz.*, the effect of structure upon affinity, by determining the enzyme-inhibitor dissociation constants, *i.e.*, the $K_I$ values[5] of selected series of competitive inhibitors. Since there is reason to believe that the mode of combination with the active site of the enzyme is the same for D- and L-enantiomorphs of the general formula $R_1CHR_2R_3$[3] we have, in an attempt to obtain information that would be pertinent to the bonding of both D- and L-compounds, determined the enzyme-inhibitor dissociation constants of two series of competitive inhibitors derived from D-tryptophan, *i.e.*, where $R_2$ = β-indolylmethyl and remains invariant. The first was a series of acyl-D-tryptophanamides, *i.e.*, $R_1$ variant with $R_3$ constant, and the second, a series of derivatives of acetyl-D-tryptophan, *i.e.*, $R_3$ variant with $R_1$ constant. The selection of derivatives of tryptophan rather than those of tyrosine or phenylalanine was based upon the knowledge that the affinity of the enzyme for the β-indolylmethyl side chain is considerably greater than that for either the *p*-hydroxybenzyl or benzyl side chains.[4,6] In order to provide information relative to the case where $R_3$ = H several derivatives of tryptamine were also evaluated.

The specific substrate used in this study was nicotinyl-L-tryptophanamide for which $K_S$ = $2.7 \times 10^{-3}$ *M*[5]. All experiments were performed at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* in respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer in the presence of an enzyme concentration corresponding to 0.208 mg. of protein nitrogen per ml. reaction mixture. In view of the fact that in every case the reaction was limited to 30% hydrolysis, it is permissible to ignore inhibition by one of the hydrolysis products,[5] and to formulate the systems in terms of equations (1) and (2)

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

$$E_f + I_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \qquad (2)$$

where $K_S = (k_2 + k_3)/k_1$ and $K_I = k_5/k_4$

The results of these experiments are summarized in Figs. 1–4 and Tables I–III. To facilitate discussion we have included in Tables I and II previous data pertinent to the two series under consideration. The $K_I$ values in Figs. 2–4 were calculated from $1/v_0$ *versus* $1/[S]_0$ plots based upon equation (3)[7]

$$\frac{1}{v} = \frac{K_S}{V}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V} \qquad (3)$$

and it will be noted that in every case the inhibition is competitive in nature. While the data for the four inhibitors summarized in Fig. 1, *i.e.*, *p*-methoxybenzoyl-D-tryptophanamide, acetyl-D-tryptophan ethyl ester, *p*-nitrobenzoyl-D-tryptophanamide and benzoyl tryptamine when plotted according to equation (3) also gave straight lines suggesting competitive inhibition, the apparent $K_I$ values so calculated were significantly less than $0.5 \times 10^{-3}$ *M*. Since it is probable that the enzyme concentration in these systems is of the order of $0.05 \times 10^{-3}$ *M*,[8] the specific enzyme concentration with respect to the inhibitor in each case is $E'_I = [E]/K_I = 0.1$, *i.e.*, probably beyond the upper limit for zone A.[9,10] Thus these systems

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

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

(3) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).

(4) H. T. Huang and C. Niemann, *ibid.*, **73**, 1555 (1951).

(5) H. T. Huang and C. Niemann, *ibid.*, **73**, 1541 (1951).

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

(7) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).

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

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

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

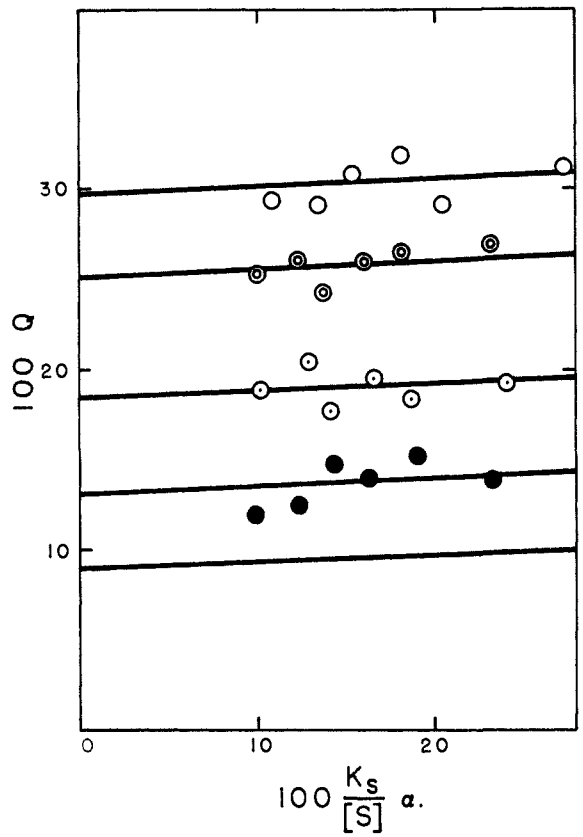



Fig. 1.—Plot of equation (10) for the determination of the $K_I$ values of certain competitive inhibitors: $Q = [I]/[(\alpha^{-1} - 1)[S]/K_S]$; [E] = 0.208 mg. protein-nitrogen per ml.; [S] varied from 5 to 20 × $10^{-3}$ *M* of nicotinyl-L-tryptophanamide; O, [I] = 0.25 × $10^{-3}$ *M* *p*-methoxybenzoyl-D-tryptophanamide; ⓪, [I] = 0.40 × $10^{-3}$ *M* acetyl-D-tryptophan ethyl ester; ⊙, [I] = 0.25 × $10^{-3}$ *M* *p*-nitrobenzoyl-D-tryptophanamide; ●, [I] = 0.20 × $10^{-3}$ *M* benzoyltryptamine. Base line previously determined *cf.* ref. 9; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

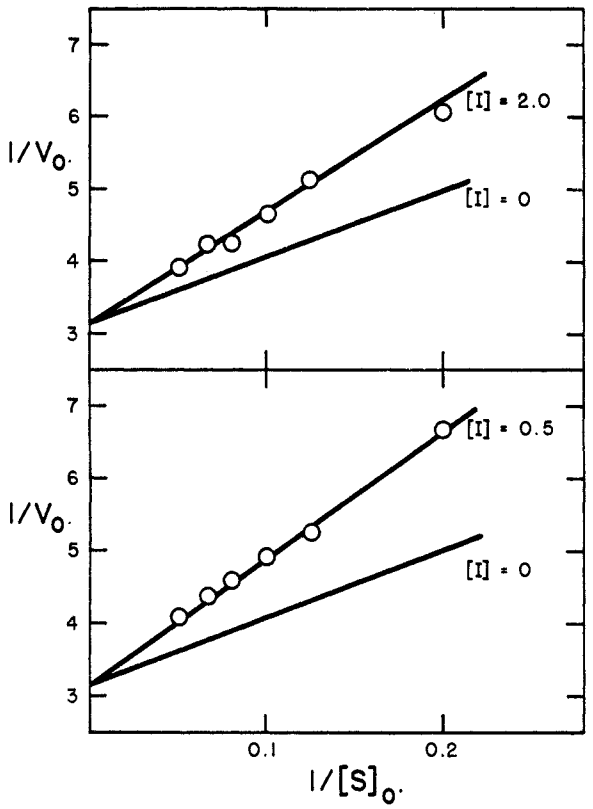
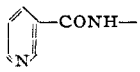
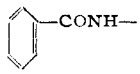
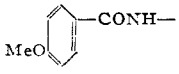
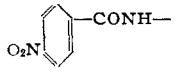



Fig. 2.—Plot of equation (3) for the determination of the $K_I$ values of a second group of competitive inhibitors: $[S]_0$ in $10^{-3}$ *M* of nicotinyl-L-tryptophanamide; $v_0$ in $10^{-3}$ *M* per min.; upper half, [I] = 2.0 × $10^{-3}$ *M* trifluoroacetyl-D-tryptophanamide; lower half, [I] = 0.50 × $10^{-3}$ *M* benzoyl-D-tryptophanamide; $[E]_0$ corresponds to 0.208 mg. protein-nitrogen per ml.; 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

TABLE I

KINETIC CONSTANTS OF A SERIES OF COMPETITIVE INHIBITORS DERIVED FROM D-TRYPTOPHANAMIDE

| Inhibitor | $R_1$ | $K_1$[a] | $-\Delta F°$[b] | Ref. | $pK_a$[c] |
|---|---|---|---|---|---|
| D-Tryptophanamide | $-NH_3^+$ or $NH_2$ | 3.2 | 3400 | 3 | |
| Acetyl-D-tryptophanamide | $CH_3CONH-$ | 2.7 | 3500 | 4 | 4.76 |
| Trifluoroacetyl-D-tryptophanamide | $CF_3CONH-$ | 2.5 | 3550 | | <0 |
| Nicotinyl-D-tryptophanamide |  | 1.4 | 3890 | 4 | 4.87 |
| Benzoyl-D-tryptophanamide |  | 0.48 | 4520 | | 4.20 |
| *p*-Methoxybenzoyl-D-tryptophanamide |  | .30 | 4800 | | 4.47 |
| *p*-Nitrobenzoyl-D-tryptophanamide |  | .18 | 5110 | | 3.42 |

[a] Units of $10^{-3}$ *M*. [b] Cal. per mole. [c] Of the acid providing the acyl group, taken from Lange's "Handbook of Chemistry," 7th Ed., Handbook Publishers Inc., Sandusky, Ohio, 1949.

are similar to that previously studied in detail with nicotinyl-L-tryptophanamide as the specific substrate and acetyl-D-tryptophan methyl ester as the competitive inhibitor[8]; *viz.*, all of these systems are in zone A with respect to the specific substrate

TABLE II

KINETIC CONSTANTS OF A SERIES OF COMPETITIVE INHIBITORS DERIVED FROM ACETYL-D-TRYPTOPHAN

| Inhibitor | $R_2$ | $K_1$[a] | $-\Delta F°$[b] | Ref. |
|---|---|---|---|---|
| Acetyl-D-tryptophan | $-CO_2^-$ | 4.8 | 3160 | 3 |
| Acetyl-D-tryptophanamide | $-CONH_2$ | 2.7 | 3500 | 4 |
| Acetyl-D-tryptophanmethylamide | $-CONHMe$ | 1.7 | 3780 | 3 |
| Acetyl-D-tryptophanhydrazide | $-CONHNH_2$ | 0.75 | 4260 | |
| Acetyl-D-tryptophan isopropyl ester | $-COOCHMe_2$ | .58 | 4450 | |
| Acetyl-D-tryptophan ethyl ester | $-COOEt$ | .25 | 4910 | |
| Acetyl-D-tryptophan methyl ester | $-COOMe$ | .089 | 5520 | 9 |

[a] Units of $10^{-3}$ *M*. [b] Cal. per mole.

TABLE III

KINETIC CONSTANTS OF A SERIES OF COMPETITIVE INHIBITORS DERIVED FROM TRYPTAMINE

| Inhibitor | $K_1$[a] | $-\Delta F°$[b] |
|---|---|---|
| Tryptamine | 2.5 | 3550 |
| Acetyltryptamine | 1.8 | 3740 |
| Trifluoroacetyltryptamine | 0.95 | 4090 |
| Benzoyltryptamine | 0.13 | 5300 |

[a] In units of $10^{-3}$ *M*. [b] Cal. per mole.

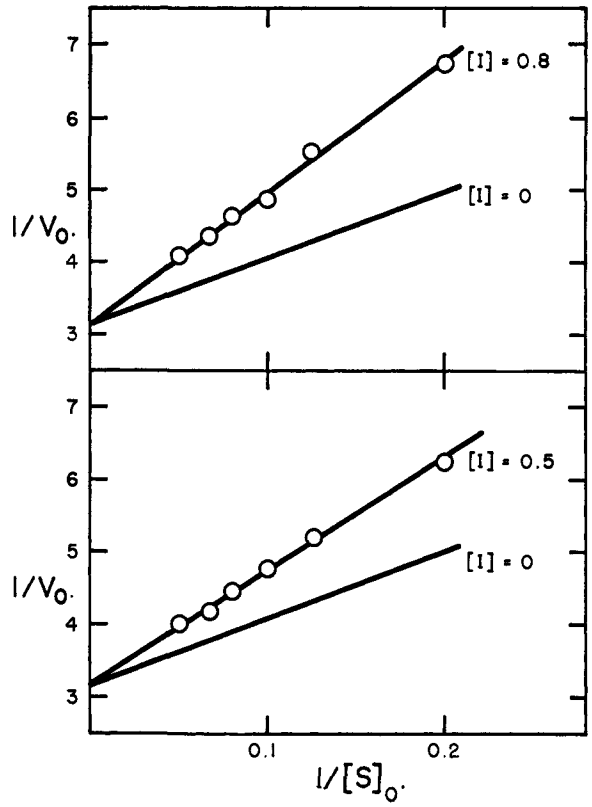



Fig. 3.—Plot of equation (3) for the determination of the $K_I$ values of a third group of competitive inhibitors: $[S]_0$ in $10^{-3}$ $M$ of nicotinyl-L-tryptophanamide; $v_0$ in $10^{-3}$ $M$ per min.; upper half, [I] = 0.80 × $10^{-3}$ $M$ acetyl-D-tryptophanhydrazide; lower half, acetyl-D-tryptophan isopropyl ester; [E] corresponds to 0.208 mg. protein-nitrogen per ml.; 0.02 $M$ tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

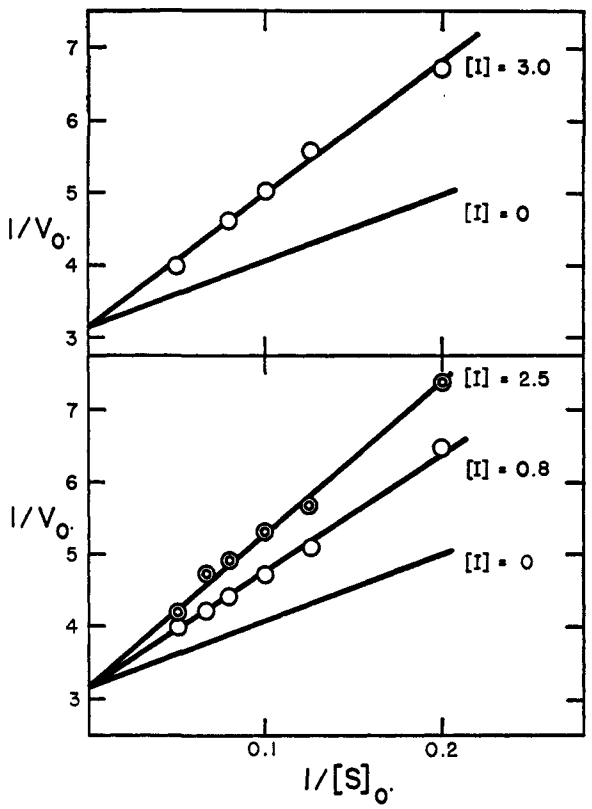



Fig. 4.—Plot of equation 3 for the determination of the $K_I$ values of a fourth group of competitive inhibitors: $[S]_0$ in $10^{-3}$ $M$ of nicotinyl-L-tryptophanamide; $v_0$ in $10^{-3}$ $M$ per min.; upper half, [I] = 3.0 × $10^{-3}$ $M$ tryptamine; lower half, ⓞ, [I] = 2.5 × $10^{-3}$ $M$ acetyltryptamine; O, [I] = 0.80 × $10^{-3}$ $M$ trifluoroacetyltryptamine; [E] corresponds to 0.208 mg. protein-nitrogen per ml.; 0.02 $M$ tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer.

and in zone B with respect to the competitive inhibitor. Accordingly, these data have been analyzed in terms of equation (4) by plotting[8]

$$\frac{[I]}{(\alpha^{-1} - 1)[S]/K_S - 1} = K_I + \frac{\alpha[E]K_S}{[S]} \qquad (4)$$

$Q = [I]/[(\alpha^{-1} - 1)[S]/K_S - 1]$ *versus* $\alpha K_S/[S]$ where $\alpha = v/V = [ES]/[E]$. Using the slope (equal to [E]) determined previously[8] the best parallel lines were drawn in order to evaluate the four $K_I$ values. The values so obtained are about 10–20% lower than the apparent $K_I$ values calculated from the corresponding $1/v_0$ *versus* $1/[S]_0$ plots.

For the case where $R_1$ is an acylamino group it has been suggested[11-14] that the attachment between the enzyme and this particular structural component may be one or more hydrogen bonds. The inhibitors listed in Table I were selected with the thought of providing data relevant to this hypothesis. If the imino hydrogen atom of the $R_1$ group functions as the donor in a hydrogen bond between the enzyme and this particular structural component one would expect a significant and progressive increase in affinity as the acidity of the imino hydrogen atom is increased, *i.e.*, as the strength of the parent acid of the acyl group is increased. This has not been observed (*cf.* Table I). Similarly, if the acceptor function of the carbonyl oxygen atom is the important factor in the attachment, then one would expect the order of affinity with the three benzoyl derivatives given in Table I to be that associated with decreasing electron availability at the benzene ring, *i.e.*, *p*-methoxybenzoyl > benzoyl > *p*-nitrobenzoyl. The actual order observed, however, is *p*-nitrobenzoyl > *p*-methoxybenzoyl > benzoyl. Finally, if the attachment involves both the donor and acceptor properties of the amido group, it is difficult to see why there should be such a marked increase in affinity in going from acetyl to nicotinyl, or in going from benzoyl to *p*-methoxybenzoyl to *p*-nitrobenzoyl (*cf.* Table I). It is, therefore, concluded that intermolecular hydrogen bond formation between the $R_1$ group and its complementary center, $\rho_1$, on the enzyme is not a significant factor in the over-all combination process.

The data given in Table I suggest that the interaction between $R_1$ and $\rho_1$ may depend, in large measure, upon van der Waals forces. However, other factors are required in order to explain the substantial increase in affinity in going from nicotinyl to benzoyl and from *p*-methoxybenzoyl

(11) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).
(12) S. Kaufmann and H. Neurath, *ibid.*, **21**, 437 (1949).
(13) S. Kaufmann and H. Neurath, *J. Biol. Chem.*, **181**, 623 (1949).
(14) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

to *p*-nitrobenzoyl. One such factor may be the degree of hydration of the acyl group. It is reasonable to assume that in the process of formation of the intermediate complex the two reacting molecules, *i.e.*, enzyme and specific substrate, or competitive inhibitor, must necessarily approach to within a sufficiently close range to permit the operation of whatever forces are responsible for the formation of the complex. Thus water molecules, on the catalytically active site and on the specific substrate or competitive inhibitor will have to be eliminated, at least in part, during the combination process. However, the work involved in the elimination of water molecules at the active site of the enzyme is very likely to be the same for the formation of the respective intermediate complexes for a series of structurally similar trifunctional inhibitors, and therefore may be considered as a constant factor in the current discussion. Thus for two inhibitors of approximately the same size and shape, such as benzoyl- and nicotinyl-D-tryptophanamide, the one containing the more hydrophilic acyl group, *i.e.*, the nicotinyl compound, will have more water molecules to be eliminated and hence will require the expenditure of more work in the combination process. This appears to be reflected in the smaller free energy of binding of the $\alpha$-chymotrypsin–nicotinyl-D-tryptophanamide complex, *i.e.*, 3890 cal. per mole, as compared with the corresponding benzoyl complex, *i.e.*, 4520 cal. per mole.

In respect to the combination of the $R_3$ group with its complementary center $\rho_3$ it follows from the data given in Table II that in the series $-CO_2- < -CONH_2 < -CONHNH_2 < -CO_2R$ (R = Me, Et, isoPr) the affinity appears to increase as the carbonyl character of the –CO– function becomes more pronounced. That other factors must be involved is indicated by the greater affinity of the methylamide relative to the amide and the suggestion that a steric factor is operative in the case of the three homologous esters where the greatest affinity is shown by the first member of the series.

It is a necessary condition of the three point attachment theory that compounds containing two, or only one of the three structural components, should be capable of functioning as competitive inhibitors and inhibition by bifunctional compounds of the type $R_2CH_2R_3$, has been reported.[13-15] It is evident from the data summarized in Table III that the series of acyltryptamines, $R_1CH_2R_2$, where $R_1$ = acylamino or amino, and $R_2$ = $\beta$-indolylmethyl, are excellent competitive inhibitors of $\alpha$-chymotrypsin. The fact that the affinity of these compounds is considerably higher than the corresponding acyl-D-tryptophanamides is consistent with the expectation that with the two bifunctional inhibitors under discussion the $R_1$ and $R_2$ groups can interact with the complementary $\rho_1$ and $\rho_2$ centers to give a better fit and with less strain than in the case of a trifunctional compound. Thus while the increase in $-\Delta F^\circ$, in cal. per mole, in going from amino to acetamido to trifluoroacetamido to benzamido is 190 to 350 to 1210 in the tryptamine series, the same structural changes in the acyl-D-tryptophanamide series are accompanied by increases in $-\Delta F^\circ$ of only 100 to 150 to 970. As with the acylated D-tryptophanamides the observed order of affinity, *i.e.*, benzoyl > trifluoroacetyl > acetyl is suggestive of a van der Waals interaction between enzyme and inhibitor.

(15) H. Neurath and J. A. Gladner, *J. Biol. Chem.*, **188**, 407 (1951).

## Experimental[16,17]

**Nicotinyl-L-tryptophanamide** (I).—Prepared as previously described.[18]

**Trifluoroacetyl-D-tryptophanamide** (II).—To a solution of D-tryptophanamide in 100 ml. of ethyl acetate, obtained by the action of concentrated aqueous potassium carbonate on 1.6 g. of the amide hydrochloride,[3] was added 1.2 ml. of trifluoroacetic anhydride,[19] and the reaction mixture allowed to stand at room temperature for one-half hour with occasional shaking. 1.0 ml. of additional trifluoroacetic anhydride was then introduced and after standing for another one-half hour the clear solution was extracted with 50 ml. of 10% aqueous sodium bicarbonate. The non-aqueous phase was dried over calcium sulfate, evaporated *in vacuo* and the sirup crystallized by rubbing with ether, to give 0.6 g. of (II), m.p. 162°, fine silky needles, after two recrystallizations from aqueous methanol, $[\alpha]^{23}D$ −22° (*c* 0.5% in methanol).

*Anal.* Calcd. for $C_{13}H_{12}O_2N_3F_3$ (299): C, 52.2; H, 4.0; N, 14.1. Found: C, 52.3; H, 4.2; N, 14.3.

**Benzoyl-D-tryptophanamide** (III).—Ammonolysis of 3 g. of crude benzoyl-D-tryptophan methyl ester, obtained by the benzoylation of crude D-tryptophan methyl ester,[20] gave 2 g. of (III), m.p. 202–203°, fine needles, after two recrystallizations from a mixture of methanol and ether. Recrystallization of (III) from aqueous methanol gave dense clusters of fine needles, m.p. 191.5°. The two forms are readily interconvertible by recrystallization from the appropriate solvent; $[\alpha]^{24}D$ +26° (*c* 1% in methanol).

*Anal.* Calcd. for $C_{18}H_{17}O_2N_3$ (307): C, 70.3; H, 5.6; N, 13.7. Found: C, 70.4; H, 5.7; N, 13.6.

***p*-Methoxybenzoyl-D-tryptophanamide** (IV).—Ammonolysis of 3 g. of crude *p*-methoxybenzoyl-D-tryptophan methyl ester, obtained by acylation of crude D-tryptophan methyl ester[20] with anisoyl chloride, gave 2.1 g. of (IV), m.p. 203.5°, soft silky needles, after two recrystallizations from a methanol–ether mixture; $[\alpha]^{25}D$ +47° (*c* 0.5% in methanol).

*Anal.* Calcd. for $C_{19}H_{19}O_3N_3$ (337): C, 67.7; H, 5.7; N, 12.5. Found: C, 67.6; H, 5.8; N, 12.3.

***p*-Nitrobenzoyl-D-tryptophanamide** (V).—Ammonolysis of 3 g. of crude *p*-nitrobenzoyl-D-tryptophan methyl ester, obtained by the acylation of crude D-tryptophan methyl ester[20] with *p*-nitrobenzoyl chloride, gave 2 g. of (V), m.p. 219°, silky light yellow needles, after two recrystallizations from methanol; $[\alpha]^{25}D$ +54° (*c* 0.25% in methanol).

*Anal.* Calcd. for $C_{18}H_{16}O_4N_4$ (352): C, 61.4; H, 4.6; N, 15.9. Found: C, 61.4; H, 4.8; N, 15.5.

**Acetyl-D-tryptophanhydrazide** (VI).—A solution of 1 g. of acetyl-D-tryptophan methyl ester[4] in 5 ml. of methanol containing 0.5 g. of hydrazine hydrate was warmed for 10 minutes at 60°. After standing at room temperature overnight the slightly brownish precipitate was recovered and recrystallized three times from a mixture of methanol and ethyl acetate to give 0.5 g. of (VI), m.p. 201–202°, clusters of short thin needles; $[\alpha]^{23}D$ −20° (*c* 1% in methanol).

*Anal.* Calcd. for $C_{13}H_{16}O_2N_4$ (260): C, 60.0; H, 6.2. Found: C, 59.9; H, 6.2.

**Acetyl-D-tryptophan Ethyl Ester** (VII).—Resolution of the free ester from 15 g. of DL-tryptophan ethyl ester hydrochloride with $\alpha$-chymotrypsin, gave 4.5 g. of crude sirupy D-ester. Acetylation of the latter compound with acetic anhydride in ethyl acetate solution in the presence of aqueous sodium bicarbonate gave 4.0 g. of a crude product, which after two recrystallizations from ethyl acetate gave

(16) Microanalyses by Dr. A. Elek.

(17) All melting points are corrected.

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

(19) Prepared by Dr. Henry Shine of this Laboratory.

(20) M. Brenner, E. Sailer and V. Kocher, *Helv. Chim. Acta*, **31**, 1909 (1948).

(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

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[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.

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

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