TABLE I

CALCULATION OF THE BINDING OF CHLORIDE BY BOVINE SERUM ALBUMIN IN 0.15 *M* SODIUM CHLORIDE AT 0°

*p*H				7.00		5.40	3.20
G. albumin per 100 ml.	0	2	4	6	8	4	4
$u_{Cl} \times 10^5$	−36.19	−34.40	−32.72	−31.10	−29.40	−32.62	−29.59
$u_{Na} \times 10^5$	21.59	20.84	20.11	19.38	18.65	20.10	20.10[a]
$u_P \times 10^5$	...	−4.55	−4.41	−4.26	−4.11	−2.75	4.00[b]
$\bar{v}_{Cl}$(eq. 6)		6.3	7.4	7.7	8.2	8.8	29
$\bar{v}_{Cl} - \bar{v}_{Na}$(eq. 10)		5.5	6.5	6.6	7.1	8.4	32
$\bar{v}_{Cl}$(other methods[c])				8		11	31

[a] Assumed. [b] R. A. Alberty, *J. Phys. Colloid Chem.*, **53**, 114 (1949). [c] G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 540 (1950).

(6) and (10) are given in Table I. The experimental uncertainty in $\bar{v}$ is of the order of 10% so that the variation in binding with protein concentration at *p*H 7 is not to be considered significant. For comparison, the values calculated from the graph given by Scatchard, Scheinberg and Armstrong for human serum albumin (ref. 5, p. 539) are given in the last line, and it is seen that the agreement is completely satisfactory. The constants in their equation were determined by the dialysis method and the e.m.f. method and allowance was made for two classes of binding sites assuming that the ratio of intrinsic constants is the same as that determined for thiocyanate ion.[21] It is especially interesting that the large increase in binding of chloride at low *p*H which was originally suggested by the titration data of Tanford[22] is confirmed by the moving boundary method. Calculations of binding with equation (7) by making reasonable assumptions as to u_P and n show that this equation yields very nearly the same results as equation (6).

(22) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

Discussion

The moving boundary method for the study of protein–ion interaction has the disadvantage that rather large quantities of the protein are required and very careful mobility determinations must be made. One characteristic of the calculations, however, is that all apparatus factors cancel so that if the same limb of the same Tiselius cell is used for all experiments some possible errors cancel. An advantage of the moving boundary method is that it may be used to study the interactions of proteins with other proteins, nucleic acids or polysaccharides, cases in which the dialyses and e.m.f. methods are not applicable.

Acknowledgments.—The authors are indebted to Dr. Harry Svensson for originally suggesting that it is the constituent mobility which is obtained in experiments of this type. This work was supported by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Wisconsin Alumni Research Foundation.

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The Enzyme–Inhibitor Dissociation Constants of α-Chymotrypsin and Three Enantiomorphic Pairs of Competitive Inhibitors[1]

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

The enzyme–inhibitor dissociation constants of α-chymotrypsin and three enantiomorphic pairs of competitive inhibitors derived from tryptophan have been evaluated, at 25° and *p*H 7.9, and it has been found that for each pair of inhibitors the affinity of the enzyme for the D-isomer is greater than that for the L-isomer. The significance of this observation is discussed.

The α-chymotrypsin-catalyzed hydrolysis of specific substrates may be formulated in terms of the Michaelis–Menten intermediate complex theory[3–7] and if consideration is limited to the enzyme and specific substrate[8] the over-all reaction may be visualized as proceeding *via* two consecutive steps, one, the reversible combination of the specific substrate and the enzyme to form a characteristic intermediate enzyme–substrate complex, and two, the subsequent transformation of this complex into enzyme and reaction products. A competitive inhibitor of the above reaction may be regarded as a substance which is capable of participation in only the first step, *i.e.*, reversible combination with the enzyme at the same reactive site involved in the combination of the enzyme with a specific substrate, though it is clear that the distinction between a specific substrate and a competitive inhibitor may

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

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

(3) H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950).

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

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

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

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

(8) It is obvious that in the hydrolytic reaction the enzyme and specific substrate are not the only reactants since either water or hydroxyl ion must also participate in the reaction. However, as all hydrolytic experiments are usually conducted under conditions wherein these latter "non-specific substrates" are present in great excess little can be said at present in regard to their manner of participation.

frequently be one of degree rather than of quality since specific substrates which are hydrolyzed at a rate too slow to be measured may be regarded, for all practical purposes, as competitive inhibitors.

In view of the above it is essential, in a consideration of the relation between enzymatic activity and the molecular structure of specific substrates, to recognize the existence of two distinct series of relationships, one, the relation between the structure of a specific substrate and the affinity of the enzyme for that substrate, and two, the relation between the structure of a specific substrate and the susceptibility to hydrolysis of the intermediate enzyme–substrate complex. A structural change in a specific substrate which may result in an enhancement of the affinity of the enzyme for that substrate may or may not lead to an increase in the susceptibility to hydrolysis of the corresponding enzyme–substrate complex. It is evident that accurate and coherent information concerning these two separate, though not necessarily independent, processes is required before the nature of the forces involved in these two processes can be described and an understanding gained as to the mode of action of α-chymotrypsin.

Although present analytical methods have provided data relative to the affinity of α-chymotrypsin for competitive inhibitors,[3-7] *i.e.*, K_I values,[9] and the susceptibility to hydrolysis of the enzyme–substrate complexes,[3-7] *i.e.*, k_3 values,[9] because of the composite character of the constant K_S[9] there is at present no unambiguous way whereby the affinity of α-chymotrypsin for the various specific substrates can be evaluated from kinetic data, based upon the determination of the rate of disappearance of the specific substrate or the rate of appearance of a reaction product, except for the special case where the K_S values of two substrates are in the inverse order of the respective k_3 values.[10]

A consideration of the K_S and K_I values of several acylated D- and L-tryptophanamides[4] and D- and L-tyrosinamides[5] has led to the speculation that for the specific substrates of this series $k_2 >> k_3$ and $K_S = k_2/k_1$, *i.e.*, for all practical purposes K_S may be taken as an apparent equilibrium constant and hence an affinity constant.[4,5] In order to obtain further information along these lines the enzyme–inhibitor dissociation constants, *i.e.*, the K_I values, of three enantiomorphic pairs of competitive inhibitors of α-chymotrypsin were evaluated. These inhibitors were derived from D- or L-tryptophan and although two of them possessed the L-configuration, and thus can function as specific substrates, their susceptibility to hydrolysis was low enough to permit their employment as competitive inhibitors in the present study. The results of these experiments, all 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 with nicotinyl-L-tryptophanamide as the specific substrate, are summarized in Table I and Figs. 1–4. In view of the fact that in all experiments the reactions were not allowed to proceed beyond 30% hydrolysis it is permissible to ignore inhibition of the hydrolytic reaction by one of the hydrolysis products,[4,5] 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 $K_I = k_5/k_4$.

TABLE I

ENZYME–INHIBITOR DISSOCIATION CONSTANTS[a]

Inhibitor	K_I[b]	$-\Delta F°$[d]
Acetyl-D-tryptophan	4.8	3160
Acetyl-L-tryptophan[c]	17.5	2390
D-Tryptophanamide	3.2	3400
L-Tryptophanamide	6.3	3000
Acetyl-D-tryptophanmethylamide	1.7	3780
Acetyl-L-tryptophanmethylamide	4.8	3160

[a] At 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer with nicotinyl-L-tryptophanamide as the specific substrate. [b] In units of 10^{-3} *M*, probable accuracy ±5–8%. [c] *Cf.* ref. 4. [d] In calories per mole at 25° to the nearest 10 calories.

It will be seen from the data given in Table I and Figs. 1–4 that in every case the inhibition is competitive in nature and for each enantiomorphic pair of competitive inhibitors the affinity of the enzyme for the D-isomer is greater than that for the L-isomer. The K_I values reported by Kaufman and Neurath[11] for α-chymotrypsin and benzoyl-D- and L-phenylalanine also indicate a greater affinity of the enzyme for the D-isomer despite the lack of close agreement between the K_I values for the D and L-isomers and that for the DL-mixture. The competitive inhibition of the α-chymotrypsin-catalyzed hydrolysis of nicotinyl-L-tryptophanamide, at 25° and *p*H 7.9, by both D- and L-tryptophanamide presents a rather formidable obstacle to the concept of the multiple specificity of α-chymotrypsin as advanced by Fruton and Bergmann,[12] and it appears that this hypothesis will have to be abandoned. A second hypothesis that is incompatible with the facts disclosed in this communication is the so-called "hypothesis of the essential center."[13] This hypothesis, which was devised to explain the antipodal specificity of the proteolytic enzymes, predicts, for α-chymotrypsin,[13] as well as for the other proteolytic enzymes, that in general only the L-isomer of a molecule otherwise capable of functioning as a substrate will combine with the enzyme. The fact that it has been found, in the three cases investigated, that α-chymotrypsin possesses a greater affinity for the D-isomer than for the L-isomer of an enantiomorphic pair of competitive inhibitors, would appear to render this second hypothesis untenable particularly in view of the accessory fact that the L-isomer of two of the three pairs of enantiomorphic competitive inhibitors investigated, *i.e.*, L-

(9) For definitions of symbols see ref. 4.

(10) J. E. Snoke and H. Neurath, *Arch. Biochem.*, **21**, 351 (1949).

(11) S. Kaufman and H. Neurath, *J. Biol. Chem.*, **181**, 623 (1949).

(12) J. S. Fruton and M. Bergmann, *ibid.*, **145**, 253 (1942).

(13) J. S. Fruton, in D. E. Green, "Currents in Biochemical Research," Interscience Publishers, Inc., New York, N. Y., 1946, pp. 129–131.

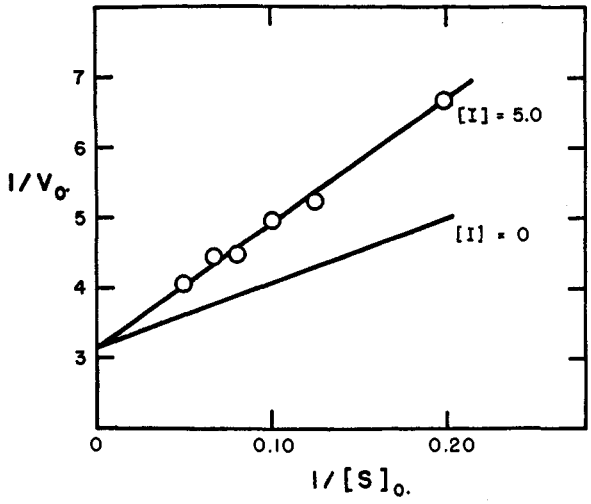

Fig. 1.—Inhibition of hydrolysis of nicotinyl-L-tryptophanamide by acetyl-D-tryptophan; [E] = 0.208 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} M.

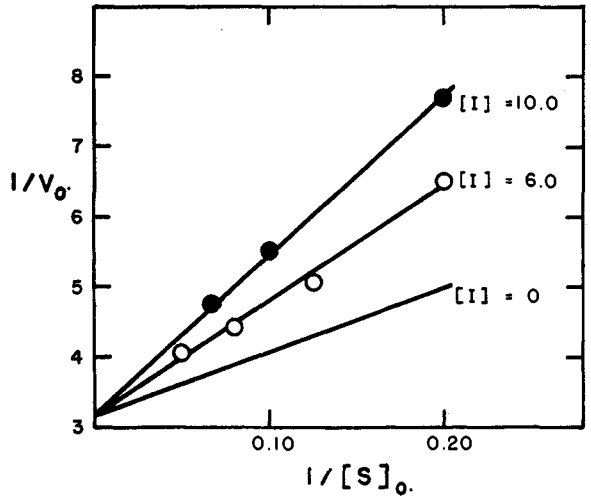

Fig. 2.—Inhibition of hydrolysis of nicotinyl-L-tryptophanamide by L-tryptophanamide; [E] = 0.208 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} M.

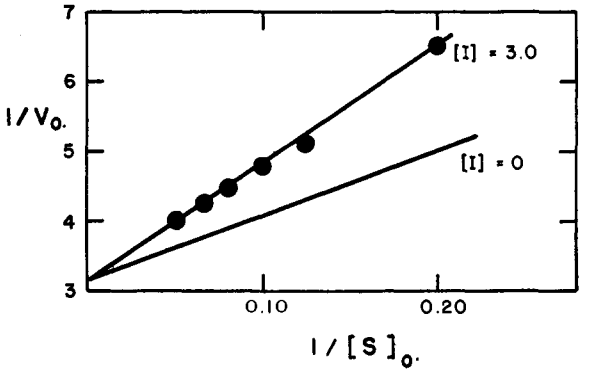

Fig. 3.—Inhibition of hydrolysis of nicotinyl-L-tryptophanamide by D-tryptophanamide; [E] = 0.208 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} M.

tryptophanamide and acetyl-L-tryptophanmethylamide, can function as specific substrates. The low susceptibility to hydrolysis, at 25° and pH 7.9, of the enzyme–substrate complexes derived from these specific substrates is without effect upon the validity of the argument since the hypothesis under discussion relates to the initial or combination stage of the over-all reaction rather than to the subsequent transformations of the enzyme–substrate complex.

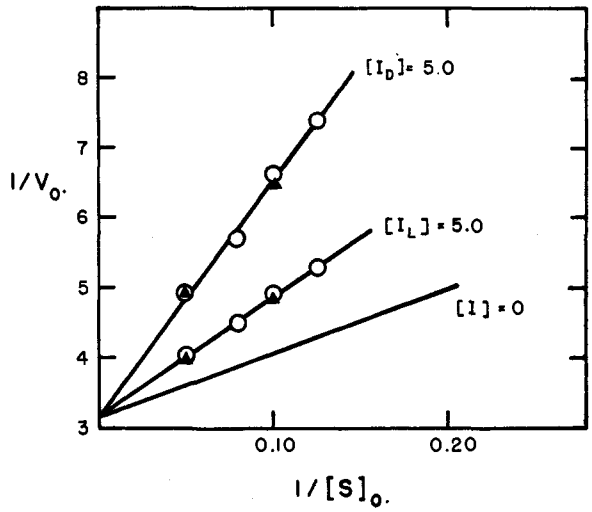

Fig. 4.—Inhibition of hydrolysis of nicotinyl-L-tryptophanamide by acetyl-D- and L-tryptophanmethylamide; [E] = 0.208 mg. protein nitrogen per ml., [S] and [I] in units of 10^{-3} M.

Reasonably reliable data are now available in respect to the K_S or K_I values of six enantiomorphic pairs of compounds which individually are capable of interaction with α-chymotrypsin either as specific substrates or as competitive inhibitors. These data are summarized in Table II. All of the specific substrates and competitive inhibitors listed in Table II may be described by the general formula $R_1CHR_2R_3$ where, in the cases at hand, R_1 is either an acetamido-, nicotinamido- or an amino group,[14] R_2 a β-indolylmethyl- or a p-hydroxybenzyl-group, and R_3 a carbamido-, N-methylcarbamido-, or a

TABLE II

RATIOS OF APPARENT AFFINITY CONSTANTS OF D- AND L-ISOMERS

Compounds	$K_{S,I}$[a]	$(K_{S,I})_L/(K_I)_D$
Acetyl-L-tryptophanamide	5.3[b,d]	2.0
Acetyl-D-tryptophanamide	2.7[c,d]	
Nicotinyl-L-tryptophanamide	2.7[b,d]	2.0
Nicotinyl-D-tryptophanamide	1.4[c,d]	
L-Tryptophanamide	6.3[c]	2.0
D-Tryptophanamide	3.2[c]	
Acetyl-L-tryptophan	17.5[c,d]	3.65
Acetyl-D-tryptophan	4.8[c]	
Acetyl-L-tryptophanmethylamide	4.8[c]	2.8
Acetyl-D-tryptophanmethylamide	1.7[c]	
Acetyl-L-tyrosinamide	30.5[b,e]	2.5
Acetyl-D-tyrosinamide	12.0[b,e]	

[a] K_S or K_I in units of 10^{-3} M. [b] K_S value at 25° and pH 7.9. [c] K_I value at 25° and pH 7.9. [d] *Cf.* ref. 4. [e] *Cf.* ref. 5.

(14) Titration data have indicated that the ammonium group in a protonated D- or L-tryptophanamide has a pK'_A of 7.5 ± 0.1. Therefore the K_I values of both D- and L-tryptophanamide at pH 7.9 are necessarily functions of the concentrations of the protonated and unprotonated species at this pH.

carboxylate[15] group. It will be noted that when R_2 and R_3 are held constant and R_1 is varied there is no significant change in the ratio $(K_{S,I})_L/(K_I)_D$. However, where R_1 and R_3 are held constant and R_2 varied, or R_1 and R_2 held constant and R_3 varied a change in the magnitude of the ratio $(K_{S,I})_L/(K_I)_D$ is observed. The constancy of the ratio of $(K_{S,I})_L/(K_I)_D$ for the three enantiomorphic pairs of compounds derived from β-indolylpropionamide, *i.e.*, where only R_1 is variant, is reflected in a comparison of the $K_{S,I}$ values of the free amino,[16] acetamido- and nicotinamido- derivatives of the L-series, *i.e.*, 6.3:5.3:2.7 (2.3:2.0:1.0) and of the D-series, *i.e.*, 3.2:2.7:1.4 (2.3:2.0:1.0). If these regularities are not fortuitous, the simplest explanation is that, one, the K_S values of acetyl-L-tryptophanamide and nicotinyl-L-tryptophanamide are actually approximate equilibrium constants, *i.e.*, k_3 is too small to influence the magnitude of K_S and $K_S \doteq k_2/k_1$ and, two, that a given structural change in R_1 produces an almost identical change in the affinity of α-chymotrypsin for the D- and the L-compounds. Thus the relative affinity of α-chymotrypsin for the D- and L-isomers of an enantiomorphic pair is altered only when the nature of the characteristic amino acid side chain, *i.e.*, R_2, or that of the susceptible group, *i.e.*, R_3, is changed. It should be emphasized that while the above interpretation of K_S appears to be valid for all of the specific substrates of the amide type that have been studied in detail, sufficient data are not available to warrant its extension to specific substrates of high susceptibility such as the esters.

If it is true that K_S may be regarded as an affinity constant then it is reasonable to assume that the K_I for L-tryptophanamide can be taken as the approximate K_S value for this compound as a specific substrate. On the basis of the above assumption and the observed rate of hydrolysis of the substrate in question it is estimated that, at 25° and *p*H 7.9, k_3 for L-tryptophanamide is approximately 0.006×10^{-3} *M*/min./mg. protein nitrogen/ml. In view of the fact that, at 25° and *p*H 7.9, k_3 for acetyl L-tryptophanamide is 0.6×10^{-3} *M*/min./mg. protein nitrogen/ml.[4] it appears that whereas replacement of an amino group[16] by an acetamido group increases the affinity constant of the enzyme for the specific substrate by only about 10%, the same structural change results in an enhancement of the susceptibility to hydrolysis of the corresponding enzyme substrate complex by a factor of approximately 100. Since a similar comparison can be made for the pair acetyl-L-tryptophanmethylamide–acetyl-L-tryptophanamide and in the absence of any evidence for a consistent relationship between the affinity of the enzyme for all specific substrates and the susceptibility to hydrolysis of the corresponding enzyme–substrate complexes it is important to recognize that a given structural change in a specific substrate may have dissimilar effects upon the two stages of the over-all reaction.

Although the suggestion that α-chymotrypsin possesses a greater affinity for a competitive inhibitor of the D-configuration than for the enantiomorphic substrate of the L-configuration[4] has not been rigorously established the evidence that has now been accumulated in favor of this premise is sufficient to warrant a discussion of its implications at a molecular level. Without making any assumptions as to the nature of the forces which are responsible for the formation of the enzyme–substrate complex, it appears reasonable on the basis of present knowledge[3-7] to postulate for α-amino acid derivatives that, one, a specific substrate or a competitive inhibitor of the L-configuration is bonded to the active site on the enzyme at three centers ρ_1, ρ_2 and ρ_3 complementary to the three groups R_1, R_2 and R_3 in the molecule $R_1CHR_2R_3$, and two, that in both L-specific substrate and D-competitive inhibitor group R_2, *i.e.*, the characteristic amino acid side chain, is bonded to the complementary center ρ_2. While it may be argued that some competitive inhibitors need be bonded to only one center and others to only two centers at the active site of the enzyme the fact that when the nature of any of the three structural components R_1, R_2 and R_3 of a competitive inhibitor possessing the D-configuration is changed the affinity of the enzyme for the inhibitor is also altered makes it obligatory to postulate bonding to at least three centers for competitive inhibitors of this type. Therefore, in the extreme case, there are only two ways in which a competitive inhibitor of the D-configuration can be oriented at the active site of the enzyme if the characteristic amino acid side chain, R_2, is bonded to the complementary center ρ_2 for both D- and L-enantiomorphs, *i.e.*, one, with the three groups R_1, R_2 and R_3 bonded to the complementary centers ρ_1, ρ_2 and ρ_3, respectively, *i.e.*, the same as for specific substrates and competitive inhibitors of the L-configuration, and two, with the three groups R_1, R_2 and R_3 bonded to the three centers ρ_3, ρ_2 and ρ_1, respectively. In view of the observation that a given structural change in the nature of R_1 and R_3 appears to produce a change in the same direction in the affinity of the enzyme for both the D- and L-components of an enantiomorphic pair, *cf.* Table II, it is probable that the mode of combination is that given in one above, *i.e.*, both D- and L-enantiomorphs are bonded to the same complementary centers *via* the same R groups. Contrary to previous impressions,[17] and possibly because of the fact that in the case at hand one of the four groups bonded to the asymmetric carbon atom is a hydrogen atom, it can be shown, with the aid of molecular models, that the goodness of fit of the three R groups to the three hypothetical complementary centers is not greatly different for D- and L-enantiomorphs. In this respect it is of interest to note that for any of the enantiomorphic pairs of D- and L-competitive inhibitors or of D-competitive inhibitors and L-specific substrates for which data are available the observed difference in the real or apparent $-\Delta F^0$ values of the D- and L-enantiomorphs is less than 1 kcal. Since the enzyme–inhibitor complexes derived from the D-enantiomorphs appear to be more stable than the enzyme–inhibitor or enzyme–substrate complexes derived from the

(15) The pK'_A of acetyl-D- or L-tryptophan has been found to have a value of $3.85 \doteq 0.1$ at 25°. Therefore, at *p*H 7.9 ionization of the carboxyl group is essentially complete.

(16) As indicated previously[14] it is not known at present whether the group is actually an amino-group or an ammonium group.

(17) *Cf.* H. N. Rydon, *Biochemical Society Symposia*, **1**, 40 (1948).

corresponding L-enantiomorphs it may be inferred that in the case of the D-enantiomorphs the orientation of the R groups is the more favorable for facile bonding to the respective complementary centers. Furthermore, from a consideration of the spatial distribution of the three groups R_1, R_2 and R_3 it may be reasoned that when the groups R_1 and R_2 are bonded to their respective complementary centers ρ_1 and ρ_2 the bonding of the R_3 group of a D-competitive inhibitor with its respective complementary center ρ_3 is achieved with relative ease whereas the orientation of the R_3 group of an L-competitive inhibitor or an L-specific substrate is such that bonding with the respective complementary center ρ_3 is accompanied by a considerable amount of strain. It is hoped that further information relative to the affinity and susceptibility to hydrolysis of enantiomorphic pairs of compounds wherein the nature of the R_3 group is varied over more suitable limits will be of aid in disclosing the actual mechanism involved in the hydrolysis of the enzyme–substrate complex. Such studies are now in progress.

Experimental[18,19]

Acetyl D-tryptophan (I).—Hydrolysis of 3.4 g. of acetyl-D-tryptophan methyl ester[4] with 14 ml. of *N* aqueous sodium hydroxide, gave 2 g. of I, shiny plates, m.p. 180–181°, after three recrystallizations from water; $[\alpha]^{25}D$ −29.6 ± 1° (*c* 1.23% in water containing an equivalent amount of sodium hydroxide).

Anal. Calcd. for $C_{13}H_{14}O_3N_2$ (246): C, 63.4; H, 5.7; N, 11.4. Found: C, 63.8; H, 5.7; N, 11.4.

L-Tryptophanamide Hydrochloride (II).—The free ester from 10 g. of L-tryptophan methyl ester hydrochloride was dissolved in 50 ml. of methanol previously saturated with ammonia at 0° and the solution allowed to stand at 25° for 2 days. After evaporation the sirupy residue was dissolved in ethyl acetate, and the free amide converted to the hydrochloride by passing dry hydrogen chloride gas into the solution. The crude hydrochloride was collected and recrystallized twice from a mixture of methanol and ethyl acetate to give 6.6 g. of II, tiny prisms, m.p. 254–255° with decomposition; $[\alpha]^{24}D$ +23.5 ± 0.5° (*c* 2% in water).

Anal. Calcd. for $C_{11}H_{12}ON_3 \cdot HCl$ (240): C, 55.2; H, 5.9; N, 17.5. Found: C, 55.2; H, 5.8; N, 17.5.

D-Tryptophanamide Hydrochloride (III).—Ammonolysis of 10 g. of crude D-tryptophan methyl ester[4] and conversion of the amide to the hydrochloride, gave 6 g. of III, fine prisms, m.p. 254–255° with decomposition, after two recrystallizations from a methanol–ethyl acetate mixture; $[\alpha]^{25}D$ −23.0 ± 0.5° (*c* 2% in water).

Anal. Calcd. for $C_{11}H_{12}ON_3 \cdot HCl$ (240): C, 55.2; H, 5.9; N, 17.5; Cl, 14.7. Found: C, 55.0; H, 5.8; N, 17.2; Cl, 14.4.

(18) All melting points are corrected.

(19) Microanalyses by Dr. A. Elek.

Acetyl-L-tryptophanmethylamide (IV).—A solution of 2 g. of acetyl-L-tryptophan methyl ester[4] in 50 ml. of 25% aqueous methylamine and 25 ml. of methanol was allowed to stand at room temperature for 3 days. After evaporation *in vacuo*, the gummy residue was crystallized by rubbing with a mixture of ethyl acetate and ether. The crude material was collected and recrystallized twice from water (charcoal) to give 1.0 g. of IV, tiny dense prisms, m.p. 184–185°. The methylamide was more soluble in ethyl acetate than the corresponding amide; $[\alpha]^{25}D$ +27.9 ± 1° (*c* 0.26% in water); $[\alpha]^{25}D$ +20 ± 1° (*c* 1% in methanol).

Anal. Calcd. for $C_{14}H_{17}O_2N_3$ (259): C, 64.9; H, 6.6; N, 16.2. Found: C, 64.9; H, 6.7; N, 16.2.

Acetyl-D-tryptophanmethylamide (V).—Transformation of 1 g. of acetyl-D-tryptophan methyl ester[4] to the corresponding methylamide, gave 0.6 g. of V, dense prisms, m.p. 185–186°; $[\alpha]^{25}D$ −27.9 ± 1° (*c* 0.26% in water); $[\alpha]^{25}D$ −20 ± 1° (*c* 0.5% in methanol).

Anal. Calcd. for $C_{14}H_{17}O_2N_3$ (259): C, 64.9; H, 6.6; N, 16.2. Found: C, 65.0; H, 6.7; N, 16.1.

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 in aqueous solution[3] 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. In experiments where either D- or L-tryptophanamide hydrochloride was present, it was found advisable to add sufficient sodium hydroxide to half neutralize the amount of hydrochloride introduced in order to place the final *p*H of the system in the region desired. From the K_S value of nicotinyl-L-tryptophanamide,[4] and the data presented in Table I it can be appreciated that the conditions of our experiments were such as to permit the reactions to proceed under zone A conditions.[20,21] The α-chymotrypsin used in this study was an Armour preparation, lot no. 90402, of bovine origin. With [S] = 10×10^{-3} *M* and [E] = 0.208 mg. protein nitrogen per ml. L-tryptophanamide and acetyl-L-tryptophanmethylamide was hydrolyzed to less than 2% in 1 hour. In view of the fact that in all of the experiments used for the evaluation of the respective K_I values the time interval was 30–40 minutes it is clear that neither of the above compounds was hydrolyzed to any significant degree under the latter conditions. With [E] = 0.208 mg. protein nitrogen per ml. and a time interval of 21 hours carefully controlled experiments indicated that, at 25° and *p*H 7.9, L-tryptophanamide was hydrolyzed to an extent of 10% when [S] = 10×10^{-3} *M* and 14% when [S] = 5×10^{-3} *M*. When [S] = 10×10^{-3} *M* acetyl-L-tryptophanmethylamide was hydrolyzed to an extent of less than 2%. Under the same conditions no hydrolysis could be detected when acetyl-DL-phenylalaninethylamide, glycyl-DL-phenylalaninmethylamide or glycyl-DL-phenylalaninbenzylamide were used as substrates. The titration experiments used for the determination of the pK'_A values reported in this communication were performed solutions 0.01 *M* with respect to the acid or base and 0.1 *M* in sodium chloride. The values given were the average of duplicate experiments with the respective and D- L-compounds.

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