Interaction of Aromatic Compounds with α-Chymotrypsin. II. Combination of the Isomeric Indole Carboxamides and Carboxylate Ions with the Active Site*

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ABSTRACT: A series of six nuclear-substituted indole carboxylates and carboxamides have been evaluated as inhibitors of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinehydroxamide in aqueous solutions, at 25.0°, *p*H 7.60, and 0.20 M in sodium chloride, with the aid of a *p*H-stat. All compounds acted as competitive inhibitors, with K_I values for the carboxylates (position indicated in parentheses): 4.4 ± 1.0 (2), 4.5 ± 0.9 (3), 11 ± 3 (4), 2.8 ± 0.4 (5), 0.60 ± 0.09 (6), and 8.6 ± 2.1 mM (7). The K_I values for the carboxamides were 0.7 ± 0.2 (2), 1.3 ± 0.2 (3), 4.2 ± 1.0 (4), 1.3 ± 0.2 (5), 0.60 ± 0.09 (6), and 0.98 ± 0.14 mM (7). From the absolute values and the ratios of these numbers a model of the active site is developed which includes an anionic group near the oriented 1- position of the indole nucleus and a neutral group near the 4- position. This interpretation is consistent with data previously obtained for a series of 1-naphthylamine sulfonates.

A large number of aromatic compounds combine with the active site of α-chymotrypsin and under certain circumstances can function as fully competitive inhibitors of reactions catalyzed by this enzyme (Wallace *et al.*, 1963; Platt and Niemann, 1963). Such compounds are useful probes for exploring the topography of the active site of α-chymotrypsin.

Inhibition constants can be evaluated with relative ease and frequently can be interpreted as true thermodynamic dissociation or association constants. However, the subsequent interpretation of such constants in terms of topographical features of the active site of the enzyme requires that attention be given to the ground states of the various inhibitor molecules. An observed difference in K_I can reflect, for example, a difference in solubility (Miles *et al.*, 1963) rather than reveal some structural feature of the enzyme.

For a pair of inhibitory optical antipodes there is no question that the standard free energies calculated from their respective inhibition constants can be interpreted directly in terms of topographical features of the site with which they combine. However, only a limited number of experiments can be performed which compare pairs of optical antipodes, and the rigor inherent in comparison of the behavior of such inhibitory pairs must be sacrificed to obtain useful results.

The study described in this communication stems from the proposition that comparison of the inhibition constants of selected sets of positional isomers, in this case the isomeric indole carboxamides and the corresponding carboxylate anions, can lead to useful information about the topography of the active site of α-chymotrypsin. In the absence of information about the intimate nature of the active site of the enzyme we prefer the approach taken in this study to the alternative that requires the *ad hoc* assumption that the active site is necessarily apolar in nature and that combination of the inhibitor with it is invariably associated with a change in the ground state of the inhibitor when it is transferred from a dilute aqueous solution to the presumed apolar active site, a thesis developed in the interpretation of antigen-antibody reactions (Karush, 1962) and more recently by Miles *et al.* (1963) with respect to the interaction of aromatic compounds with α-chymotrypsin.

The knowledge that indole is one of the more effective aromatic inhibitors of α-chymotrypsin-catalyzed reactions (Huang and Niemann, 1953; Wallace *et al.*, 1963) led us to center attention on the isomeric indole carboxamides and carboxylic acids. Indole-2-carboxylic acid was prepared by the Johnson *et al.* (1945) modification of the Reissert (1897) procedure and by the Rydon and Tweddle (1955) modification of the Fischer (1886) indole synthesis. The amide was obtained from the acid via the acid chloride (Johnson *et al.*, 1945). Indole-3-carboxylic acid and carboxamide were prepared from the polymeric indole-3-carbonyl chloride of Peterson *et al.* (1958). The benzene-substituted indole carboxylic acids were obtained by hydrolysis of the corresponding cyanoindoles (Uhle, 1949; Singer and Shive, 1955b) which were prepared from the corresponding chloroindole-2-carboxylic acids (Uhle, 1949; Rydon and Tweddle, 1955). The analogous amides were obtained by hy-

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TABLE I: Evaluation of the Inhibition Constants of the Isomeric Indole Carboxamides and Carboxylate Anions.[a]

Inhibitor	[S] (mM)	[E] (μM)[b]	[I] (mM)	K_I (mM)	k_o (sec^{-1})
Indole	5.1–36.0[c]	3.7–5.3	1.81	0.63 ± 0.10	0.65 ± 0.10
Indole-2-carboxylate	10.3–71.8[d]	5.1	2.25	4.5 ± 1.4	2.09 ± 0.12
	8.8–52.7[d]	5.0	4.50	5.7 ± 1.6	1.50 ± 0.12
	10.3–61.6[d]	5.3	7.54	4.2 ± 0.7	2.43 ± 0.32
Indole-2-carboxamide	10.3–61.5[d]	5.2	0.52	0.79 ± 0.28	2.14 ± 0.24
	10.3–41.2[e]	5.1	1.04	0.64 ± 0.13	2.51 ± 0.66
Indole-3-carboxylate	8.8–52.9[f]	5.3	4.62	7.6 ± 2.8	1.79 ± 0.22
	5.9–35.1[g]	5.3	5.12	4.0 ± 0.7	1.82 ± 0.11
	8.0–40.0[g]	5.2	6.72	4.9 ± 1.1	2.00 ± 0.44
Indole-3-carboxamide	8.8–52.8[g]	5.0	1.89	1.30 ± 0.25	1.44 ± 0.11
	5.8–58.8[d]	5.3	2.02	1.37 ± 0.25	1.68 ± 0.26
	5.9–35.3[d]	5.1	3.01	1.24 ± 0.20	1.23 ± 0.19
Indole-4-carboxylate	5.9–35.1[g]	5.0	3.98	11.4 ± 3.8	1.72 ± 0.02
	5.8–35.0[d]	5.2	13.32	10.2 ± 2.0	1.77 ± 0.19
Indole-4-carboxamide	5.9–35.2[d]	5.0	1.97	4.5 ± 1.4	1.90 ± 0.07
	5.8–35.0[g]	5.0	6.07	4.0 ± 0.7	1.91 ± 0.11
Indole-5-carboxylate	5.8–35.0[d]	4.9	4.02	3.0 ± 0.5	1.66 ± 0.06
	5.8–34.9[g]	5.0	10.72	2.6 ± 0.3	2.17 ± 0.15
Indole-5-carboxamide	5.9–35.1[d]	4.9	1.40	1.35 ± 0.27	1.61 ± 0.12
	5.9–35.2[g]	5.0	2.03	1.33 ± 0.22	1.41 ± 0.08
Indole-6-carboxylate	5.9–35.1[d]	5.0	1.97	1.44 ± 0.23	1.86 ± 0.08
	5.1–30.5[d]	4.9	4.05	1.48 ± 0.17	1.59 ± 0.26
Indole-6-carboxamide	5.9–35.2[d]	5.0	0.98	0.60 ± 0.09	1.66 ± 0.17
	5.9–35.1[d]	5.2	2.01	0.60 ± 0.08	1.61 ± 0.17
Indole-7-carboxylate	5.8–35.0[g]	5.1	4.06	9.0 ± 2.8	1.77 ± 0.08
	5.9–35.6[g]	5.0	10.79	8.2 ± 1.4	1.73 ± 0.11
Indole-7-carboxamide	5.8–34.9[d]	5.3	0.98	0.98 ± 0.14	1.70 ± 0.12

[a] All inhibitors evaluated against α-*N*-acetyl-L-tyrosinehydroxamide in aqueous solutions at 25.0°, *p*H 7.60, and 0.20 M in sodium chloride. From twenty-five experiments conducted under these conditions with values of [S] = 5.1–46.4 mM and [E] = 5.0–5.3 μM, values of K_o = 51.1 ± 2.5 mM and k_o = 2.17 ± 0.07 sec^{-1} were obtained for the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinehydroxamide. [b] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [c] Twenty-two experiments with [S] within the range specified. [d] Twelve experiments with [S] within the range specified. [e] Ten experiments with [S] within the range specified. [f] Nine experiments with [S] within the range specified. [g] Eleven experiments with [S] within the range specified.

drolysis of the corresponding cyanoindoles using the procedure of Galat (1948).

The α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinehydroxamide, with its readily determinable reaction kinetics and freedom from significant substrate and enzyme blank reactions, was employed for the determination of the inhibition constants of indole and the isomeric indole carboxamides and carboxylate anions. The sodium α-*N*-acetyl-L-tyrosinehydroxamate used as the substrate was prepared by a modification of the procedure of Hogness and Niemann (1953).

Results

All of the experiments reported in this communication were conducted in aqueous solutions at 25.0°, *p*H 7.60, and 0.20 M in sodium chloride.

The kinetic parameters of α-*N*-acetyl-L-tyrosinehydroxamide were reevaluated for systems containing only enzyme, substrate, and sodium chloride. Twenty-five experiments conducted at substrate concentrations from 5.1 to 46.4 mM and enzyme concentrations from 5.0 to 5.3 μM led to values of K_o = 51.1 ± 2.5 mM and k_o = 2.17 ± 0.07 sec^{-1}, assuming a molecular weight of 25,000 and nitrogen content of 16.5% for the enzyme. These values are in reasonable agreement with those determined earlier (Kurtz and Niemann, 1962).

For systems containing added inhibitor the inhibition constants K_I were computed from the relation,

$$[S][E]/v_o = (K_o/k_o)(1 + [I]/K_I) + [S]/k_o \quad (1)$$

which assumes fully competitive inhibition. The results so obtained are summarized in Table I.

Fully competitive inhibition requires the value of k_o to be invariant in the absence or presence of added inhibitor. It is evident from the data given in Table I that the inhibition of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinehydroxamide by indole is not fully competitive. However, for all of the isomeric indole carboxamides and carboxylate anions the inhibition can be viewed as being essentially fully competitive since the variation in the values of k_o is within the limits of experimental error encountered in sets of independent experiments. This view is reinforced by the constancy of K_I values, within the limits of experimental error, for any inhibition when calculated from experiments at different inhibitor concentrations.

Discussion

The behavior of α-*N*-acetyl-L-tyrosinehydroxamide and indole resembles that of methyl hippurate and indole (Huang and Niemann, 1953), and differs from that of α-*N*-nicotinyl-L-tryptophanamide and indole, where the inhibition is fully competitive (Huang and Niemann, 1953). The conclusion that the inhibition of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl-L-tyrosinehydroxamide by any of the isomeric indole carboxamides and carboxylate anions is fully competitive, whereas that encountered with indole involves the formation of a ternary complex of enzyme, substrate, and inhibitor, suggests that the combining site of the enzyme is sufficiently restrictive that binary or ternary complex formation can be determined by the presence or absence of a carboxamide or carboxylate group on the inhibitor molecule when α-*N*-acetyl-L-tyrosinehydroxamide is employed as the substrate. This view of a site of restricted dimensions is supported by the observation that the values of K_I for the various isomeric indole carboxamides and carboxylate anions vary with the position of the substituent. If the periphery of the site were unobstructed or if there were no preferred orientation of the indole ring in the complex, one would expect all of the carboxamides to have the same value of K_I and all of the carboxylate anions to have a common value of K_I different from that of the carboxamides. Since this is not observed, it follows that the topography of the combining site is restricted and that the various isomeric inhibitors function as keys whose access to the lock (site) varies with the position of the substituent.

The preferred values of K_I for the isomeric indole carboxamides and carboxylate anions are given in Table II. From these data we can now approach the problem of the differing ground states of the isomeric indole carboxylate anions and the corresponding carboxamides. It will be noted that while the values of K_I for the isomeric indole-4-, 5-, and 6-carboxamides and the corresponding carboxylate anions vary by a factor of approximately 20 within this group, the ratios of the value of K_I for a given carboxylate anion over that of the corresponding carboxamide are essentially constant, indicating an equipotential electrostatic field in this region. For the foregoing derivatives the value of the ratio is 2.6 ± 0.3, corresponding to a difference in values of $-\Delta F^\circ_{298}$ of *ca.* 0.6 kcal, an energy difference corresponding to observed energies of hydration of anions (Benjamin and Gold, 1954). From this observation we infer that when the isomeric indole-4-, 5-, and 6-carboxylate anions combine with the active site, minimal electrostatic interactions are encountered and that the difference in values of $-\Delta F^\circ_{298}$ of *ca.* 0.6 kcal reflects a constant difference in the ground states of carboxylate anion and corresponding carboxamide.

TABLE II: Preferred Values of K_I for the Isomeric Indole Carboxamides and Carboxylate Anions.[a]

Inhibitor	K_I (mM)	$K_{I_{CO_2^-}}/K_{I_{CONH_2}}$
Indole-2-carboxylate	4.4 ± 1.0	
Indole-2-carboxamide	0.7 ± 0.2	6.3 ± 2.4
Indole-3-carboxylate	4.5 ± 0.9	
Indole-3-carboxamide	1.3 ± 0.2	3.5 ± 0.9
Indole-4-carboxylate	11. ± 3.	
Indole-4-carboxamide	4.2 ± 1.0	2.6 ± 1.1
Indole-5-carboxylate	2.8 ± 0.4	
Indole-5-carboxamide	1.3 ± 0.2	2.2 ± 0.5
Indole-6-carboxylate	1.5 ± 0.2	
Indole-6-carboxamide	0.60 ± 0.09	2.5 ± 0.5
Indole-7-carboxylate	8.6 ± 2.1	
Indole-7-carboxamide	0.98 ± 0.14	8.8 ± 2.5

[a] In aqueous solutions at 25.0°, *p*H 7.60, and 0.20 M in sodium chloride.

Although the ratios of the values of K_I for the indole-6-, 5-, and 4-carboxylate anions and those of the corresponding carboxamides are essentially constant, the absolute values of K_I for both carboxylate anion and carboxamide increase uniformly as one proceeds in a clockwise manner from the 6- to the 4- positions. We suggest that rotation of the indole nucleus about an axis normal to the plane of the bicyclic ring system is excluded and that there is in the sector of the site adjacent to 5- and 4- positions of the fixed indole nucleus an uncharged obstruction that becomes increasingly prominent as one proceeds from the 6- to the 5- to the 4- positions of the indole. The low K_I values for the 2-, 6-, and 7-substituted carboxamides are all very similar to K_I for indole (Huang and Niemann, 1953); this suggests that the neutral substituent group is free of obstruction in these positions.

In that sector of the site adjacent to the 2- and 7- positions of the indole nucleus the high ratios of $K_{I_{CO_2^-}}/K_{I_{CONH_2}}$ in Table II indicate that there is an anionic structural feature on the periphery of the site that exerts its influence largely through an electrostatic interaction. It appears to be centered close to the 1- position of the indole nucleus and may assist in preventing rotation of the nucleus through hydrogen bonding with the acidic indole NH hydrogen atom present in that position.

The interpretation of the K_I values of the 3-substituted indoles is a more difficult matter. The marked

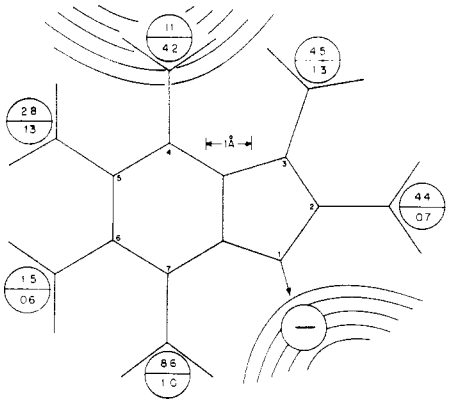

FIGURE 1: Schematic representation of indole derivatives at the active site of α-chymotrypsin. Numbers in circles refer to the observed inhibition constants for carboxylate (top) and carboxamide (bottom) groups at the position indicated. Interaction between the acidic hydrogen at position 1 and a negative charge on the enzyme surface is also indicated (see text).

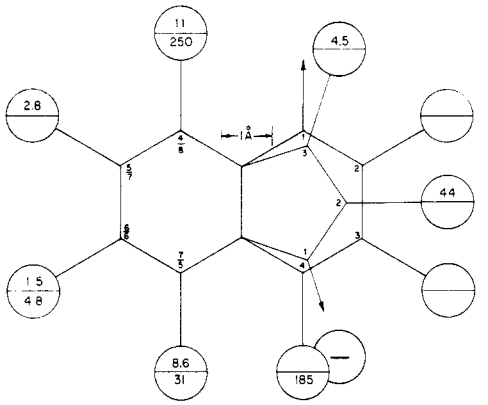

FIGURE 2: Schematic representation of indole carboxylate 1-naphthylamine sulfonate derivatives bound to the active site of α-chymotrypsin. Numbers in circles refer to the observed inhibition constants for carboxylate (top) and sulfonate (bottom) groups at the position indicated. Interaction between the acidic hydrogen at indole position 1 and a negative charge on the enzyme surface is also indicated (see text).

susceptibility of the 3- position of indole toward electrophilic attack indicates that this position is subject to strong polar influences by the indole nitrogen. It is thus reasonable that 3- substituents should exert the greatest resonance and resonance-polar effects on the acidity of the 1-hydrogen. If the acidity of this hydrogen enhances complex formation, electron withdrawal at the 3- position should stabilize the complex. The $CONH_2$ group, by virtue of its electrical neutrality, should be a more efficient electron withdrawer than the CO_2^- group. The fact that the ratio of K_I for the carboxylate ion to that of the carboxamide is somewhat greater in the 3- position than the observed minimum value of 2.5 may be owing, in part, to polar effects that are transmitted through the aromatic ring more efficiently than in other positions. The magnitudes of these effects, as opposed to direct field effects, are difficult to determine. Both the 3-carboxamide and carboxylate ion appear to be subject to repulsion by the uncharged structural feature of the active site near the 4- position of the indole ring.

The general topographical features of the site with which indole derivatives combine is illustrated in Figure 1. The most notable feature is the general aspect of an open-ended valley at one end of which there are no indicated obstructions of any kind. From these observations one can now predict that the indole molecule can be substituted in the 6- position by either charged or uncharged groups without influence on binding of the substituted molecule other than that associated with a change in ground states.

The inhibition constants of four 1-naphthylamine sulfonates were determined by Wallace *et al.* (1963). For aqueous solutions at 25.0°, *p*H 7.90, and 0.1 M in sodium chloride the following values were obtained: 4-sulfonate, K_I = 185 ± 70 mM; 5-sulfonate, K_I = 31 ± 2 mM; 6-sulfonate, K_I = 4.8 ± 0.1 mM; 8-sulfonate, K_I = 250 mM. It is reasonable to assume that the 1-naphthylamine sulfonates combine with the same site occupied by the indole derivatives. These data for the 1-naphthylamine sulfonates are reasonably consistent with either of two orientations with the active site. The first is illustrated in Figure 2. The most notable feature of this mode of combination is that the basic amino groups of these compounds are not placed in the approximate position occupied by the acidic NH groups of the indole derivatives but are in a position roughly corresponding to the 3- position of the indole ring. The second possible orientation is the mode of combination most closely corresponding to that of the indole nucleus, with the amino group near the anionic site.

We favor the first orientation on the basis of the following observation. For the indole derivatives K_I 4-carboxylate > K_I 3-carboxylate, while for the 1-naphthylamine derivatives K_I 4-sulfonate > K_I 5-sulfonate. The mode of combination of Figure 2 places the 4-sulfonate closer to the anionic site than the 5-sulfonate, while the alternative orientation would subject the 5-sulfonate to the greater steric hindrance.

Quinoline derivatives, which also contain a basic nitrogen, may also combine with the active site in an orientation that is opposite to that of the indoles. According to Wallace *et al.* (1963), the K_I of quinoline-4-carboxylate is 104 mM while the inhibition constant for the corresponding amide is 8.4 ± 1.0 mM. This large difference in binding ability between the anionic and neutral species may indicate strong repulsion by the anionic site. Furthermore, benzo[*f*]quinoline (K_I = 0.063 mM) is a much stronger inhibitor than either benzo[*c*]quinoline (K_I = 0.73 mM) or benzo[*h*]quinoline (K_I = 0.70 mM). If the orientation of the benzoquinolines corresponds to that of the indoles, benzo[*f*]quinoline would be subject to the most severe steric interaction with the active site.

TABLE III: Melting Points and Analytical Data Relating to the Synthetic Inhibitors.

Compound	Melting Point[a] Found (deg)	Melting Point[a] Reported (deg)	Carbon Calcd (%)	Carbon Found (%)	Hydrogen Calcd (%)	Hydrogen Found (%)	Nitrogen Calcd (%)	Nitrogen Found (%)
Indole-2-carboxylic acid	200.5–202.0	203–204[b]	67.1	67.1	4.4	4.4	8.7	8.7
Indole-3-carboxylic acid	235–239	247 (dec)[c]	67.1	67.0	4.4	4.5	8.7	8.6
Indole-4-carboxylic acid	212–214	213–214[d]	67.1	67.0	4.4	4.4	8.7	8.7
Indole-5-carboxylic acid	209.5–211.0	208–209[e]	67.1	67.2	4.4	4.5	8.7	8.9
Indole-6-carboxylic acid	250–252	243–244[f]	67.1	66.8	4.4	4.5	8.7	8.5
Indole-7-carboxylic acid	206–212	198–199[g]	67.1	67.2	4.4	4.5	8.7	8.6
Indole-2-carboxamide	234.0–235.5	234–235[h]	67.5	67.3	5.0	5.2	17.5	17.7
Indole-3-carboxamide	204.0–205.5	201[i]	67.5	67.4	5.0	5.4	17.5	17.6
Indole-4-carboxamide	141–142		67.5	67.5	5.0	5.0	17.5	17.5
Indole-5-carboxamide	165.5–167.0		67.5	67.2	5.0	4.9	17.5	17.4
Indole-6-carboxamide	188.5–190.5		67.5	67.7	5.0	5.1	17.5	17.6
Indole-7-carboxamide	205–207		67.5	67.6	5.0	5.2	17.5	17.7

[a] All melting points are corrected. [b] Johnson *et al.* (1945). [c] Placed in heating bath at 240° and heated at a rate of 3°/min; Shaw *et al.* (1958). [d] Uhle (1949). [e] Singer and Shive (1955a). [f] Kermack (1924). [g] Singer and Shive (1955b). [h] Leete *et al.* (1955). [i] Sanna (1934).

The foregoing evidence is certainly not conclusive and our assignment of the orientation of the 1-naphthylamines and quinolines is tentative. It is still necessary to study the effects of sulfonation in the 2-, 3-, and 7- positions of 1-naphthylamine and to compare the observed K_I values with those of neutral molecules of corresponding shape (e.g., 1-naphthylamine sulfonamides). We would predict, on the basis of the orientation of Figure 2, that:

$$K_I \text{ 6-sulfonate} < K_I \text{ 7-sulfonate} < K_I \text{ 8-sulfonate}$$

and

$$K_I \text{ 2-sulfonate} < K_I \text{ 3-sulfonate} < K_I \text{ 4-sulfonate}$$

We would further predict that 1-naphthylamine-8-sulfonamide has a larger K_I value than any of its positional isomers. A similar study is necessary for the quinoline carboxylates and quinoline carboxamides in order to determine whether the data for the 4-derivatives reflect an anionic repulsion or a large ground-state difference between the carboxylate ion and the carboxamide in aqueous solution.

Experimental

All the experiments reported in this communication were conducted with the aid of a *p*H-stat (Applewhite *et al.*, 1958; Kurtz and Niemann, 1962). The kinetic parameters were evaluated as in earlier studies using a Datatron 220 computer (Abrash *et al.*, 1960).

Inhibitors. Reagent grade indole (Matheson) was recrystallized twice from water. The analytical data and melting points of the indole carboxylic acids and indole carboxamides are given in Table III.

Substrate. Sodium α-*N*-acetyl-L-tyrosinehydroxamate was prepared according to the method of Hogness and Niemann (1953), mp 190–190.5°, $[\alpha]_D^{25} = 34.8°$.

Anal. Calcd for $C_{11}H_{13}O_4N_2Na$ (260.2): C, 50.8; H, 5.0; N, 10.8. Found: C, 50.9; H, 5.0; N, 10.7.

α-Chymotrypsin. An Armour preparation of crystalline salt-free bovine α-chymotrypsin, lot 283, was used in all kinetic studies.

Kinetic Studies. The procedures employed have been described by Applewhite *et al.* (1958) and in particular by Kurtz and Niemann (1962). Experimental details are given in Table I.

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The Acyl-Enzyme Dimer of Chymotrypsin*

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ABSTRACT: Titration of the active sites of α- and β-chymotrypsin at *p*H values 2–4 by the specific substrate *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester shows the presence of some catalytically inactive but rapidly activatable enzyme species, the percentage of which depends on the total protein concentration. Conversely, at *p*H 7–8 all of the enzyme is in the active form. The effect of the protein concentration on the active-inactive enzyme equilibrium indicates strongly that a dimer is formed. A number of pieces of evidence indicates that the dimer is an acyl-enzyme in which one molecule of enzyme acylates a second molecule: (1) the time dependence of the activation of the dimer; (2) the effect of *p*H on the monomer-dimer equilibrium; (3) inhibition of the dimerization by specific inhibitors of chymotrypsin; (4) the effect of protein concentration on the monomer-dimer equilibrium (see equation 6); and (5) the effect of *p*H and ionic strength on the rate of conversion of dimer to monomer which parallels that of a deacylation reaction. The dimer of chymotrypsin provides the ultimate acyl-enzyme, a naturally occurring compound consisting of two protein components.

Much evidence supports the hypothesis that chymotrypsin undergoes a reversible dimerization. This evidence is based mainly on physical measurements such as sedimentation, light scattering, diffusion, and the like, which determine an apparent molecular weight of the system (Schwert, 1949; Schwert and Kaufman, 1949; Schwert, 1951; Schwert and Kaufman, 1951; Smith and Brown, 1952; Frenkel, 1952; Steiner, 1954; Massey *et al.*, 1955; Egan *et al.*, 1957; Tinoco, 1957; Rao and Kegeles, 1958; Bethune and Kegeles, 1961; Winzor and Scheraga, 1964). A recent X-ray analysis of α-chymotrypsin crystals indicates that two enzyme molecules occupy one unit cell, strongly suggesting a crystalline dimeric form of the enzyme (Blow *et al.*, 1964).

This large mass of physical information does not, however, shed any light on the forces involved in the dimerization or on the mechanism of the reaction. From the point of view of enzyme chemistry, the most important point concerns the possibility of the involvement of the "active site" of the enzyme in the dimerization. On this subject contradictory evidence exists: some workers claim that neither chymotrypsinogen, DFP-inhibited chymotrypsin, nor photooxidized chymotrypsin dimerizes (Schwert, 1949; Egan *et al.*, 1957); others affirm the contrary (Schwert, 1951; Smith and Brown, 1952; Massey *et al.*, 1955). A kinetic investigation of the influence of dimerization on the catalytic activity of α-chymotrypsin did not result in a definitive mechanism of dimerization (Martin and Niemann, 1958).

The inability of the previous, mainly physical, methods to specify the mechanism of dimerization demanded a new method of approach to this problem. Such an approach was found accidentally during the titration of the active site of α-chymotrypsin by *N*-acetyl-DL-tryptophan *p*-nitrophenyl ester at *p*H values between 2 and 4 (Kézdy *et al.*, 1964). During such titrations we observed that an α-chymotrypsin solution at *p*H 4 contains some catalytically inactive but rapidly activatable enzyme species, the percentage of which depends on the total

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