Interaction of Aromatic Compounds With α-Chymotrypsin*

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One hundred thirty-six compounds, principally mono-, bi- and tricyclic fused ring aromatic compounds, have been examined as inhibitors of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-valine methyl ester. Their behavior as inhibitors has been summarized in the form of ten postulates which also provide information about the general nature of the active site of the enzyme. One of these compounds, benzo[*f*]quinoline, is the most effective inhibitor discovered to date and another, 9-aminoacridine, offers promise of being an effective tool in the further definition of the structural specificity of α-chymotrypsin.

It is common knowledge that many aromatic compounds combine with α-chymotrypsin and function as inhibitors of reactions catalyzed by this enzyme. Hence such compounds are useful probes for exploring the topography of the active site of the enzyme.

In the past, attention has been largely centered on benzenoid compounds related to the common aromatic α-amino acids (Neurath and Schwert, 1950; Foster *et al.*, 1955; Foster and Niemann, 1955a). There is relatively little information available about the behavior of other aromatic systems or of the simpler benzene derivatives. The present investigation was undertaken as the first step in a systematic examination of the interaction of aromatic compounds with α-chymotrypsin, and was intended as a survey to disclose areas worthy of further study. While not exhaustive with respect to any particular group of compounds, a large number of compounds have been examined as inhibitors of the α-chymotrypsin-catalyzed hydrolysis of a single substrate, acetyl-L-valine methyl ester, under comparable experimental conditions, thus permitting the detection of structure-reactivity correlations that otherwise might not have been observed.

EXPERIMENTAL

Inhibitors.—The source of the inhibitors used in this study is indicated in Tables I through VII. Compounds identified by the letter A were obtained from commercial sources. These compounds were the best available grade and were used without further purification. (N.B.: All tables appear at the end of the article.) Compounds identified by the letter B were obtained from commercial preparations by fractional distillation under reduced pressure in an atmosphere of nitrogen. Compounds identified by the letter C were obtained by recrystallization of commercial preparations. Recrystallization was usually accompanied by decolorization with activated carbon. The naphthalene disulfonic acids and quinoline-8-sulfonic acid were recrystallized and used as the sodium salts. The naphthylamine sulfonic acids were dissolved in aqueous sodium hydroxide, the solutions were decolorized with activated carbon, sufficient sodium hydrosulfite was added to discharge the pink color, and the solutions were acidified to *p*H 2. This process was repeated until colorless crystalline products were obtained. Compounds identified by the letter D were obtained as indicated in the following paragraphs.

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Propionanilide, *N* - benzylacetamide, *N* - benzyl - α-chloroacetamide, *N*-benzylpropionamide, *N*-β-phenethylacetamide, and *N*-β-phenethyl-α-chloroacetamide were prepared by the following procedure. Two equivalents of the amine were dissolved in an excess of absolute ethyl ether, the solution was cooled in an ice-salt bath, and one equivalent of the appropriate acyl chloride was added dropwise with vigorous stirring. The precipitated amine hydrochloride was removed by filtration; the filtrate was washed first with dilute aqueous hydrochloric acid, then with dilute aqueous sodium bicarbonate, and finally twice with water before it was dried over anhydrous magnesium sulfate. The dried solution was evaporated *in vacuo*, the residual solid, or oil, was taken up in a small volume of benzene, the solution was decolorized with activated carbon and filtered, and the filtrate was diluted with ten times its volume of ligroin. The precipitated amide was collected and recrystallized three times from a mixture of benzene and ligroin. The amides which did not contain chlorine were recrystallized an additional two times from diisopropyl ether.

2-Styryl-4-aminoquinoline was prepared as described by Albert and Royer (1949). The hydrochloride was twice recrystallized from aqueous ethanol.

Quinoline-4-carboxylic acid was obtained by oxidation of 4-styrylquinoline with potassium permanganate (Elderfield and Siegel, 1951).

D- and L-3-carboxydihydroisocarbostyril were prepared as described by Hein and Niemann (1962).

2-Methyl-3-hydroxyquinoxaline was obtained by reaction of *o*-phenylenediamine, dissolved in boiling 1 N aqueous acetic acid, with an excess of ethyl pyruvate. The crude product was recrystallized twice from aqueous ethanol.

Reaction of phthalaldehydic acid and hydrazine hydrate in boiling water gave phthalazone, mp 182° after recrystallization from 75% aqueous ethanol.

2-Amino-4-hydroxypteridine was obtained as tan-colored needles by the addition of two equivalents of glyoxal-sodium bisulfite to a boiling solution of 2,5,6-triamino - 4 - hydroxypyrimidine sulfate monohydrate (Korte and Barkemeyer, 1956) in 1 N aqueous hydrochloric acid.

Xanthopterin was prepared as described by Korte and Barkemeyer (1956).

N-Methylindole was synthesized by the procedure of Julian and Printy (1949) and *N*-methyloxindole by that of Stollé (1930). The former compound was purified by distillation at 1 micron in a closed system.

Phthalimidine was prepared as described by Graebe (1888).

The method of Bistrzycki and Przeworski (1912) was used for the preparation of 2-hydroxymethylbenzimidazole and 2-carboxybenzimidazole. Both com-

pounds were recrystallized twice from water. The latter compound was esterified by the method of Brenner and Huber (1953) to give 2-carboxymethylbenzimidazole, mp 187°. D-Glucobenzimidazole, mp 215° after two recrystallizations from water, was prepared by the procedure of Moore and Link (1940) and 2-(β-hydroxyethyl)benzimidazole, mp 153.5° after three recrystallizations from water, was prepared by the method of Bachman and Heisey (1949).

9-Aminoanthracene was prepared by the method of Meisenheimer and Connerade (1903).

The ring closure of 3'-aminodiphenylamine-2-carboxylic acid was conducted as described by Albert (1951) to give 1- and 3-aminoacridine.[1] The products were separated as directed and the hydrochlorides were purified by repeated recrystallization from a mixture of ethanol and ethyl ether. 2-Aminoacridine was prepared by the procedure of Albert and Ritchie (1941) and 9-aminoacridine was prepared as described by the same authors (1942). The hydrochloride of the latter compound was prepared by passing dry hydrogen chloride into an ethanolic solution of the base. The precipitated hydrochloride was recrystallized three times from 75% aqueous ethanol. Where the hydrochlorides were obtained they were used as such in the inhibition studies.

9-Acridone was prepared as described by Albert (1951) and 9-acetamidoacridine was prepared by the procedure of Albert and Goldacre (1943) as modified by Wilkinson and Finor (1946). The final product, mp 276°, was recrystallized twice from ethanol.

9-Aminoacridine was acylated with chloroacetic anhydride and the resulting 9-chloroacetamidoacridine was recrystallized from ethanol. This product was transformed into the corresponding 9-iodoacetamidoacridine by the procedure of Jacobs, Heidelberger, and Rolf (1919) and the iodo compound, mp > 300°, was recrystallized from a mixture of acetone and water.

Anal. Calcd. for $C_{15}H_{11}ON_2I$ (362.2): C, 49.7; H, 3.1; N, 7.7; I, 35.0. Found: C, 49.8; H, 3.1; N, 7.7; I, 34.9.

Dibenzo[*f,h*]quinoline, mp 174° after recrystallization from 0.1 N acetic acid, was prepared as described by Krueger and Mosettig (1940).

Sparteine hydrochloride was obtained, as hygroscopic crystals, from the commercially available sulfate by reaction of the latter salt with one equivalent of aqueous barium chloride.

Compounds identified by the letter E are substances whose inhibition constants and preparation were reported previously.

Substrate.—Acetyl-L-valine methyl ester was prepared essentially as described by Applewhite, *et al.* (1958b). The neutralization of the acetic anhydride after the acylation step is best accomplished by heating the mixture on a steam bath and adding just enough water to facilitate reaction. The residue, after removal of the chloroform, is taken up in hot diisopropyl ether, the solution is treated with Norite and filtered, and the product is caused to crystallize by adding hexane to the filtrate. Recrystallization of the product is facilitated by dissolving it in diisopropyl ether, adding boiling hexane just to the point of incipient precipitation, and allowing the solution to cool slowly to room temperature. The large prismatic needles so obtained are collected, washed with hexane, and dried *in vacuo* to give acetyl-L-valine methyl ester, mp 62–63°, $[\alpha]_D^{25} = -48.5 \pm 0.3°$ (*c*, 10% in water).

[1] The Chemical Abstracts system of nomenclature is used in this communication.

Enzyme.—Crystalline, bovine, salt free, α-chymotrypsin, Armour Lot No. T-97207, was employed in all experiments.

Kinetic Studies.—All kinetic studies were conducted with a *p*H-stat (Neilands and Cannon, 1955) following the procedure described by Applewhite, *et al.* (1958a,b). All reaction systems were maintained at 25.0 ± 0.1° and *p*H 7.90 ± 0.02, and were 0.10 M in sodium chloride. In every case the recorder tracing of the extent of reaction vs. time were essentially linear thus permitting direct evaluation of values of v_o, the so-called initial velocities. Values of K_I were obtained from the relation

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

where v_o is the initial velocity in the presence of the inhibitor and K_o = 112 mM and k_o = 0.151 sec^{-1} for the uninhibited reaction (Waite and Niemann, 1962). Values of K_I were taken as the enzyme-inhibitor dissociation constants for fully competitive inhibition unless otherwise noted.

Results

One hundred thirty-six compounds have been examined for their ability to combine with the active site of α-chymotrypsin in a manner competitive with the combination of a representative trifunctional substrate of this enzyme. Wherever possible the enzyme-inhibitor dissociation constants have been determined. These data are summarized in Tables I to VII inclusive. Of the one hundred three dissociation constants listed in these tables only ten are previously published values. These have been included because of their pertinence to those of closely related compounds considered in this study.

Discussion

Aromatic compounds are useful probes for determining the topography of the active site because the flat and rigid aromatic nucleus is conformationally determinate. However, this structural feature does not ensure that all aromatic compounds will combine with the active site of the enzyme in precisely the same mode. As with substrates of this enzyme (Hein and Niemann, 1961, 1962), it is reasonable to expect that a number of aromatic compounds may combine with the active site in several modes. Under these circumstances the observed inhibition constant K_I will be related to the dissociation constants of the several complexes by the equation

$$1/K_I = \sum_j^n 1/K_{I_j} \quad (2)$$

Only if one of the several dissociation constants, K_{I_j}, is smaller by an order of magnitude than all others will the dissociation constant of the most stable complex be essentially equal to the value of K_I. Thus in the interpretation of values of K_I it is necessary to consider which of the several loci at the active site are implicated in the combination of a given inhibitor as well as the nature of the interaction at the locus (or loci) so involved.

The information at hand does not lead directly to the positive identification of the locus (or loci) participating in the binding of any given inhibitor. In the absence of such knowledge it shall be assumed, as a first approximation, that the interaction of aromatic compounds with one of the several loci is more favorable, by at least an order of magnitude, than with all

others. It is further assumed that the favored locus is the ρ_2 locus involved in the binding of the side chains of acylated aromatic α-amino acid ester type model substrates (Hein and Niemann, 1961, 1962). It is recognized that the above assumptions will not be universally valid. In fact, their validity is necessarily restricted to limit cases associated with what may be termed monofunctional inhibitors, i.e., those compounds which because of their structures interact with no more than one locus in any given interaction with the active site. Because it appeared likely that simple monosubstituted benzene derivatives might best approximate monofunctional inhibitors, attention was first centered on this class of compounds.

Derivatives of Benzene.—The results obtained from examination of thirty-eight derivatives of benzene are summarized in Table I. For thirty-four it was possible to obtain values of K_I. Of this number, thirty-three contain a benzenoid nucleus. Of the thirty-three, twenty-six are compounds bearing no formal charge under the conditions used for the evaluation of K_I. For this latter group the values of K_I range from 3.4 mM to 25 mM, with an arithmetic mean of 8.6 mM. If one were to select a single value of K_I for simple, monosubstituted, uncharged, benzenoid compounds a value of 8 ± 5 mM would be a reasonable choice. Thus, the binding of these compounds with the active site of α-chymotrypsin is associated with a value of $-\Delta F^*_{298}$ of 3.0 ± 0.5 kilocalories. That the magnitude of this value is dependent, at least in part, on the presence of a benzenoid nucleus may be inferred from a comparison of the inhibition constants of phenol, 6.4 mM, and cyclohexanol, 75 mM. In this instance the value of $-\Delta F^*_{298}$ = 3.0 kcal, for the aromatic compound is twice that of its hydroaromatic analog, i.e., 1.5 kcal. Although aromaticity, or a consequence thereof, is implicated, the nature of the interaction of simple aromatic compounds with the active site remains obscure. Such combination may involve dipole-dipole interaction, van der Waals (London) forces, charge transfer type intermolecular bonding, hydrophobic bonding (Nemethy and Scheraga, 1962), and in certain cases direct intermolecular hydrogen bonding, with all types of interactions subject to perturbation by steric and coulombic factors. It is probable that the degree of participation of each of the above attractive forces will vary with the nature of the inhibitor, making it difficult to identify the particular force or forces involved except in certain limit cases.

For phenol and cyclohexanol it could be argued that aromaticity is to be associated with coplanarity and that for an aromatic-hydroaromatic pair the aromatic member, with an assumed greater effective area of contact in its interaction with the active site, will have the lower inhibition constant. It will be seen later that the validity of this argument is supported by the still lower inhibition constants of certain polycyclic aromatic compounds. However, the lack of data for saturated alicyclic compounds other than cyclohexanol robs the above argument of much of its force.

Turning to compounds containing a benzene nucleus it is noteworthy that benzene, with a value of $-\Delta F^*_{298}$ = 2.2 kcal, is a poorer inhibitor than toluene, or any of the other uncharged monosubstituted benzene derivatives listed in Table I. The lower inhibition constants of the derivatives containing electron-releasing substituents, e.g., toluene, phenol, anisole, and aniline, might suggest the presence of an electron deficient locus at the active site. However, there are in Table I examples of aromatic carboxamides (electron accepting substituent) and the analogous anilides (electron donating substituent). Since their inhibition constants are essentially indistinguishable, as are those of compounds of the type $C_6H_5(CH_2)_nCONH_2$, where n = 0, 1, 2, and 3, we may conclude that charge transfer bonding is not a dominant factor in determining the magnitude of the inhibition constants unless the locus, or the site, is hermaphroditic and contains both a π acid and a π base.

For a hermaphroditic site an inhibitor that is a π base would presumably interact at one locus that is electron deficient, and one that is a π acid would interact at a second locus that is electron rich. With a trifunctional substrate, as employed in the present study, both types of inhibitors would lead to fully competitive inhibition and their inhibition constants could be similar in magnitude. Only when bifunctional substrates which differ in their binding modes are found will it be possible to distinguish between the above two hypothetical types of inhibitors.

It is not profitable to discuss other possible types of interactions at this time because the lack of significant variability of the inhibition constants of the simple, uncharged, monosubstituted benzene derivatives listed in Table I does not permit recognition of limit cases for particular types of interactions. It is evident that a greater variety of uncharged benzene derivatives must be examined, and experiments must be performed in which pairs of inhibitors are placed in competition with each other and in which sets of inhibitors are separately evaluated against a series of bifunctional substrates. Such experiments are currently in progress.

The inability of *p*-benzoquinone to function as an inhibitor at a concentration of 40 mM might be taken as an indication that π-complex formation is not an important factor in the interaction of aromatic molecules with the active site of the enzyme. However, this conclusion is premature in view of the limited amount of information that is presently available.

The behavior of simple, anionic, monosubstituted benzene derivatives has been examined by Kaufman and Neurath (1949), Neurath and Gladner (1951), Huang and Niemann (1952), and Foster and Niemann (1955a,b). These investigators have suggested that interaction of these compounds with the active site of the enzyme involves a coulombic repulsion between the negatively charged carboxylate group and a negative charge at the active site. It is also known that the inhibition constants determined in systems containing a phosphate buffer are substantially lower than those determined in systems buffered with tris(hydroxymethyl)aminomethane and hydrochloric acid (Foster and Niemann, 1955b). The values given in Table I for the carboxylate ions are for systems containing the uni-univalent buffer.

The data given in Table I for the five anionic inhibitors suggest that a negative charge is within several angstroms of the locus interacting with the benzene nucleus. There is also evidence of the existence of other charged groups in the same region. However, their nature and disposition cannot be specified at this time because of the conformational indeterminacy of the inhibitor molecules.

Derivatives of Naphthalene.—Several naphthalene derivatives, including β-(α-naphthyl)propionate, were examined as inhibitors of α-chymotrypsin by Neurath and Gladner (1951) and Loewus and Briggs (1952). However, because of the choice of compounds and the use of a phosphate buffer there was no indication that certain naphthalene derivatives might combine with the active site of α-chymotrypsin more effectively than their benzene counterparts.

TABLE I
DERIVATIVES OF BENZENE[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Hydrocarbons			
1	Benzene	B	25 ± 3[b]	4 Expts. with [I] = 8 mM
2	Toluene	B	13 ± 1[b]	4 Expts. with [I] = 3.5 mM
	Alcohols and phenols			
3	Phenol	A	6.4 ± 0.8[c]	6 Expts. with [I] = 3, 5, 10, and 50 mM
4	Cyclohexanol	A	75 ± 9[d]	4 Expts. with [I] = 2.5, 5, 10, and 20 mM
5	Benzyl alcohol	A	5.8 ± 1.1	4 Expts. with [I] = 6, 10, 20, and 50 mM
6	2-Phenylethanol	A	4.0 ± 0.1	3 Expts. with [I] = 3, 5, and 10 mM
7	3-Phenylpropan-1-ol	A	4.3 ± 0.1	3 Expts. with [I] = 1, 2, and 4 mM
8	2-Phenoxyethanol	B	7.7 ± 0.9[e]	3 Expts. with [I] = 5, 7.5, and 10 mM
	Ethers			
9	Anisole	A	8.4 ± 0.6	3 Expts. with [I] = 3, 5, and 7 mM
	Ketones and quinones			
10	*p*-Benzoquinone	A	—	No inhibition with [I] = 40 mM
	Amines			
11	Aniline	B	6.6 ± 0.2	3 Expts. with [I] = 2, 5, and 7.5 mM
12	*N*-Methylaniline	B	6.3 ± 0.1	3 Expts. with [I] = 2, 4, and 7.5 mM
13	*N*-Dimethylaniline	B	3.4 ± 0.3	3 Expts. with [I] = 1, 2.5, and 3.75 mM
14	*N*-Ethylaniline	B	6.6 ± 0.2	2 Expts. with [I] = 2 and 3.75 mM
15	*N*-Diethylaniline	B	—	Too insoluble to evaluate
16	Diphenylamine	A	—	Too insoluble to evaluate
	Ammonium salts			
17	Benzylammonium chloride	A	22 ± 5	3 Expts. with [I] = 1, 2, and 4 mM
18	β-Phenethylammonium chloride	A	48 ± 1[f]	3 Expts. with [I] = 10, 15, and 50 mM
	Anilides			
19	Formanilide	A	3.9 ± 0.2	3 Expts. with [I] = 4, 10, and 20 mM
20	Acetanilide	E	13 ± 3[g]	Value of Foster and Niemann (1955a)
21	Propionanilide	D	6.6 ± 0.4	2 Expts. with [I] = 3 and 5 mM
22	Benzanilide	A	—	Too insoluble to evaluate
	Carboxamides			
23	Benzamide	E	10 ± 2[g]	Value of Foster and Niemann (1955a)
24	Phenylacetamide	E	15 ± 3[g]	Value of Foster and Niemann (1955a)
25	β-Phenylpropionamide	E	7 ± 2[g]	Value of Foster and Niemann (1955a)
26	γ-Phenylbutyramide	E	12 ± 3[g]	Value of Foster and Niemann (1955a)
27	*N*-Benzylacetamide	D	7.5 ± 1.0	3 Expts. with [I] = 6, 10, and 15 mM
28	*N*-Benzyl-α-chloroacetamide	D	4.9 ± 0.1	3 Expts. with [I] = 2.5, 5, and 10 mM
29	*N*-Benzylpropionamide	D	6.3 ± 0.4	2 Expts. with [I] = 5 and 10 mM
30	*N*-β-Phenethylacetamide	D	11.4 ± 0.1	2 Expts. with [I] = 15 and 25 mM
31	*N*-β-Phenethyl-α-chloroacetamide	D	9.9 ± 0.1	2 Expts. with [I] = 5 and 10 mM
	Carboxylates[h]			
32	Benzoate	E	150 ± 50[g]	Value of Foster and Niemann (1955a)
33	Phenylacetate	E	200 ± 50[g]	Value of Foster and Niemann (1955a)
34	β-Phenylpropionate	E	25 + 5[g]	Value of Foster and Niemann (1955a)
35	γ-Phenylbutyrate	E	60 ± 10[g]	Value of Foster and Niemann (1955a)
	Sulfonamides			
36	Benzenesulfonamide	A	4.3 ± 0.4	2 Expts. with [I] = 5 and 10 mM
37	*p*-Sulfanilamide	C	15.4 ± 3.0	3 Expts. with [I] = 3, 5, and 7.5 mM
	Sulfonates			
38	Benzenesulfonate	C	70	1 Expt. with [I] = 10 mM

[a] As inhibitors of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride unless otherwise noted. [b] Value determined by J. R. Rapp (1962). [c] Huang and Niemann (1953) give a value of 7.0 mM for this compound when evaluated against nicotinyl-L-tryptophanamide in aqueous solutions at 25.0°, *p*H 7.90, and 0.02 M in tris(hydroxymethyl)aminomethane. [d] Huang and Niemann (1953) give a value of 80 mM for this compound when evaluated against the system described in footnote *c*. [e] Neurath and Gladner (1951) give a value of 5.8 mM for this compound when evaluated against acetyl-L-tyrosinamide in aqueous solutions at 25.0°, *p*H 7.80, and 0.1 M in a phosphate buffer. [f] Stated to be an inhibitor by Kaufman and Neurath (1949) but K_I not evaluated by them because of experimental difficulties. [g] Reaction system same as described in footnote *c*. [h] Neurath and Gladner (1951) give K_I values for twelve carboxylate ions including the four listed in Table I. However, their values were obtained for reaction systems 0.1 M in phosphate. Foster and Niemann (1955b) have shown that the K_I values of anionic bifunctional inhibitors determined in the presence of phosphate ion are substantially lower than when determined in the reaction system described in footnote *c*. Hence, the values of Neurath and Gladner (1951) are not comparable to those given in Table I and are not reproduced in this table.

TABLE II
DERIVATIVES OF NAPHTHALENE[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Phenols			
1	α-Naphthol	A	0.2 ± 0.02	5 Expts. with [I] = 0.1, 0.2, 0.4, 0.6, and 2 mM
2	1,3-Dihydroxynaphthalene	A	1.4 ± 0.2	3 Expts. with [I] = 0.2, 1, and 2 mM
3	*p*-Hydroxybiphenyl	A	0.25 ± 0.02	3 Expts. with [I] = 0.1, 0.2, and 0.5 mM
	Quinones			
4	*o*-Naphthoquinone	A	—	Too insoluble to evaluate
	Amines			
5	α-Naphthylamine	A	0.30	1 Expt. with [I] = 2 mM
6	β-Naphthylamine	A	0.25	1 Expt. with [I] = 0.93 mM
	Carboxylates[b]			
7	β-Naphthoate	A	1.4	1 Expt. with [I] = 6.5 mM
	Sulfonates			
8	β-Naphthalenesulfonate[c]	A	1.84 ± 0.27	1 Expt. with [I] = 1.58 mM
9	2,7-Naphthalenedisulfonate	C	400	1 Expt. with [I] = 40 mM
10	2,6-Naphthalenedisulfonate	C	130 ± 25	3 Expts. with [I] = 25, 75, and 125 mM
11	1,5-Naphthalenedisulfonate	C	—	No inhibition with [I] = 125 mM
12	1,6-Naphthalenedisulfonate	C	—	No inhibition with [I] = 125 mM
	Aminosulfonates			
13	1-Naphthylamine-4-sulfonate	C	185 ± 70	2 Expts. with [I] = 20 and 37.5 mM
14	1-Naphthylamine-5-sulfonate	C	31 ± 2	2 Expts. with [I] = 20 and 37.5 mM
15	1-Naphthylamine-6-sulfonate	C	4.8 ± 0.1	3 Expts. with [I] = 5, 10, and 20 mM
16	1-Naphthylamine-8-sulfonate	C	250	1 Expt. with [I] = 10 mM, limited soly.
17	2-Naphthylamine-1-sulfonate	C	41 ± 2	2 Expts. with [I] = 20 and 37.5 mM
18	2-Naphthylamine-6-sulfonate	C	11 ± 0.2	2 Expts. with [I] = 10 and 25 mM

[a] As inhibitors of the α-chymotrypsin catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride unless otherwise noted. [b] Neurath and Gladner (1951) give the following values for compounds evaluated in aqueous solutions at 25.0°, *p*H 7.8, and 0.1 M in a phosphate buffer: β-(1-naphthyl)propionate, K_I = 4.0 mM; β-(1-naphthyl)ethylmalonate, K_I = 55 mM. Loewus and Briggs (1952) obtained a value of K_I = 3.8 mM for the former compound from equilibrium dialysis experiments under comparable conditions. It is not known how these values of K_I would compare with those determined in the absence of phosphate. [c] Inhibition demonstrated to be fully competitive by examination of reaction for [S] = 10, 15, 20, 30, 40, 50, and 70 mM.

In Table II are listed eighteen derivatives of naphthalene and values of K_I for all but three of these compounds. One striking feature is the low value of K_I for α-naphthol and α- and β-naphthylamine. When these three compounds are compared with phenol and aniline it is seen that $-\Delta F^*_{298}$ is increased from *ca.* 3 kcal for the latter two compounds to *ca.* 5 kcal for the naphthalene derivatives. It is instructive that the same increase is observed when phenol is compared with *p*-hydroxybiphenyl. From these observations we conclude that the locus with which benzene derivatives combine is sufficiently extensive to permit additional positive interaction with aromatic nuclei of greater surface area than benzene. There is no evidence that a limit has been reached with either naphthalene or biphenyl.

The remainder of the compounds listed in Table II were selected to provide information about the behavior of disubstituted naphthalene derivatives in which one or both substituents were negatively charged. It will be seen that the K_I values of β-naphthoate and β-naphthalene sulfonate are very much lower than those of the corresponding benzene derivatives. However, the increase in $-\Delta F^*_{298}$ of 1.6 ± 0.2 kcal is reasonably close to the difference of 2 kcal noted above for the corresponding uncharged phenols and amines. Thus, with these anionic monosubstituted naphthalene derivatives, there is no evidence as yet that the inhibitor molecules can orient themselves at the active site in such a manner as to avoid a coulombic repulsion, e.g., by rotation of the molecule through 180°. Even though the coulombic repulsion is associated with a decrease in binding energy, the mode in which it is encountered, with the monosubstituted compounds, is still more favorable for combination than any other mode.

If disubstituted naphthalene derivatives are arranged in order of increasing relative distance between the points of substitution on the naphthalene nucleus, one arrives at the sequence given in Table VIII. If the derivative is a disulfonate one finds, for the derivatives for which data are available, that the values of K_I decrease as the relative distance between the points of substitution increases, i.e., the *amphi*-disubstituted compound has the lowest dissociation constant. However, the value of this constant is substantially greater than that of the monosubstituted compound.

These observations suggest that less effective combination may arise because of enhanced electron withdrawal from the nucleus or, more likely, that interactions other than those involved in binding of the monosubstituted derivatives are being encountered in the case of the disubstituted compounds, and that the latter lead to a decrease in binding energy. It is not known whether these latter interactions are purely steric or are a result of coulombic repulsions.

It will be seen from the data given in Table VIII that the naphthalene disulfonates are relatively poor probes for examining the topography of the active site in the vicinity of the locus with which they interact because the values of K_I are indeterminably high. For this reason attention was directed to the naphthylaminesulfonates where it was anticipated that lower values of K_I would be encountered.

Examination of the K_I values of the six naphthylaminesulfonates listed in Tables II and VIII might lead one to believe that the relation concerning distance between points of substitution on the naphthalene

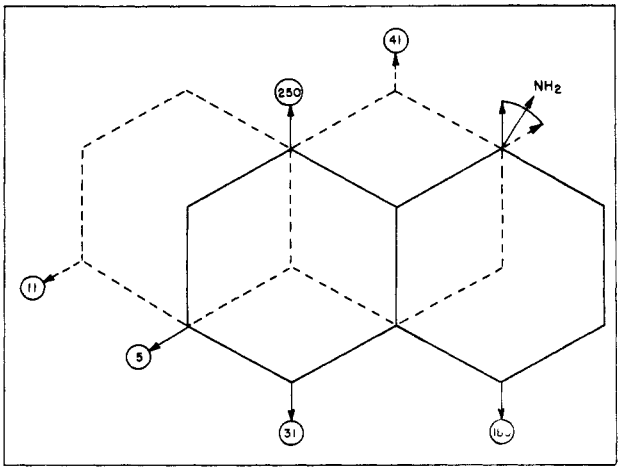

FIG. 1.—Possible disposition of the α- and β-naphthylaminesulfonates at the ρ_2 locus. Solid lines α-derivative, dotted β.

nucleus and values of K_I observed for the naphthalenedisulfonates is not valid for the naphthylaminesulfonates. However, this is partly an illusion arising from the fact that four of the six naphthylaminesulfonates are derivatives of α-naphthylamine and the remaining two of β-naphthylamine. If attention is restricted to the α-naphthylaminesulfonates one finds, for the compounds examined, that the values of K_I decrease as the distance between the points of substitution of the naphthalene nucleus increases. This empirical generalization, although based upon incomplete information, provides a clue to the disposition of these molecules at the locus with which they combine.

The values of K_I for α- and β-naphthylamine are lower, by about an order of magnitude, then that of β-naphthalenesulfonate. Therefore we assume that with the naphthylaminesulfonates the amino group interacts with a sublocus and in every case this interaction will make a greater contribution to the total binding energy than will that involving a sulfonate group. With the former interaction and that of the naphthalene nucleus with its sublocus providing the major positive contributions to the over-all binding energy we further assume that these two interactions will tend to determine the disposition of all naphthylaminesulfonates at the locus with which they combine, i.e., probably the ρ_2 locus defined previously. Thus we may expect topographical features adjacent to this locus to be revealed, in terms of values of K_I, by perturbation of an intrinsic binding capacity arising from interaction of a sulfonate group in various positions with such features.

For the α-naphthylaminesulfonates we may postulate a situation such as that depicted in Figure 1. In this representation a perturbing interaction is represented, in a vector sense, by an arrow and a circled number, the latter being the value of K_I in units of mM. There are data for four of the seven α-naphthylaminesulfonates. From these data it is seen that no interaction involving a sulfonate group leads to an increase in binding energy over that of α-naphthylamine itself. There are two prominent obstacles to combination located in the *peri* and *para* positions, the least prominent in the *amphi* position, hence the empirical rule noted above, which can now be regarded as being somewhat fortuitous. The nature of the obstacles is unknown. They could arise from either steric or coulombic repulsions. The gross topography of the locus is evident from Figure 1. Even though the mapping is incomplete, a function for the obstacles in the *peri* and *para* positions is evident. They markedly restrict the ways in which the molecule can be oriented at the locus if effective combination is to be achieved.

The β-naphthylaminesulfonates present a more difficult problem because only two values of K_I are available. The disposition given in Figure 1 was selected largely because of three postulates, i.e., a common sublocus for interaction with the amino group of both α- and β-naphthylamine derivatives, maximum overlap of the area occupied by α-naphthylamine, and minimization of interference with obstacles revealed by the K_I values of both the α- and β-naphthylaminesulfonates. One feature of the orientation of the β-naphthylamine nucleus requires comment. In Figure 1 a representation has been adopted which requires deformation of the direction of the interaction involving the amino group. This is not an obligatory requirement since a displacement of the nucleus along the longer axis of the "valley" would be a reasonable alternative. At this time there are insufficient data to decide between these two possibilities and others which may be equally plausible. In any event there is now a model sufficiently explicit so that relatively few experiments can be decisive, e.g., a predicted value of K_I for 2-naphthylamine-5-sulfonate of *ca.* 10 mM, for 2-naphthylamine-4-sulfonate of *ca.* 50 mM, and those of 1-naphthylamine-7-sulfonate and 2-naphthylamine-3-sulfonate to be comparable to or smaller than that of 1-naphthylamine-6-sulfonate. Such experiments are contemplated as well as those required to explore the "ends of the valley."

Derivatives of Pyridine and the Azanaphthalenes.—In working with the compounds considered in Tables I and II it became evident that lack of water solubility would soon prove to be an effective barrier to studies with the higher molecular weight compounds. Therefore an exploratory study was made of the behavior of derivatives of pyridine and the azanaphthalenes. Thirty-three such compounds are listed in Table III. Values of K_I were obtained for twenty-four.

Huang and Niemann (1953) give a value of K_I = 50 mM for pyridine. With improved methods the present study leads to a value of 28 ± 2.5 mM. Thus, pyridine and benzene are comparable with respect to their ability to combine with the active site of α-chymotrypsin. The observation that the K_I value of 2,4,6-trimethylpyridine is but a third of that of pyridine again suggests that a charge-transfer type of interaction may be involved. If this were the case one would expect K_I values of a set of mono-, di-, and trisubstituted pyridines to be in the order pyridine > 2-methylpyridine > 2,4-dimethylpyridine > 2,4,6-trimethylpyridine. A test of this prediction is contemplated.

The K_I values of 2- and 4-hydroxypyridine are very large and could arise from the zwitterionic structures assigned to these substances (Albert, 1959). In a sense they are similar to benzoate ion in that they also contain a formal negative charge in close proximity to the nucleus, a situation associated earlier with high K_I values.

In the series 2-, 3-, and 4-aminopyridine the value of K_I decreases linearly with increasing pK_A' of the conjugate acid (Brown *et al.*, 1955) in a relationship which does not include pyridine. This observation suggests that with the aminopyridines effective combination is dependent upon protonation of the cyclic hetero atom and localization of the positive charge on the amino group.

It will be seen from the data given in Table III that quinoline and isoquinoline are effective inhibitors of α-chymotrypsin, and in this respect are equal or supe-

TABLE III

DERIVATIVES OF PYRIDINE AND THE AZANAPHTHALENES[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Pyridines			
1	Pyridine	A	28 ± 2.5	4 Expts. with [*I*] = 10, 20, 40, and 60 mM
2	2,4,6-Trimethylpyridine	B	10 ± 3	2 Expts. with [*I*] = 4 and 8 mM
3	Nicotine	A	—	Buffers reaction system
	Hydroxypyridines			
4	2-Hydroxypyridine	A	110 ± 12	3 Expts. with [*I*] = 37.5, 50, and 75 mM
5	4-Hydroxypyridine	A	—	No inhibition with [*I*] = 5 mM, limited soly.
	Aminopyridines			
6	2-Aminopyridine	A	9.4 ± 1.0	3 Expts. with [*I*] = 5, 10, and 20 mM
7	3-Aminopyridine	A	12.3 ± 0.4	3 Expts. with [*I*] = 5, 10, and 20 mM
8	4-Aminopyridine	A	2.9 ± 0.1	3 Expts. with [*I*] = 2.5, 5, and 10 mM
	Quinolines			
9	Quinoline	B	0.6	1 Expt. with [*I*] = 0.89 mM
10	Isoquinoline	B	0.32 ± 0.05	4 Expts. with [*I*] = 0.3, 0.5, 1, and 2 mM
11	2-Methylquinoline	B	1.5 ± 0.1	4 Expts. with [*I*] = 0.5, 1, 1.5, and 2 mM
12	4-Methylquinoline	B	2.3 ± 0.4	3 Expts. with [*I*] = 1.5, 2, and 4 mM
13	7-Methylquinoline	B	0.7 ± 0.04	4 Expts. with [*I*] = 0.3, 0.5, 1, and 2 mM
	Hydroxyquinolines			
14	2-Hydroxyquinoline	A	0.87 ± 0.1	3 Expts. with [*I*] = 0.5, 1, and 2 mM
15	8-Hydroxyquinoline	A	0.77 ± 0.02	3 Expts. with [*I*] = 0.2, 0.5, and 2 mM
16	Quinoline-2-carbinol	A	—	Too insoluble to evaluate
17	Quinoline-4-carbinol	A	12.2 ± 0.4	3 Expts. with [*I*] = 2, 4, and 7.5 mM
18	Quinine	A	—	No inhibition with [*I*] = 0.75 mM, limited soly.
	Aminoquinolines			
19	2-Aminoquinoline	A	1.3 ± 0.1	3 Expts. with [*I*] = 0.5, 1.5, and 2.5 mM
20	3-Aminoquinoline	A	2.3 ± 0.2	2 Expts. with [*I*] = 0.5 and 1.5 mM
21	4-Aminoquinoline	A	1.1 ± 0.05	3 Expts. with [*I*] = 0.4, 0.8, and 1.5 mM
22	2-Styryl-4-aminoquinoline	D	0.26 ± 0.05	3 Expts. with [*I*] = 0.1, 0.2, and 0.4 mM
	Quinoline carboxamides			
23	Quinoline-4-carboxamide	E	8.4 ± 1.0[b]	
	Quinoline carboxylates			
24	Quinoline-4-carboxylate	D	104[c]	1 Expt. with [*I*] = 55.7 mM
	Quinoline sulfonates			
25	Quinoline-8-sulfonate	C	177 ± 50	4 Expts. with [*I*] = 30, 100, 200, and 400 mM
	Quinolinium bases			
26	Ethylquinolinium iodide	A	43	1 Expt. with [*I*] = 20 mM
	Dihydroisocarbostyrils			
27	D-3-carboxydihydroisocarbostyril	D	—	No inhibition at [*I*] = 7.5 mM
28	L-3-carboxydihydroisocarbostyril	D	—	No inhibition at [*I*] = 7.5 mM
	Bisazanaphthalenes			
29	Quinoxaline	A	5	1 Expt. with [*I*] = 10 mM
30	2-Methyl-3-hydroxyquinoxaline	D	—	No inhibition with [*I*] = 0.5 mM, limited soly.
31	Phthalazone	D	2.95 ± 0.15	3 Expts. with [*I*] = 2, 3, and 4 mM
	Tetrazanaphthalenes			
32	2-Amino-4-hydroxypteridine	D	—	No inhibition with [*I*] = 0.5 mM, limited soly.
33	Xanthopterin	D	—	Too insoluble to evaluate

[a] As inhibitors of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride unless otherwise noted. [b] Value determined by R. A. Bernhard (1955) from inhibition of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide in aqueous solutions at 25.0°, *p*H 7.6, and 0.3 M in tris(hydroxymethyl)aminomethane. Inhibition found to be fully competitive from analysis of data obtained with [*S*] = 5, 10, 20, 30, and 40 mM and [*I*] = 5.0 mM in quinoline-4-carboxamide, mp 178°. [c] Value determined by W. Jackson (1962) from inhibition of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinhydroxamide in aqueous solutions at 25.0°, *p*H 7.6, and 0.2 M in sodium chloride. Inhibition is fully competitive.

rior to indole, a compound long regarded as one of the better reversible inhibitors of this enzyme. In view of the character of the inhibition observed with indole and methyl hippurate (Huang and Niemann, 1953; Applewhite *et al.*, 1958a) the need for comparable studies with quinoline and isoquinoline is clearly indicated.

The properties of the three methylquinolines are of interest in terms of the representation given in Figure 1. If it is assumed that quinoline and α-naphthylamine are similarly disposed at a common locus the fact that the value of K_I for 7-methylquinoline is practically identical with that of quinoline affords modest support for the disposition assigned to β-naphthylamine, or the one arising from displacement of the nucleus without distortion of the direction of interaction involving the amino group. The values of K_I for 2- and 4-methylquinoline suggest the presence of obstacles to combination in the 2- and 4-positions, with that in the 4-position being the greater of the two. In this instance steric

TABLE IV
DERIVATIVES OF INDANE, INDOLE, ISOINDOLE, IMIDAZOLE, AND BENZIMIDAZOLE[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Indanes			
1	Indene	A	—	Too insoluble to evaluate
2	1-Indanone	A	1.88 ± 0.2	3 Expts. with [I] = 0.5, 1, and 2 mM
3	1,3-Indandione	A	2.4 ± 0.6	3 Expts. with [I] = 0.5, 1.5, and 2 mM
4	1,2,3-Indantrione hydrate	A	2.7 ± 0.2	3 Expts. with [I] = 1, 2, and 4 mM
	Indoles			
5	Indole	C	0.80 ± 0.20	Value of Foster and Niemann (1955a)
6	*N*-Methylindole	D	0.80 ± 0.02	3 Expts. with [I] = 0.3, 0.5, and 0.75 mM
7	*N*-Methyloxindole	D	0.87 ± 0.03	3 Expts. with [I] = 0.5, 1, and 2 mM
8	7-Azaindole	A	1.33 ± 0.2	5 Expts. with [I] = 0.5, 1, 2, 3, and 4 mM
	Isoindoles			
9	Phthalimidine	D	2.02 ± 0.2	5 Expts. with [I] = 0.5, 1, 2, 3, and 4 mM
10	Phthalimide	A	—	No inhibition with [I] = 10 mM, limited soly.
	Imidazoles			
11	Imidazole	A	45	1 Expt. with [I] = 50 mM
	Benzimidazoles			
12	Benzimidazole	A	3	1 Expt. with [I] = 3 mM
13	2-Hydroxymethylbenzimidazole	D	3.4 ± 0.7	4 Expts. with [I] = 1, 2, 3, and 5 mM
14	2-(β-Hydroxyethyl)-benzimidazole	D	1.35 ± 0.1	5 Expts. with [I] = 0.25, 0.5, 1, 2, and 4 mM
15	2-(D-Gluco)-benzimidazole	D	1.36 ± 0.04	3 Expts. with [I] = 0.25, 0.5, and 1 mM
16	Benzimidazole-2-carboxylate	D	5.4 ± 1.3	4 Expts. with [I] = 2, 3, and 4 mM
17	2-Carboxymethylbenzimidazole	D	5.6 ± 0.7	4 Expts. with [I] = 2, 3, 4, and 6 mM

[a] As inhibitors of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride.

factors appear to override electronic effects arising from the electron donating methyl groups.

The inhibition constants of quinoline, 2-hydroxyquinoline, and 8-hydroxyquinoline are essentially indistinguishable, a situation very different from that observed for the analogous pyridine derivatives. Here again doubly competitive experiments involving the hydroxypyridines and hydroxyquinolines will be required to determine whether these two classes of inhibitors interact at the same or different loci.

The relatively high value of K_I for quinoline-4-carbinol, which probes farther than 4-methylquinoline, is consistent with the presence of the obstacle previously located in the 4-position. The inability of quinine to function as an inhibitor is no surprise after consideration of the representation given in Figure 1.

It was noted previously that the three aminopyridines have K_I values lower than that of pyridine. This feature is reversed with the aminoquinolines, where the K_I values are higher than those of quinoline or α- or β-naphthylamine. The pK_A' values for the conjugate acids of 2-, 3-, and 4-aminoquinoline are 7.25, 4.86, and 9.08, respectively (Brown *et al.*, 1955). Therefore with 3- and 4-aminoquinoline one encounters two extreme situations. With 3-aminoquinoline the normal, unprotonated species is encountered at *p*H 7.90. With 4-aminoquinoline the cyclic hetero atom is protonated and the positive charge localized on the amino group. 2-Aminoquinoline lies in an intermediate position with the observed value of K_I being composite and hence not readily interpreted. Comp rison of the K_I values of quinoline-4-carboxamide and quinoline-4-carboxylate ion clearly demonstrates the marked effect of a negative charge in the *para* position to the cyclic hetero atom. Thus, with 4-aminoquinoline bearing a positive charge in the 4-position it is not surprising that its K_I value is lower than that of the uncharged 3-aminoquinoline. The fact that 2-styryl-4-aminoquinoline has a value of K_I lower than that of 4-aminoquinoline, or quinoline, informs us that there is an additional area available for interaction with aromatic nuclei beyond that surveyed with either the naphthalene or quinoline derivatives.

The high value of K_I for quinoline-8-sulfonate is consistent with the representation given in Figure 1 and provides further justification for placing quinoline and α-naphthylamine in a common category.

The remaining data given in Table III are instructive in that they suggest that derivatives of bis- and tetrazanaphthalenes offer little promise of being more useful probes than those considered previously. It is not clear whether this property is a consequence of their π-deficient nuclei or arises because of other reasons.

Derivatives of Indane, Indole, Isoindole, Imidazole, and Benzimidazole.—In Table IV there are seventeen derivatives of indane, indole, isoindole, and benzimidazole. Having at hand extensive information about the inhibitory properties of all of the isomeric indole carboxylate ions and carboxamides (Abrash, 1961), our intent was to examine derivatives of other aromatic nuclei containing fused five- and six-member rings. While a number of these latter derivatives will be useful in specific cases, none was superior to indole as an inhibitor of α-chymotrypsin. The more significant features of this group are seen in a comparison of the K_I values of indole, *N*-methylindole and *N*-methyloxindole, indole and benzimidazole, and 2-carboxy- and 2-carboxymethylbenzimidazole.

Pyrimidines, Purines, Nucleosides, and Nucleotides.—The observations of Hofstee (1960) on the formation of soluble stoichiometric complexes of nucleic acids and α-chymotrypsin led us to examine a set of representative pyrimidines, purines, nucleosides, and nucleotides as inhibitors of this enzyme. Our results are consistent with Hofstee's observation that the inhibitory properties of yeast RNA are greatly decreased when the nucleic acid is subjected to prior treatment with ribonuclease. We conclude that the structural units of the nucleic acids have little tendency to combine with the active site of α-chymotrypsin.

TABLE V

PYRIMIDINES, PURINES, NUCLEOSIDES, AND NUCLEOTIDES[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Pyrimidines			
1	Cytosine	A	—	No inhibition with $[I]$ = 10 mM
2	Uracil	A	—	No inhibition with $[I]$ = 10 mM
	Purines			
3	Adenine	A	—	No inhibition with $[I]$ = 5 mM
4	Guanine	A	—	Too insoluble to evaluate
5	Hypoxanthine	A	—	No inhibition with $[I]$ = 5 mM
6	Xanthine	A	—	Too insoluble to evaluate
	Nucleosides			
7	Cytidine	A	—	No inhibition with $[I]$ = 10 mM
8	Uridine	A	—	No inhibition with $[I]$ = 10 mM
9	Adenosine	A	—	No inhibition with $[I]$ = 10 mM
10	Guanosine	A	—	Too insoluble to evaluate
11	Inosine	A	—	No inhibition with $[I]$ = 5 mM
12	Xanthosine	A	—	Too insoluble to evaluate
	Nucleotides			
13	Adenosine monophosphate	A	—	No inhibition with $[I]$ = 10 mM
14	Adenosine triphosphate	A	—	No inhibition with $[I]$ = 5 mM

[a] As inhibitors of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride.

Derivatives of Anthracene, Azanthracene, Azaphenanthrene, and the Like.—The lower K_I values of α-naphthylamine and quinoline, 0.3 and 0.6 mM, relative to those of aniline and pyridine, 6.6 and 28 mM, suggested the desirability of extending our studies to include representative tricyclic aromatic compounds. Eighteen such compounds are listed in Table VI. As anticipated, 9-aminoanthracene was too insoluble to evaluate as an inhibitor in aqueous systems. However, this difficulty did not arise in the case of the acridines and several other tri- and tetracyclic systems containing nitrogen as a cyclic hetero atom.

The formal addition of a benzo nucleus to pyridine to produce quinoline is associated with an increase in $-\Delta F^*_{298}$ of *ca.* 2.4 kcal in over-all binding energy. The addition of a second benzo nucleus to give acridine leads to a further increase in $-\Delta F^*_{298}$ of only *ca.* 0.5 kcal. Thus, it might be concluded that with acridine the area of the locus available for facile combination has been exceeded. However, as will be shown shortly, this conclusion is premature because with acridine and its derivatives the inhibitor may be no longer molecularly dispersed. Interpretation of the inhibition constant of acridine must be deferred until it can be shown that acridine is monomeric under the conditions employed in its evaluation as an inhibitor, or, if it is present as a micelle, that the dissociation constant of the micelle is substantially greater than the true enzyme-inhibitor dissociation constant.

The 1-, 2-, 3-, and 9-aminoacridines present a situation comparable to that encountered previously with the isomeric aminopyridines and aminoquinolines. The pK_A' values of the first group of compounds are 6.0, 5.9, 8.0, and 10.0, respectively. Thus, with 3-aminoacridine at *p*H 7.90 both the base and conjugate acid are present in substantial amounts and the value of K_I is composite. With 9-aminoacridine the conjugate acid with the charge localized at the amino group is the predominant species.

In the absence of additional information the K_I values obtained for the isomeric aminoacridines are confusing, particularly when it is recalled that 4-aminoquinoline has the lowest K_I value of the three isomeric aminoquinolines examined. Furthermore, if the cyclic hetero atom in 4-aminoquinoline and 9-aminoacridine occupies the same position at the locus, one might have expected that 9-aminoacridine would have a lower K_I value than acridine because of a coulombic attraction between the 9-ammonium group and the anionic structural feature of the site located in the *para* position to the cyclic hetero atom of the combining molecule. Instead, a K_I value so high as to be indeterminable is observed. This anomaly has been resolved (Wallace and Peterson, 1962). However, its solution has required studies too extensive to be reported here, other than to comment that the behavior of 9-aminoacridine can be explained provided it is recognized that this compound is present in the reaction system as a cationic micelle and not as the molecularly dispersed species.

The relatively high value of K_I for quinacrine(3-chloro - 9 - (ω - diethylamino - α - methylbutylamino)-7-methoxyacridine) could arise because of the formation of a relatively stable micelle or because of the bulky group in the 9 position. Even though the behavior of 9-acetamidoacridine would favor the second alternative we are unable to decide between the two possibilities at the present time largely because the pK_A' of the conjugate acid of quinacrine is *ca.* 7.9 (Albert, 1951) and hence K_I is composite.

The behavior of proflavine (3,6-diaminoacridine) and acriflavine (a mixture of proflavine and 3,6-diamino-10-methylacridine) is intriguing because the K_I values indicate that 3,6-diamino-10-methylacridine may be more effective than 1-acetyl-2-(L-tyrosyl)hydrazine as an inhibitor of α-chymotrypsin-catalyzed reactions. This latter substance, with a K_I value of 0.074 mM (Kurtz and Niemann, 1961), was the most effective reversible inhibitor known up to the time of this study.

The fact that acriflavine is a mixture makes it difficult to identify the inhibitory species. However, it is known (Albert, 1951) that the conjugate acid of proflavine has a pK_A' of 9.65 and that of the conjugate acid of 3,6-diamino-10-methylacridine is greater than 12. Thus in aqueous solutions at *p*H 7.90 the predominant species will be the conjugate acids in which the charge is not localized at a particular atom but may be pictured as being disposed in a line subtending the two amino groups and passing through the cyclic nitrogen atom. This situation could lead to an interaction with

TABLE VI

DERIVATIVES OF ANTHRACENE, AZANTHRACENE, AZAPHENANTHRENE, AND THE LIKE[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Anthracenes			
1	9-Aminoanthracene	D	—	Too insoluble to evaluate
	Acridines			
2	Acridine	A	0.22 ± 0.01	6 Expts. with [I] = 0.1, 0.25, and 0.5 mM
3	1-Aminoacridine	D	0.34 ± 0.01	3 Expts. with [I] = 0.1, 0.2, and 0.4 mM
4	2-Aminoacridine	D	0.22 ± 0.01	3 Expts. with [I] = 0.1, 0.2, and 0.4 mM
5	3-Aminoacridine	D	0.23 ± 0.01	3 Expts. with [I] = 0.1, 0.2, and 0.4 mM
6	9-Aminoacridine	D	—	No inhibition with [I] = 15 mM, limited soly.
7	9-Acridone	D	—	Too insoluble to evaluate
8	Quinacrine[b]	A	—	No inhibition with [I] = 1.5 mM, limited soly.
9	9-Acetamidoacridine	D	—	No inhibition with [I] = 0.1 mM, limited soly.
10	9-Iodoacetamido-acridine	D	—	Too insoluble to evaluate
11	Proflavine[c]	A	0.13 ± 0.04	3 Expts. with [I] = 0.5, 1, and 2 mM
12	Acriflavine[d]	A	0.08 ± 0.003	3 Expts. with [I] = 0.5, 1, and 2 mM
13	3,9-Diamino-7-ethoxyacridine (RIVANOL)	A	13.5 ± 2.0	3 Expts. with [I] = 2, 3, and 6 mM
	Phenazines			
14	Phenazine	A	—	Too insoluble to evaluate
	Azaphenanthrenes			
15	Benzo[c]quinoline[e]	A	0.23 ± 0.03	3 Expts. with [I] = 0.1, 0.2, and 0.4 mM
16	Benzo[f]quinoline[f]	A	0.063 ± 0.008	10 Expts. with [I] = 0.05 to 0.4 mM
17	Benzo[h]quinoline[g]	A	0.70 ± 0.03	3 Expts. with [I] = 0.2, 0.3, and 0.4 mM
18	o-Phenanthroline	A	15.1 ± 1.4	3 Expts. with [I] = 2, 5, and 7.5 mM
19	Dibenzo[f,h]quinoline	D	—	No inhibition with [I] = 0.01 mM, limited soly.

[a] As inhibitors of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, pH 7.90, and 0.10 M in sodium chloride. [b] 3-Chloro-9-(ω-diethylamino-α-methylbutylamino)-7-methoxyacridine. [c] 3,6-Diaminoacridine. [d] A mixture of 3,6-diaminoacridine and 3,6-diamino-10-methylacridine. [e] 3:4-Benzoquinoline. [f] 5:6-Benzoquinoline. [g] 7:8-Benzoquinoline.

the locus in which the acridine nucleus in proflavine and acriflavine is so oriented as to place the linearly disposed positive charge in juxtaposition to the anionic feature of the site previously located in the 4 position of quinoline when the latter molecule is at the locus. If this situation prevails one would expect that a 3,6-diaminoacridine with a sulfonate group in the 8 position would have a high K_I value and ones with the sulfonate group in the 5 or 7 position would have very much lower K_I values.

3,9-Diamino-7-ethoxyacridine appears to be another, but less extreme, example of the situation encountered earlier with 9-aminoacridine. As before, more information is required to fully account for the magnitude of the K_I value.

The results obtained with acridine and its derivatives led us to the isomeric benzoquinolines. It will be recalled that K_I = 0.22 mM for acridine. As one departs from the linear array of acridine with its centrally located pyridine nucleus and proceeds through the series benzo[c]-, benzo[f]-, and benzo[h]quinoline, one finds K_I values of 0.22, 0.23, 0.063, and 0.70 mM, respectively. While all these compounds are notable inhibitors, benzo[f]quinoline is the most effective one discovered to date. In this instance $-\Delta F^*_{298}$ = 6 kcal.

That benzo[f]quinoline is approximately four times more effective than acridine or benzo[c]quinoline is not surprising in view of the rule developed earlier for naphthalene derivatives: benzo substitution involving the *amphi* position with respect to the nitrogen atom would be expected to lead to lower K_I values, cf. Figure 1. We expect that when benzo[g]quinoline is examined, its K_I value will approach that of benzo[f]-quinoline. More notable is the order of magnitude difference between benzo[f] and benzo[h]quinoline. In both compounds the pyridine ring is terminal. However, in benzo[h]quinoline the position of the terminal benzo group may be viewed as being *syn* to the nitrogen atom, whereas in benzo[f]quinoline the disposition is *anti*. With reference to Figure 1 it appears that with the nitrogen atom positioned as in α-naphthylamine, or quinoline, effective combination of the aromatic nuclei can proceed along a broadly curved swath proceeding downward from the reference nitrogen atom in a clockwise or counterclockwise direction. The behavior of dibenzo[f,h]quinoline, while obscured by the lack of solubility of this compound in water, is also indicative of the relatively poor combining properties of a *syn* disposition even when coupled with an *anti* array. o-Phenanthroline bears a formal resemblance to benzo[h]quinoline. The additional cyclic nitrogen atom leads to an order of magnitude increase in the value of K_I. The reason for this behavior is not obvious but could be due to a more π-deficient nucleus. At pH 7.9 neither of the nitrogen atoms would be protonated (Lee *et al.*, 1948). If both electronic and steric factors were operative during combination with the active site one would expect p-phenanthroline to have a K_I value smaller than that of o-phenanthroline, but greater than that of benzo[f]quinoline.

POSTULATES

The results described in the preceding paragraphs are sufficiently extensive to permit additional comment on the nature of the interaction of aromatic molecules with the active site of α-chymotrypsin. They will be presented as postulates.

1. *For interactions at a single locus aromatic compounds are more effective inhibitors than their hydroaromatic analogs.* The validity of this postulate is dependent upon the observation that phenol is an order

of magnitude more effective than cyclohexanol as an inhibitor of α-chymotrypsin. It is imperative that further studies be undertaken along these lines.

2. *Monosubstituted benzene derivatives containing a polarizable but uncharged substituent are more effective inhibitors than the parent hydrocarbon.* This empirical generalization, which appears to be valid for benzene derivatives, but not necessarily for those of di-, tri-, and polycyclic aromatic hydrocarbons, is based upon the uniform behavior of twenty-four uncharged monosubstituted benzene derivatives.

3. *The active site of α-chymotrypsin may be hermaphroditic in the sense that one locus may be electron deficient and a second electron rich.* This speculation stems from the observation that uncharged monosubstituted benzene derivatives bearing either electron donating or electron withdrawing substituents have essentially indistinguishable inhibition constants. As noted previously the separate examination of pairs of such inhibitors in competition with selected structurally deficient bifunctional substrates should provide a suitable test of this postulate.

4. *The active site of α-chymotrypsin has a negative charge in close proximity to at least one of the loci involved in interaction with aromatic molecules.* The relatively large inhibition constants of benzoate and benzene sulfonate ion, and those of other anionic inhibitors, provide support for this postulate. The results of other studies on the possible disposition of the negative charge will be reported in separate communications.

5. *All other factors being equal, a structure which provides a larger planar area of interaction than another will result in a greater binding energy and, therefore, a lower value of K_I.* This postulate is in principle equivalent to postulate 5 of Hein and Niemann (1962). For polycyclic aromatic compounds the values of K_I vary not only with the substituent but also with the size of the ring system, generally decreasing in the order monocyclic > bicyclic > tricyclic. Since the difference between the K_I values for the bi- and tricyclic compounds is generally less than between mono- and bicyclic compounds, it appears that a requirement for a minimum coplanar surface area may be associated with given levels of effectiveness as an inhibitor, at least where the interaction involves but one of the several loci present at the active site. Thus pyridine or benzene derivatives with a coplanar surface area of *ca.* 17 sq A are not as effective as quinoline or naphthalene derivatives which have an area of *ca.* 28 sq A. These in turn are poorer inhibitors than the acridines and azaphenanthrenes with planar surface areas of *ca.* 38 sq A. Increasing the area of the phenol molecule from 17 sq A to 34 sq A by adding a second benzene nucleus to form *p*-hydroxybiphenyl reduces the value of K_I from 6.4 to 0.25 mM. Although the latter molecule is not necessarily coplanar, there is no reason to assume that it cannot be so when combined with the locus with which it interacts. Again, increasing the surface area of 4-aminoquinoline from 28 sq A to 50 sq A for 4-amino-2-styrylquinoline decreases K_I from 1.3 to 0.26 mM. Finally, quinoline (28 sq A) has a K_I value of 0.6 mM whereas benzo[*f*]quinoline (38 sq A) has a K_I value of 0.063 mM. Providing there are no significant steric influences, as is obviously the case with benzo[*h*]quinoline, the effect discussed above is seen to be general throughout Tables I through VI. Identical requirements have been found by Albert (1949) for the effectiveness of similar compounds as antibacterial agents. If, for α-chymotrypsin, a value of K_I = 0.10 mM is arbitrarily chosen as the dividing point between inhibitors of two degrees of effectiveness, a necessary but not sufficient requirement is established for a minimum coplanar surface area of 28 sq A for the more effective inhibitors, provided, of course, that interactions with other loci are not involved.

6. *Orientation of aromatic compounds at the locus with which they combine is achieved in part through interaction of a polarizable substituent with a sublocus.* The similarity in behavior of comparable derivatives of naphthylamine and quinoline leads one to the proposition that interaction of the amino group in the former compounds and the cyclic nitrogen atom in the latter with a localized but still undefined feature of the locus is responsible for the common orientation of these compounds. This interaction may involve either proton donation or acceptance. Participation of a charged group is not excluded.

7. *The presence of two or more polarizable substituents in an unsymmetrically substituted molecule may lead to a multiplicity of modes of combination when the interaction involving any one substituent is not dominant.* This postulate is a corollary of the previous one and is largely untested.

8. *The locus with which aromatic compounds frequently combine is surrounded by an unsymmetrically disposed array of structural features which interact with appropriately positioned substituents of the combining molecule. These interactions, if steric, make a negative contribution, and, if coulombic, either a positive or negative contribution to the over-all binding energy.* The behavior of the naphthylaminesulfonates and other derivatives leads one to this postulate.

9. *Effective combination of a polycyclic aromatic compound does not require a completely coplanar complementary surface at the locus. What is required is that the binding energy gained by subtending a greater area of the locus be larger than that lost by the presence within the locus of minor steric obstructions.* If the locus is a curved valley the floor need not be completely flat. However, spurs of the adjacent ridges will be avoided. One is led to this postulate largely because of the information presented in Figure 1 and the behavior of the benzoquinolines.

10. *The locus with which aromatic molecules frequently combine while predominantly flat has greater length than breadth and with reference to its longest dimension is not straight but curved.* This topographical deduction arises from data obtained for the benzoquinolines and that presented in Figure 1.

The preceding ten postulates generalize many of the detailed observations discussed previously. They should serve as a useful guide for future inhibition studies. In order to emphasize the limited scope of the present study as well as its usefulness we wish to comment on the behavior of several of the compounds listed in Table VII.

The fact that coumarin has a value of K_I = 0.67 mM, comparable to that of indole serves to illustrate that the nitrogen containing heterocyclic ring systems have no special virtues except perhaps water solubility. The behavior of fluorescein and of cresol red, which are comparable to phenol as inhibitors, might have been predicted from postulate 10. However, it is noteworthy that the K_I values of these compounds are more suggestive of phenol than of benzoate and benzenesulfonate. Sparteine was examined because of its rigid nonplanar shape. Its lack of inhibitory properties is a confirmation of the general validity of postulate 1. The properties of cholic acid may be obscured because of micelle formation. Those of choline and γ-butyrolactone were expected. Trichloroacetylglycine methyl ester remains the only known completely aliphatic inhibitor which is reasonably effective (Wolf and Niemann, 1963).

TABLE VII
MISCELLANEOUS COMPOUNDS[a]

No.	Inhibitor	Source	K_I (mM)	Remarks
	Oxygen heterocycles			
1	Phthalide	A	1.42 ± 0.2	5 Expts. with [I] = 0.2, 0.5, 1, 1.5, and 2 mM
2	Coumarin	A	0.67 ± 0.07	4 Expts. with [I] = 0.2, 0.5, 1, and 2 mM
3	Fluorescein	A	10.2 ± 0.6	3 Expts. with [I] = 2, 4, and 7.5 mM
	Others			
4	Cresol red	A	4.67 ± 1.7	2 Expts. with [I] = 5 and 25 mM
5	Sparteine	D	—	No inhibition with [I] = 10 mM, limited soly.
6	Cholic acid	A	—	No inhibition with [I] = 80 mM, limited soly.
7	Choline	A	—	No inhibition with [I] = 75 mM
8	γ-Butyrolactone	A	—	No inhibition with [I] = 75 mM

[a] As inhibitors of the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-valine methyl ester in aqueous solutions at 25.0°, *p*H 7.90, and 0.10 M in sodium chloride.

As stated in the introduction, the purpose of this investigation was to provide the basis for a detailed study of the interaction of aromatic compounds with the active site of α-chymotrypsin. It is believed that this purpose has been achieved and direction has been given to future inhibition studies. This has not been the only result because it is now possible to use the data contained herein for other purposes. For example, if faced with the problem of selecting a water-soluble substrate containing a quinoline nucleus in the side chain one would have reason to investigate acetyl-β-(2-quinoline-6-sulfonic acid)alanine methyl ester before others.

TABLE VIII
DISTANCE BETWEEN POINTS OF SUBSTITUTION ON THE NAPHTHALENE NUCLEUS AND VALUES OF K_I[a]

Positions[b]	Relative Distance[c]	K_I (mM) NDS[d]	K_I (mM) NAS[e]
1:2	1.00		41[g]
1:3, 2:4, 1:8	1.73		250
1:4	2.00		185
1:7, 2:8, 1:5	2.65	—[f]	31
1:6, 2:5	3.00	—[f]	4.8
2:7	3.46	400	
2:6	3.61	130	11

[a] Of naphthalenedisulfonates and naphthylaminesulfonates. [b] For the naphthylaminesulfonates first number indicates position of amino group and second that of sulfonate group unless otherwise noted. For the disulfonates 1:3 = 2:4, 1:7 = 2:8, and 1:6 = 2:5. [c] Approximate values based upon assumption of uniformity of bond angles and distances in the naphthalene nucleus. [d] Naphthalenedisulfonates. [e] Naphthylaminesulfonates. [f] No inhibition with [I] = 125 mM. [g] 2-Naphthylamine-1-sulfonate.

REFERENCES

Abrash, H. I. (1961), Ph.D. thesis, California Institute of Technology, Pasadena.
Albert, A. (1949), *Brit. J. Exp. Pathol. 30*, 159.
Albert, A. (1951), The Acridines, London, Arnold.
Albert, A. (1959), Heterocyclic Chemistry, London, Univ. of London Press, p. 57.
Albert, A., and Goldacre, R. (1943), *J. Chem. Soc. 1943*, 454.
Albert, A., and Ritchie, B. (1941), *J. Soc. Chem. Ind. 60*, 120.
Albert, A., and Ritchie, B. (1942), *Org. Syn. 22*, 5.
Albert, A., and Royer, R. (1949), *J. Chem. Soc. 1949*, 1803.
Applewhite, T. H., Martin, R. B., and Niemann, C. (1958a), *J. Am. Chem. Soc., 80*, 1457.
Applewhite, T. H., Waite, H., and Niemann, C. (1958b), *J. Am. Chem. Soc. 80*, 1468.
Bachman, G., and Heisey, L. V. (1949), *J. Am. Chem. Soc. 71*, 1987.
Bernhard, R. A. (1955), Ph.D. thesis, California Institute of Technology, Pasadena.
Bistrzycki, A., and Przeworski, G. (1912), *Ber. 45*, 3483.
Brenner, M., and Huber, W. (1953), *Helv. Chim. Acta 36*, 1109.
Brown, H. C., McDaniel, O. H., and Häfliger, O. (1955), *in* Braude, E. A., and Nachod, F. C., eds., Determination of Organic Structures by Physical Methods, New York, Academic.
Elderfield, R. C., and Siegel, M. (1951), *J. Am. Chem. Soc. 73*, 5622.
Foster, R. J., and Niemann, C. (1955a), *J. Am. Chem. Soc. 77*, 3370.
Foster, R. J., and Niemann, C. (1955b), *J. Am. Chem. Soc. 77*, 3365.
Foster, R. J., Shine, H. J., and Niemann, C. (1955), *J. Am. Chem. Soc. 77*, 2378.
Graebe, C. (1888), *Ann. 247*, 291.
Hein, G., and Niemann, C. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1341.
Hein, G. E., and Niemann, C. (1962), *J. Am. Chem. Soc. 84*, 4487, 4495.
Hofstee, B. H. J. (1960), *Biochim. Biophys. Acta 44*, 194.
Huang, H. T., and Niemann, C. (1952), *J. Am. Chem. Soc. 74*, 5963.
Huang, H. T., and Niemann, C. (1953), *J. Am. Chem. Soc. 75*, 1395.
Jackson, W. (1962), Unpublished data obtained in these laboratories.
Jacobs, W. A., Heidelberger, M., and Rolf, I. P. (1919), *J. Am. Chem. Soc. 41*, 470.
Julian, P. L., and Printy, H. C. (1949), *J. Am. Chem. Soc. 71*, 3206.
Kaufman, S., and Neurath, H. (1949), *J. Biol. Chem. 181*, 623.
Korte, F., and Barkemeyer, H. (1956), *Ber. 89*, 2400.
Krueger, J., and Mosettig, E. (1940), *J. Org. Chem. 5*, 313.
Kurtz, A. N., and Niemann, C. (1961), *Biochim. Biophys. Acta 53*, 324.
Lee, T. S., Kolthoff, I. M., and Leussing, D. L. (1948), *J. Am. Chem. Soc. 70*, 2348.
Loewus, M. W., and Briggs, D. R. (1952), *J. Biol. Chem. 199*, 857.
Meisenheimer, J., and Connerade, E. (1903), *Ann. 330*, 165.
Moore, S., and Link, K. P. (1940), *J. Org. Chem. 5*, 642.
Neilands, J. B., and Cannon, M. D. (1955), *Anal. Chem. 27*, 29.
Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.
Neurath, H., and Gladner, J. A. (1951), *J. Biol. Chem. 188*, 407.

Neurath, H., and Schwert, G. (1950), *Chem. Rev. 46*, 69.
Rapp, J. R. (1962), Unpublished data obtained in these laboratories.
Stollé, von R. (1930), *J. Prakt. Chem. 128*, 1.
Waite, H. R., and Niemann, C. (1962), *Biochemistry 1*, 250
Wallace, R. A., and Peterson, R. (1962), Unpublished studies conducted in these laboratories.
Wilkinson, J. H., and Finor, I. L. (1946), *J. Chem. Soc. 1946*, 115.
Wolf, J. P., III, and Niemann, C. (1963), *Biochemistry 2*, 493.

The Effect of Aprotic Dipolar Organic Solvents on the Kinetics of α-Chymotrypsin-Catalyzed Hydrolyses*

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The kinetics of several α-chymotrypsin-catalyzed hydrolyses have been determined in mixtures of water and three dipolar aprotic solvents, dioxane, acetone, and acetonitrile. The effect of the organic solvent on k_2 (the acylation constant), k_3 (the deacylation constant), k_{cat} (the catalytic or turnover constant), K_m (the Michaelis constant) and k_2/K_m was determined. The major effect of the organic solvent is to increase K_m (or decrease k_2/K_m) with only a small (lowering) effect on the rate constants, k_2, k_3, and k_{cat}. Correlations of the kinetic data with simple competitive inhibition by the organic solvent or with the dielectric constant of the medium are only qualitatively successful. However, a treatment involving the combination of these two factors affords a quantitative correlation of the kinetic data up to 15% organic solvent. The order of competitive inhibitors for α-chymotrypsin is: dioxane > acetonitrile > acetone > methanol. The concentration of active sites of an α-chymotrypsin solution is not affected by 32% dioxane-water, as measured by spectrophotometric titration with *N-trans*-cinnamoylimidazole.

In our continuing studies on the kinetics and mechanism of hydrolyses catalyzed by proteolytic enzymes, it was of interest to investigate the effect of dipolar aprotic solvents on the kinetics of such reactions. Investigations of the effect of such solvents on two groups of kinetic constants of α-chymotrypsin-catalyzed hydrolyses were carried out: (1) the effect on the turnover constant k_{cat} and the Michaelis constant K_m(app) (defined by equation 1); and (2) the effect on the acylation rate constant k_2/K_m (defined by equation 2).

$$E + S \underset{}{\overset{K_m(app)}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \qquad (1)[1]$$

$$E + S \overset{K_m}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (2)$$

$$ES' + P_1$$

The effect of organic solvents on the kinetics of chymotrypsin reactions has been noted in the literature previously. Kaufman *et al.*, (1949) reported a linear decrease in the "proteolytic coefficient" (k_{cat}/K_m) with increasing methanol concentration for the α-chymotrypsin-catalyzed hydrolysis of glycyl-L-tyrosinamide and benzoyl-L-tyrosine ethyl ester. Further, Kaufman and Neurath (1949) studied the α-chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosinamide at three methanol concentrations. From water to 5.15 M methanol, K_m was found to exhibit a 2.5-fold increase while k_{cat} remained constant. A plot of $1/K_m$ vs. methanol concentration was found to be linear. In the chymotrypsin-catalyzed hydrolysis of methyl hydrocinnamate in three aqueous methanol solutions up to 25% methanol by weight, it was found that K_m increases while k_{cat} decreases with increasing methanol concentration (Barnard and Laidler, 1952). A dielectric constant effect was suggested as a partial explanation of the results. More recently a marked inhibition of the same reaction by methanol was rationalized on the basis of a specific interaction between the enzyme and methanol (Stein and Laidler, 1959).

An extensive study of the effect of several organic solvents on the kinetics of the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate was reported by Applewhite, *et al.* (1958). In acetone-water and dioxane-water solutions, it was found that the value of k_{cat} remains constant whereas the value of K_m increases with increasing solvent concentration. These data could not be explained on the basis of simple competitive inhibition or of a dielectric constant effect. A model to explain the data was suggested consisting of the partitioning of the substrate between two phases, the hydrated enzyme and the aqueous-aprotic solvent. In this study, it was also found that methanol increases K_m while it decreases k_{cat}.

In methanol-water solutions, ambiguous results can be obtained from the fact that the protic organic solvent, methanol, can participate directly in the enzymatic reaction, as well as exert a generalized solvent effect.[2] The direct participation of methanol and other alcohols in enzymatic processes has been well documented (McDonald and Balls, 1956; Koshland and Herr, 1957; Bender and Glasson, 1960). The participation of methanol in the enzymatic process results in the conversion of the hydrolysis reaction to a

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[1] K_m(app) of eq. (1) is related to K_m of equation (2) by the relationship: $K_m(app) = [k_3/(k_2 + k_3)]K_m$ (Gutfreund and Sturtevant, 1956). K_m (app) and K_m will not be further differentiated in this paper.

[2] The results cited are probably not ambiguous, because in those reactions cited acylation is probably largely or solely rate-controlling. Under these circumstances, the direct participation of methanol is of no consequence.