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The Interaction of Trypsin with Neutral Substrates and Modifiers*

Barbara M. Sanborn† and George E. Hein‡

ABSTRACT: In an attempt to assess the role of the positive charge in trypsin specificity, the hydrolysis constants of neutral substrates were compared with their charged analogs. Replacement of the side chain of arginine by that of citrulline and of lysine by that of heptyline resulted in decreases of 10^2–10^6 in k_0/K_0. Enzyme treated to remove chymotrypsin impurities did not lose activity toward these substrates. Trypsin inactivated with diisopropylphosphofluoridate or 1-chloro-3-tosylamido-7-amino-2-heptanone failed to hydrolyze the neutral substrates. Ternary complex formation involving the enzyme, a positively charged molecule, and a neutral molecule was indicated by the following data. (1) The rate of hydrolysis of a given substrate was affected in a qualitatively different manner by modifiers of different charge types at equivalent $[M]/K_i$ ratios: phenol was a competitive inhibitor of benzoylcitrulline methyl ester hydrolysis while phenylguanidinium chloride was a noncompetitive inhibitor. (2) A given modifier affected the hydrolysis of different substrate charge types in a qualitatively different manner: phenol was a competitive inhibitor of benzoylcitrulline methyl ester hydrolysis and a noncompetitive inhibitor of benzoylarginine methyl ester hydrolysis. An auxiliary binding site to which neutral molecules preferentially bind is thus implicated.

This paper seeks to confirm and extend earlier observations on the relationship between the hydrolysis of neutral substrates by trypsin and evidence for a second or auxiliary binding site for this enzyme (Sanborn and Hein, 1967). Early work on enzymatic cleavage catalyzed by trypsin established its high specificity for bonds in which L-lysine or L-arginine contributed the carbonyl function (Bergmann *et al.*, 1939). These original conclusions on specificity were supported by subsequent studies on ester and amide derivatives (Neurath and Schwert, 1950). More recently, however, reports of neutral substrates hydrolyzed by trypsin have appeared (Inagami and Sturtevant, 1960; Inagami and Mitsuda, 1964; Cohen and Petra, 1967).

That trypsin specificity is highly dependent upon the length of the side chain containing the positive charge has been shown by Elmore and coworkers using lysine and arginine homologs (Baines *et al.*, 1964; Baird *et al.*, 1965; Elmore *et al.*, 1967). Inhibitory capacity is also dependent upon the nature of the side chain and its orientation with respect to the positive charge (Mares-Guia and Shaw, 1965, 1967; Inagami, 1964; Geratz, 1966, 1967).

Evidence for an auxiliary binding site which can accommodate both positively charged (Trowbridge *et al.*, 1963) and neutral (Howard and Mehl, 1965) molecules and result in increased catalysis has been reported. The present study seeks to further clarify the role of the positive charge in trypsin specificity by investigating combinations of charged substrates (arginine (Ia) and lysine (IIa) derivatives) and neutral analogs (citrulline (Ib) and heptyline (IIb) derivatives). In the process of studying the behavior of positively charged and neutral modifiers toward these substrates, information has been gained about the nature of an auxiliary binding site.

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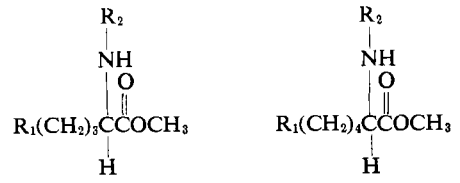
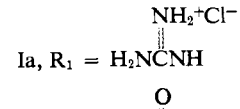
† Public Health Service predoctoral fellow, 1965–1967. This paper was taken from the thesis submitted in partial fulfillment of the requirements for the degree of Ph.D., Boston University, 1968. Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. 02154.

‡ Present address: Elementary Science Study, Education Development Center, Newton, Mass. 02160.



R_2 = tosyl, benzoyl

R_2 = tosyl, acetyl



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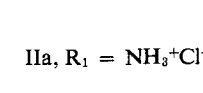
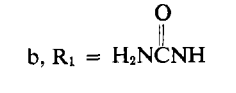


IIa, R_1 = $NH_3^+Cl^-$



b, R_1 = H_2NCNH

b, R_1 = CH_3

Experimental Procedures

Heptyline Substrates. The heptyline derivatives were prepared as follows. DL-Heptyline (2-aminoheptanoic acid) was made by the condensation of *n*-pentyl bromide with ethyl acetamidomalonate in 70% yield, mp 270° dec (Albertson, 1946). Chloroacetyl-DL-heptyline was formed by addition of chloracetyl chloride to the DL-acid in 2 N sodium hydroxide. It was isolated by acidifying the solution with hydrochloric acid and extracting with ethyl acetate. Evaporation of the solvent and recrystallization of the resulting solid from ether gave chloroacetylheptyline (mp 100–101°).

Enzymatic resolution of the DL compound was then carried out (Greenstein, 1957). A solution of the chloracetyl derivative (20.8 g) in water was brought to pH 6.9 with lithium hydroxide, diluted to 500 ml, divided into two portions, and placed in a constant-temperature bath at 38°. To each portion was added 10 mg of acylase (Nutritional Biochemical Co.) followed by 3 mg on each of 4 successive days. The solutions were then acidified with glacial acetic acid until the precipitate disappeared, clarified with carbon, and filtered. Ethanol was added and the solutions were chilled. The resulting precipitates were collected and yielded 6.0 g of crude L-heptyline (88% yield). This was refluxed with 50 ml of 95% ethanol and filtered hot. The insoluble material had a specific rotation of $[\alpha]_D^{25}$ +34.1° (*c* 1.26, 6 N HCl).

L-Heptyline methyl ester was prepared by esterification of L-heptyline with thionyl chloride in methanol (Boissonas *et al.*, 1958). The resulting solid melted at 120–122°. Acetylation according to Applewhite *et al.* (1958) using acetic anhydride resulted in colorless crystals melting at 31.5–32.5° when recrystallized twice from isopropyl ether in the freezer, $[\alpha]_D^{25}$ −20.8° (*c* 0.78, ethanol).[1]

Benzoyl-L-heptyline methyl ester was prepared from 9.1 g of L-heptyline methyl ester by reaction with benzoyl chloride (Applewhite *et al.*, 1958). The resulting material was dissolved in benzene and applied to a 1 × 15 cm silica gel column which was then washed with benzene and finally chloroform. Infrared spectra of the effluents were used to locate the desired product which was recrystallized from petroleum ether: mp 43.5–44.5°, $[\alpha]_D^{25}$ −12.0° (*c* 0.86, 6 N HCl). *Anal.* Calcd for $C_{15}H_{21}NO_3$: C, 68.4; H, 7.98; N, 5.32. Found: C, 68.3; H, 7.96; N, 5.21.[2]

Other Substrates. Acetylglycine methyl ester was prepared from acetylglycine by esterification with thionyl chloride (Wolf and Niemann, 1959). Vacuum distillation and recrystallization from methanol–isopropyl ether gave a solid melting at 56–57.5°.

Esterification of N^α-acetyl-D-lysine (Cyclo Chemical Corp.) with thionyl chloride resulted in an oil which solidified after washing five times with dry ether and drying *in vacuo* over phosphorous pentoxide at room temperature for 1 week. Acetyl-D-lysine methyl ester hydrochloride was hygroscopic and was weighed out under nitrogen in a dry box, $[\alpha]_D^{25}$ +22.2° (*c* 2.04, water). *Anal.* Calcd for $C_9H_{19}ClN_2O_3$: C, 42.0; H, 7.40; N, 10.9. Found: C, 42.13; H, 7.44; N, 10.8.

N^α-Hippuryl-L-lysine methyl ester hydrochloride was prepared by esterification of hippuryllysine (Mann Research Corp.). The resulting oil was crystallized from isopropyl ether at −15° and dried *in vacuo* at room temperature for 36 hr. The elemental analysis of the crystalline material was reasonable for the monohydrate. *Anal.* Calcd for $C_{16}H_{26}ClN_3O_5$: C, 51.2; H, 6.93; N, 11.2. Found: C, 51.9; H, 6.95; N, 11.3.

N^α-Acetyl-L-lysine methyl ester hydrochloride and N^α-benzoyl-L-lysine methyl ester hydrochloride (Cyclo Chemical Corp.) were washed with dry ether, dried *in vacuo*, and weighed out under nitrogen.

Chromatographically pure benzoylarginine methyl ester hydrochloride was purchased from Mann Research Laboratories and used as received. N^α-Tosyl-L-ornithine methyl ester hydrochloride was obtained from Cyclo Chemical Corp. as were TLCK[3] and TPCK.

Benzoylcitrulline methyl ester was synthesized as described previously (Sanborn and Hein, 1967). N^α-Toluenesulfonyl-L-citrulline was prepared from citrulline by reaction with toluenesulfonyl chloride (McDonald and Balls, 1956), mp 175–176.5° (58%). Esterification was achieved by treatment with thionyl chloride. The resulting oil was dissolved in chloroform and washed successively with cold 1 N sodium hydroxide, 1 N hydrochloric acid, and saturated sodium bicarbonate. The solution was dried over magnesium sulfate and filtered; the solvent was evaporated *in vacuo*. The oil obtained was crystallized with difficulty from isobutyl alcohol–isopropyl ether (2:1) upon scratching: mp 99.5–100°, $[\alpha]_D^{25}$ +20.7° (*c* 1.55, 6 N HCl). *Anal.* Calcd for $C_{14}H_{21}N_3O_5S$: C, 49.0; H, 6.13; N, 12.2. Found: C, 49.09; H, 6.24; N, 12.01.

Modifiers. Ethylamine (70% in water) and *n*-butylamine were used as received. The concentrations were determined by titration with hydrochloric acid to a

[1] Jones *et al.* (1965) report [α] +22.6° (*c* 1.5, methanol) but have communicated that this is the rotation for the D enantiomer.

[2] Analyses by Spang Microanalytical Laboratory, Ann Arbor, Mich.

[3] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BAME, N^α-benzoyl-L-arginine methyl ester; BCME, N^α-benzoyl-L-citrulline methyl ester; TOME, N^α-tosyl-L-ornithine methyl ester; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, (1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

methyl red end point. Phenol (Merck, reagent) was used without further purification. In some instances the concentration was determined by the method of Fritz and Keen (1953).

Phenylurea was synthesized from aniline and sodium cyanate as described by Vogel (1962a), mp 144–145.5°. Toluenesulfonamide was prepared according to Vogel (1962b), mp 136–136.5°.

Phenylguanidinium nitrate was prepared according to the method of Bannard *et al.* (1958) from 1-guanyl-3,5-dimethylpyrazole nitrate and aniline. The nitrate salt was converted into a hydrated carbonate salt (mp 128–137°) *via* the sulfate (Smith, 1924) and generated as the hydrochloride *in situ* by the addition of hydrochloric acid. Elemental analysis of the carbonate was consistent with one-half molecule of water per carbonate. *Anal.* Calcd for $C_{15}H_{21}N_6O_{3.5}$: C, 52.7; H, 6.16; N, 24.6. Found: C, 52.3; H, 6.33; N, 24.7.

Enzymes and Kinetic Procedure. The trypsin used in these studies was twice crystallized and salt free (Worthington Biochemical Corp., lot no. 6402). Dialysis against 0.001 N hydrochloric acid and lyophilization removed diffusable protein but did not affect the nature of the reactions in any way. Diisopropylphosphoryltrypsin was purchased from Worthington. Tosyllysylmethyltrypsin was prepared according to the method of Shaw *et al.* (1965).

The kinetics of hydrolysis were followed on a difunctional recording titrator (International Instrument Co.) at 25 ± 0.1° under a stream of nitrogen. All experiments were performed in a 10-ml volume in 0.1 M potassium chloride at pH 7.0 unless otherwise indicated. At low substrate concentrations a 100-ml volume was used to increase reproducibility. Enzyme solutions were prepared fresh daily and stored in ice; the concentration was determined by weight or absorbance at 280 mμ using the optical factor 0.651 mg ml^{-1} (OD unit)$^{-1}$ and assuming a molecular weight of 24,000 (Trowbridge *et al.*, 1963). Rate constants reported were calculated on the basis of total enzyme concentration. Carbon dioxide free water was used in all solutions.

Irreversible inactivation by TLCK was followed using the procedure of Shaw *et al.* (1965) in 10% dioxane.

Results

Kinetic Treatment. A minimum general reaction scheme including ternary complex formation involving enzyme, substrate, and modifier is given by eq 1. The

$$\begin{array}{ccccc} E + S & \overset{K_0}{\rightleftharpoons} & ES & \overset{k}{\longrightarrow} & E + P_1 \\ + & & + & & \\ M & & M & & \\ \uparrow\downarrow K_i & & \uparrow\downarrow \alpha K_i & & \\ EM + S & \overset{\alpha K_0}{\rightleftharpoons} & EMS & \overset{\beta k}{\longrightarrow} & E + P_1 + P_2 \\ \downarrow \gamma k & & & & \\ E + P_2 & & & & \end{array} \qquad (1)$$

steady-state rate expression for this scheme simplifies to

$$v = \frac{k[E_0][S]}{K_0(1 + [M]/K_i) + [S](1 + [M]/\alpha K_i)} \qquad (2)$$

when M is not a substrate (or γk is much smaller than k) and βk is negligible compared with k (*i.e.*, EMS is not converted into products at any appreciable rate). Should EMS decompose to give products, a plot of the reciprocal rate *vs.* [M] would not be linear. Deviations from linearity were not observed within experimental error in inhibition studies over tenfold ranges in [M], and βk was therefore neglected in the calculations. An increase rather than a decrease in reaction rate with increasing [M] could be evidence that βk is not zero in all cases.

Data in the form $1/v$ *vs.* [M] at a constant [S] value were evaluated using a computer program which determined the best common intercept of three least-squares lines (the x coordinate of which is $-K_i$) and the error in K_i (Hein and Powell, 1967). α is a function of the ratio of the y coordinate of the common point and the y intercept for a given line. If α is one, M binds as well to ES as it does to E (noncompetitive behavior). An α value less than 1 could indicate a cooperative interaction which enhances the binding of M to ES (uncompetitive or coupling behavior; Webb, 1963a). When $1 < \alpha < 20$, mixed inhibition occurs, this implies an effect of M on S binding or *vice versa*. α values greater than 20 indicate effectively competitive inhibition. It has been shown that a negative α is also an indication of competitive behavior and reflects the limit of the computer program for these kind of data (Sanborn, 1968). One advantage of the computer program is that it permits objective assessment of both the type of inhibition observed and the errors involved. All data on substrates were fitted to least-squares lines from which the appropriate constants and their probable errors were determined.

Hydrolysis of Neutral Substrates. Table I lists the results for the hydrolysis of neutral substrates and the comparison with analogous charged substrates. The absence of the positive charge has a marked effect on the kinetic constants in all four cases. Also listed in Table I are other compounds studied as substrates for trypsin during the course of this investigation. Changes in the kinetic constants compared with a specific substrate are of the same order of magnitude for neutral compounds, the D enantiomer of a charged substrate, and for ornithine, a positively charged substrate possessing a shorter side chain than lysine. Acetylglycine methyl ester, which is lacking both the positive charge and the hydrocarbon side chain, is an even poorer substrate.

The hydrolysis of uncharged substrates is not due to chymotrypsin impurities in the trypsin preparation. Incubation of the enzyme with a 40-fold molar excess of TPCK did not affect the rate of hydrolysis of BAME or BCME. Similar treatment of chymotrypsin resulted in loss of all catalytic activity toward BCME and acetyltyrosine ethyl ester.

Trypsin treated with diisopropylphosphofluoridate to phosphorylate a catalytically essential serine (Balls

TABLE I: Substrates of Trypsin.

Compounds	Charge[a]	K_0 (M)	k_0 (sec^{-1})	k_0/K_0 (M^{-1} sec^{-1})
N^{α}-Benzoyl-L-arginine methyl ester hydrochloride[b] (6)[c]	+	$9.6 \pm 0.4 \times 10^{-6}$	9.3 ± 0.6	9.7×10^{5}
N^{α}-Benzoyl-L-citrulline methyl ester (8)	0	$4.1 \pm 1.3 \times 10^{-2}$	0.14 ± 0.05	3.5
N^{α}-Tosyl-L-arginine methyl ester hydrochloride[d]	+	1.3×10^{-5}	60	4.8×10^{6}
N^{α}-Tosyl-L-citrulline methyl ester[e] (1)	0	$9.1 \pm 1.6 \times 10^{-2}$	0.39 ± 0.07	4.3
N^{α}-Acetyl-L-lysine methyl ester hydrochloride (3)	+	$2.5 \pm 0.01 \times 10^{-4}$	52 ± 2	2.1×10^{5}
N-Acetyl-L-heptyline methyl ester[f] (1)	0	$4.9 \pm 1.7 \times 10^{-2}$	0.44 ± 0.16	9.0
N^{α}-Benzoyl-L-lysine methyl ester hydrochloride (1)	+	$5.5 \pm 1.9 \times 10^{-5}$	1.8 ± 0.7	3.3×10^{4}
N-Benzoyl-L-heptyline methyl ester[g] (1)	0	$1.0 \pm 0.2 \times 10^{-4}$	$9.4 \pm 0.7 \times 10^{-3}$	9.0×10^{1}
N^{α}-Hippuryl-L-lysine methyl ester hydrochloride	+	$1.7 \pm 0.2 \times 10^{-4}$	76 ± 6.6	4.4×10^{5}
N^{α}-Tosyl-L-ornithine methyl ester hydrochloride[h]	+	$1.9 \pm 0.05 \times 10^{-2}$	1.4 ± 0.4	7.4×10^{1}
N^{α}-Acetyl-D-lysine methyl ester hydrochloride	+	$2.0 \pm 0.1 \times 10^{-3}$	$7.3 \pm 0.7 \times 10^{-2}$	3.6×10^{1}
Acetylglycine methyl ester	0	1.4 ± 0.2	$4.1 \pm 0.8 \times 10^{-2}$	2.9×10^{-2}

[a] Net ionic charge on substrate at pH 7. [b] 100-ml volume. [c] The numbers in parentheses indicate the number of separate experiments included in the least-squares calculations. [d] Trowbridge *et al.* (1963). [e] 15% acetonitrile. [f] 6% acetonitrile. [g] 18% acetonitrile; $K_0 = 1.5 \pm 0.4 \times 10^{-4}$ M and $k_0 = 3.0 \pm 0.3 \times 10^{-2}$ in 38% acetonitrile. [h] pH 6.75.

and Jansen, 1952), or TLCK to alkylate a specific histidine residue (Shaw *et al.*, 1965) does not hydrolyze BCME. Thus the same residues are implicated in the catalytic mechanism of hydrolysis for both types of substrates.

The above results are of interest but are not entirely unexpected since trypsin is known to show some activity toward neutral substrates. What was unusual about these compounds was the lack of competitive behavior exhibited between substrates and modifiers of different charge types.

Positive Substrates and Positive Modifiers. As might be expected, positively charged substrates and modifiers showed competitive behavior. Phenylguanidine hydrochloride was the competitive inhibitor of tosylornithine methyl ester ($K_i = 1.0 \pm 0.1 \times 10^{-4}$ M, pH 6.75) and of hippuryllysine methyl ester ($K_i = 7.4 \pm 0.8 \times 10^{-5}$ M). These results compare favorably with the competitive behavior of phenylguanidine sulfate and benzoylarginine *p*-nitroanilide, $K_i = 7.2 \times 10^{-5}$ M (pH 8.2) (Mares-Guia and Shaw, 1965). Similarly N^{α}-acetyl-D-lysine methyl ester was a competitive inhibitor of BAME ($K_i = 1.6 \pm 3.7 \times 10^{-4}$ M).

Neutral Substrates and Neutral Modifiers. BCME was inhibited competitively by phenylurea ($K_i = 1.1 \pm 0.1 \times 10^{-1}$ M, 25% dioxane) and by toluenesulfonamide ($K_i = 9.6 \pm 0.4 \times 10^{-3}$ M). Figure 1 shows the effect of phenol on BCME hydrolysis. Competitive inhibition is not immediately distinguished from mixed inhibition in this type of plot but was indicated from the computer results ($K_i = 5.4 \pm 0.6 \times 10^{-2}$ M, $\alpha = -1$).

Positively Charged Substrates and Neutral Modifiers. Phenylurea, a competitive inhibitor of BCME, had a slight activating effect on BAME under comparable conditions and virtually no effect on acetyllysine methyl ester or tosylornithine methyl ester hydrolysis as shown in Table II. Toluenesulfonamide also had a slight acti-

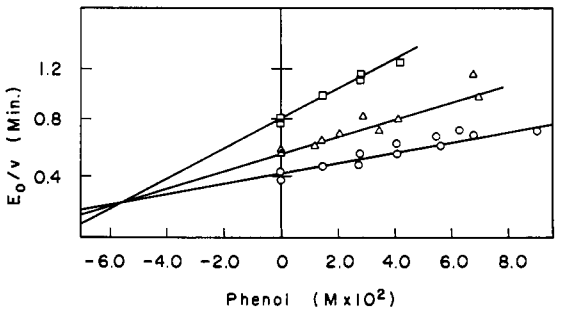


FIGURE 1: The effect of phenol on the hydrolysis of benzoylcitrulline methyl ester. Substrate concentrations are (□) 0.69×10^{-2} M, (△) 1.08×10^{-2} M, and (○) 1.54×10^{-2} M.

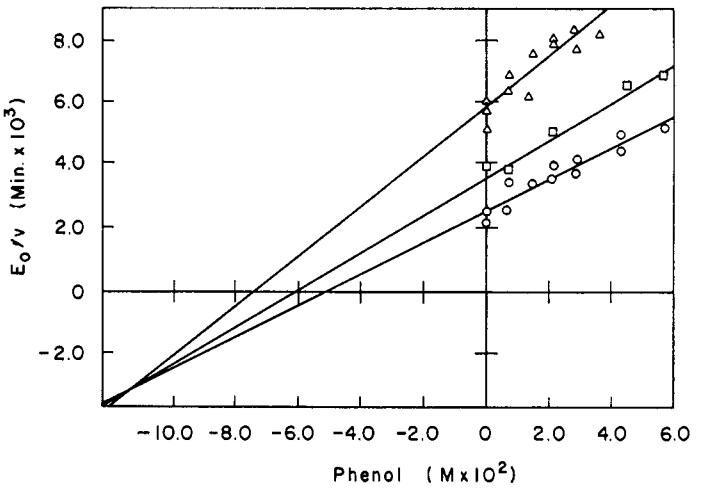


FIGURE 2: The effect of phenol on the hydrolysis of benzoylarginine methyl ester in a 100-ml volume. Substrate concentrations are (△) 0.76×10^{-5} M, (□) 1.03×10^{-5} M, and (○) 3.30×10^{-5} M.

vating effect on BAME. This is to be distinguished from the activation of tosylarginine methyl ester hydrolysis observed by Howard and Mehl (1965) under conditions of substrate saturation. In the present case the primary substrate site is not saturated and toluenesulfonamide might have been expected to act as a competitive inhibitor.

In contrast to its effect on BCME hydrolysis, phenol showed at least noncompetitive behavior and perhaps even uncompetitive behavior (Webb, 1963a) with BAME. Figure 2 shows a representative sampling of the data. The lines are generated by the computer as described. The inhibition constant, $K_i = 12 \pm 1.4 \times 10^{-2}$ M, is higher than that observed with BCME; α calculated from the three lines was 0.44, 0.37, and 0.38.

TABLE II: The Effect of Phenylurea on the Hydrolysis of Trypsin Substrates.

Concn of Phenylurea (M × 10^2)	Obsd Rel Rate	Calcd Rel Rate Assuming Competitive Behavior
Tosylornithine methyl ester (7.71×10^{-3} M)[a]		
0.00	1.00	1.00[b]
5.52	0.88	0.77
6.90	0.90	0.74
8.28	0.91	0.70
9.67	0.92	0.66
10.9	0.89	0.64
Acetyllysine Methyl Ester (6.1×10^{-4} M)[c]		
0.00	1.00	1.00[d]
0.31	1.08	0.96
0.92	0.87	0.90
1.23	0.91	0.88
1.54	0.88	0.83
1.84	0.89	0.81

[a] pH 6.8, 10% acetonitrile. [b] Calculated assuming $K_0 = 1.2 \times 10^{-2}$ M, $k_0 = 0.77$ sec^{-1}, $K_i = 1.2 \times 10^{-1}$ M. [c] 25% dioxane. [d] Calculated assuming $K_0 = 2.5 \times 10^{-4}$ M, $k_0 = 52$ sec^{-1}, and $K_i = 1.2 \times 10^{-1}$ M.

While acetyl-D-lysine methyl ester, a slowly hydrolyzed substrate of trypsin (see Table I) is effectively a competitive inhibitor of BAME hydrolysis, BCME does not behave in this manner (Sanborn and Hein, 1967). Neither is BCME competitive with acetyl-L-lysine methyl ester. Also, acetylglycine methyl ester has no effect on BAME hydrolysis.

Neutral Substrates and Positively Charged Modifiers. Phenylguanidine hydrochloride has been reported to show noncompetitive behavior with BCME ($K_i = 2.0 \pm 0.3 \times 10^{-4}$ M, $\alpha = 0.92 \pm 0.08$, 0.87 ± 0.07, and 0.77 ± 0.10, 25% dioxane) (Sanborn and Hein, 1967). Ethylamine, a competitive inhibitor of benzoylarginine ethyl ester ($K_i = 6.2 \times 10^{-2}$ M) (Inagami, 1964), is a noncompetitive inhibitor of BCME as shown in Figure 3 ($K_i = 6.6 \pm 1.1 \times 10^{-2}$ M, $\alpha = 0.58 \pm 0.25$, 0.53 ± 0.26, and 0.54 ± 0.26, pH 6.65). Butylamine, however, is a competitive inhibitor ($K_i = 5.4 \times 10^{-4}$ M) with a constant somewhat smaller than reported by Inagami (1964), $K_i = 1.7 \times 10^{-3}$ M.

The Effect on Irreversible Inactivation. As shown in Figure 4, phenylguanidine slows the rate of inactivation of trypsin by the active site specific agent TLCK. If the action is strictly competitive and if autolysis is neglected, the observed second-order rate constant, k, will be replaced by the expression $k/(1 + [M]/K_i)$. Using this expression, a K_i of 3.6×10^{-4} M was calculated at pH 6.0 in 10% dioxane. This is in reasonable agreement with the K_i values obtained against substrates considering the changes in conditions. Figure 4 also shows that phenylurea had virtually no effect on the action of TLCK. This behavior is consistent with the observed failure of phenylurea to compete with positively charged substrates.

Discussion

The Effect on the Kinetic Constants of Replacement of the Positive Charge with a Neutral Isostere. Removal of the positive charge from trypsin substrates results in a greater than 100-fold increase in K_0 (or decrease in k_0/K_0). This is considerably more than the 8- to 30-fold

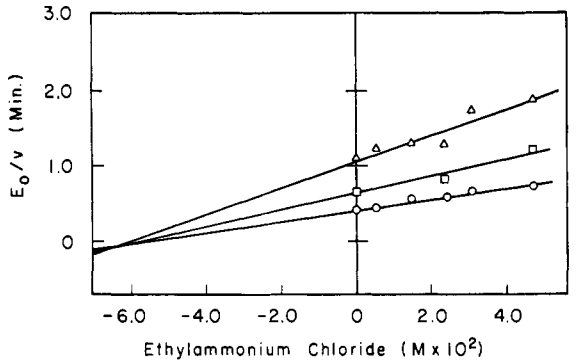


FIGURE 3: The effect of ethylammonium chloride on the hydrolysis of benzoylcitrulline methyl ester at pH 6.75. Substrate concentrations are (▲) 0.53 × 10^{-2} M, (□) 0.80 × 10^{-2} M, and (○) 1.34 × 10^{-2} M.

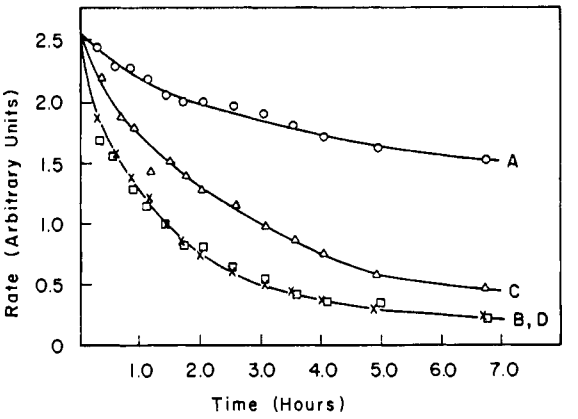


FIGURE 4: The effect of phenylguanidinium chloride and phenylurea on the inactivation of trypsin by TLCK at pH 6.05 in 0.2 M Tris-maleate buffer containing 10% dioxane. Solutions are 6.8 × 10^{-6} M in trypsin, 20 × 10^{-6} M in TLCK, 1.9 × 10^{-4} M in phenylguanidinium chloride, and 3.1 × 10^{-2} M in phenylurea as follows. Line A: (○) trypsin; line B: (×) trypsin and TLCK; line C (△) trypsin, TLCK, and phenylguanidine; and line D: (□) trypsin, TLCK, and phenylurea.

increases observed in similar comparisons for a variety of systems, notably hapten–antibody interactions, acetylcholinesterase substrate specificity, and sarcosine oxidase specificity (Webb, 1963b). The present study indicates that for trypsin the neutral substrates are preferentially binding somewhere other than the site for positively charged molecules. This may be reflected in the larger changes in K_0.

Evidence for Ternary Complex Formation. The presence of a second binding site on trypsin has been suggested previously by observations under conditions in which the primary binding site is saturated (Trowbridge *et al.*, 1963; Howard and Mehl, 1965). These conditions were necessary because the materials used were substrates or products which would interact at both sites, the auxiliary binding site having a much larger K_0 than the primary site. Toluenesulfonamide was not such a substance, however, and in the present study it was shown not to affect BAME hydrolysis when [S] $\cong K_0$. This means that toluenesulfonamide *preferentially* binds to a second binding site, indicating a specificity requirement there also.

The evidence for ternary complex formation when neutral and positively charged molecules are involved can be summarized in several ways. First, the rate of hydrolysis of a given substrate is affected in a *qualitatively* different manner by modifiers of different charge types at equivalent [M]/K_i values. Table III shows the comparative data for the effect of phenylguanidine and phenylurea on both tapes of substrates. Although tosylornithine methyl ester, a positively charged substrate, has hydrolysis constants more similar to those of BCME, a neutral substrate, than BAME, it is affected by modifiers in a manner similar to that of BAME. The qualitative difference in modifier behavior is thus consistent with charge classifications rather than substrate superiority. Second, a given modifier affects the hydrolysis of substrate charge types in a qualitatively different manner as shown in Table IV. Again deviations from competitive behavior occur between molecules of different charge types. Third, the action of the specific irreversible inhibitor TLCK was unaffected by the presence of phenylurea but inhibited by phenylguanidine. Phenylurea has been shown to interact with trypsin since it is a competitive inhibitor of BCME. Therefore the above result is consistent with the model for ternary complex formation derived from hydrolysis kinetics. Fourth, these conclusions obtained by kinetic means have been substantiated by the technique of equilibrium dialysis. As discussed in detail elsewhere (Sanborn and Bryan, 1968), the effect of phenol on the binding of [^{3}H]phenylguanidinium ion to trypsin is not consistent with competitive behavior.[4]

The Location of the Auxiliary Binding Site. Several pieces of evidence point to the spatial proximity of the primary and auxiliary binding sites. The fact that hydrolysis of neutral substrates requires the same catalytic residues points to one catalytic site on trypsin and hence limits the location of the binding site for neutral molecules. This fact, together with the observation that neutral compounds can have no effect, can inhibit hydrolysis of charged substrates noncompetitively or can enhance binding while inhibiting hydrolysis, suggests that overlap between the sites is a function of the nature of the molecules occupying each.

This postulate is specifically supported by the following evidence. Phenylurea has little effect on acetyllysine methyl ester hydrolysis. Increasing the acyl group to benzoylglycyl(hippuryllysine methyl ester) results in

[4] A referee has suggested that the apparent disparity in K_i (1.6 × 10^{-4} M) and K_0 (2 × 10^{-3} M) determined for acetyl-D-lysine methyl ester as an inhibitor of BAME and as a substrate, respectively, can be rationalized if it is assumed that most or all of the modes of binding of the D-lysine derivative to tyrpsin interfere with the productive binding mode (Hein and Niemann, 1962) of BAME, whereas the stronger binding modes of the lysine compound do not interfere with its own productive mode. The productive binding mode for the D substrate would place the lysine side chain in the "neutral" binding site. If this area allows a looser fit of the substrate, it is conceivable that some nonproductive modes with the more favored (and perhaps more restricted) lysine side-chain-positive binding site interaction might not interfere with the productive model.

TABLE III: The Behavior of Different Modifier Types with the Same Substrate.

Substrate	Charge[a]	Modifier (K_i) 0 Phenylurea	Modifier (K_i) + Phenylguanidinium Chloride
N^{α}-Benzoyl-L-arginine methyl ester hydrochloride	+	At 1–10 × 10^{-2} M,[b] no effect	7.2 × 10^{-5} M,[e] competitive inhibition
N^{α}-Tosyl-L-ornithine methyl ester hydrochloride	+	At 5–10 × 10^{-2} M,[c] no effect	1.0 × 10^{-4} M,[f] competitive inhibition
N^{α}-Benzoyl-L-citrulline methyl ester	0	1.1 × 10^{-1} M,[d] competitive inhibition	2.0 × 10^{-4} M,[d] α = 0.9, 0.8, noncompetitive inhibition

[a] Net ionic charge on substance at pH 7. [b] 8–12% acetonitrile. [c] 10% acetonitrile (pH 6.80). [d] 25% dioxane. [e] (Mares-Guia and Shaw, 1965) (pH 8.15) against benzoyl-DL-arginine *p*-nitroanilide. [f] pH 6.75.

TABLE IV: The Behavior of Different Substrate Types with the Same Modifier.[a]

Modifier	Charge	Substrates (K_i) + BAME	Substrates (K_i) 0 BCME	Others
Phenylguanidinium nitrate	+	7.2 × 10^{-5} M,[b] competitive inhibition	2.0 × 10^{-4} M,[c] α = 0.9, noncompetitive inhibition	TOME,[d] 1 × 10^{-4} M (+) competitive inhibition
Ethylammonium chloride	+	6.2 × 10^{-2} M,[e] competitive inhibition	6.6 × 10^{-2} M, α = 0.5, noncompetitive inhibition	
Phenol	0	12 × 10^{-2} M, α = 0.4, noncompetitive inhibition	5.4 × 10^{-2} M,[e] competitive inhibition	
Phenylurea	0	At 1–10 × 10^{-2} M,[f] no effect	1.1 × 10^{-1} M, competitive inhibition	TOME, at 5–10 × 10^{-2} M[g] (+) no effect
p-Toluenesulfonamide	0	At 0.1–63 × 10^{-3} M, no effect	9.6 × 10^{-3} M, competitive inhibition	

[a] BAME, benzoylarginine methyl ester; BCME, benzoylcitrulline methyl ester; TOME, tosylornithine methyl ester; BApNA, benzoyl-DL-arginine *p*-nitroanilide. Charges are net ionic charges at pH 7. [b] (Mares-Guia and Shaw, 1965) (pH 8.15), BApNA. [c] 25% dioxane. [d] pH 6.75. [e] (Inagami, 1964), pH 6.6, ethyl ester. [f] 8–12% acetonitrile. [g] 10% acetonitrile (pH 6.80).

competitive behavior by phenylurea (K_i = 2.7 ± 0.3 × 10^{-1} M). The overlap of sites could be along the acyl chain which is the peptide chain in a natural substrate.

At present there is little direct evidence to suggest that the neutral binding site and the site involved in activation by an additional substrate molecule are the same. Phenylurea does not appear to decrease substrate activation of tosylarginine methyl ester, however.

Gorecki and Shalitin (1967) report that uncharged compounds which have the ability to form hydrogen bonds are readily hydrolyzed by trypsin. Both ethyl α-acetamidoadipamate, $H_2NCO(CH_2)_3CH(NHCOCH_3)$-$COOC_2H_5$, and *N*-acetyl-*S*-acetamidomethylcysteine ethyl ester, $H_2NCOCH_2SCH_2CH(NHCOCH_3)COO$-$C_2H_5$, are substrates. The authors state that benzamide, benzylamine, and butylamine are competitive inhibitors of these compounds. In the present study, butylamine was the only positively charged inhibitor that gave competitive kinetics with BCME. It remains to be seen whether the above substrates show strictly competitive behavior with all inhibitors or *vice versa*. This may be another indication that the interaction between binding sites is severely dependent upon the structure of the molecules occupying them.

Implications of These Findings. The possible location of auxiliary binding sites in the region occupied by the peptide chain of a natural substrate is important in

view of reported anomalies in polypeptide hydrolyses (Maroux *et al.*, 1966; Plapp *et al.*, 1967; Bachmayer *et al.*, 1968). Cooperative interactions determined by the environment of a given bond could lead to reactions unpredicted by the conventional specificity considerations.

Inconsistencies in Inagami's picture (Inagami and Murachi, 1964) of the activation of acetylglycine ethyl ester hydrolysis by amines (Erlanger and Castleman, 1964) can be understood in terms of the present picture. The neutral substrate does not act as a competitive inhibitor of BAME and thus presumably is oriented like a BCME molecule. The amines fit into the primary substrate binding site and their degree of interaction with the second site is a function of their structure. Erlanger and Castleman have found the action of amines to be complex while Heidberg *et al.* (1967) have evidence that long aliphatic amines bind in two hydrophobic regions on the enzyme.

The similarity of chymotrypsin and trypsin in structure (Smillie and Hartley, 1966) and in mechanism (Bender and Kézdy, 1965) can be expanded to include auxiliary binding sites since chymotrypsin appears to possess one also. Substrate activation (Trowbridge *et al.*, 1963), activation by 9-aminoacridine (Wallace *et al.*, 1966), ank stereospecific activation by methyl L-(2-trimethylammonium iodide-4-methyl) pentanoate (Ponzi and Hein, 1966) point to a site for positively charged molecules distinct from the hydrophobic substrate site. Again the type of behavior noted changes to competitive as the *N*-acyl chain of substrates is increased (D. Ponzi and G. E. Hein, unpublished data). It is interesting that the two types of sites should have reversed specificity in the two enzymes.

Another proteolytic enzyme, subtilisin, shows behavior which is quite similar to that exhibited by trypsin (Glazer, 1967). Indole, phenol, and hydrocinnamate are competitive inhibitors of acetyltyrosine methyl ester hydrolysis but noncompetitive inhibitors of benzoylarginine ethyl ester hydrolysis. However, benzoylarginine is reported to be competitive with acetyltyrosine ethyl ester. The phenomenon of auxiliary binding sites may prove to be quite general as more of the traditional "one-site" enzymes are examined for secondary specificity requirements.

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The Binding of Phenylguanidinium Ion and Phenol to Trypsin*

Barbara M. Sanborn† and William P. Bryan

ABSTRACT: The technique of equilibrium dialysis was employed to investigate the nature of the interaction of phenylguanidinium ion and phenol with trypsin. [^{3}H]Phenylguanidinium ion bound at one site per catalytically active trypsin molecule with a dissociation constant of 1.4×10^{-4} M. Phenol affected the binding of phenylguanidine in a manner which was not competitive with $K_i = 4.3 \times 10^{-2}$ M. These results are consistent with the proposed model for ternary complex formation involving an auxiliary binding site on trypsin.

In a previous publication (Sanborn and Hein, 1968), kinetic evidence was presented for an auxiliary site on trypsin to which at least some neutral molecules preferentially bind. Confirmation of the existence of ternary complex formation was sought using the technique of equilibrium dialysis.

Equilibrium dialysis has been used to study the binding of virtual substrates to chymotrypsin and has yielded information on the groups involved in binding as well as thermodynamic data (Doherty and Vaslow, 1952; Vaslow, 1958; Loewus and Briggs, 1952; Johnson and Knowles, 1966). Competitive dialysis, where the displacement of a strongly bound molecule by a more weakly bound molecule is followed, has been employed to investigate the interaction of inhibitors with chymotrypsin (Weiner *et al.*, 1966). Most recently, the binding of thionine to trypsin, chymotrypsin, and modified forms of these enzymes has been reported (Glazer, 1967).

Experimental Details

Procedure. Lucite dialysis cells (E-1) of 2-ml total capacity were purchased from Technilab Instruments, Los Angeles, Calif. Squares of dialysis tubing (Visking Co., 1.87 stainless steel dialysis) were cut from tubing boiled for 7 hr in distilled water, stored in the cold, and soaked in the appropriate buffer for at least 1 hr prior to use. Eight cells were mounted on a Lucite holder and rotated in a Tamson-refrigerated bath at $10 \pm 0.1°$ for 7 hr unless otherwise indicated.

At the conclusion of the experiment, three 0.1-ml samples were withdrawn from each chamber of a given cell, added to 25 ml of Bray's (1960) scintillation solution, and counted in a Packard 3202 liquid scintillation spectrometer. Aliquots were reproducible within 1%; total counts were reproducible within 3%. Both an external standard provided with the counter and an internal [^{3}H]water standard (New England Nuclear Corp.) were used to normalize the counts.

The data were analyzed by the method of least squares with a t distribution percentage point of 0.05 and a constraint to retain 90% of the data. The errors are probable errors.

Materials. For this study, two structural analogs showing the kinetic behavior for compounds of different charge types predicted by the previous study were sought. Phenylurea and phenylguanidinium ion seemed likely choices of an isosteric pair since they each showed competitive behavior with substrates of the same charge type but deviated from it with substrates of a different charge type. Solubility considerations, however, necessitated the use of phenol in place of phenylurea. Phenol and phenylurea show similar kinetic behavior as modifiers of the trypsin-catalyzed hydrolyses of neutral and positively charged substrates (Sanborn and Hein, 1968).

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† Public Health Service predoctoral fellow, 1965–1967. This paper was taken from the thesis submitted in partial fulfillment of the requirements for the degree of Ph.D., Boston University, 1968. Present address: Graduate Department of Biochemistry, Brandeis University, Waltham Mass. 02154.