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Substrate Activation of Trypsin*

CLARENCE G. TROWBRIDGE,† ARLENE KREHBIEL,‡ AND MICHAEL LASKOWSKI, JR.

From the Department of Chemistry, Purdue University, Lafayette, Indiana

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The initial rates of hydrolysis of D- and L-*p*-toluene-sulfonyl arginine methyl ester by trypsin were examined over a 100,000-fold range of substrate concentration. The hydrolysis of neither substrate fits simple Michaelis-Menten kinetics, but can be fitted to a scheme based on the assumption that there are complexes with both one and two molecules of substrate bound to the enzyme. In such a scheme both the binary and ternary complexes decompose to products, the latter at a much higher rate than the former. Evidence is presented which shows that the concentration dependence of the rate is not due to impure enzyme preparations. The analysis of D–L mixtures shows that both substrates compete for the same active site and further that the D isomer is capable of activating the hydrolysis of the L substrate.

The discovery that trypsin and thrombin catalyze the hydrolysis of both optical isomers of α-*N*(*p*-toluene)-sulfonyl arginine methyl ester (TAM)[1] (Laskowski *et al.*, 1958) led to a further investigation of these substrates as part of a study of the specificity of trypsin and chymotrypsin. L-TAM has been widely used as a substrate for trypsin, and several workers have made kinetic analyses of the system (Scheraga *et al.*, 1958; Ronwin, 1959; Martin *et al.*, 1959). All these workers report simple Michaelis-Menten behavior.[2] Work reported in this paper shows that trypsin/TAM does not follow simple Michaelis-Menten kinetics; the results are compatible with the formation of binary and ternary enzyme-substrate complexes, both capable of decomposing to products, the latter at a greater rate (substrate activation) than the former.

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† This paper was taken from the thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, Purdue University, 1963, by Clarence G. Trowbridge. Present address: Department of Chemistry, Emory University, Atlanta 22, Ga.

‡ Present address: Biochemical Laboratory for Cancer Research, Marquette University, School of Medicine, Milwaukee 3, Wis.

[1] Abbreviations used in this paper are as follows: TAM, α-*N*(*p*-toluene)sulfonyl arginine methyl ester; DIP, diisopropyl phosphoryl; DIPF, diisopropyl phosphofluoridate; L-BAE, α-*N*(benzoyl)-L-arginine ethyl ester; L-ATE, N-acetyl-L-tyrosine ethyl ester.

Experimental

Materials.—Both TAM isomers were prepared from the corresponding L- and D-arginine hydrochloride

[2] Martin (1962) later published some data which show a rate-concentration relation not predicted by his constants, but which is in agreement with the analysis given in this paper.

(Bergmann *et al.*, 1939). Two crystallizations from *n*-butanol gave white crystals, mp 147–148° for both isomers. For typical preparations, $[\alpha]_D^{20}$ is −14.36° for L-TAM and +14.17° for the D isomer (1-dm tube, c = 0.76% in 0.2 M KCl, 0.05 M $CaCl_2$, *p*H 8.0). Total hydrolysis of TAM preparations was 96% of theory or better. Most of the work reported here was done with trypsin purchased from the Worthington Corporation, lot no. TRLSF 6105 (trypsin I). The results obtained with trypsin I were compared with a Nutritional Biochemicals Corporation preparation, lot no. 4980 (trypsin II), and with a highly purified trypsin which was a gift of Professor M. Laskowski, Sr., Marquette University, Milwaukee, Wis. (trypsin III). Trypsin III was prepared from the crystalline trypsin-pancreatic inhibitor complex, and, although its activity was lower than that of the commercial preparations I and II, the rigorous preparative procedure made it unlikely that any enzyme other than trypsin was present. Diisopropyl phosphoryl trypsin (DIP trypsin) was a gift from Dr. E. F. Jansen, U. S. Department of Agriculture, Albany, Calif. Enzyme stock solutions were prepared at 0.1% concentration in 0.2 M KCl, 0.05 M $CaCl_2$ and stored at 3°. The concentration of trypsin solutions was calculated from the absorption at 280 mμ, using 0.651 mg ml^{-1} $(OD\ unit)^{-1}$ as an optical factor (Worthington, 1961); the molecular weight of trypsin was taken to be 24,000 (Kay *et al.*, 1961). Deionized, carbon dioxide–free water and reagent-grade chemicals were used.

Procedure.— The kinetic data were obtained from initial rates determined by the *p*H-stat method. A Radiometer TTT-1 automatic titrator was coupled with an Ole Dich syringe drive-recorder; 0.02 M, 0.05 M, 0.10 M, or 0.20 M KOH was used as titrant, depending upon the experimental conditions. The hydrolysis "blank" was determined and its contribution deducted from the total observed rate. The nonenzymic hydrolysis was found to be first order in substrate and in hydroxyl ion. Enzyme and titrant concentrations were chosen so that the hydrolysis blank was never more than 10% of the total observed rate. Reaction volume in most cases was 10 ml, but for the very dilute substrate concentrations the volume was as much as 800 ml.

CO_2 must be rigorously excluded from large-volume reactions. Comparison of rates for the same concentration of substrate obtained from different reaction volumes but using the same mass of enzyme showed that no systematic errors were introduced by the use of large volumes. Reactions were followed under a nitrogen atmosphere at 25.0 ± 0.1°; the medium contained 0.05 M $CaCl_2$ and 0.2 M KCl. At higher substrate concentrations constant ionic strength was maintained by varying the KCl concentration, although no effects due to ionic strength (in the range 0.35 to 0.55 M) were detected. The *p*H-stat results were confirmed over a much narrower concentration range with a spectrophotometric technique (Hummel, 1959); *p*H 8.0 was maintained with 0.1 M tris(hydroxymethyl)aminomethane buffer, the calcium chloride being kept at 0.05 M. The initial rates were measured with a Cary 14 recording spectrophotometer at 245 mμ. Cell length was varied from 0.1 to 10.0 cm to permit maximum extension of the substrate concentration range.

Results

Extension of the L-TAM concentration beyond the usual limits for a trypsin/substrate analysis produced the curved Eadie plot shown in Figure 1. The straight line in Figure 1 is calculated from constants which are representative of those given in the literature for this system. A reasonable fit of the experimental data can be made as tangents to the Eadie plot at several places, if the substrate concentration range is sufficiently restricted. The curve in Figure 1 represents only part of the substrate concentration range studied; the L-TAM concentration was varied from 2.0 × 10^{-6} M to 0.2 M to permit a complete analysis of the system. For D-TAM[3] a narrower concentration range was sufficient. Figure 2 shows the dependence of the tryptic hydrolysis of L-TAM upon the substrate concentration at *p*H 8.0[4] for the entire range studied; Figure 3 presents the same information for D-TAM. The semi-log plots are used to permit presentation of the complete data range in one figure; these plots are analogous to the usual representation of titration data (Edsall and Wyman, 1958). The form of Figures 2 and 3 suggests the following possible interpretations: (1) the trypsin preparations are mixtures of two "simple" enzymes, both having TAM as a substrate; (2) the trypsin preparations are homogeneous but each trypsin molecule has two separate catalytic sites,[5] (3) the trypsin preparations are homogeneous and have only one catalytic site per molecule, but (*a*) the mechanism involves an enzyme modifier (unknown) and the resulting system is a nonequilibrium one (Botts and Morales, 1953), or, (*b*), the modifier is another molecule of substrate. In such a case the behavior shown in

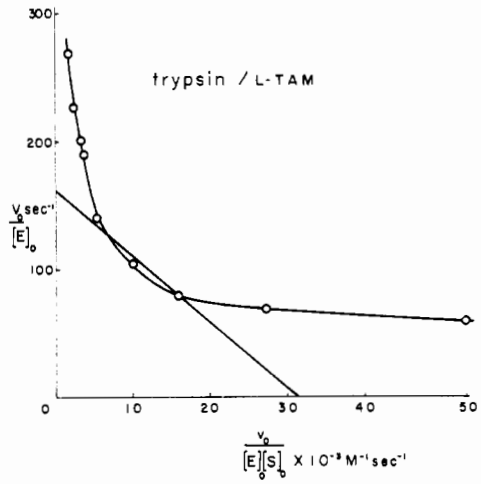

FIG. 1.—An Eadie plot of some trypsin/L-TAM data (open circles). The straight line is calculated from representative values of k_L and K_L taken from the literature (k_L = 160 sec^{-1}, K_L = 5.0 × 10^{-3} M). *p*H 8.0, 0.2 M KCl, 0.05 M $CaCl_2$ at 25.0 ± 0.1°, trypsin preparation I.

[3] The discovery that D-TAM is hydrolyzed by trypsin was made by Ehrenpreis and Laskowski at Cornell University (Laskowski *et al.*, 1958). The investigation of the lack of absolute stereospecificity in trypsin is continuing; it has been delayed by complications arising from the substrate activation behavior exhibited by trypsin. We have also found that both L- and D-lysine ethyl esters are hydrolyzed by trypsin. On the other hand no hydrolysis of the D isomer was observed by Riedel and Wunsch (1959), who used the substrate α-*N*-(benzoyl)-DL-arginine naphthylamide.

[4] The complex dependence of the initial rate upon substrate concentration was found to persist down to *p*H 5, the lowest *p*H used (Trowbridge, 1963).

[5] The possibility that our results are due to polymerization of trypsin is discounted because of the low enzyme concentrations used and because the initial rates were proportional to enzyme concentration at both high and low substrate concentrations.

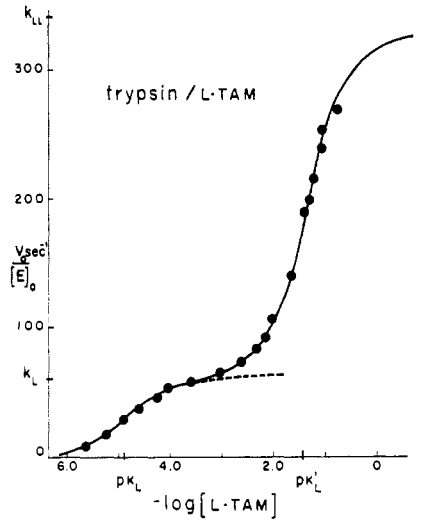

FIG. 2.—The dependence of the tryptic hydrolysis of L-TAM upon substrate concentration. pH 8.0, 0.2 M KCl, 0.05 M $CaCl_2$, at 25.0 ± 0.1°, trypsin preparation I. The curve through the data is calculated from equation (3) and the parameters for L-TAM given in Table III. These parameters were determined from the linear plots shown in Figures 4 and 5. The inflections indicated give pK_L and pK_L' (on the $-\log$ [L-TAM] axis); the values of k_L and k_{LL} are shown on the $V_0/[E]_0$ axis. The broken curve would result if $k_{LL} = 0$ and $K_L' = \infty$.

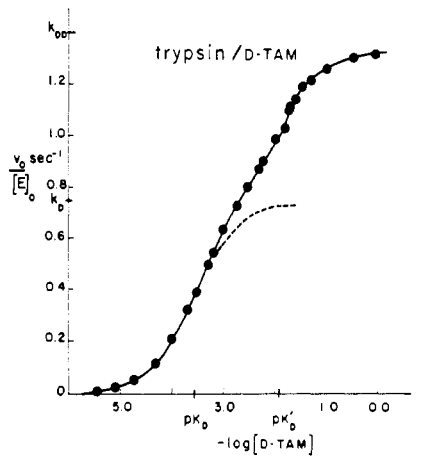

FIG. 3.—The dependence of the tryptic hydrolysis of D-TAM upon substrate concentration. pH 8.0, 0.2 M KCl, 0.05 M $CaCl_2$. at 25.0 ± 0.1°, trypsin preparation I. The curve through the data is calculated from equation (3) and the D-TAM parameters in Table III. These parameters were determined from linear plots for D-TAM, of the same form as Figures 4 and 5. The inflections indicated give pK_D and pK_D' (on the $-\log$ [D-TAM] axis); the values of k_D and k_{DD} are shown on the $V_0/[E]_0$ axis. The broken curve would result if $k_{DD} = 0$ and $K_D' = \infty$.

Figures 2 and 3 would arise in both equilibrium and nonequilibrium systems. If the form of Figures 2 and 3 is due to a mixture of two enzymes, the positions of the plateau and of the asymptote depend upon the relative amounts of the two enzymes present, as well as upon their respective V_{max} values.

Comparison of Three Trypsin Preparations.—Besides preparation I, two other trypsin preparations were examined (see Materials). Preparation II was a different commercial material but gave the same substrate concentration dependence as I, apart from a difference in activity (Table I). Preparation III was of considerable interest because of its high purity, and it was used to study the L-TAM hydrolysis from 5 × 10^{-6} M to 0.1 M in substrate. The same concentration dependence was again obtained, correcting for the difference in activity between I and III. It therefore appears that the relationship between rate and substrate concentration is the same for trypsin preparations from different sources, which makes it unlikely that there are two enzymes always present in the same proportion in different samples of trypsin.

TABLE I

COMPARISON OF TRYPSIN PREPARATIONS

Ratio of the rate to the rate at 1.00 × 10^{-3} M L-TAM.[a]

[L-TAM] × 10^3 M	Trypsin I	II[b]	III[c]	DIP-Trypsin[d]
1.00	1.00	1.00	1.00	1.00
10.0	1.63	1.65	1.55	1.63
25.0	2.17	2.22	2.24	2.21
50.0	3.08	3.12	3.12	3.02

[a] At pH 8.0 in 0.2 M KCl, 0.05 M $CaCl_2$, 25.0 ± 0.1°. [b] The ratio $(V_0/[E]_0$, trypsin II$)/(V_0/[E]_0$, trypsin I$) = 0.7 \pm 0.05$. [c] The ratio $(V_0/[E]_0$, trypsin III$)/(V_0/[E]_0$, trypsin I$) = 0.85 \pm 0.03$. [d] The ratio $(V_0/[E]_0$, DIP-trypsin$)/(V_0/[E]_0$, trypsin I$) = 0.028 \pm 0.002$.

Diisopropyl Phosphoryl Trypsin.—It has been shown that diisopropyl phosphofluoridate (DIPF) reacts with trypsin in a 1:1 mole ratio (Jansen and Balls, 1952), the resultant DIP-trypsin being catalytically inactive. A DIP-trypsin preparation usually has some residual activity (Cohen *et al.*, 1962), and we wished to know if the residual activity would have the same characteristics as that of an untreated trypsin preparation. The DIP-trypsin used had a residual activity 3% of that of the trypsin I used in this investigation, but Table I shows that the dependence of this residual activity upon the substrate concentration is the same as that shown in Figure 2. This result means that a molecule of trypsin which has reacted with DIPF remains inactive at all concentrations of L-TAM, and that the trypsin which has not reacted with DIPF has the same properties as the active fraction of trypsins I, II, and III. The result indicates again that the trypsin preparations are not mixtures of two enzymes, since it is extremely unlikely that such two enzymes would show identical reactivities toward DIPF and extremely different ones toward TAM.

The Effect of Calcium Ion.—Since it has been shown by Green *et al.* (1952) that calcium ion activates trypsin-catalyzed hydrolyses, it is of interest to ascertain if the complex substrate dependence observed here is in some manner dependent upon the presence of calcium ion. Table II shows that the complex rate dependence is observed both in the presence and in the absence of calcium ion. On the basis of these limited data it is not possible to decide how the parameters K_s and K_s' are affected; Inagami and Sturtevant (1960) have demonstrated that K_s for trypsin/L-BAE is approximately halved by addition of 0.05 M $CaCl_2$.

Evaluation of the Kinetic Parameters.—The data represented by Figures 2 and 3 have been fitted (see below) to equation (1), where $b/d > a/c$ (substrate

$$\frac{V_0}{[E]_0} = \frac{a[S] + b[S]^2}{1 + c[S] + d[S]^2} \qquad (1)$$

activation). Such a form can arise from many distinct mechanisms, which although chemically different are indistinguishable on the basis of steady-state kinetic experiments (Botts, 1958; Dalziel, 1957; Laidler, 1958). These alternative mechanisms fall into the three broad classes outlined before. On the basis of the evidence already presented (see also DL Mixtures,

TABLE II

EFFECT OF CALCIUM ION

Ratio of the rate to the rate at 1.00×10^{-3} M L-TAM.[a]

[L-TAM] × 10^3 M	0.05 M $CaCl_2$	No $CaCl_2$[b]
1.00	1.00	1.00
10.0	1.63	1.57
25.0	2.17	2.25

[a] At *p*H 8.0 in 0.2 M KCl, at 25.0 ± 0.1°, preparation I. [b] The ratio $(V_0/[E]_0$, 0.05 M $CaCl_2)/(V_0/[E]_0$, no $CaCl_2)$ is 1.36 ± 0.05.

High Concentration) we feel that the rate-substrate concentration relation (Figures 2 and 3) is not caused by impure enzyme preparations or by the presence of two separate catalytic sites on the trypsin molecule. There remains the class of mechanisms involving either an unknown modifier (other than the substrate) in a nonequilibrium system (Botts and Morales, 1953; Laidler, 1958) or the participation of two (or more) substrate molecules with one (or more) of these acting as modifier in quasi or nonquasi equilibrium situations. It is at present not possible to give the parameters of equation (1) an unequivocal interpretation, since their interpretation depends upon the mechanism chosen. At the same time it seems barren simply to determine the parameters without comment or interpretation. We have therefore chosen to interpret the data on the basis of the following assumptions: (1) Binary and ternary complexes can exist. Both kinds can decompose to products, but the ternary complex does so faster than do the binary complexes. (2) All the complexes are in quasi equilibrium; for the sake of generality it is supposed that there are two binary complexes (*ES* and *SE*). These are of course indistinguishable by steady-state kinetic experiments (Edsall and Wyman, 1958). A possible mechanism which fits this general scheme is that in which a molecule of substrate binds to the acyl enzyme and modifies its rate of hydrolysis. In this case *SES* would be a complex in which one substrate molecule is dissociably bound and the other substrate molecule is contributing the acyl moiety to the acyl enzyme. This mechanism leads to steady state rate expressions of considerable complexity, but of the same general form as equation (1). Foster (1961) has shown that indole promotes the deacylation of acetyl α-chymotrypsin; this can be taken to be in favor of the substrate–acyl enzyme intermediate suggested above. Wolf and Niemann reported substrate activation for the system α-chymotrypsin/acetyl glycine methyl ester (1959), but have apparently subsequently concluded that the system can be described by simple Michaelis-Menten kinetics (1963).

We now proceed to evaluate the kinetic parameters. In so doing we shall assign to them the meaning they would have if the equilibrium assumption (outlined above) were known to be correct. We therefore define the two dissociation constants as shown in equation (2)

$$K_s = \frac{[E][S]}{[ES] + [SE]}, \quad K_s' = \frac{([ES] + [SE])[S]}{[SES]} \qquad (2)$$

(Edsall and Wyman, 1958). These definitions allow equation (1) to be written as equation (3), where k_{ss}

$$\frac{V_0}{[E]_0} = \frac{k_s[S]/K_s + k_{ss}[S]^2/K_sK_s'}{1 + [S]/K_s + [S]^2/K_sK_s'} \qquad (3)$$

is the first-order rate constant for the decomposition of *SES* to products, and k_s is defined by the relation shown in equation (4). A system described by (3)

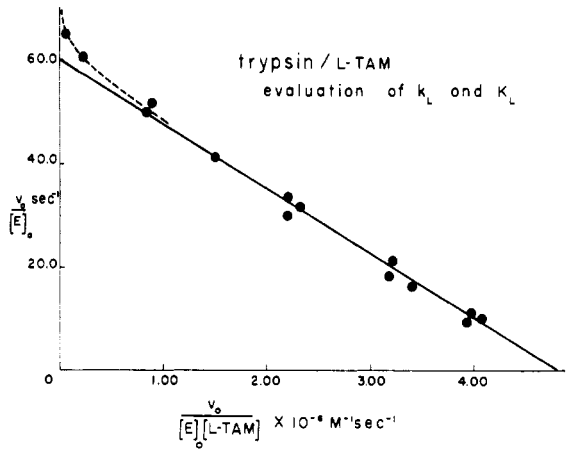

FIG. 4.—Evaluation of k_L and K_L for trypsin/L-TAM. *p*H 8.0, 0.2 M KCl, 0.05 M $CaCl_2$, 25.0 ± 0.1°, trypsin preparation I. The slope is $-K_L$ (equation 5) and the $V_0/[E]_0$ intercept is k_L. The broken curve shows the onset of complex rate dependence as [L-TAM] increases.

$$k_s = \frac{k_{ES}[ES] + k_{SE}[SE]}{[ES] + [SE]} \qquad (4)$$

will generate a curved Eadie plot of the type shown in Figure 1, if $k_{ss} > k_s$. The limiting values of the slope at the extremes of substrate concentration for such a system are given by equations (5) and (6).

$$\lim_{[S] \to 0} \left\{ \frac{d(V_0/[E]_0)}{d\{V_0/([E]_0[S])\}} \right\} = \frac{K_sK_s'}{K_s - K_s'} \qquad (5)$$

$$\lim_{[S] \to \infty} \left\{ \frac{d(V_0/[E]_0)}{d\{v_0/([E]_0[S])\}} \right\} = -K_s' \left\{ \frac{k_{ss} - k_s}{k_{ss}} \right\} \qquad (6)$$

Since the data indicate that $K_s << K_s'$ (see Figures 2 and 3), equation (5) is applied to the linear low concentration limiting Eadie plot (Fig. 4) by assuming that the slope gives K_s directly. This assumption is subject to test and has proved to be quite satisfactory. The $V_0/[E]_0$ intercept of this limiting straight line gives k_s. Although (6) can be similarly applied to the high concentration data, experimental limitations make it difficult to ascertain that limiting linearity had been achieved. To avoid this weakness, equation

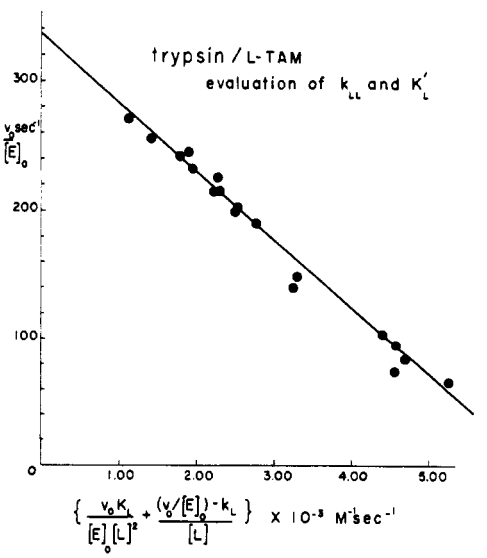

FIG. 5.—Evaluation of high concentration trypsin/L-TAM data (see equation 7). *p*H 8.0, 0.2 M KCl, 0.05 M, $CaCl_2$, at 25.0 ± 0.1°, trypsin preparation I. The slope is $-K_L'$, the $V_0/[E]_0$ intercept is k_{LL}.

TABLE III

PARAMETERS FOR THE TRYPTIC HYDROLYSIS OF L- AND D-TAM[a]

(sec^{-1})[b]		(M)		(M)^2	
k_L[c]	60	K_L[e]	1.25×10^{-5}		
k_D[c]	0.74	K_D[e]	2.7×10^{-4}		
k_{LL}	340	K_L'[e]	5.3×10^{-2}	K_LK_L'	6.62×10^{-7}
k_{DD}	1.4	K_D'[e]	1.25×10^{-2}	K_DK_D'	3.37×10^{-6}
k_{DL}[d]	156			K_{DL}[f]	5.85×10^{-7}

[a] At *p*H 8.0 in 0.2 M KCl, 0.05 M $CaCl_2$ at 25.0 ± 0.1°, preparation I. [b] The absolute values of these rate constants depend upon the activity of the trypsin used, but their relative values do not. Lebowitz and Laskowski (1962) have shown that preparation I is approximately 61% active. [c] Defined by equation (4). [d] Defined by equation (12). It is shown (see Evaluation of Kinetic Parameters) that essentially only L-TAM is hydrolyzed in D–L mixtures. [e] Defined by equation (2). [f] Defined by equation (11).

(3) is rewritten in the linear form as equation (7). Since K_s and k_s are now known, a plot of $V_0/[E]_0$ vs. the quantity in brackets on the left-hand side of (7) gives K_s' from the slope and k_{ss} from the $V_0/[E]_0$ intercept. Figure 5 illustrates the use of equation (7)

$$\frac{V_0}{[E]_0} = -K_s' \left\{ \frac{V_0 K_s/[E]_0}{[S]^2} + \frac{(V_0/[E]_0) - k_s}{[S]} \right\} + k_{ss} \quad (7)$$

with the trypsin/L-TAM data.[6] The parameters thus obtained from the extremes of substrate concentration give a good fit to the experimental data throughout the entire concentration range for both L- and D-TAM. Table III contains the constants for both isomers of TAM, and the curves through the data in Figures 2 and 3 are calculated from these parameters.

Mixtures of L- *and* D-*TAM, Low Concentration.*—If the preceding analysis of trypsin/TAM is correct, one should be able to observe simple competitive behavior between L- and D-TAM at sufficiently low substrate concentrations (so that only binary complexes exist). Under such conditions, the initial rate for a mixed system is given by equation (8), where the

$$\frac{V_0}{[E]_0} = \frac{k_L[L]/K_L + k_D[D]/K_D}{1 + [L]/K_L + [D]/K_D} \quad (8)$$

subscript letters denote to which enzyme/substrate complex the constant applies. Table III shows that the rate due to D-TAM hydrolysis in an L- and D-TAM mixture can be made negligible by a suitable choice of conditions; then, neglecting the contribution of D-TAM to the total observed rate, equation (8) can be rewritten in the linear form (equation 9) (Inagami and Sturtevant, 1960).

$$\frac{V_0}{[E]_0} = -\left\{ \frac{K_L}{K_D(K_L + [L])} \right\} \frac{V_0[D]}{[E]_0} + \frac{k_L[L]/K_L}{1 + [L]/K_L} \quad (9)$$

In a plot of $V_0/[E]_0$ vs. $V_0[D]/[E]_0$ at constant [L-TAM], the slope is given by the quantity in brackets in equation (9). Such plots for two different [L-TAM]'s are shown in Figure 6; the straight lines through the experimental points are calculated from the appropriate constants in Table III, and the fit is taken to demonstrate that the two substrates show simple competitive behavior.

[6] The linearity of the data in Figure 5 emphasizes that terms in $[S]^3$ are needed in neither the numerator nor denominator of equation (3). On the basis of our assumed model this means that the second substrate-binding site can be saturated and that no kinetically significant third site exists.

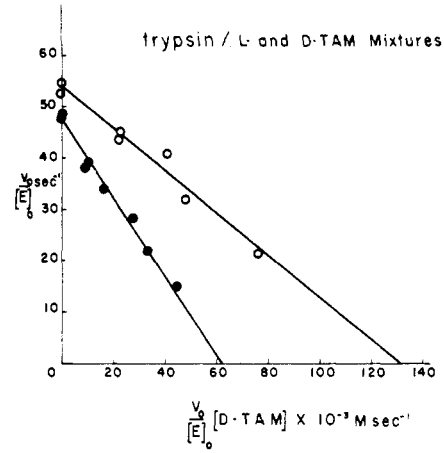

FIG. 6.—The inhibition of L-TAM at two concentrations by D-TAM (see equation 9). Open circles 1.00×10^{-4} M L-TAM, closed circles 5.00×10^{-5} M L-TAM. The straight lines through the data are calculated from k_L, K_L, and K_D in Table III. *p*H 8.0, 0.2 M KCl, 0.05 M $CaCl_2$ at 25.0 ± 0.1°, trypsin preparation I.

High Substrate Concentration.—At higher substrate concentrations the rate for substrate mixtures cannot be expected to follow the simple relation (8) because terms involving $[L]^2$ and $[D]^2$ (arising from L*E*L and D*E*D) should be included. Further, a logical extension of the model predicts that some fraction of the enzyme molecules will form ternary complexes in which one molecule of each isomer participates in the complex. Reasoning in the manner which led to equation (3), equation (10) is written directly by analogy. The

$$\frac{V_0}{[E]_0} = \frac{k_L[L]/K_L + k_{LL}[L]^2/K_LK_L' + k_D[D]/K_D + k_{DD}[D]^2/K_DK_D' + k_{DL}[D][L]/K_{DL}}{1 + [L]/K_L + [L]^2/K_LK_L' + [D]/K_D + [D]^2/K_DK_D' + [D][L]/K_{DL}} \quad (10)$$

only new parameters in (10) are K_{DL} and k_{DL}, where the former is defined by equation (11), and k_{DL} is given

$$K_{DL} = \frac{[E][L][D]}{[DEL] + [LED]} \quad (11)$$

by equation (12). K_{DL} is analogous to the product

$$k_{DL} = \frac{k_{DEL}[DEL] + k_{LED}[LED]}{[DEL] + [LED]} \quad (12)$$

K_sK_s'. It is not possible to calculate K_{DL} from the previously determined constants without making arbitrary assumptions about the microscopic dissociation constants (Edsall and Wyman, 1958), because the K_s' are not defined for dissociation from mixed ternary complexes. The experimental determination of the DL parameters is based on the linear form (13), which is a rearrangement of (10). A plot of $V_0/[E]_0$ vs. the quantity in brackets on the right side of equation (13)

$$\frac{V_0}{[E]_0} = -K_{DL}\left\{ \frac{\left[\frac{V_0 A}{[E]_0}\right] - B}{[D][L]} \right\} + k_{DL} \quad (13)$$

$$A = 1 + [L]/K_L + [L]^2/K_LK_L' + [D]/K_D + [D]^2/K_DK_D'$$

$$B = k_L[L]/K_L + k_{LL}[L]^2/K_LK_L' + k_D[D]/K_D + k_{DD}[D]^2/K_DK_D'$$

gives $-K_{DL}$ as the slope and k_{DL} as the $V_0/[E]_0$ intercept. The applicability of (13) was tested by measuring the initial hydrolysis rates of D- and L-TAM mix-

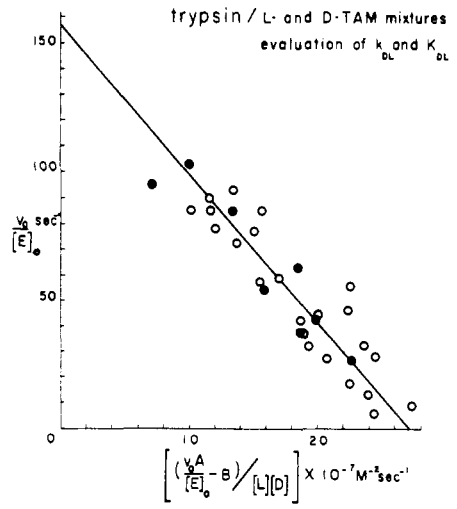

FIG. 7.—Evaluation of k_{DL} ($V_0/[E]_0$ intercept) and K_{DL} (slope = $-K_{DL}$), see equation (13). *p*H 8.0, 0.2 M KCl, 0.05 M $CaCl_2$ at 25.0 ± 0.1°, trypsin preparation. I. The filled circles represent initial rate measurements; open circles obtained from rates determined at intervals during an extended hydrolysis (see DL mixtures, High Concentration).

tures; Figure 7 shows the resultant fit of the data. The location of a point is determined by both the absolute and relative concentrations of the two substrates, and for this reason it is possible for different choices of substrate concentration to give the same total observed rate. The analysis gives k_{DL} = 156 sec^{-1}, $K_{DL} = 5.85 \times 10^{-7}$ M^2; it should be pointed out that the abscissa of Figure 7 arises from small differences ($\{V_0A/[E]_0\} - B$) and is therefore a very sensitive test of the data. Furthermore, calculation of the abscissa assumes the correctness of all the previously determined parameters in Table III. Although the scattered fit increases the uncertainty in the evaluation of k_{DL} and K_{DL}, it does argue in favor of the correctness of equation (10). This of course proves only the validity of the general kinetic form, and not of any detailed mechanism.

The evaluation of the "mixed" parameters makes possible another test for a mixture of two enzymes. The initial rate expression for a mixture of two enzymes which are hydrolyzing two competing substrates is given in equation (14), where the subscript numbers

$$\frac{V_0}{[P]_0} = \frac{rg_{1L}[L]/K_{1L} + rg_{1D}[D]/K_{1D}}{1 + [L]/K_{1L} + [D]/K_{1D}} + \frac{(1-r)g_{2L}[L]/K_{2L} + (1-r)g_{2D}[D]/K_{2D}}{1 + [L]/K_{2L} + [D]/K_{2D}} \quad (14)$$

denote enzyme 1 or 2, and the subscript letters indicate L- or D-TAM.

The *g*'s are first-order rate constants for the decomposition of E_1L, E_1D, E_2L, and E_2D to products; the concentration of enzyme 1 is given by $[E_1]_0 = r\,[P]_0$, where $[P]_0$ is the total enzyme concentration. By combining the terms on the right-hand side of (14), regrouping as coefficients of [L], [D], and [D][L], and by comparing term by term with equation (10), the following relations are seen:

$$\frac{1}{K_L} = \frac{1}{K_{1L}} + \frac{1}{K_{2L}}; \quad K_LK_L' = K_{1L}K_{2L}$$

$$k_L = \frac{rg_{1L}K_{2L} + (1-r)g_{2L}K_{1L}}{K_{1L} + K_{2L}}$$

$$k_{LL} = rg_{1L} + (1-r)g_{2L}$$

(Corresponding relations hold for the D-TAM parameters.)

$$k_{DL,\,calcd} = \frac{K_{1D}K_{2L}[rg_{1L} + (1-r)g_{2D}] + K_{1L}K_{2D}[rg_{1D} + (1-r)g_{2L}]}{K_{1L}K_{2D} + K_{1D}K_{2L}}$$

$$\frac{1}{K_{DL,\,calcd}} = \frac{1}{K_{1L}K_{2D}} + \frac{1}{K_{1D}K_{2L}} \quad (15)$$

The calculated cross-term constants in (15) compare with the experimentally determined DL parameters as follows: K_{DL}/K_{calcd} = 3.7; k_{DL}/k_{calcd} = 2.4. The calculated parameters are substantially different from those evaluated with equation (13), and this result reinforces our earlier conclusion that the observed dependence of the initial rates upon substrate concentration is not an artifact due to the presence of two simple enzymes in the trypsin preparations (or to two separate catalytic sites on trypsin).

Attainment of the steady state for the mixed systems is confirmed by the fact that changing the order of mixing of the three reagents (trypsin, L-TAM, and D-TAM) does not alter the result shown in Figure 7. A further check on experimental validity is based on the observation that these systems do not show inhibition by products. Several different trypsin/L- and D-TAM mixtures were allowed to proceed to approximately 80% completion based on the L-TAM concentration; rates were measured at intervals of time and calculated on the assumption that all enzymic hydrolysis was due to splitting of the L isomer. Data obtained in this manner give the same results as initial rates determined at corresponding substrate concentrations. The fact that D-TAM hydrolysis remains negligible when the DL cross term is significant means that in the mixed ternary complexes the L isomer accounts for nearly all the substrate hydrolyzed. This fact, together with the observation that $k_{LL} > k_{DL} > k_L$ (Table III), indicates that D-TAM is capable of activating the hydrolysis of L-TAM, but to a lesser degree than L-TAM itself.

DISCUSSION

An increasing body of evidence shows that the rates of enzyme-catalyzed hydrolyses frequently depend upon the substrate concentration in a manner not described by the classical Michaelis-Menten equation. Schwert and Eisenberg (1949) reported that the system trypsin/benzoyl-L-arginine amide was inhibited by benzoyl-L-arginine, but that the type of inhibition depended upon the concentration of the inhibitor. Lumry *et al.* (1951) found substrate inhibition for the system carboxypeptidase/carbobenzoxyglycyl-L-tryptophan, the rate at high substrate concentration approaching a nonzero limiting value. The indole inhibition of α-chymotrypsin-catalyzed hydrolyses of its "specific" substrates has been found to depend upon the substrate used (Huang and Niemann, 1953). Awad (1959) found that indole and β-phenyl propionate act as competitive inhibitors for the acylation of chymotrypsin by *p*-nitrophenyl acetate, but double the rate of deacylation of the acetyl enzyme. He suggested that binding of an aromatic moiety to the enzyme enhances the catalysis and that the aromatic residue can be supplied by a separate molecule (a second substrate, or modifier). Bargoni *et al.* (1960) have published the observation that trypsin is activated by histidine and methionine at low concentration (< 10^{-3} M) but inhibited at higher concentrations (0.1 M). Arginine also activated trypsin, while chymotrypsin is activated by arginine, lysine, and

aspartic and glutamic acids. They also found that pancreatic amylase is activated by glycine, lysine, ornithine, and arginine.

Kurtz and Niemann (1961) have concluded that ternary complexes are formed in solutions containing the inhibitor 1-acetyl-2-(L-tyrosyl) hydrazine, chymotrypsin, and acetyl-L-valine methyl ester. In correspondence with the earlier work with indole (Huang and Niemann, 1953), they find that the type of inhibition depends upon the substrate used. Adler and Kistiakowsky (1962) have examined the kinetics of pig liver esterase acting upon butyrate esters, and conclude that the mechanism involves a molecule of enzyme to which two substrate molecules are bound. Graae and Hansen (1961) have studied the kinetics and reaction products resulting from the action of chymotrypsin on tyrosine ethyl ester; they conclude that intermediates exist in which more than one molecule of substrate or product is bound to one enzyme molecule.

McFadden and Laskowski (1956) established that trypsin catalyzes the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (L-ATE), a "specific" substrate for α-chymotrypsin. Inagami and Sturtevant (1960) studied this system, as well as the action of α-chymotrypsin upon α-*N*(benzoyl)-L-arginine ethyl ester (L-BAE), in their examination of "nonspecific" catalyses by those two enzymes. We have found (Trowbridge, 1963) that at low concentrations ($< 10^{-3}$ M) L-TAM competitively inhibits chymotrypsin/L-ATE with $K_I = 2.2 \times 10^{-4}$ M; at higher L-TAM concentrations noncompetitive behavior develops. Analysis of α-chymotrypsin/L-TAM shows that there is a marked substrate activation, but in the limiting low concentration range $k_L = 0.005$ sec^{-1} and $K_L = 2.2 \times 10^{-4}$ M. Although Inagami and Sturtevant (1960) show a linear Eadie plot for α-chymotrypsin/L-BAE, extension of the substrate concentration range establishes that this system shows a slight substrate activation; trypsin/L-BAE also exhibits a slight substrate activation, with the limiting low concentration ($< 10^{-3}$ M) data giving $k_2 = 15$, $K_s = 3.5 \times 10^{-6}$ M. The noncompetitive action of α-chymotrypsin upon mixtures of L-ATE with L-TAM or L-BAE appears to be an exception to Niemann's rule (Kurtz and Niemann, 1961) that α-chymotrypsin will not show noncompetitive behavior with an inhibitor and a specific substrate (here, L-ATE). In view of all the evidence cited above and of the Results section of this paper, it appears reasonable to conclude that modification of simple Michaelis-Menten kinetics by substrate (or substrate analog) is a widespread phenomenon.

Almond and Niemann (1960) have enumerated the sources of systematic error in the measurement of enzyme-catalyzed reactions, and their effects upon the analysis of data. In particular they show the various curvatures which these errors can cause in the commonly used linear plots. Thus they suggest that great care be exercised in proposing nonlinear behavior. However, after great care has been taken in the collection of data, curvature in such plots has been reported. We therefore suggest that apparently linear data must be carefully examined (principally by extension of substrate concentration range, see Figure 1) to insure that curvature does not in fact exist; the limited solubility of many acyl amino acid derivatives and the complications encountered at very low substrate concentrations can make the detection of deviations from linearity difficult.

Many workers have from time to time questioned the assumption of a single catalytic site on trypsin and α-chymotrypsin. Northrop (1948) reported that mixtures of gelatin and casein were hydrolyzed more rapidly than was either protein separately at the same concentration used in the mixture. Castenada-Agullo and Del Castillo (1958a,b) showed that both trypsin and α-chymotrypsin hydrolyze casein-gelatin mixtures in a noncompetitive manner. They found that mixtures of the two proteins (both at a concentration sufficient to saturate the enzyme) were hydrolyzed at rates always greater than that of the faster of the separate reactions. As the protein concentrations were increased, the hydrolysis of gelatin-casein mixtures was in some cases faster than the sum of the separate reactions. Similar noncompetitive effects were observed by these authors in mixtures of the same proteins with benzoyl arginine esters, using either trypsin or α-chymotrypsin. The authors concluded that either the enzymes do not form stable complexes with their substrates, or there is more than one active site on these enzymes.

It is our view that the noncompetitive behavior of the protein mixtures can be explained in the same way as the hydrolysis of L- and D-TAM, and that such kinetics can occur without two separate catalytic sites. We suggest that one of the denatured substrate proteins acts as an activator of the hydrolysis of the other.

Because of steric considerations it is unlikely that trypsin combines simultaneously with two native protein molecules. Nonetheless the presence of two substrate binding sites may play a significant role in proteolysis. The geometry of the sites and of the native protein substrate may be such that both substrate binding sites can be filled by the same protein molecule. Such an interaction could (in analogy with our results) lead to a much faster hydrolysis than if there were only a single interaction.

This concept may well be of value in explaining the unusually high specificity of proteolytic enzymes in activating precursors of biologically active proteins (*e.g.*, zymogen-enzyme, fibrinogen-fibrin conversion). In such reactions one or a few of the many susceptible bonds in the protein are hydrolyzed much more rapidly than the remaining chemically equivalent bonds.

Appendix

Another rate expression, equation (16), which might be expected to fit the results shown in Figure 1, was also tested. Such a form results if the simple Michaelis-Menten path to products is allowed, but a second path to products is provided by a bimolecular reaction between the enzyme-substrate complex and a free substrate molecule. The dependence of the initial rate upon the substrate concentration is then given by equation (16), where k_2 is the rate constant for the

$$\frac{V_0}{[E]_0} = \frac{k_s[S]/K_s + k_2[S]^2/K_s}{1 + [S]/K_s} \qquad (16)$$

bimolecular reaction between the enzyme-substrate complex and a free substrate molecule. When the enzyme is saturated with substrate ($[S] >> K_s$), equation (16) becomes equation (17).

$$\frac{V_0}{[E]_0} = k_s + k_2[S] \qquad (17)$$

Equation (17) requires that a plot of $V_0/[E]_0$ vs. $[S]$ become a straight line at high substrate concentration, with the $V_0/[E]_0$ intercept $= k_s$ and slope $= k_2$. Figure 8 shows such plots for both L- and D-TAM. It is clear that neither system is described by (17), although as K_s' becomes larger (less favorable substrate binding) it is more difficult to distinguish between equations (16) and (3). For a sufficiently large K_s',

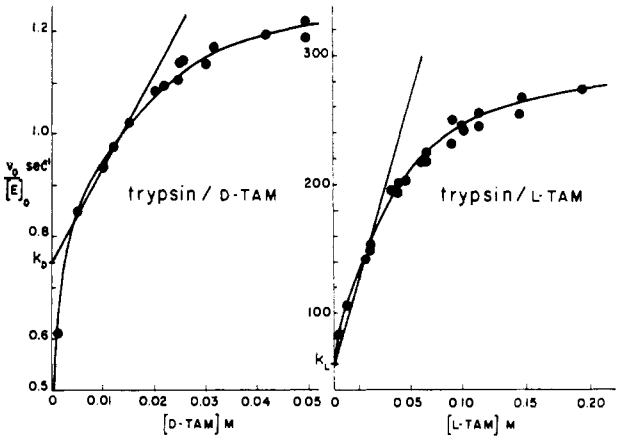

FIG. 8.—Dependence of initial rate upon substrate concentration for L- and D-TAM. The straight lines are drawn through k_D and k_L (equation 17) and show that the data do not fit such a relation. *p*H 8.0, 0.2 M KCl, 0.05 $CaCl_2$ at 25.0 ±0.1°, trypsin preparation I.

the two cases (i.e., ternary complex formation vs. bimolecular reaction) would be indistinguishable by this test.

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