Titration of Active Centers in Thrombin Solutions. Standardization of the Enzyme*

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ABSTRACT: Human thrombin was shown to catalyze the hydrolysis of nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate and *N*-benzyloxycarbonyl-L-lysinate with formation of an acylthrombin as intermediate in the reaction. The rapid formation and relatively slow hydrolysis of this intermediate made it possible to determine the concentration of active sites in the enzyme solution. Then a simple rate assay with a more conventional thrombin substrate, N^{α}-benzoyl-L-arginine ethyl ester, was employed to standardize the activity of the enzyme for routine use.

Hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester by thrombin yields a sigmoid pH rate profile with pK_i = 6.45, which implicates the participation of histidine side chains in catalysis. The previously observed kinetic similarities between thrombin, trypsin, and chymotrypsin are underlined by the absolute values of measured rate constants.

The determination of the molarity of active centers of an enzyme solution is one of the necessary steps before the enzyme can be studied by meaningful kinetic and mechanistic analysis. Since the most singular property of enzymes is their ability to catalyze reactions and since this catalytic property is often destroyed by physical and chemical agents without disrupting the primary structure of the enzyme, the knowledge of the concentration of active centers, as determined by kinetic methods, is usually more important than that of the concentration of protein molecules.

In recent years, several esteroproteolytic enzymes have been shown to react with their substrates in two kinetically significant steps (for a review of the subject see, *e.g.*, Bender and Kézdy, 1965) with the formation of an acyl-enzyme intermediate (ES′) according to the scheme

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow[H_2O]{k_3} E + P_2$$
$$+$$
$$P_1$$

where the symbols E, S, and P stand for the concentrations of enzyme, substrate, and products; K_S is the dissociation constant of the enzyme–substrate adsorption complex (ES), and k_2 and k_3 are the rate constants of acylation and deacylation, respectively.

It has been shown that if the initial concentration of enzyme (E_0) is much smaller than that of the substrate (S_0) the amount of P_1 produced in time t can be described (Kézdy and Bender, 1962) by eq 1.

$$P_1 = \frac{k_{cat}E_0S_0}{S_0 + K_M} + E_0\left[\frac{\frac{k_2}{k_2 + k_3}}{1 + K_M/S_0}\right]^2 \times \left(1 - e^{-\frac{(k_2 + k_3)S_0 + k_3K_S}{K_S + S_0}t}\right) \quad (1)$$

The experimentally measured Michaelis constant (K_M) is defined as $K_M = K_Sk_3/(k_2 + k_3)$ and the maximal turnover rate constant (k_{cat}) as $k_{cat} = k_2k_3/(k_2 + k_3)$.

At high values of t the exponential term approaches zero, and the production of P_1 can be described as a linear function of t

$$P_1 = At + \pi$$

where $A = k_{cat}E_0S_0/(S_0 + K_M)$ and the intercept

$$\pi = E_0\left[\frac{\frac{k_2}{k_2 + k_3}}{1 + \frac{K_M}{S_0}}\right]^2 \quad (2)$$

The experimental demonstration of such an intercept has a dual meaning. On one hand it shows the existence of an acyl-enzyme intermediate and, on the other, it permits (provided $k_2 \gg k_3$, as is the case with *p*-nitrophenyl ester substrates; see Discussion) evaluation of the enzyme concentration (E_0).

If indeed $k_2 \gg k_3$, eq 2 can be expressed as

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$$\frac{1}{\sqrt{\pi}} = \frac{1}{\sqrt{E_0}} + \frac{K_M}{\sqrt{E_0}} \times \frac{1}{S_0} \qquad (3)$$

Thus, by plotting $1/\sqrt{\pi}$ *vs.* $1/S_0$ for a series of experiments with constant enzyme concentration, one obtains a straight line, the intercept of which with the ordinate yields $1/\sqrt{E_0}$ and with the abscissa $-K_M$.

Once the effective (or functional) molar concentration of the enzyme solution is known, it may be readily standardized in terms of customary rate assays. A rate-assay determination of enzyme concentration provides a more flexible experimental tool in that it usually requires a much smaller amount of enzyme and less complicated equipment.

In the present paper we provide experimental evidence to show that the reaction of human thrombin with *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (CTNE)[1] proceeds by the above postulated two-step mechanism, allowing us to determine the concentration of active centers in solution. With the help of the calculated concentration value we standardized a specific rate assay using N^{α}-benzoyl-L-arginine ethyl ester (BAEE) and thus developed a procedure for measuring the active-center concentration of thrombin solutions with a single hydrolytic rate assay.

The choice of CTNE as substrate was dictated by the fact that it is a nitrophenyl ester ($k_2 \gg k_3$ and its hydrolysis can be easily measured spectrophotometrically) and that it is one of the most specific substrates for thrombin (Lorand *et al.*, 1962). Quite similar reasons dictated the choice of BAEE (Sherry and Troll, 1954) as substrate for rate assays. Finally, since thrombin-catalyzed reactions apparently require the presence of a base with pK_i of about 7 (inferred from Figure 2a of Sherry and Troll, 1954), hydrolysis of CTNE was studied at pH 5 to slow down the reaction as much as possible. Thrombin solutions seem to denature quite fast below pH 5.

Materials

CTNE was a gift from Dr. C. J. Martin of the Chicago Medical School (see Martin *et al.*, 1959), and it was dissolved in acetonitrile. Chromatographically pure BAEE hydrochloride was purchased from Mann Research Laboratories, New York, N. Y. (batch No. 707-H 2065); a stock solution of this ester was made up in 0.2 M sodium acetate–acetic acid buffer of pH 4. N^{α}-Benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CLNE), mp 151°, was a product of Cyclo Chemical Corp., Los Angeles, Calif.; a solution of this ester was prepared in 90% aqueous acetonitrile. All buffers were made up with analytical grade chemicals.

Human thrombin was obtained by a method (Miller and Copeland, 1962, 1965) consisting of the following steps: (a) extraction of lyophilized Cohn Fraction III with chloroform–methanol (2:1) to denature impurities; (b) extraction of the prothrombin; (c) two absorptions of the zymogen on barium citrate, decomposing each precipitate with IRC-50-Na^+ and at the same time removing impurities; (d) removal of excess sodium citrate and soluble barium citrate; (e) quantitative activation to thrombin; (f) ion-exchange chromatography; and (g) Sephadex G-200 chromatography. The enzyme preparation used in these experiments contained 9200 Iowa clotting units (Warner *et al.*, 1936; Ware and Seegers, 1949) and 5750 NIH clotting units (U. S. Standard Thrombin Lot B-2) per mg of protein (the latter based on the Folin–phenol reaction). The final thrombin product contained 66% of the total prothrombin clotting units available in the original extract, and 93% of the total units were accounted for when measurements of discarded fractions were included. Thus alteration of the prothrombin and thrombin activities during purification may have been insignificant.

The thrombin used in the present study had no detectable plasmin or plasminogen activity, as indicated by the analyses of Dr. A. J. Johnson of the New York University Medical Center. Incidentally, the data presented in Table VII of the present paper also fully substantiate the absence of plasmin.

The clotting activity was stable for 2 days at 25° and for at least 3 months at −10°. The BAEE-hydrolyzing activity of our stock thrombin solution was shown to remain constant for at least 6 days of storage at −20°. It gave BAEE-hydrolytic rates (see below) of 197×10^{-5} AU/sec before and 201×10^{-5} AU/sec after storage.

Trypsin was obtained from Mann Laboratories (G 1945; 9700 BAEE units/mg). Soybean trypsin inhibitor (R 372–111) was purchased from Armour and Co., Kankakee, Ill. Plasmin was a product of the Michigan State Health Department (10.1 Remmert and Cohen units/ml).

Methods

For standardization of measurements of protein concentration a 1-ml sample of thrombin was dialyzed overnight against two 500-ml portions of 0.15 M sodium chloride; its optical density was measured in 0.1 N sodium hydroxide at 280 mμ in a 1-cm cuvet and its nitrogen content was determined with the aid of micro-Conway diffusion after Kjeldahl digestion (Chibnall *et al.*, 1943).

Based on a 17.0% nitrogen content, determined on three similar human thrombin preparations, the extinction coefficient for a 1% protein solution gave $A^{1\%}_{280\ m\mu,\ 1\ cm} = 16.2$ AU. Measurement of the apparent absorbance of the same solution at 340 mμ indicated that correction for light scattering at 280 mμ should be less than $^1/_{100}$ of the 16.2 value. The latter may be compared with the 19.5 AU reported for bovine thrombin (Winzor and Scheraga, 1964).

Our stock thrombin solution was calculated to contain 0.31% protein. Absorbance changes during reac-

[1] Abbreviations used in this work: cbz, carbobenzoxy; CTNE, *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester; BAEE, N^{α}-benzoyl-L-arginine ethyl ester; CLNE, *N*-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester.

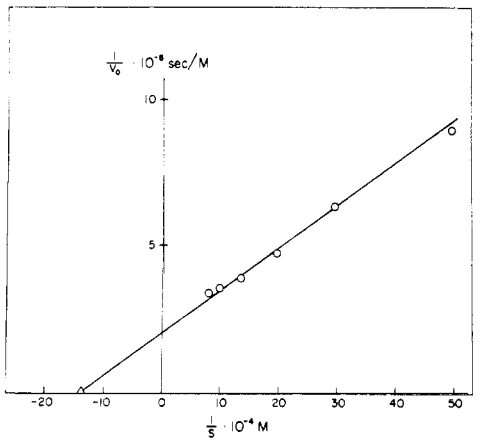

FIGURE 1: Lineweaver–Burk plot for the hydrolysis of cbz-L-tyrosine *p*-nitrophenyl ester by thrombin (2.28×10^{-6} M).

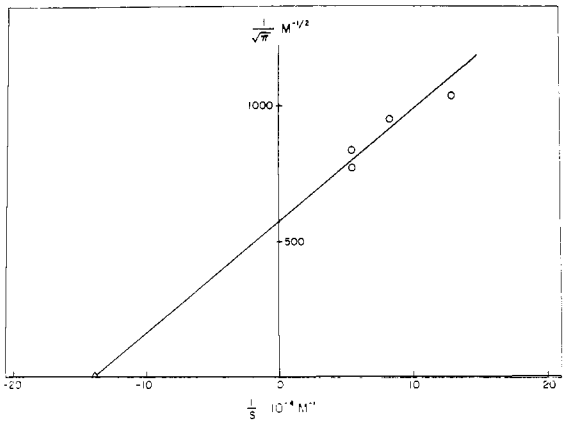

FIGURE 2: Initial "burst" in the reaction of cbz-L-tyrosine *p*-nitrophenyl ester with thrombin (3×10^{-6} M) plotted according to eq 3.

tion were measured on a Cary 14 recording spectrophotometer, equipped with a thermostated cell compartment, a 0–0.1-AU slide wire and a 20 cm/min chart-speed motor.

In calculating product formation, the following differential molar extinction coefficients were used: CTNE *vs.* hydrolysis products at pH 5.0, $\Delta\epsilon_{340} = 6220$; BAEE *vs.* hydrolysis products in the pH range of 5 and 10, $\Delta\epsilon_{255} = 808$; CLNE *vs.* hydrolysis products at pH 5, $\Delta\epsilon_{340} = 6250$.

In the "direct titration" experiments, the substrate solution was added to the buffer solution in a 1-cm quartz cuvet and the initial absorbance, as well as possible nonenzymatic hydrolysis of the substrate, was measured for a few minutes. Then the appropriate amount of enzyme solution measured onto the tip of a stirring rod from a micropipet was admixed and the reading of the enzymatic reaction was started. The time elapsed between the addition of the enzyme and the beginning of recording was carefully determined and usually did not exceed 5 sec. In the "inverse titrations" the enzyme solution was added first and its absorbance recorded, and the substrate was admixed subsequently. For the duration of the experiments the nonenzymatic rates of hydrolysis of substrates were less than 1% of those catalyzed by the enzyme; thus no correction was required.

Results

Reaction of CTNE. Production of *p*-nitrophenol with CTNE substrate was measured in all experiments at 25°, 1.6% acetonitrile, 0.2 M sodium acetate–acetic acid buffer, pH 5.02, 340 mμ. Figure 1 shows the Lineweaver–Burk (1934) type of plot obtained from a single rate curve with initial CTNE concentration of 1.8×10^{-5} M and 41.6 times diluted human thrombin stock solution. This yields $K_M = 7.15 \times 10^{-6}$ M.

In Table I, the amount of the initial observed "burst" is reported as a function of substrate concentration, using a 31.5-fold diluted stock thrombin. These data are plotted according to eq 3 in Figure 2. Using the above value of K_M for the intercept with the abscissa, the experimental points yield a straight line which makes it possible to evaluate the concentration of the active sites in the stock enzyme solution: $E_0 = 9.5 \times 10^{-5}$ M. Results presented in Table II and Figures 3a and 3b show that the value of π is proportional to the enzyme concentration, as is also the rate of the turnover reaction. The experimental results clearly indicate that the thrombin-catalyzed hydrolytic reaction indeed proceeds by a mechanism described by eq 1.

TABLE I: Titration of Thrombin with *N*-Benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (S_0).[a]

Mode of Titration	$S_0 \times 10^6$ (M)	$\pi \times 10^4$ (AU_{340})	$\pi \times 10^6$ (M)
Inverse	18.4	88	1.42
Inverse	12.0	68	1.09
Inverse	7.7	60	0.96
Direct	18.4	103	1.66

[a] The kinetic "burst" (π) of nitrophenol production is given as a function of substrate concentration. For experimental details see text.

Reaction of CLNE. Figure 4 presents the Lineweaver–Burk (1934) plot for the thrombin-catalyzed hydrolysis of CLNE at pH 5.02 (0.2 M sodium acetate–acetic acid), 1.6% acetonitrile, and 25°. Production of *p*-nitrophenol was measured at 340 mμ at initial CLNE concentration of 2.55×10^{-4} M and thrombin concentration (as calculated by means of the "burst" in CTNE hydrolysis) of 1.54×10^{-6} M. Initial "burst" was also readily observable with the CLNE substrate. However, the rapid-

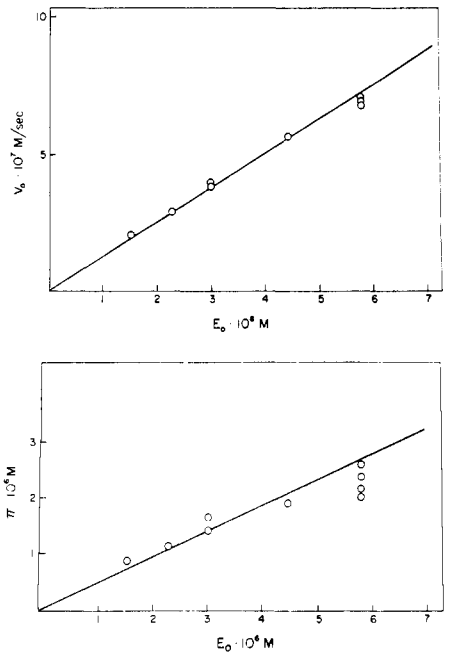

FIGURE 3: Hydrolysis of cbz-L-tyrosine *p*-nitrophenyl ester (S_0 = 1.8 × 10^{-5} M) in presence of varying concentrations of thrombin. (a) Initial steady-state rates; (b) initial "burst."

TABLE II: The Kinetic "Burst" (π) and the Initial Velocity (V_0) of Hydrolysis of *N*-Benzyloxycarbonyl-L-tyrosine *p*-Nitrophenyl Ester (S_0 = 1.8 × 10^{-5} M) as a Function of Thrombin (E) Concentration.[a]

Mode of Titration	$E \times 10^6$ (M[b])	$V_0 \times 10^7$ (M/sec)	$\pi \times 10^6$ (M)
Direct	2.27	2.89	1.14
Inverse	5.80	7.00	2.30
Inverse	5.80	6.88	2.41
			2.65
Inverse	4.44	5.67	1.93
Inverse	3.00	3.89	1.42
Direct	3.00	3.92	1.66
Inverse	5.80	7.17	2.20
Direct	1.53	2.03	0.88

[a] For experimental details see text. [b] Based on the calculated E_0 = 9.5 × 10^{-5} M concentration for the enzyme stock solution (obtained from Figure 2), various dilutions of thrombin were used.

ity of the reaction considerably decreased the accuracy of the measurements. Table III shows that, within considerable experimental error, the obtained π values agree with the calculated ones based on the titration of active centers with CTNE.

Reaction of BAEE. The reaction of BAEE with human thrombin was measured at pH values between 5 and 9.35, at 25°, with 10^{-3} M initial concentration of BAEE, by following changes in absorption at 255 mμ (see Schwert and Takenaka, 1955). The results are summarized in Table IV. The variation of k_{cat} as a function of pH fits correctly the usual sigmoid relation

$$k_{cat} = \frac{k_{cat,lim}}{1 + \frac{[H^+]}{K_i}}$$

where $[H^+]$ is the hydrogen ion concentration, $k_{cat,lim}$ is the maximal obtainable catalytic rate, and K_i is the ionization constant of a group in the enzyme which has a pK_a of 6.45 and is apparently required for catalysis.

In the light of these results we chose the following conditions for the standardization of the rate assay:

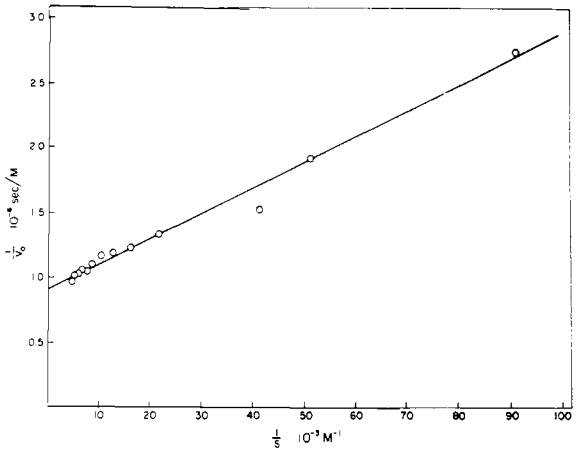

FIGURE 4: Lineweaver–Burk plot for the hydrolysis of α-cbz-L-lysine *p*-nitrophenyl ester by thrombin (1.54 × 10^{-6} M).

TABLE III: Kinetic "Burst" (π) in the Hydrolysis of N^{α}-Benzyloxycarbonyl-L-lysine *p*-Nitrophenyl Ester (S_0) by Thrombin ("Direct" Titration).[a]

$S_0 \times 10^4$ (M)	$E_0 \times 10^6$ (M)	π_{calcd} × 10^4 (AU$_{340}$)	π_{obsd} × 10^4 (AU$_{340}$)
2.55	1.54	96	140 ± 40
2.55	1.54	96	75 ± 25
2.39	7.19	450	390 ± 50

[a] For experimental details see text. The concentration of enzyme (E_0) and the expected "burst" (π_{calcd}) were calculated from the thrombin-catalyzed hydrolysis of *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester as shown in Figure 2.

TABLE IV: Hydrolysis of N^{α}-Benzoyl-L-arginine Ethyl Ester (S_0) by Thrombin (E).[a]

Buffer	pH	$E \times 10^7$ (M)	$S_0 \times 10^4$ (M)	k_{cat} (sec^{-1})	$K_M \times 10^5$ (M)
0.2 M sodium acetate	5.02	33.1	9.79	0.79	50
M/15 sodium phosphate	7.07	3.11	10.1	11.9	4.05
0.2 M Tris hydrochloride	7.97	3.47	10.1	17.15	4.76
0.2 M Tris hydrochloride	8.75	3.11	10.1	16.7	5.58
0.2 M sodium carbonate	9.35	3.11	10.1	16.0	2.24

[a] For experimental conditions see text. The concentration of enzyme is based on the measured kinetic "burst" with *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester substrate as shown in Figure 2. Apparent Michaelis constants (K_M) and molar turnover rates (k_{cat}) are presented. The limiting turnover rate ($k_{cat,lim}$) averages to be 16.5 sec^{-1}.

0.2 M Tris hydrochloric acid buffer of pH 7.97, 10^{-3} M BAEE, 25°, and 255 mμ. The results in Table V and Figure 5 show that, over a 100-fold range of enzyme concentration, the initial rate of hydrolysis of BAEE is a linear function of the enzyme concentration. A rate of 10^{-3} AU/sec is obtained with 8.10^{-8} M enzyme in solution.

TABLE V: Standardization of Thrombin in Terms of the N^{α}-Benzoyl-L-arginine Ethyl Ester Hydrolytic Rate Assay (V_0; initial velocity).[a]

$E_0 \times 10^9$ (M)	$V_0 \times 10^5$ (AU_{255}/sec)	$E_0 \times 10^9$ (M)	$V_0 \times 10^5$ (AU_{255}/sec)
603	738	154	197
456	578	62.1	80
306	392	6.13	5.75

[a] Initial ester concentration is 10^{-3} M; pH 7.97, 0.2 M Tris hydrochloride buffer; 25°. The concentration of thrombin (E) is based on the measured kinetic "burst" with *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester substrate as shown in Figure 2.

TABLE VI: Action of Soybean Trypsin Inhibitor (STI) on the Thrombin- and Trypsin-Catalyzed Hydrolysis of N^{α}-Benzoyl-L-arginine Ethyl Ester.[a]

[Thrombin] $\times 10^7$ (M)	[Trypsin] $\times 10^3$ (g/l.)	$V_{0,BAEE} \times 10^5$ (AU_{255}/sec)	
		No Inhibitor	STI; 2.9×10^{-2} (g/l.)
0	7.9	281	6
1.32	0	143	142
1.32	7.9	430	144

[a] 24.5 ± 0.5°; 0.19 M Tris HCl; pH 8.0; $[BAEE]_0 = 1.0 \times 10^{-3}$ M. Initial rates (V_0) of BAEE hydrolysis were measured at 255 mμ in a Zeiss M4Q III recording spectrophotometer with 1-cm light path 1 min after mixing the enzymes and inhibitor. Bovine trypsin was dissolved in 0.001 M hydrochloric acid. Human thrombin was in 0.15 M sodium chloride; its molarity was determined by the kinetic "burst" with CTNE.

While such a standardization of enzyme activity is valid for thrombin solutions which do not contain other BAEE-hydrolyzing impurities, it is not immediately obvious that the described rate assay could be readily adopted to measure thrombin activity in the presence of other BAEE-hydrolyzing enzymes. Since thrombin is derived from blood serum, trypsin and plasmin might be considered as likely contaminants. However, as shown in Tables VI and VII, measuring the BAEE hydrolytic rate with added soybean trypsin inhibitor permits a true evaluation of thrombin activity even in the presence of excess amounts of trypsin or plasmin. Such differential inhibition of nonthrombin impurities extends the usefulness of the BAEE hydrolytic assay for estimating thrombin activity in less pure systems.

Discussion

The experimental evidence presented in this paper bears out the assumption that thrombin catalyzes the hydrolysis of synthetic ester substrates by a two-step mechanism, preceded by a fast complex formation in equilibrium with the free enzyme and substrate. The intermediate can be identified as an aminoacyl-thrombin, which is likely to involve the diisopropyl phosphofluoridate sensitive serine in the enzyme (Miller and Van Vunakis, 1956; Gladner and Laki, 1956).

Moreover, the pH dependence of the reaction of thrombin with BAEE gives a sigmoid curve having a pK_i of 6.45, very similar to the $pK_i = 6.30$ obtained for the hydrolysis of BAEE by trypsin (Gutfreund, 1955; J. V. Killheffer, Jr., unpublished data). Even more strik-

TABLE VII: Action of Soybean Trypsin Inhibitor (STI) on the Thrombin- and Plasmin-Catalyzed Hydrolysis of N^{α}-Benzoyl-L-arginine Ethyl Ester.[a]

[Thrombin] $\times 10^7$ (M)	[Plasmin] (R + C units/ml)	$V_{0,BAEE} \times 10^5$ (AU_{255}/sec) No Inhibitor	$V_{0,BAEE} \times 10^5$ (AU_{255}/sec) STI; 2.9 $\times 10^{-2}$ (g/l.)
1.32	0	157[b]	155[b]
0	0.326	148	0
1.32	0.326	288	145

[a] 26.5 ± 0.5°; 0.19 M Tris HCl; pH 8.0; $[BAEE]_0 = 1.0 \times 10^{-3}$ M; 1.6% glycerol. Details of experiment same as described in Table VI. Human thrombin was dissolved in 0.15 M sodium chloride. Human plasmin (Michigan lot 8) was supplied in 50% glycerol. [b] No glycerol.

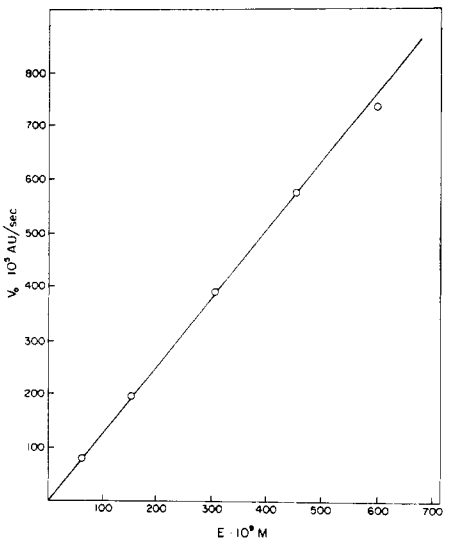

FIGURE 5: Standardization of human thrombin in terms of the velocity of hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester.

ing are the quantitative similarities in the rate constants of thrombin, trypsin, and α-chymotrypsin, as shown in Table VIII (Lorand *et al.*, 1962; Bender *et al.*, 1964; F. J. Kézdy, unpublished data). Thus not only the mechanism but also the kinetic specificity of these three enzymes must be very similar if not identical.

Once the concentration of active centers in thrombin solution has been determined with the use of the kinetic "burst" during hydrolysis of CTNE, this knowledge could be utilized to standardize the activity of the enzyme by a BAEE hydrolytic rate assay. We found that 1 mole of enzyme hydrolyzed 16.5 moles of substrate/sec at 25° and pH $\geq$ 8. Thus, 1 BAEE unit of enzyme is equal to 1.1×10^{-9} mole of enzyme as expressed in the recommended units (Report of the Commision on Enzymes, 1961); *i.e.*, 1 unit of human thrombin is that amount of enzyme which hydrolyzes 1 μmole of BAEE/min at 25° and pH $\geq$ 8.

Knowing the functional molarity and the protein concentration of the thrombin solution used, we calculate that the maximum limit for the molecular weight of human thrombin is 32,600. This calculation assumes thrombin to contain 17% N and possess one active center/molecule of protein. Further, if the purity of the enzyme preparation used would be less than 100%, the true molecular weight would be smaller accordingly.

Throughout our kinetic treatment we assumed $k_2 \gg k_3$. Several arguments sustain this assumption: (a) identical "burst" within experimental errors in the hydrolyses of both CTNE and CLNE; (b) the rate constants for hydrolyzing CTNE and CLNE by thrombin are almost identical with the rate constants obtained for the hydrolysis of these substrates by trypsin and chymotrypsin (for these enzymes it has already been shown that $k_2 \gg k_3$); (c) with the use of the kinetic "burst" and assuming $k_2 \gg k_3$, we obtain a reasonable value for the molecular weight of thrombin (Magnusson, 1965).

Since thrombin was shown to effect the conversion

TABLE VIII: A Comparison of the Kinetic Constants of the Hydrolysis of Ester Substrates by Human Thrombin (HT), Porcine and Bovine Trypsins (TR), and Bovine α-Chymotrypsin (CHT).[a]

Substrate	pH	k_{cat} (sec^{-1}) HT	TR bovine	TR porcine	CHT	$K_M \times 10^6$ (M) HT	TR bovine	TR porcine	CHT
CTNE	5.02	0.22	1.04	0.65	1.47	7.2	8.8	8.4	0.32
CLNE	5.02	0.72	0.98			21	5.7		
BAEE	8.75	16.5	16.0	24		56	10.9	4	

[a] Apparent Michaelis constants (K_M) and molar turnover rates (k_{cat}) are shown for *N*-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (CTNE), N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CLNE), and N^{α}-benzoyl-L-arginine ethyl ester (BAEE).

of fibrinogen to fibrin by a process of limited hydrolysis in which about 3% of the substrate protein is removed as fibrinopeptides (Lorand, 1951, 1952), it makes good chemical sense to postulate that in the fibrinogen–thrombin reaction, too, an acyl-enzyme intermediate would form.

$$\text{fibrinogen} + \text{thrombin} \rightleftarrows [\text{fibrinogen–thrombin}] \longrightarrow \underset{+\ \text{fibrin}}{H_2N\text{–fibrinopeptidyl–CO–thrombin}} \xrightarrow{H_2O} \text{fibrinopeptide} + \text{thrombin}$$

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