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Comparison of the Esterase Activities of Trypsin, Plasmin, and Thrombin on Guanidinobenzoate Esters. Titration of the Enzymes*

T. Chase, Jr., and Elliott Shaw

ABSTRACT: The acyl-enzyme intermediate, *p*-guanidinobenzoyl-trypsin, has been shown to deacylate unusually slowly, permitting the use of *p*-nitrophenyl *p'*-guanidinobenzoate as a convenient titrant of trypsin. It is now found that thrombin and plasmin, key enzymes in blood clotting physiology which have a trypsin-like esterase specificity, can also be titrated by *p*-nitrophenyl *p'*-guanidinobenzoate, showing similar "burst" behavior. This indicates the formation of an acyl-enzyme intermediate in normal plasmin catalyzed reactions, as has been established for the other two enzymes, and strengthens the hypothesis that all three proteolytic enzymes have evolved from a common ancestral form. The kinetic properties of the esterase action of trypsin thrombin, and plasmin on the ethyl and *p*-nitropheny esters of *p'*-guanidinobenzoic acid have been characterized. Noteworthy quantitative differences have been found distinguishing thrombin from trypsin and plasmin: the first named is the slowest acylated and the most rapid in deacylation. The esterase action of thrombin appeared even more disparate from the other two proteolytic enzymes in its relative ease of hydrolysis of the isomeric ester, *p*-nitrophenyl *m'*-guanidinobenzoate. These findings suggest that the active center of thrombin has greater geometric adaptibility to simple substrates than that of trypsin and plasmin.

We have recently reported on the use of *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (*p*-NPGB[1]) as a titrant for trypsin (Chase and Shaw, 1967); we wish to extend these observations and describe the reaction of this and related compounds with the proteolytic enzymes of the blood clotting system, plasmin and thrombin. Current interest in these enzymes derives not only from their very important physiological role but also from the possibility that such specialized enzymes may have evolved from trypsin or from a common

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[1] The following abbreviations are used: DIP-trypsin, diisopropylphosphoryl-trypsin; E*p*GB, ethyl *p*-guanidinobenzoate·HCl; GB, guanidinobenzoyl (as, *p*-guanidinobenzoyl-trypsin); *m*-NPGB, *p*-nitrophenyl *m'*-guanidinobenzoate·HCl; *p*-NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate·HCl; NPZL, *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate·HCl; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone·HCl.

ancestral precursor (Neurath *et al.*, 1967). A comparison of their kinetic properties is essential to an evaluation of their similarities and differences and to an eventual reconstruction of their evolutionary history. At the same time, useful methods of titration could be of great value in studying structure and function relationships of the individual enzymes. The advantages of specific titration procedures over rate assays for the determination of absolute concentrations of solutions of enzymes have recently been emphasized (Bender *et al.*, 1966). The former have as absolute standard an easily purified small molecule rather than "pure" enzyme, and avoid the large number of variables involved in rate assays. Different naturally occurring or artificially modified forms of an enzyme may show different rates of catalytic action (*cf.* Schroeder and Shaw, 1968), while a titration measures all functionally active species, and thus in studies on enzyme modification allows discrimination between the modification and the destruction of active sites (Ray *et al.*, 1960; Koshland *et al.*, 1962). This problem can be made more complex by the possibility of loss of one catalytic property relative to another, as in the case of thrombin, where clotting ability appears to be less stable than esterase activity (Lanchantin *et al.*, 1967); however, a single active center is involved. Also, comparison of functional molarity determined by a titration procedure with molarity of total protein (based on the protein content of a solution and the molecular weight as determined by physical methods) may indicate the purity of the enzyme preparation and the number of active sites per enzyme molecule.

Examination of the specificities of thrombin (Sherry and Troll, 1954) and plasmin (Troll *et al.*, 1954) in the hydrolysis of simple esters has shown them to be similar to that of trypsin. As a consequence, some of the titrants recently developed for trypsin have already been examined for suitability in the titration of thrombin and plasmin. Thus, Kézdy *et al.* (1965) have used *p*-nitrophenyl N^{α}-benzyloxycarbonyl L-lysinate hydrochloride (NPZL) and *p*-nitrophenyl-L-tyrosinate as titrants for thrombin; postburst (steady state) hydrolysis of the lysine ester at their recommended pH, 5.0, is too rapid for accurate determination of the burst, and in both cases the burst π must be determined at several substrate concentrations and a plot of $1/\sqrt{\pi}$ *vs.* 1/[S] extrapolated to infinite substrate concentration in order to determine the true enzyme concentration $[E]_0$.[2] Such a requirement limits the routine use of the titrant. Elmore and Smyth (1968) have reported on the use of N^{α}-methyl-N^{α}-(*p*-toluenesulfonyl)-L-lysine β-naphthyl ester and *p*-nitrophenyl N^{2}-acetyl-N^{1}-benzylcarbazate for the titration of trypsin; neither of these compounds reacted with thrombin at a satisfactory rate.[3] Tanizawa *et al.* (1968) have recently reported on the use of *p*-nitrophenyl *p'*-amidinobenzoate hydrochloride as a titrant for trypsin; the reaction of this compound with thrombin is under investigation in this laboratory.

Titration Theory. Bender *et al.* (1966, 1967) have developed kinetic expressions for the titration of enzymes which catalyze reactions according to the three-step sequence

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} P_1 + ES' \xrightarrow{k_3} E + P_2 \quad (1)$$

where E is enzyme, S is substrate, E · S is the (adsorptive) enzyme–substrate complex, ES' is the product of the stoichiometric reaction of the enzyme with substrate, liberating 1 equiv of P_1 (here *p*-nitrophenol), and P_2 is the finally released other product (here *p*-guanidinobenzoate ion). Assuming $[S]_0 > [E]_0$, the expression for the formation of *p*-nitrophenol is

$$[P_1] = \frac{k_{cat}[E]_0[S]_0 t}{[S_0] + K_{m\ app}} + [E_0]\left[\frac{k_{cat}/k_3}{1 + K_{m\ app}/[S]_0}\right]^2(1 - e^{-bt}) \quad (2)$$

where $k_{cat} = k_2k_3/(k_2 + k_3)$, $K_{m\ app} = K_sk_3/(k_2 + k_3)$, and $b = k_3 + k_2/[1 + (K_s/[S]_0)]$. When $k_2 \gg k_3$, b is the first-order rate constant of the presteady-state reaction at a given substrate concentration $[S]_0$. At high values of t the exponential term approaches zero (completion of the presteady-state reaction) and the amount of *p*-nitrophenol produced may be expressed as the observed burst, π, plus the steady-state production, $k_{cat}[E]_0[S]_0t/(K_{m\ app} + [S]_0)$. The burst π is determined by extrapolating the line of steady-state production to time zero. If $k_2 \gg k_3$ and $[S]_0 \gg K_{m\ app}$, π will not be affected significantly by substrate concentration and is approximately equal to $[E]_0$; if only the first assumption holds, π is dependent upon substrate concentration as described in eq 3, and

$$\pi = [E]_0\left[\frac{k_{cat}/k_3}{1 + K_{m\ app}/[S]_0}\right]^2, \text{ or } \frac{1}{\sqrt{\pi}} = \frac{k_2 + k_3}{k_2\sqrt{[E]_0}} + \frac{(k_2 + k_3)K_{m\ app}}{k_2\sqrt{[E]_0}}\frac{1}{[S]_0} \quad (3)$$

must be determined at several substrate concentrations to provide a plot of $1/\sqrt{\pi}$ *vs.* 1/[S], whose 1/[S] = 0 intercept is $1/\sqrt{[E]_0}$. This is the case with *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate hydrochloride as a titrant of trypsin and thrombin (Bender *et al.*, 1965; Kézdy *et al.*, 1965). It is thus desirable to determine k_2, k_3, and $K_{m\ app}$ in order to show that both conditions hold and a titration at a single substrate concentration is a valid measurement of $[E]_0$; alternatively, π may be measured at several substrate concentrations to see whether there is any observable dependence on substrate concentration.

[2] Compare the similar correction in the use of *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate hydrochloride as titrant for trypsin (Bender *et al.*, 1965, 1966).

[3] While these studies were in progress, Baird and Elmore (1968) were independently studying the reaction of plasmin and thrombin with *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride, with results somewhat different from ours (see Discussion).

The coefficient b above arises from the expression for rate of formation of the acyl-enzyme

$$\frac{d[ES']}{dt} = a - b[ES'] \quad (4)$$

where $a = k_2[E]_0/[1 + (K_s/[S_0])]$. Integration (Bender *et al.*, 1967) yields

$$bt = \ln \frac{a}{a - b[ES']}, \text{ or } -bt = \ln \frac{[E]_0 - [1 + (k_3/k_2)(1 + K_s/[S]_0)][ES']}{[E]_0} \quad (5)$$

If $k_2 \gg k_3$, the coefficient of [ES'] reduces to 1, and b may be determined as the slope of a plot of ln $([E]_0 - [ES'])/[E]_0$ *vs.* time. In the acylation of plasmin and thrombin by *p*-NPGB, the presteady-state reaction may be observed directly at very low *p*-NPGB concentration ($2–10 \times 10^{-6}$ M); $[E]_0 - [ES']$ is determined as the difference between the amount of *p*-nitrophenol expected by extrapolation of the steady-state line back to the time of observation, $\pi + k_{cat}[E]_0[S]_0t/(K_{m\,app} + [S]_0)$, and the actual amount of *p*-nitrophenol formed. In the acylation of plasmin by ethyl *p*-guanidinobenzoate hydrochloride (E*p*GB) $[E]_0 - [ES']$ is simply the amount of enzyme remaining active on NPZL in a rate assay. In both cases $[E]_0 - [ES']/[E]_0$ is the fraction of enzyme remaining active.

Assuming $k_2 \gg k_3$, the expression for b may be rearranged to give

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s}{k_2[S]_0} \quad (6)$$

Having determined b at various substrate concentrations, a plot of $1/b$ *vs.* $1/[S]$ has intercept $1/k_2$ and slope K_s/k_2. K_s and k_2 for the reactions of thrombin and plasmin with *p*-NPGB and plasmin with E*p*GB were determined by this procedure.

In the case of trypsin the presteady-state reaction with *p*NPGB is too rapid for observation with ordinary instrumentation. But in the presence of a competitive inhibitor eq 6 becomes

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s(1 + [I]/K_i)}{k_2[S]_0} \quad (7)$$

Thus at high inhibitor concentrations ($[I]/K_i \gg 1$) the presteady-state reaction is slowed enough to become observable. If b is determined at constant substrate concentration and varying inhibitor concentration, a plot of $1/b$ *vs.* [I] has intercept $1/(k_2) + K_s/(k_2[S]_0)$ and slope $K_s/(k_2K_i[S]_0)$. K_i being known, $K_s/(k_2[S]_0)$ can be found and shown to be much less than $1/k_2$; thus k_2 can be determined from the intercept and K_s from the slope. K_s and k_2 for the reaction of trypsin with *p*-NPGB were determined by this method using the competitive inhibitor benzamidine hydrochloride, whose K_i for competition with the acylation of trypsin with E*p*GB has already been determined (Mares-Guia and Shaw, 1967).

In the acylation of thrombin by E*p*GB, k_2 is *not* much greater than k_3, and eq 5 may not be simplified. Under these conditions acylation is not complete, and an equilibrium is reached where the rates of acylation and deacylation are equal. Thus $d[ES']/dt = 0$, and eq 4 may be developed to yield

$$k_2[E]_0 = [k_2 + k_3(1 + K_s/[S]_0)][ES'] \quad (8)$$

or

$$[E]_0/[ES'] = 1 + k_3/k_2 + k_3K_s/k_2[S]_0 \quad (9)$$

Thus if the ratio of acyl enzyme (total enzyme minus active enzyme) to total enzyme is determined at equilibrium in various substrate concentrations, a plot of $[E]_0/[ES']$ *vs.* $1/[S]_0$ will have intercept $1 + k_3/k_2$ and slope k_3K_s/k_2. If k_3 is known independently (see below), k_2 and K_s can be determined.

Determination of k_3. In order to determine $K_{m\,app}$ it is necessary to know k_3, the rate constant of deacylation. When $[S]_0 \gg K_{m\,app}$ and $k_2 \gg k_3$, the expression for postburst (steady-state) production of *p*-nitrophenol, $[P_1] = k_{cat}[E]_0[S]_0t/([S]_0 + K_{m\,app})$, reduces to $[P_1] = k_3 \cdot [E]_0t$. However, as has been previously noted (Chase and Shaw, 1967), the observed postburst production of *p*-nitrophenol can include a nonspecific catalysis of hydrolysis of the reagent, presumably by the imidazole and amino groups of the protein. Any contamination by a nonspecific esterase would also appear here.

Therefore, k_3 is best determined by actual isolation of the acyl-enzyme (by gel filtration to remove excess *p*-NPGB), incubation under the conditions used for titration, and observation of the recovery of enzymic activity (by titration or by rate assay). A plot of ln $[ES']/[E]_0$ *vs.* time will have slope $-k_3$; [ES'] is determined as $[E]_0 - [E]$. Comparison of k_3 thus determined with the rate constant *p*-nitrophenol production, k_{pb}, at a given *p*-NPGB concentration will indicate whether nonspecific catalysis of reagent hydrolysis is important at that concentration ($k_{pb} > k_3$). If k_{pb} is large but k_3 is small, as is the case with plasmin, it may be advisable to use a lower *p*-NPGB concentration.

Experimental Procedure

Materials and Methods. Bovine thrombin was purified from Parke-Davis topical thrombin by Dr. George Glover of this laboratory, using a combination of the procedures of Magnusson (1965) and Baughman and Waugh (1967). Human thrombin, prepared by the method of Miller and Copeland (1965), was the kind gift of Dr. Kenneth Woods. Urokinase-activated human plasmin was the kind gift of Dr. Kenneth C. Robbins; it had been activated in 25% glycerol with 0.3% urokinase (Robbins *et al.*, 1965), and was stored at −20° in 0.03 M Tris–0.01 M lysine, pH 8.8, 25% glycerol. Streptokinase-activated (Robbins *et al.*, 1965) human plasmin was also the gift of Dr. Kenneth C. Robbins; it was stored in 0.05 M Tris–0.02 M lysine, pH 9,

25% glycerol. Spontaneously activated (in 50% glycerol) human plasmin was the kind gift of Dr. Alan E. Johnson. β-Trypsin (the single-chain form (Schroeder and Shaw, 1968)) was purified from twice-crystallized salt-free lyophilized bovine trypsin (Worthington Biochemical Corp.) by chromatography on sulfoethyl-Sephadex (Schroeder and Shaw, 1968). DIP-trypsin was prepared as previously described (Chase and Shaw, 1967). Thrombin was inactivated with 1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride by incubation of the enzyme with a 0.01 M (saturated) solution of the inhibitor in 0.025 M potassium phosphate (pH 7.0)–1.0 M NaCl at 25° for 3 hr (1.1% of the activity on NPZL remained after this treatment) followed by dialysis against three 14-l. changes of deionized water at 4° for 36 hr; the protein precipitated upon dialysis but redissolved on dilution in 0.1 M sodium Veronal.

p-Guanidinobenzoic acid hydrochloride was prepared from *p*-aminobenzoic acid and NH_4SCN as described by Beyerman and Bontekoe (1953); *m*-guanidinobenzoic acid hydrochloride was prepared by the same method (mp 238–240°). Ethyl *p*-guanidinobenzoate hydrochloride was prepared by HCl-catalyzed esterification of the free acid (Beyerman and Bontekoe, 1953). *p*-Nitrophenyl *p'*-guanidinobenzoate hydrochloride was synthesized as previously described (Chase and Shaw, 1967); *p*-nitrophenyl *m'*-guanidinobenzoate hydrochloride (*m*-NPGB) was synthesized by the same procedure (mp 215–217°). *Anal.* Calcd for $C_{14}H_{13}ClN_4O_4$ (mol wt 336.74): C, 49.93; H, 3.89; Cl, 10.52; N, 16.63. Found (by Alfred Bernhardt, Mulheim, Germany): C, 49.97; H, 4.04; Cl, 10.30; N, 16.61. 1-Chloro-3-tosylamido-7-amino-2-heptanone hydrochloride (TLCK) was synthesized as previously described (Shaw *et al.*, 1965). Benzamidine hydrochloride and *m*-aminobenzoic acid were products of Aldrich Chemical Co.; *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate was a product of Cyclo Chemical Co. Other chemicals were of best reagent grade. Bio-Gel P-2 was a product of Bio-Rad Laboratories, Richmond, Calif. Water was distilled water passed through a Barnstead demineralizer and stored in glass.

Total protein in a solution of trypsin and guanidinobenzoyl-trypsin was determined by a modified phenol method (Sutherland *et al.*, 1949; Rabinowitz and Pricer, 1962), using as standard a solution of β-trypsin whose concentration of total protein was determined by amino acid analysis. The guanidinobenzoyl-enzyme absorbs approximately 50% more light at 280 mμ than the free enzyme (Mares-Guia and Shaw, 1967), and since the observed absorbance is a function both of the exact extinction coefficient of the acyl-enzyme and of the amount acylated, substantial uncertainties are introduced if total protein concentration is measured by ultraviolet absorption. With plasmin the increase on acylation (assumed the same as with trypsin, $\Delta E_{M(280)} \simeq 15{,}240$ (Mares-Guia and Shaw, 1967)) is much smaller in relation to the total molar absorption ($E_{M(280)} \simeq 180{,}500$); determinations of total protein by ultraviolet adsorption and by the phenol method, using in both cases as standard a solution whose concentration was determined by amino acid analysis (Dr. K. C. Robbins, personal communication), were in agreement within 1%. Concentration of thrombin was determined by the phenol method or by ultraviolet absorption, using for bovine thrombin $E^{1\%}_{280}$ 19.5 (Winzor and Scheraga, 1964); no correction for light scattering (Baughman and Waugh, 1967) was made, but solutions of absorbance > 2.0 were diluted for absorbance determination, and no deviation from Beer's law at lower absorbances was noted.

Inactivation of plasmin and thrombin was followed by a rate assay with NPZL in which 0.05 ml of a 0.01 M solution of the substrate in 95% CH_3CN–5% H_2O was added to 2.95 ml of 0.25 M sodium maleate buffer (pH 6.8) in each cuvet (thermostated at 25°) of a Beckman DB double-beam spectrophotometer equipped with variable range optical density converter and chart recorder. The solutions were mixed (1.67×10^{-4} M NPZL) with a polyethylene plunger and enzyme (25 μl) immediately added to the sample cuvet; after mixing the solutions production of *p*-nitrophenol was followed for 1 min at 402 mμ. Since in all cases only relative activity was sought, no conversion into micromoles per minute was carried out.

The clotting activity of bovine thrombin was determined as described by Ware and Seegers (1949). The clotting activity of human thrombin and the proteolytic activity of plasmin were reported by the donors.

Enzyme Titrations. Titrations were carried out in the Cary 15 double-beam recording spectrophotometer, essentially as previously described (Chase and Shaw, 1967), except for the omission of $CaCl_2$ from the Veronal buffer for titrations of bovine thrombin (purified in phosphate buffer). Human thrombin, however, tends to precipitate in the absence of calcium. Since plasmin and thrombin are not normally available in such large quantities as trypsin, the 0–0.1 slide wire of the Cary spectrophotometer was used, and the *p*-NPGB concentration was reduced to 5×10^{-5} M (10^{-5} M in some titrations of plasmin) to reduce errors resulting from unequal additions of *p*-NPGB to the reference and sample cuvet and from the relatively rapid postburst *p*-nitrophenol production with plasmin.

Determination of K_s and k_2. For the reaction of thrombin and urokinase-activated plasmin with *p*-NPGB and *m*-NPGB these constants were determined by titrating low concentrations of the enzymes with low concentrations of substrate in the Beckman DB spectrophotometer. Enzyme concentrations were 2.36×10^{-6} M plasmin, 2.02×10^{-6} M human thrombin, and 1.54×10^{-6} M bovine thrombin; lower concentrations were used when [*p*-NPGB] $< 4 \times 10^{-6}$ M. $([E]_0 - [ES'])/[E]_0$ was determined as described above (Titration Theory) at various points of the presteady-state reaction (which takes 20–60 sec for completion at low *p*-NPGB). The operational first-order rate constant b for each substrate concentration was obtained by a computer-performed least-squares treatment of the data according to the equation $\ln([E]_0 - [ES']/[E]_0) = -bt$; $1/k_2$ and K_s/k_2 were then determined as the intercept and slope of a plot of $1/b$ *vs.* 1/[*p*-NPGB] (here and in all plots the line best fitting the points was found by a computer-performed least-squares procedure and is the line

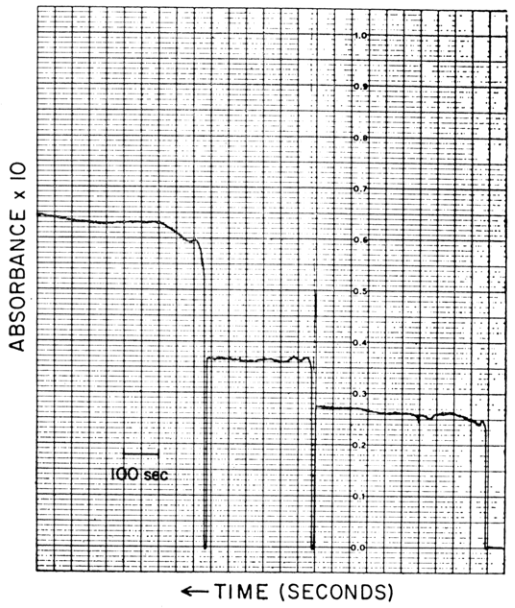

FIGURE 1: Titration of (from right) 0.02, 0.03, and 0.05 ml of a solution of streptokinase-activated human plasmin. The procedure was as described in text, using 10^{-5} M *p*-NPGB. Extrapolation of the absorbance to time zero (the chart line just to the right of the initial rise in absorbance) corresponds to enzyme concentrations of 1.51×10^{-6}, 2.145×10^{-6}, and 3.765×10^{-6} M, respectively. Another titration of 0.05 ml of enzyme solution (not shown) gave a value of 3.90×10^{-6} M; the stock solution is thus $7.51 \pm 0.23 \times 10^{-5}$ M.

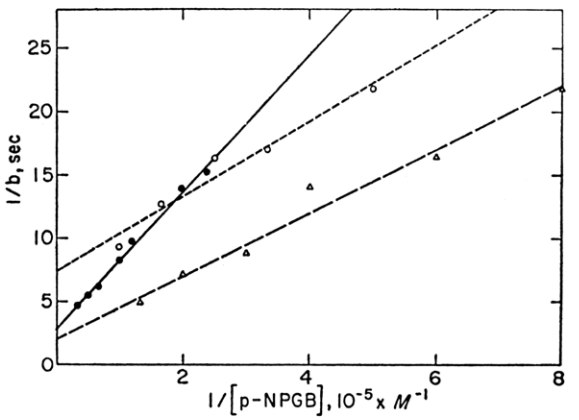

FIGURE 2: Determination of k_2 and K_s for the reaction of plasmin (Δ), human thrombin (●), and bovine thrombin (○) with *p*-NPGB. The first-order rate constants *b* at various *p*-NPGB concentrations were determined as described in the text.

shown in the figures; the uncertainties reported in the tables represent the standard deviation of the mean provided by this procedure, without reference to the error of each individual measurement or computation of *b*). For the reaction of trypsin with *p*-NPGB the reaction was carried out in the Beckman DB double-beam spectrophotometer, using 10^{-4} M *p*-NPGB and varying concentrations of benzamidine hydrochloride, the constant *b* for each benzamidine concentration determined as above, and k_2 and K_s determined as described above (Titration Theory) from a plot of $1/b$ *vs.* benzamidine concentration, using $K_i = 1.28 \times 10^{-5}$ M (Mares-Guia and Shaw, 1967). For the reaction of plasmin with E*p*GB the enzyme (1×10^{-6} M) was incubated with varying concentrations of E*p*GB in 0.5 M Tris-Cl (pH 8.15) at 25° (Mares-Guia and Shaw, 1967); at various times samples were withdrawn for assay of remaining activity ([E] = $[E]_0$ − [ES′]) with NPZL as described above. The data were treated in the same manner as for the reaction with *p*-NPGB. For the reaction of thrombin with E*p*GB the enzyme (1.5×10^{-5} M human thrombin, 1.9×10^{-6} M bovine thrombin) was inclubated with various concentrations of E*p*GB under the same conditions as for plasmin, but measurement of activity with NPZL was continued until the activity was constant; $[E]_0/[ES']$ was then plotted *vs.* 1/[E*p*GB] and k_3/k_2 and K_sk_3/k_2 determined as described above (Titration Theory). With a knowledge of k_3 from deacylation studies (see below) k_2 could then be found.

In all kinetic studies (except measurement of k_{pb}) a single preparation of each enzyme was used; in the case of plasmin this was the urokinase-activated sample.

Determination of k_3. Solutions of trypsin and human thrombin were diluted to 1 ml with 0.1 M sodium Veronal (pH 8.3)–0.02 M $CaCl_2$ and an excess of *p*-NPGB (2.5–3 equiv, in dimethylformamide) was added to acylate the enzyme. The solution was then filtered through a column (0.9 × 23 cm) of Bio-Gel P-2 and equilibrated with 0.1 M sodium Veronal (pH 8.3)–0.02 M $CaCl_2$, and 5 ml of eluate was collected after the void volume (6 ml). The eluate was incubated at 25°; the trypsin eluate was made 0.006 M in benzamidine hydrochloride to prevent autolysis. At intervals (time measured from when all the acyl-enzyme had sunk into the gel bed) the molarity of active enzyme was titrated with *p*-NPGB as described above. The same procedure was followed with plasmin and bovine thrombin, but for bovine thrombin $CaCl_2$ was omitted from the diluent and column buffer and for plasmin the diluent and column buffer were 0.04 M potassium diethylmalonate (pH 8.3)–0.01 M lysine. Several attempts to observe the deacylation of guanidinobenzoyl-plasmin in Veronal–lysine buffer were without success.

The concentration of activable enzyme, $[E]_0$, in the case of trypsin and plasmin was determined from the total protein concentration of the eluate and the per cent activity of the enzyme sample used. After 50-hr incubation the concentration of free (reactivated) trypsin was 98.3% of that expected from the total protein concentration and previous activity. The concentration of activable thrombin was determined as the final molarity of enzyme active to *p*-NPGB titration; this was 117% of that expected on the basis of total protein and initial activity of human thrombin, but only 76% with bovine thrombin.

From these data the percentage of acyl-enzyme remaining at each time, $[ES']/[E]_0$, was calculated and the slope $-k_3$ of the plot of ln $([ES']/[E]_0)$ *vs.* time was determined.

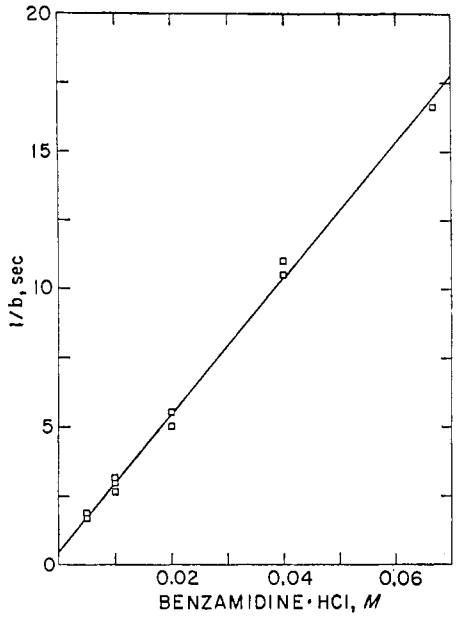

FIGURE 3: Determination of k_2 and K_s for the reaction of trypsin with *p*-NPGB. The first-order rate constants *b* were determined at various benzamidine hydrochloride concentrations as described in the text; *p*-NPGB concentration, 10^{-4} M.

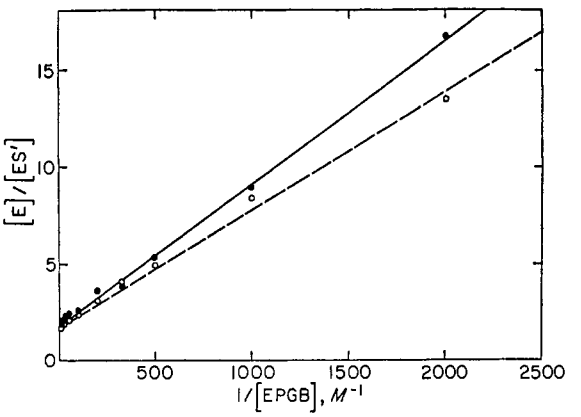

FIGURE 4: Determination of k_2 and K_s for the reaction of human thrombin (●) and bovine thrombin (○) with E*p*GB. The values for $[E]_0/[ES']$ were determined from the fraction of enzyme remaining active to NPZL at each E*p*GB concentration, as described in the text.

The empirical rate constant of postburst nitrophenol production, k_{pb}, was determined from the rate of postburst nitrophenol production by different amounts of enzyme at various *p*-NPGB concentrations, corrected for any catalysis of reagent hydrolysis by the buffer which the enzyme was stored in, and divided by the total molar concentration of enzyme (active and inactive) in the sample. With thrombin the molarity of active enzyme was used in the calculation, since the available evidence indicates (see below) that the postburst nitrophenol production is true turnover without a nonspecific contribution. The rate constant k_3 for the deacylation of *m*-guanidinobenzoylthrombin was determined as the V_{max} found by a Lineweaver–Burk (1934) treatment of the observed rates of steady-state nitrophenol production at various concentrations of *m*-NPGB (conditions as in the determination of k_2 and K_s).

Dependence of burst size on *p*-NPGB concentration was observed with plasmin and thrombin only when [*p*-NPGB] $< 5 \times 10^{-6}$ M, and was not observed at all with trypsin; hence, no correction for such an effect need be made at the *p*-NPGB concentration normally used for titration (5×10^{-5} M).

Results

Enzyme Titration. A sample of bovine thrombin, freshly chromatographed on IRC-50 and cellulose phosphate (Magnusson, 1965; Baughman and Waugh, 1967), was determined by the above procedure to be $1.445 \pm 0.007 \times 10^{-5}$ M in active enzyme. Assuming a molecular weight of 36,600 (Baughman and Waugh, 1967), this corresponds to 0.53 mg of active enzyme per ml; since the total protein concentration was 0.56 mg/ml (A_{280} 1.10), the sample was 95% active. It had a clotting activity of 1980 NIH units/mg of protein, 88% of the activity (2200 NIH units/mg) reported for pure thrombin (Baughman and Waugh, 1967). It should be emphasized that samples of "purified" thrombin may contain material reacting with *p*-NPGB but not able to clot fibrinogen, either because of contamination with another esterase or because of degradation of thrombin with loss of clotting ability (Seegers *et al.* (1958) and observations in this laboratory).

A sample of human thrombin was determined by the above procedure to be $2.02 \pm 0.15 \times 10^{-4}$ M in active enzyme; assuming a molecular weight of 32,600 (Kézdy *et al.*, 1965), this corresponds to 6.6 mg of active enzyme/ml. Since the sample was 12.9 mg of total protein/ml, the sample was 51.0% active. It had a clotting activity of 4651 Iowa units/mg of protein, 48.5% of the activity (10,600 Iowa units/mg of protein) of the purest human thrombin reported (Miller and Copeland, 1965).

A sample of urokinase-activated human plasmin was determined by this procedure to be $8.64 \pm 0.51 \times 10^{-5}$ M in active enzyme. Assuming a molecular weight of 75,400 (Barlow *et al.*, 1969), this corresponds to 6.51 mg of active enzyme/ml; since the sample was 8.6 mg of total protein/ml, this corresponds to 75.7% active enzyme. The proteolytic activity was 31.7 casein units (Rennert and Cohen, 1949) per mg of protein.

A sample of streptokinase-activated human plasmin was determined by this procedure to be $7.51 \pm 0.23 \times 10^{-5}$ M in active enzyme (Figure 1). Assuming molecular weight 75,400, this corresponds to 5.66 mg of active enzyme per ml; since the sample was 9.0 mg of total protein/ml, this corresponds to 62.9% active enzyme. The proteolytic activity was 22.1 casein units (Rennert and Cohen, 1949) per mg.

A sample of spontaneously activated human plasmin was determined by this procedure to be $0.353 \pm 0.007 \times 10^{-5}$ M, corresponding to 0.269 mg of active enzyme/ml.

TABLE I: Kinetic Constants of the Reaction of Trypsin, Plasmin, and Thrombin with E*p*GB and *p*-NPGB.[a]

Enzyme	Reaction with E*p*GB		Reaction with *p*-NPGB		Deacylation
	k_2, $10^3 \times$ sec^{-1}	K_s, $10^3 \times$ M	k_2, sec^{-1}	K_s, $10^6 \times$ M	k_3, $10^5 \times$ sec^{-1}
Trypsin, bovine	4.64	0.536	1.95 ± 0.61	0.61 ± 0.19	3.40 ± 0.16
Plasmin, human	2.35 ± 0.68	13.5 ± 3.9	0.46 ± 0.19	11.3 ± 4.9	0.525 ± 0.05
Thrombin, human	1.02 ± 0.12	8.4 ± 0.9	0.35 ± 0.03	18.6 ± 1.9	88 ± 3
Thrombin, bovine	1.19 ± 0.18	7.3 ± 1.0	0.13 ± 0.02	3.95 ± 0.76	98 ± 5

[a] The values for the reaction of trypsin with E*p*GB are from Mares-Guia and Shaw (1967); the other values, with standard deviations from the least-squares treatment, were determined as described in the text and shown in Figures 2–5.

Since the sample was 1.41 mg of total protein/ml, this corresponds to 19.2% active plasmin. The proteolytic activity was 10 casein units/mg of protein.

Determination of k_2, K_s, and k_3. Plots of the experimental data according to eq 6 (Figure 2), 7 (Figure 3), and 9 (Figure 4) give straight lines, validating the assumptions of eq 1. The slight curvature at high E*p*GB concentrations in Figure 4 may be due to competitive inhibition of NPZL hydrolysis by E*p*GB in the aliquot of enzyme–E*p*GB solution used for assay.

Similarly, the recovery of activity by spontaneous deacylation of the acyl-enzymes follows first-order kinetics, as shown in Figure 5. The thrombins deacylate far more rapidly than trypsin, essentially complete reactivation being accomplished in 1.5 hr as against 50 hr for trypsin. Plasmin deacylates still more slowly, and complete recovery of activity was not achieved. Guanidinobenzoyl-trypsin may also be completely deacylated by incubation in 3.33 M $NH_2OH \cdot HCl$ (pH 6.8) for 75 min, at which time it reaches maximal activity (90–95% of activity before acylation), but the hydroxylamine-treated enzyme, like hydroxylamine-treated nonacylated enzyme, showed kinetic differences (high k_{pb}) from untreated or spontaneously deacylated trypsin, suggesting that other modifications have taken place.

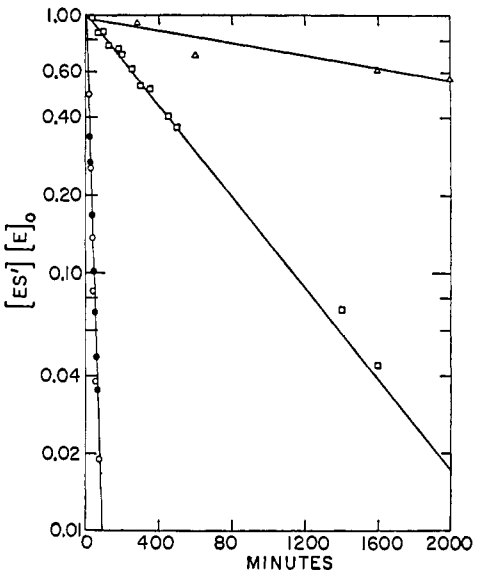

FIGURE 5: Deacylation of guanidinobenzoyl-plasmin (Δ), guanidinobenzoyl-trypsin (□), guanidinobenzoyl-human thrombin (●), and guanidinobenzoyl-bovine thrombin (Ö). The points represent the fraction of activatable enzyme remaining acylated at the indicated time, determined by *p*-NPGB titration of the deacylated enzyme. The lines for plasmin and trypsin are the theoretical lines calculated by application of a least-squares procedure; the deacylation of the thrombins is too rapid to be shown accurately on this scale.

The kinetic constants thus determined are summarized in Table I. It is clear that *p*-NPGB is most specific for trypsin and least specific for thrombin, with plasmin intermediate in rate of acylation, though with a high K_s like thrombin and a low k_3 like trypsin. The thrombins, as might be expected, are closely similar, though the lower K_s and k_2 of bovine thrombin for *p*-NPGB suggests a difference near the catalytic region where the *p*-nitrophenyl group might be bound.

"Postburst" Nitrophenol Production. After the burst of *p*-nitrophenol produced by reaction of 1 equiv of *p*-NPGB with each reactive site, a slow linear increase of absorbance is seen. As previously noted (Chase and Shaw, 1967), this can result either from deacylation of some of the acyl-enzyme (completion of the normal catalytic reaction) and reaction of the active site with more *p*-NPGB ("specific nitrophenol production") or from catalysis of hydrolysis of the active ester *p*-NPGB by groups (most probably the imidazole ring of histidine) in other parts of the enzyme surface ("nonspecific nitrophenol production"). They may be distinguished as follows: nonspecific nitrophenol production is proportional to *p*-NPGB concentration as well as enzyme concentration, and is unaffected by destruction of the active site by other reagents (DFP, TLCK); specific nitrophenol production is unaffected by *p*-NPGB concentration, is abolished by abolition of the active site with DFP or TLCK, and should be approximately equal to the rate constant of deacylation, k_3, determined by observation of the deacylation of the isolated acyl-enzyme. If the observed rates of nitrophenol production at various *p*-NPGB concentrations

TABLE II: Rate Constants of Postburst Nitrophenol Production by Trypsin, Urokinase-Activated Plasmin, and Human and Bovine Thrombin.[a]

Enzyme	k_{pb}, $10^4 \times sec^{-1}$	k_{1st}, $10^4 \times sec^{-1}$	k_{2nd}, $M^{-1} sec^{-1}$
Trypsin, bovine	0.48	0.176 ± 0.078 (9)	0.629 ± 0.034
DIP-trypsin	0.22	−0.078 ± 0.050 (7)	0.607 ± 0.017
Plasmin, human	11.8	−0.048 ± 0.043 (10)	24.0 ± 4.2
Thrombin, human	17.9	17.83 ± 0.690 (22)	−0.08 ± 0.10
Thrombin, bovine	8.1	7.85 ± 0.42 (15)	0.25 ± 0.28

[a] The constants k_{1st} and k_{2nd} are the intercept and slope, respectively, of the lines best fitting the points of Figures 6 and 7, as determined by a least-squares procedure; the number of values used is given, as in the interest of clarity not all are shown in the figures. From these values the average observed value of k_{pb} at [*p*-NPGB] = 5×10^{-5} M, the typical concentration used in titration, has been calculated.

are divided by the enzyme concentration and the resultant operational rate constants, k_{pb}, are plotted *vs.* *p*-NPGB concentration, they should describe a straight line whose intercept is the first-order (in enzyme) rate constant, k_{1st}, of specific nitrophenol production and whose slope is the second-order (first-order in enzyme and in *p*-NPGB) rate constant, k_{2nd}, of nonspecific nitrophenol production.

As shown in Figures 6 and 7, the observed rate constants k_{pb} do fall on straight lines; the constants derived from them are summarized in Table II. In the case of plasmin (Figure 7) the nonspecific nitrophenol production is large, and specific nitrophenol production is not observable, as expected from the very small k_3 (Table I).

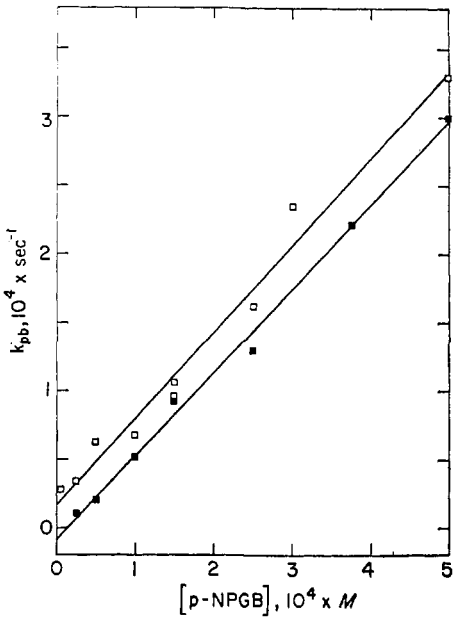

FIGURE 6: "Postburst" nitrophenol production by trypsin (□) and DIP-trypsin (■) as a function of *p*-NPGB concentration. Operational rate constants, k_{pb}, were determined from the rate of change of absorbance at 410 mμ after the burst (if any), using 4.25×10^{-6} M (total protein) β-trypsin or 1.255×10^{-5} M DIP-trypsin.

The thrombins present the reverse case: k_{2nd} is not statistically significant, and k_{1st} is approximately equal to the k_3 determined by observing the deacylation of the isolated acyl-enzyme. (That k_{1st} is slightly lower than k_3 in the case of trypsin and bovine thrombin is not unexpected, since the temperature was controlled at 25° in deacylation studies, but was slightly lower in determination of postburst nitrophenol production; also, k_{1st}, if equal to k_3, should be derived using the concentration of active enzyme rather than of total protein; this being

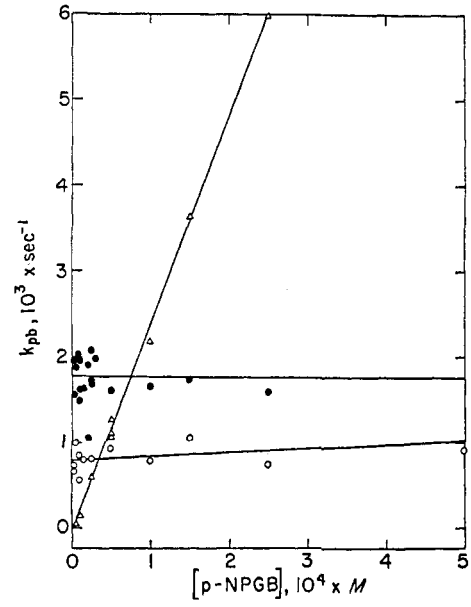

FIGURE 7: "Postburst" nitrophenol production by urokinase-activated plasmin (Δ), human thrombin (●), and bovine thrombin (○) as a function of *p*-NPGB concentration. Operational rate constants were determined as in Figure 6, using 1.14×10^{-6} M (total protein) plasmin, 1.74×10^{-6} M (active) human thrombin, or 1.32×10^{-6} M (active) bovine thrombin; thrombin points below 5×10^{-5} M *p*-NPGB are derived from the postburst rates found in experiments to determine k_2 and K_s, and some points used in calculating the theoretical lines shown have been omitted in the interests of clarity.

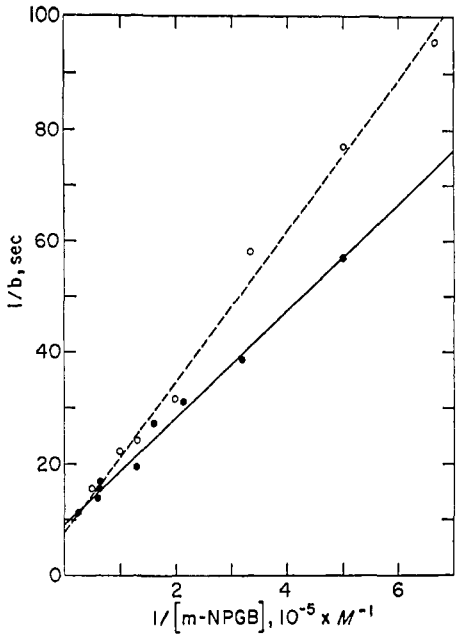

FIGURE 8: Determination of k_2 and K_s for the reaction of *m*-NPGB with human (●) and bovine (○) thrombin. The same experimental procedure was used as with *p*-NPGB.

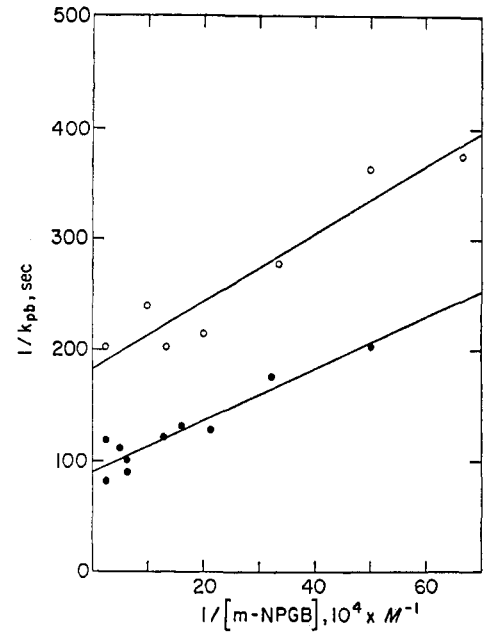

FIGURE 9: Determination of k_3 (assumed equal to the limiting value of k_{pb}) and K_m for the hydrolysis of *m*-NPGB by human (●) and bovine (○) thrombin. Values of k_{pb} at various *m*-NPGB concentrations were calculated from the steady-state rates of *p*-nitrophenol production observed in the experiments for the determination of k_2 and K_s.

smaller, k_{1st} is larger than calculated on the basis of the total protein concentration. A small systematic error is also indicated by the fact that the determined k_{1st} for DIP-trypsin is less than zero. However, no explanation is available for the observation that k_{1st} for human thrombin is about twice the k_3 found by deacylation.) Furthermore, TLCK-treated thrombin, dialyzed to remove excess reagent, releases no nitrophenol whatsoever from *p*-NPGB, indicating that hydrolysis of *p*-NPGB by thrombin occurs only at the active site. In the case of trypsin both types of catalysis are present, though small, and the difference between k_{1st} for trypsin and for DIP-trypsin, when corrected by using the concentration of active trypsin (77.6% of the total protein), is 3.29×10^{-5} sec^{-1}, satisfactorily close to the observed k_3 value of 3.40×10^{-5} sec^{-1}.

Table II also includes the values of k_{pb} at the typical *p*-NPGB concentration used in titration, 5×10^{-5} M, to illustrate considerations affecting the choice of *p*-NPGB concentration for titration. At 5×10^{-5} M postburst nitrophenol production by urokinase-activated plasmin is already high, and it is better to use 10^{-5} M *p*-NPGB, so that the extrapolation to zero time may be more accurate. Postburst nitrophenol production by the thrombins is high, but cannot be lessened by changing the *p*-NPGB concentration. Postburst nitrophenol production by changing trypsin is low, and trypsin may be titrated at [*p*-NPGB] = 10^{-4} M more accurately than either of the other enzymes.

The above data relate to urokinase-activated plasmin; it seemed possible that residual urokinase might be responsible for the relatively rapid nonspecific catalysis of hydrolysis of *p*-NPGB. Indeed k_{2nd} is lower for streptokinase-activated plasmin (5.0 ± 0.7 M^{-1} sec^{-1}; *cf.* Figure 1), but higher for spontaneously activated plasmin (58.4 ± 7.4 M^{-1} sec^{-1}). This may suggest partial exposure of the active site in plasminogen, or the presence of degraded material with more exposed histidines.

Finally, it should be reiterated that nucleophilic buffers such as Tris catalyze the hydrolysis of *p*-NPGB (Chase and Shaw, 1967), which should be taken into account when titrating a solution of enzyme so buffered; even 0.02 ml of 0.03 M Tris makes an appreciable contribution to the nonspecific postburst nitrophenol production, and it would be nearly impossible to titrate accurately a solution in Tris buffer if the enzyme concentration were so low that it was necessary to use 0.2 ml. This observation should likewise apply to the titrations of Tanizawa *et al.* (1968), who used Tris buffer at pH 8.2 and found a high rate of nonenzymic breakdown of their reagent.

Reaction with p-Nitrophenyl m'-Guanidinobenzoate Hydrochloride. *m*-NPGB is quite unlike *p*-NPGB as a substrate for trypsin and plasmin. With trypsin it shows an initial rate of nitrophenol production ($b = 1.01 \times 10^{-2}$ sec^{-1} at 10^{-4} M *m*-NPGB) only three times as fast as the steady-state rate ($k_{cat} = 3.3 \times 10^{-3}$ sec^{-1}). With plasmin production of *p*-nitrophenol is linear with time without any presteady-state burst (indicating $k_2 < k_3$); however, the reaction follows Michaelis–Menten kinetics ($K_m = 6.74 \times 10^{-4}$ M, $k_{cat} = V_{max} = 6.67 \times 10^{-2}$ sec^{-1}), suggesting that it does occur at the active site, rather than being associated with the site of nonspecific nitrophenol production (or, that site is a second true active site of different specificity). These observations indicate the high specificity of the active sites of trypsin and plasmin for the linear geometry of *p*-NPGB.

TABLE III: Kinetic Constants of the Reaction of *m*-NPGB with Human and Bovine Thrombin.

Enzyme	k_2, sec^{-1}	K_s, $10^5 \times M$	k_3, $10^3 \times sec^{-1}$	K_m (calcd), $10^6 \times M$	K_m (obsd), $10^6 \times M$
Thrombin, human	0.107 ± 0.047	1.02 ± 0.47	11.07 ± 0.73	9.6 ± 4.5	25.6 ± 4.9
Thrombin, bovine	0.122 ± 0.03	1.64 ± 0.40	5.48 ± 0.46	7.05 ± 1.8	16.7 ± 2.8

K_s, k_2, k_3 (assumed = k_{pb}(lim)), and K_m(obsd) are derived from Figures 8 and 9; K_m(calcd) is $K_{m\ app}$, calculated from k_2, K_s, and k_3.

With the thrombins, however, burst behavior is seen, similar to the burst with *p*-NPGB, though not as marked; the rates of presteady-state acylation may be obtained as with *p*-NPGB and $1/b$ plotted *vs.* 1/[*m*-NPGB] (Figure 8) to determine the rate constants for the reaction. The rate of deacylation has been assumed equal to the observed k_{pb} of postburst nitrophenol production, as it is with *p*-NPGB, but here Michaelis–Menten kinetics are observable in the variation of k_{pb} with *m*-NPGB concentration and it is necessary to plot $1/k_{pb}$ *vs.* 1/[*m*-NPGB] to obtain $k_{cat} = V_{max}$ (Figure 9). This Lineweaver–Burk (1934) plot also provides the "normal" K_m, which may be compared with $K_{m\ app}$ calculated from K_s, k_2, and k_3.

The kinetic constants are summarized in Table III. They are quite similar to those for *p*-NPGB, the chief difference being the three- to fivefold greater k_3, making *m*-NPGB less satisfactory as a titrant for thrombin than *p*-NPGB (though it might be useful to titrate thrombin in the presence of plasmin). In this respect thrombin is quite different from trypsin and plasmin, which are generally considered more similar to each other than to thrombin; the more intimate portions of the specificity site appear to be more flexible than in trypsin and plasmin. This contrasts with the greater specificity of thrombin toward protein substrates, which may involve portions of the enzyme surface more distant from the active site.

The directly determined Michaelis–Menten constants are close to those calculated from K_s, k_2, and K_3, but exceed them by a factor of about 2.5. The difference probably reflects difficulties in the exact determination of the rates of acylation and deacylation, especially at low substrate concentration. They are also dependent upon the accuracy of the determination of the burst, since this is used in the calculation of k_{pb} (moles of nitrophenol produced per mole of enzyme per second); this also is difficult to determine accurately at very low substrate concentration ($<10^{-5}$ M).

Discussion

A number of characteristics of a compound are important in assessing its usefulness as a burst titrant (Bender *et al.*, 1966; Chase and Shaw, 1967). These are: a high k_2 (rapid completion of the presteady-state reaction); a low k_3 (little turnover); a low $K_{m\ app}$, and a solubility high enough so that a substrate concentration much higher than $K_{m\ app}$ can be used; a high molar absorbance coefficient at the pH of titration for the initially released product, so that low enzyme concentrations can be measured; reasonable stability (slow nonenzymic hydrolysis) at the pH of measurement (preferably near neutrality); and good specificity (failure to react with other enzymes commonly contaminating preparations of the enzyme being titrated). *p*-NPGB is not fully specific, reacting with α-chymotrypsin (showing a burst and a high turnover) and with at least one other esterase in partially purified thrombin, as well as with trypsin, plasmin, and thrombin. It does not, however, react with subtilisin, or with papain (J. F. Kirsch, personal communication).

On other counts *p*-NPGB is significantly superior to other titrants described for trypsin and thrombin; relevant derived constants ($K_{m\ app}$ and k_2/k_3) for trypsin and human thrombin are summarized in Table IV. No data are available on the titration of plasmin and bovine thrombin with other titrants; with *p*-NPGB, $K_{m\ app}$ and k_2/k_3 are 1.3×10^{-16} M and 87,000 for plasmin and 2.9×10^{-6} M and 136 for bovine thrombin.

The single most important characteristic of *p*-NPGB is the very low value of k_3, which besides making easy the extrapolation of postburst absorbance to zero time (the actual measurement of the burst), results in a very low $K_{m\ app}$ and a high k_2/k_3 ratio, so that the size of the burst is not affected by substrate concentration, as it is with NPZL as titrant of both trypsin and thrombin. No change in burst size was seen with trypsin down to the lowest *p*-NPGB concentration used, 2×10^{-6} M, with only a 10% excess of reagent over enzyme. Some decrease in burst size was noted at *p*-NPGB concentrations below 5×10^{-6} M with plasmin and thrombin, but the early portions of the presteady-state reaction still followed first-order kinetics.

The slow rate of deacylation, even at pH 8.3, combines with the relative stability of the reagent at that pH (compared with NPZL, whose nonenzymic hydrolysis is fairly rapid even at pH 6.8) to allow titration at pH 8.3, rather than 3.0 or 5.0 as with NPZL; as a result full advantage can be taken of the high molar absorbance coefficient of *p*-nitrophenoxide ion, allowing titration of enzyme concentrations as low as 10^{-6} M. The titration could be extended to lower pH ranges by use of

TABLE IV: Comparison of Kinetic Constants of Various Titrants for Trypsin and Thrombin.[a]

Compound	pH	$\Delta\epsilon_M$	Reaction with Trypsin $K_{m\ app}$, M	k_3, sec^{-1}	k_2/k_3	$k_{cat}/K_{m\ app}$, $10^4 \times M^{-1}$ sec^{-1}	Human Thrombin $K_{m\ app}$, M	k_2/k_3
p-Nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate hydrochloride[b]	3.0	6150	1.27×10^{-6}	0.0143	27.6	0.109		
p-Nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate hydrochloride[c]	5.0	6250					2.2×10^{-4}	
N^{α}-Methyl-N^{α}-(*p*-toluenesulfonyl)-L-lysine β-naphthyl ester hydrogen bromide[d]	7.0	1690	8.8×10^{-6}	0.0583	98.3	0.656		
p-Nitrophenyl *p'*-amidinobenzoate hydrochloride[e]	4.7	8200	$\ll 5 \times 10^{-7}$	7.8×10^{-4}		>0.156		
	8.2	16600	$\ll 5 \times 10^{-7}$	0.0408		>8.2		
p-Nitrophenyl *p'*-guanidinobenzoate hydrochloride	8.3	16600	1.07×10^{-11}	3.4×10^{-5}	5.7×10^4	317.5	4.7×10^{-8}	394

[a] Values for other titrants are taken from or calculated from data in references below. Molar difference extinction coefficients are those given for the pH of titration. [b] Bender *et al.* (1965, 1966). [c] Kézdy *et al.* (1965). [d] Elmore and Smyth (1968). [e] Tanizawa *et al.* (1968).

the 2,4-dinitrophenyl and 2,4-dinitronaphthyl esters of *p*-guanidinobenzoic acid; the synthesis of these compounds is under study.

Baird and Elmore (1968) have reported that *p*-NPGB did not react with either of two samples of streptokinase-activated plasmin, obtained from Dr. P. Wallen; also, they found a value of k_2 for the reaction of bovine thrombin with *p*-NPGB 20-fold higher than our value. We have experienced no difficulty in titrating three samples of plasmin, activated by three different methods; no difference with method of activation was expected, since the same peptide bond is cleaved by all methods of activation (Summaria *et al.*, 1967) and no difference had been observed in the hydrolysis of a number of ester substrates (Sherry *et al.*, 1966). The proportion of active enzyme to total protein has ranged as high as 0.75, and is approximately proportional to specific activity in a proteolytic assay, indicating that we are indeed titrating plasmin rather than a contaminating enzyme. No explanation of the difference of our results from those of Baird and Elmore (1968) is available, other than the trivial possibility that their plasmin samples had lost all activity in transit from the donor (proteolytic activity of their samples was not reported).

The observation that plasmin is inactivated by DFP and other organophosphates (Mounter and Shipley, 1958) suggests that plasmin-catalyzed hydrolyses proceed *via* an acyl-enzyme intermediate, similar to the acyl-chymotrypsin and acyl-trypsin intermediates observed with a wide variety of substrates (Bender and Kézdy, 1965a). The behavior of plasmin with *p*-NPGB, an ester substrate, appears to establish the acyl-enzyme pathway: formation of an enzyme–substrate complex (as indicated by saturation kinetics in the initial burst reaction, Figure 2) is followed by rapid release of one equivalent of *p*-nitrophenoxide, with formation of an isolable inactive form of the enzyme, which slowly recovers activity, following first-order k netics (Figure 5). The uniqueness of the esterase action on *p*-guanidinobenzoates lies in the slowness of the deacylation, as with trypsin.

Bender and Kézdy (1965a) have emphasized the significance of the derived kinetic constant $k_{cat}/K_{m\ app}$, which is also equal to k_2/K_s (from the definitions of k_{cat} and $K_{m\ app}$; see Titration Theory above), in consideration of correlation between structure of a substrate and specificity of an enzyme for it. It is apparent from Table IV that $k_{cat}/K_{m\ app}$ for the reaction of *p*-NPGB with trypsin has a high value compared with those found with what are usually considered more specific substrates (esters of aliphatic α-acylamido amino acids); the values for the reaction of *p*-NPGB with plasmin and human and bovine thrombin, 40,500, 18,700, and 33,900, respectively, are lower than the value calculated for trypsin, but still an order of magnitude above the values for "specific" substrates of trypsin.

These high values for $k_{cat}/K_{m\ app}$ do not, of course, mean that *p*-NPGB is a "more specific" substrate; rather they reflect the unexpectedly low values of K_s. In the hydrolysis of "specific" ester substrates of chymotrypsin (Zerner *et al.*, 1964) and trypsin (Bender

TABLE V: Ratios of Kinetic Constants of Hydrolysis of Alkyl and *p*-Nitrophenyl Esters by Proteolytic Enzymes.[a]

Enzyme	Esters of	$\frac{k'_{NO_2C_6H_4}}{k'_{Me\ or\ Et}}$	$\frac{K_{s(Me\ or\ Et)}}{K_{s(NO_2C_6H_4)}}$	$\frac{k_{2(NO_2C_6H_4)}}{k_{2(Me\ or\ Et)}}$
Chymotrypsin	N^{α}-Acetyl-L-tryptophan[b]	48.5	(1)	(48.5)
	N-Acetyl-L-phenylalanine[b]	37	(1)	(37)
	Acetylglycine[c]	244	43	815
Papain	N^{α}-Benzyloxycarbonyl-L-lysine[d]	296	100–300	≥ 5
Trypsin	N^{α}-Benzyloxycarbonyl-L-lysine[e]	32	(1)	(32)
	p-Guanidinobenzoic acid	367,000	873	420
Plasmin	*p*-Guanidinobenzoic acid	43,000	1195	195
Thrombin, human	*p*-Guanidinobenzoic acid	65,000	452	340
Thrombin, bovine	*p*-Guanidinobenzoic acid	48,000	1850	113

[a] $k' = k_{cat}/K_{m\ app}$. Ratios given are for *p*-nitrophenyl ester *vs.* ethyl ester, except for acetylglycine and benzyloxycarbonyllysine, where data were available for the methyl ester but not the ethyl ester; catalytic constants for these two are not expected to differ by more than a factor of 2, and usually are very similar (Zerner *et al.*, 1964). Values in parentheses are derived from the assumption that for specific substrates of trypsin and chymotrypsin $K_{s(NO_2C_6H_4)} = K_{s(Me\ or\ Et)}$. [b] Zerner *et al.* (1964). [c] Zerner and Bender (1964). [d] Bender and Brubacher (1966). [e] Bender and Kézdy (1965).

and Kézdy, 1965b; Elmore *et al.*, 1967) differences between $k_{cat}/K_{m\ app}$ for aryl and alkyl esters have been comparatively small (Table V), and ascribable entirely to differences in k_2 commensurate with the intrinsic difference in reactivity of the esters (k_{OH}(nitrophenyl)/k_{OH}(ethyl) = 115 for acetates (Zerner *et al.*, 1964)). Thus in these cases the assumption (Zerner *et al.*, 1964; Zerner and Bender, 1964) that K_s is essentially invariant for all esters of a given acid is probably valid, implying that the alcohol group does not participate in binding.

In the case of *p*-NPGB and E*p*GB the large observed differences in $k_{cat}/K_{m\ app} = k_2/K_s$ are due not only to the expected differences in k_2, but also to large differences in K_s. No such differences have been observed with *p*-nitrophenyl or β-naphthyl esters of α-acylamido aliphatic *amino* acids ("specific" substrates) though considerable differences have been observed in the hydrolyses of esters of acetylglycine by chymotrypsin (Zerner and Bender, 1964) and esters of N^{α}-benzyloxycarbonyl-L-lysine by papain (Bender and Brubacher, 1966). The obvious explanation for a low K_s value for the *p*-nitrophenyl esters would be a contribution of the aromatic nucleus to binding, perhaps to a hydrophobic site not available to "specific" substrates; this might be expected to result in a different position of the substrate in the active site and consequent reduction of the rate of acylation (as is seen with *p*-NPGB, compared with "specific" *p*-nitrophenyl esters). The difference in K_s with esters of *p*-guanidinobenzoic acid seems large to be accounted for by such binding. The difference cannot be due to an influence of the acylation reaction on the initial binding equilibrium, even if k_{-1} and k_1 are both abnormally slow, since this would tend to *increase* the apparent K_s with the reactive ester, not decrease it (and if $k_{-1} < k_2$, the apparent k_2 would be decreased, while in fact k_2 for the nitrophenyl ester is larger in comparison to the ethyl ester than is the case with "specific" substrates (Table V)). It is possible that the effect is related to a configurational change; if *p*-NPGB, but not E*p*GB or "specific" substrates, can bind to an unfavorable configuration of the enzyme, and then be stabilized in position by a change to the configuration necessary for acylation, it might account for the observed low K_s. The question deserves further study.

It will also be noted that k_2 for *p*-NPGB is quite low for a *p*-nitrophenyl ester. From data in Bender *et al.* (1965) (k_2 = 0.395 sec^{-1} at pH 2.66, dependent upon a group of pK_a = 6.80) a limiting value for k_2 in NPZL acylation of trypsin can be calculated: $k_{2(lim)}$ = 5450. This difference is no doubt in part due to the chemical and steric deactivating effects of the aromatic nucleus in position α to the carboxyl of *p*-guanidinobenzoates on both the intrinsic reactivity of the ester and the ability of the enzymic active site to attack it (Silver and Sone, 1968), but probably mainly reflects the same unusual steric factors which so effectively hinder the deacylation (Mares-Guia and Shaw, 1967), especially if the principle of microscopic reversibility is invoked.

Finally, the possible clinical usefulness of guanidinobenzoate esters is worth considering. Thrombin and plasmin have similar specificities; it is consequently difficult to inhibit one without inhibiting the other, short of using a large protein inhibitor. But by taking advantage of the different kinetic constants of the two enzymes, such a differential inactivation is possible, at least *in vitro:* 10^{-3} M E*p*GB will essentially completely inactivate human plasmin with a half-time of about 50 min, but will inactivate human thrombin to the extent of only about 10%, because of the much higher rate of deacylation. Conversely, extensive inactivation of thrombin without inactivation of plasmin should be possible with the proper active ester of *m*-quanidinobenzoic acid. There are many complications, such as

hydrolysis of the esters by other serum esterases and possible toxic effects of the released alcohol; however, these are probably amenable to structural control. This approach to the differential inactivation of trypsin-like enzymes is under further study.

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