The Determination of the Concentration of Hydrolytic Enzyme Solutions: α-Chymotrypsin, Trypsin, Papain, Elastase, Subtilisin, and Acetylcholinesterase[1]

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Abstract: The use of rate assays is contrasted with the use of titrants to determine the absolute concentration of active sites of hydrolytic enzymes. Arguments are given which lead to the preference of a stoichiometric titration for the determination of the concentration of enzyme active sites. The absolute standard in this method is the small organic titrant rather than the enzyme which must be the absolute standard in a rate assay procedure. The mathematical basis of four methods of determining absolute enzyme concentration is presented. Three of these four methods were tested using *p*-nitrophenyl acetate and 2,4-dinitrophenyl acetate as titrants for α-chymotrypsin, showing good agreement. Of five titrants investigated for the determination of the concentration of trypsin solutions, *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride was the most precise, and the one most closely resembling a specific substrate of trypsin. *p*-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate served as a titrant for papain solutions. N-*trans*-Cinnamoylimidazole was a titrant for the enzyme subtilisin. For the titration of elastase, no suitable specific substrate was found and diethyl *p*-nitrophenyl phosphate was used. *o*-Nitrophenyl dimethylcarbamate was used for the titration of acetylcholinesterase. For each titration, it was demonstrated that the criteria necessary for a rigorous titration were met. For most enzymes, a rate assay, based on a k_{cat} calculated from the titration data, was developed as a secondary standard for the determination of enzyme concentration. A discussion of the purity of each enzyme preparation, possible impurities in the preparations, and the possible effect of these impurities on titrations and kinetics is given.

The assay of an enzyme solution historically has been tied to an operational phenomenon: an enzyme is present if catalysis of a particular chemical reaction can be detected, and the concentration of the enzyme is related to the rate of catalysis. Enzymes, however, may now be characterized by more than their catalytic behavior; namely, in terms of chemical constitution and in terms of stoichiometric chemical reactions. Thus, the usage of a "rate assay" to determine the concentration of a catalyst such as an enzyme may be questioned, a usage which is tantamount to using a rate assay for determining the concentration of a catalyst such as hydrochloric acid. This commentary is particularly true of the hydrolytic enzymes whose chemical constitution and stoichiometric chemical reactions have become reasonably well known in the past decade. It is the thesis of this paper that the accurate, simple, convenient procedures for the direct titration of the concentration of active sites of the hydrolytic enzymes chymotrypsin, trypsin, elastase, subtilisin, papain, and acetylcholinesterase, now available, offer a better approach to the determination of enzyme concentration than the rate assays of the past. Thus the definition of an enzyme used here is an intact active site which can undergo a specific stoichiometric reaction.

Methods will be presented for the determination of the concentration of active sites of certain hydrolytic enzymes. Because of the possible ambiguity with respect to the number of active sites per enzyme molecule, it is convenient to define these determinations in terms of normality of active sites. This is the object of this paper.

There are many other approaches to defining enzyme solutions. One may speak of enzyme units, enzyme activity, enzyme purity, or enzyme concentration.[3] One unit of enzyme is defined as that amount of enzyme which will catalyze the transformation of 1 μmole of substrate per minute under defined conditions.[3] The specific activity is expressed as units of enzyme per milligram of protein. The concentration of an enzyme solution is expressed as units of enzyme per milliliter. The purity of an enzyme solution is expressed as the specific activity of the preparation divided by the specific activity of the pure enzyme. These definitions were developed on the basis of the premise stated earlier that enzymes may be observed to catalyze reactions, but are not sufficiently characterized chemically for direct analysis. Of the above terms, we will not be concerned with units of enzyme nor with specific activity of an enzyme. We will, however, be concerned with the concentration of an enzyme solution which we will attempt to express in equivalents of enzyme per liter (normality). Knowing the equivalent weight of the enzymes from independent investigations and the

(1) This research was supported by grants from the National Institutes of Health.

(2) (a) Subtilisin; (b) National Institutes of Health Postdoctoral Research Fellow; (c) elastase; (d) papain; (e) trypsin; (f) chymotrypsin; (g) National Institutes of Health Predoctoral Research Fellow; (h) acetylcholinesterase.

(3) Report of the Commission on Enzymes of the International Union of Biochemistry, Pergamon Press, London, 1961, p 7; Enzyme Nomenclature: Recommendations (1964) of the International Union of Biochemistry, Elsevier Publishing Co., New York, N. Y., 1965; *Science*, 150, 719 (1965).

weight of enzyme making up a given solution, purity of an enzyme solution can then be expressed in terms of the actual normality divided by the normality calculated on a weight basis.

Rate assays for the determination of the concentration of enzyme solutions in terms of units of enzyme per milliliter have been described for many of the enzymes considered here utilizing natural or synthetic substrates.[4,5] In certain instances these assays have been converted from units of enzyme per milliliter to equivalents of enzyme per milliliter, a true concentration, by making the assumption that a reference standard of 100% pure (active) enzyme was available. There is one fundamental problem and a number of subsidiary problems associated with such a procedure.

The fundamental problem is associated with our present inability to define a 100% pure (active) enzyme. Certainly enzymes have been crystallized, shown to be homogeneous chromatographically, electrophoretically, and by ultracentrifugation. But it is difficult to accept the use of a pure enzyme as an absolute analytical standard when enzymes can adsorb impurities, when crystalline enzymes, even pure ones, contain very appreciable amounts of water which cannot be removed without denaturation of the enzyme, and when crystalline α-chymotrypsin, one of the purest and best characterized enzymes extant, is often found to contain 20–30% of impurities (including water). Analytical chemistry demands that absolute reference standards be extremely well characterized. At the present time no enzyme meets the criteria that analytical chemistry demands for such a characterization.

In addition to this fundamental problem of the absolute reference standard, rate assays are plagued with many uncertainties which limit the accuracy of the determination of an enzyme concentration. These include uncertainties due to the large number of variables in the rate assay that must be controlled such as temperature, ionic strength, cofactors, inhibitors, activators, pH, etc. The recommended procedure[3] for carrying out a rate assay is to use initial rates "in order to avoid complications due, for instance, to reversibility of reactions or to formation of inhibitory products." Furthermore, conditions are recommended in which the enzyme is saturated with substrate so that the rate approaches zero-order kinetics and a maximal velocity may be observed, or alternatively that a maximal velocity be determined by a Michaelis–Menten treatment.[3] Many of the rate assays for the enzymes under discussion violate one or more of the recommendations given above. These recommendations, even if followed, beg the question of the use of initial rates, a risky, last-resort procedure because initial rates are, many times, hard to determine and may hide a multitude of kinetic sins; beg the question of impurities in the enzyme test solution which may completely invalidate the kinetics when compared to a pure enzyme solution; and beg the question of the problems of rate assay which in some instances will not allow agreement from two laboratories to better than a factor of two. Thus, both theoretical and practical problems plague the use of rate assays for the determination of enzyme concentration.

(4) M. Laskowski, *Methods Enzymol.*, **2**, 8 (1955).
(5) H.-U. Bergmeyer, Ed., "Methods of Enzymatic Analysis," Academic Press Inc., New York, N. Y., 1963.

A number of the above problems may be obviated by the use of the "optical factor." In essence, this procedure involves a spectrophotometric determination of the concentration of protein in a given solution. Again the 100% pure (active) enzyme is used as absolute reference standard and again the problems inherent in such a procedure are not met. But in addition, the assumption must be made that the enzyme test solution under consideration is absolutely pure, using the same criteria as that for the 100% pure enzyme, and furthermore, that this solution is free from ultraviolet-absorbing contaminants, particularly of the peptide and protein variety. Since this assumption is ordinarily the point of issue in a determination of enzyme concentration, the use of the "optical factor" is a most unsatisfactory method for the determination of absolute enzyme concentration. The use of an "optical factor" involves not the determination of an *enzyme* concentration but rather the determination of a *protein* concentration. Since the two concentrations are not necessarily identical because of the usual presence of denatured enzyme, this procedure is unsatisfactory. Thus the use of an "optical factor" begs the question of the reactivity of the enzyme since the activity of the enzyme may easily be lost without any change in gross chemical structure. Thus analytical methods must be developed for the determination of the active sites of enzymes.

One of the knotty problems in dealing with enzymes is the possible presence of an enzymatically active impurity which can react with a substrate or titrant. Such extraneous reaction would be expected to occur at a rate different from that of the principal reaction, and thus should show up in the kinetics. However, it is advisable with any enzyme preparation to determine kinetics with at least two different degrees of purification against several substrates in order to determine if the observed activity is independent of purification. A further check may be made by comparing the rate of reaction of an inhibitor with the enzyme preparation to the rate of inhibition of its activity as determined with several different substrates.

The Stoichiometric Basis of the Titrations of Hydrolytic Enzymes. The stoichiometric reactions of hydrolytic enzymes with organophosphates provide the ultimate basis for the determination of the concentration of such enzyme solutions.[6] Following the discovery of these stoichiometric reactions, many other stoichiometric reactions have been observed with hydrolytic enzymes, some of which are readily adaptable for the present purposes. In general, what is required for the determination of enzyme concentration is a stoichiometric reaction which is readily and accurately observable.

But since an enzyme is a complex organic molecule, care must be taken to ensure that the stoichiometry observed and used is a meaningful stoichiometry. The primary requisite of the stoichiometry is that it must involve the active site of the enzyme. This requirement follows from the objective of these determinations, namely to titrate active sites as a true measure of active enzyme molecules. The stoichiometry may involve either a substrate,[7] an inhibitor,[8] or a coenzyme.[9]

(6) A. K. Balls and E. F. Jansen, *Advan. Enzymol.*, **13**, 321 (1952).
(7) B. Chance in "Technique of Organic Chemistry," Vol. 8, S. I. Friess and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1953, p 627, and references therein.

However, the latter two reagents do not test the primary chemical reaction of the enzyme and thus are less desirable than the former. Thus, the optimal stoichiometric reaction is a reaction at the active site of something closely resembling a normal substrate of the enzyme.

The stoichiometric basis of the present determinations of hydrolytic enzyme concentrations may be represented by eq 1 which represents the over-all pathway for

$$E + S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\longrightarrow} ES' \overset{k_3}{\longrightarrow} E + P_2 + P_1 \qquad (1)$$

α-chymotrypsin,[10] trypsin,[11] elastase,[12] subtilisin,[13] papain,[14] and acetylcholinesterase[15] reactions. The conversion of enzyme, E, into acyl-enzyme, ES′, and product one, P_1, can be considered a stoichiometric reaction with respect to an individual active site. If this process can be observed before the turnover (regeneration) of the enzyme occurs, then a direct measure of the enzyme concentration may be made.

A limiting example of the conversion of an enzyme into acyl-enzyme and P_1 without the regeneration of any enzyme is seen in the reaction of organophosphate and organosulfonate compounds with many hydrolytic enzymes, mentioned above. Such stoichiometric reactions for α-chymotrypsin, trypsin, elastase, subtilisin, acetylcholinesterase, and other cholinesterases were followed by the complete inhibition of these enzymes. Observations of the stoichiometry of the process were made by the amount of reactant (organophosphate) used up, by the liberation of P_1, in terms of hydrogen ion or *p*-nitrophenol, or by the attachment of phosphorus, isopropyl groups (from diisopropylphosphorofluoridate) or radioactivity (from a radioactively labeled organophosphate) to the protein.[4] Any of these observations of stoichiometry could be used as the basis of a procedure for the routine determination of enzyme concentration. Several disadvantages of using this approach may be noted. (1) The organophosphates are, in general, nonspecific compounds; for example, a stoichiometric reaction of bromelain and diisopropylphosphorofluoridate has been found but it does not involve the active site of this enzyme;[16] (2) except for the observation of *p*-nitrophenol (P_1), the experimental methods are both tedious and of limited accuracy.

The use of a specific substrate stems not only from the requirement that the stoichiometric reaction take place at the active site, but also from the desirability of being able to titrate the active sites of one enzyme in the presence of a number of other enzymes. Thus, the titrant for any given enzyme should optimally be modeled as closely as possible on the substrate of that enzyme to ensure reaction only at the active site and to ensure maximum selectivity between different enzymes.

The stoichiometry to be used for the titrations presented here will depend not on a pure enzyme as absolute standard, but rather on (a change in a physical property of) the substrate as absolute standard. The stoichiometric transformation will involve the reaction, $E \cdot S \rightarrow ES' + P_1$. The ultimate test of the stoichiometry, as mentioned above, is that ES′ is inactive whereas E is active enzymatically. With this test, the stoichiometry which is pertinent to an enzymatic process is demonstrated. In other words, this stoichiometry is an operational measure of the active site of the enzyme.

Probably the first direct observation of a stoichiometric production of P_1 using a substrate was in the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate.[17] Observation of the liberation of *p*-nitrophenol from this substrate led to the conclusion that the reaction proceeds in at least two distinct kinetic steps, specifically a fast initial liberation of approximately 1 mole of *p*-nitrophenol per mole of enzyme, followed by a slow turnover reaction. This observation, in itself, indicated the possibility of determining the concentration of kinetically active sites on the enzyme in an easy and accurate manner. However, the exact working conditions of a reliable experimental titration method were not estabilished.

The kinetic equations rigorously describing the hydrolysis of *p*-nitrophenyl acetate by α-chymotrypsin proved to be complex,[18,19] and furthermore the amount of phenol liberated in the initial step was shown to be a complex function of the equilibrium and rate constants involved in the reaction.[20,21] Experimental confirmation of these equations was not demonstrated. Subsequently methods were developed to titrate α-chymotrypsin with the substrate N-*trans*-cinnamoylimidazole[22] and the inhibitor 3-nitro-4-carboxyphenyl-N,N-diphenylcarbamate.[23] These methods did not, however, test the validity of the equations referred to above describing the stoichiometry during the enzymatic hydrolysis of a specific substrate.

It is the object of the present paper to describe a number of titrations of hydrolytic enzymes, which conform to the stoichiometry contained in eq 1 and which provide practical methods for the absolute determination of the normality of an enzyme solution. These determinations involve titrations of the concentrations of active sites of α-chymotrypsin, trypsin, elastase, subtilisin, papain, and acetylcholinesterase. A number

(8) T. Inagami and J. M. Sturtevant, *J. Biol. Chem.*, **235**, 1019 (1960); D. G. Doherty and F. Vaslow, *J. Am. Chem. Soc.*, **74**, 931 (1952).
(9) D. D. Ulmer, T.-K. Li, and B. L. Vallee, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1115 (1961).
(10) M. L. Bender and F. J. Kézdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964) and references therein.
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(12) T. H. Marshall, unpublished experiments.
(13) S. A. Bernhard, S. J. Lau, and H. Noller, *Biochemistry*, **4**, 1108 (1965); M. L. Begue and C. G. Miller, unpublished experiments.
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(15) I. B. Wilson, *Enzymes*, **4**, 501 (1959); R. M. Krupka and K. J. Laidler, *J. Am. Chem. Soc.*, **83**, 1458 (1961); M. L. Bender and J. K. Stoops, *ibid.*, **87**, 1622 (1965); J. K. Stoops, unpublished experiments.
(16) T. Murachi, *Biochim. Biophys. Acta*, **71**, 239 (1963); T. Murachi, T. Inagami, and M. Yasui, *Biochemistry*, **4**, 2815 (1965); T. Murachi and M. Yasui, *ibid.*, **4**, 2275 (1965).
(17) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **56**, 288 (1954).
(18) H. Gutfreund and J. M. Sturtevant, *ibid.*, **63**, 656 (1956).
(19) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).
(20) L. Ouellet and J. A. Stewart, *Can. J. Chem.*, **37**, 737 (1959).
(21) J. M. Sturtevant, *Brookhaven Symp. Biol.*, **13**, 164 (1960).
(22) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).
(23) B. F. Erlanger and F. Edel, *Biochemistry*, **3**, 346 (1964).

of these titrations involve substrates specific for the particular enzyme, and thus the titrations are accurate, experimentally facile, and specific.

Titration Theory. The kinetic equations describing eq 1 have been solved.[17,18,24,25] These equations offer several methods for the determination of the concentration of the (kinetically) active enzyme. Let us consider some of these possibilities. The first of these gives either a relative or absolute enzyme concentration, but the others give absolute enzyme concentration.

(1) The initial turnover rate of formation of *p*-nitrophenol in the enzymatic hydrolysis of a *p*-nitrophenyl ester following eq 1 may be expressed by eq 2[18]

$$V_0 = \frac{\frac{k_2k_3}{k_2 + k_3}[E]_0[S]_0}{[S]_0 + K_sk_3/(k_2 + k_3)} = \frac{k_{cat}[E]_0[S]_0}{[S]_0 + K_m(app)} \quad (2)$$

where $K_m(app)$ is the apparent Michaelis constant and k_{cat} is the catalytic rate constant, both operational parameters; K_s, k_2, and k_3 are defined by eq 1 and may be operational in certain instances.

When $[S]_0 \gg K_m(app)$, the rate reduces to

$$V_0 = k_2k_3[E]_0/(k_2 + k_3) \quad (3)$$

Furthermore, when $k_2 \gg k_3$

$$V_0 = k_3[E]_0 \quad (4)$$

In all cases V_0 is proportional to $[E]_0$ at a given $[S]_0$ and given experimental conditions. If the proportionality constant between V_0 and $[E]_0$ is known, the latter may be easily calculated from the former; if the proportionality constant is not known, a relative rate may still be calculated. Many rate assays of hydrolytic enzymes are based on this proportionality. However, as discussed above, such rate assays have severe limitations.

One approach for determining the proportionality constant between V_0 and $[E]_0$ is to determine one V_0 and one $[E]_0$. V_0 may be measured straightforwardly but a 100% pure (active) enzyme must be obtained in order to know $[E]_0$. Thus, this method depends ultimately on using a pure enzyme as absolute reference standard, with the limitations inherent in this procedure, as outlined above.

Another approach for determining the proportionality constant between V_0 and $[E]_0$ may be utilized when eq 4 is operative. The proportionality constant of eq 4, k_3, may be determined independently if the acyl-enzyme, ES′, of either a good or poor substrate is prepared. ES′ may be prepared by using an excess of substrate to effect acylation, followed by dilution of the solution so that the substrate concentration drops below $K_m(app)$ and buildup of the acyl-enzyme stops. Alternatively, acylation may be stopped by shifting the pH and/or removing the excess substrate by means of a Sephadex filtration or other methods. The decay of ES′ to E may be followed in many ways to determine the first-order constant, k_3, the most rigorous being the regeneration of E as measured with a "true" substrate of the enzyme; this reaction, is, of course, independent of the original enzyme concentration. The constant, k_3, together with the maximal velocity, $V_0 = k_3[E]_0$, then gives the absolute enzyme concentration directly. Essentially this procedure was used by Wilson and co-workers[26,27] for acetylcholinesterase, with the exception that the transformation from k_3 to $[E]_0$ was more complicated.

(2) The relationship between the slopes of the Lineweaver–Burk plots of the steady-state and presteady-state portions of reaction 1 leads to the determination of the absolute concentration of enzyme. Equation 2, describing the initial steady-state (turnover) reaction, may be transformed into

$$\frac{1}{V_0} = \frac{k_2 + k_3}{k_2k_3}\frac{1}{[E]_0} + \frac{K_s}{k_2[E]_0[S]_0} \quad (5)$$

which in terms of Michaelis parameters is identical with

$$\frac{1}{V_0} = \frac{1}{k_{cat}}\frac{1}{[E]_0} + \frac{K_m(app)}{k_{cat}[E]_0}\frac{1}{[S]_0} \quad (6)$$

The comparable equation of the presteady state is

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

if $(k_2 + k_3) > k_3K_m(app)$[18] ($b$ is the first-order rate constant of the presteady state; see eq 10). Thus the slope of eq 5 (plotted as $1/V_0$ *vs.* $1/[S]_0$) is $K_s/k_2[E]_0$ while the slope of eq 7 is K_s/k_2; by dividing the former slope by the latter, we may obtain $[E]_0$, the absolute concentration of enzyme.[28]

(3) Under certain very stringent conditions, the kinetics of acylation ($d[P_1]/dt$) may be observed under second-order conditions.[24,25,29] Under these conditions, which require that $k_2 \gg k_3$ and that $K_m(app) > [S]_0 \cong [E]_0 < K_s$, the rate of appearance of *p*-nitrophenol may be represented by

$$V = d[P_1]/dt = (k_2/K_s)([E]_0 - [P_1])([S]_0 - [P_1]) \quad (8)$$

Equation 8 may be transformed into

$$\frac{V}{[S]_0 - [P_1]} = \frac{k_2}{K_s}[E]_0 - \frac{k_2}{K_s}[P_1] \quad (9)$$

A plot of $V/([S]_0 - [P_1])$ *vs.* $[P_1]$ gives a straight line whose intercept divided by the slope is $[E]_0$, the absolute concentration of enzyme.

(4) The most common set of conditions for observation of enzymatic process, and the most important set for the determination of enzyme concentration, utilizes $[S]_0 \gg [E]_0$. Under these conditions a presteady state (acylation) and a steady state (deacylation) may be observed, the former often being too fast to measure, and the concentration of P_1 liberated in the presteady state (π) may be related to the absolute enzyme concentration. For reaction 1, when $[S]_0 \gg [E]_0$, $[P_1]$ (conveniently *p*-nitrophenol) produced in time t may be described by[18]

(24) T. Spencer and J. M. Sturtevant, *J. Am. Chem. Soc.*, **81**, 1874 (1959).

(25) F. J. Kézdy and M. L. Bender, *Biochemistry*, **1**, 1097 (1962).

(26) I. B. Wilson, M. A. Hatch, and S. Ginsberg, *J. Biol. Chem.*, **235**, 2312 (1960).

(27) I. B. Wilson and M. A. Harrison, *ibid.*, **236**, 2292 (1961).

(28) J. M. Reiner, "Behavior of Enzyme Systems," Burgess Publishing Co., Minneapolis, Minn., 1959, p 58, proposes a method for the absolute determination of enzyme concentration which is essentially a combination of enzyme concentration which is essentially a combination of eq 5 and 7. This method is limited, however, because it only applied to initial rates and only when $[E] \cong [S] \cong K_S$.

(29) M. L. Bender, G. R. Schonbaum, and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

$$[P_1] = \frac{k_{cat}[E]_0[S]_0 t}{[S]_0 + K_m(app)} + [E]_0\left[\frac{\left(\frac{k_2}{k_2 + k_3}\right)}{\left(1 + \frac{K_m(app)}{[S]_0}\right)}\right]^2 \times \left(1 - \exp\frac{-(k_2 + k_3)[S]_0 + k_3K_s}{K_s + [S]_0} t\right) \quad (10)$$

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

$$[P_1] = \pi + At \quad (11)$$

where $A = (k_{cat}[E]_0[S]_0/([S]_0 + K_m(app))$ and π may be expressed by

$$\pi = [E]_0\frac{\left(\frac{k_2}{k_2 + k_3}\right)^2}{\left(1 + \frac{k_m(app)}{[S]_0}\right)^2} \quad (12)$$

π may be easily determined experimentally since it is the intercept of a plot of $[P_1]$ *vs.* t at $t = 0$. Equation 12 may be transformed to eq 13, which indicates that

$$\frac{1}{\sqrt{\pi}} = \frac{k_2 + k_3}{k_2}\frac{1}{\sqrt{[E]_0}} + \frac{(k_2 + k_3)K_m(app)}{k_2\sqrt{[E]_0}}\frac{1}{[S]_0} \quad (13)$$

$\pi = [E]_0$ only if $k_2 \gg k_3$ and $[S]_0 \gg K_m(app)$. If the latter condition does not hold, a series of experiments at constant $[E]_0$ but varying $[S]_0$ enables one to circumvent this condition. From eq 13, a plot of $1/\sqrt{\pi}$ *vs.* $1/[S]_0$ gives a straight line whose intercept is $((k_2 + k_3)/k_3(1/[E]_0))$. If the condition is met that $k_2 \gg k_3$, the intercept of such a plot will give $1/[E]_0$ and hence $[E]_0$ directly.

The reactions of diethyl *p*-nitrophenyl phosphate and related compounds with various hydrolytic enzymes is usually carried out under conditions in which $[S]_0 > [E]_0$. However, these reactions must be treated in a special manner since no evidence for an adsorptive enzyme–substrate complex has as yet been found. Hartley and Kilby[30a] who first investigated the reaction of this reagent with chymotrypsin considered that the reaction followed eq 14 where S is the phosphate ester, ES′, is the diethyl phosphoryl-enzyme, P_1 is nitrophenol, and k_{II} is the second-order rate constant of the reaction of enzyme and phosphate ester, rather than eq 1.

$$E + S \xrightarrow{k_{II}} ES' + P_1 \quad (14)$$

However, it is known that diethyl phosphoryl-trypsin and diethyl phosphoryl-chymotrypsin spontaneously dephosphorylate.[30b,c] Therefore, the reaction should be written

$$E + S \xrightarrow{k_{II}} \underset{+\ P_1}{ES'} \xrightarrow{k_3} E + P_2 \quad (15)$$

where the symbolism is the same as before with the addition of P_2, diethyl phosphate. If one assumes that the concentration of phosphate titrant, S, is much greater than that of the enzyme and that the reaction between enzyme and phosphate occurs only at the active site of the enzyme, the relationship between π, the amount of *p*-nitrophenol liberated in the pseudo-first-order reaction, and the true enzyme concentration may be derived. The derivation utilizes two differential equations describing eq 15, $d[P_1]/dt = k_{II}[S]_0[E]$, and $d[ES']/dt = k_{II}[S]_0[E] - k_3[ES']$, and the conservation equation $[E]_0 = [E] + [ES']$, and leads to eq 16 and 17

$$\sqrt{\pi/[E]_0} = \frac{1}{1 + k_3/k_{II}[S]_0} \quad (16)$$

or

$$\sqrt{\pi/[E]_0} = \frac{1}{1 + k_3/(k_{obsd} - k_3)} \quad (17)$$

where k_{obsd} (*b*) is the pseudo-first-order rate constant when the substrate is in great excess over the enzyme, corresponding to k_{II}. From eq 17, it may be seen that $\pi = [E]_0$ when k_{obsd}, the pseudo-first-order rate constant of the inhibition reaction, is much greater than the dephosphorylation rate constant, k_3. Thus, there are three requirements for a successful titration corresponding to eq 15: (1) $[S]_0 \gg [E]_0$; (2) $k_{obsd} \gg k_3$; and (3) the titration reaction must occur at the active site and only at the active site of the enzyme.

Experimental Section

Materials. *p*-Nitrophenyl acetate was recrystallized from chloroform–hexane, mp 79.5–80.0°. 2,4-Dinitrophenyl acetate was recrystallized from the same solvent, mp 71–71.5°. *p*-Nitrophenyl isobutyrate was prepared from *p*-nitrophenol and isobutyric anhydride in pyridine, and was recrystallized four times from pentane, mp 39.5–40.2° (lit.[31] mp 39–40°). *p*-Nitrophenyl trimethylacetate was prepared from *p*-nitrophenol and trimethylacetyl chloride in pyridine, and was recrystallized twice from ethanol, mp 93.5–94.5° (lit.[32a] mp 94–95°). Upon hydrolysis in 0.05 *M* potassium hydroxide, $\Delta\epsilon$ was 18,300 ± 840 (lit.[25] ϵ of *p*-nitrophenoxide ion, 18,300).

o-Nitrophenyl dimethylcarbamate was synthesized from dimethylcarbamyl chloride and *o*-nitrophenol in pyridine. The light yellow carbamate was crystallized twice from ether–heptane solution, mp 56.7–57.0°. *Anal.* Calcd for $C_9H_{10}N_2$: C, 51.43; H, 4.80; N, 13.33. Found: C, 51.63; H, 4.82; N, 13.17.[32b] *p*-Nitrophenyl dimethylcarbamate was synthesized from dimethylcarbamyl chloride and *p*-nitrophenol in pyridine. The carbamate was crystallized three times from hot heptane, mp 107.5–108.0°. *Anal.* Calcd for $C_9H_{10}N_2$: C, 51.43; H, 4.80; N, 13.33. Found: C, 51.55; H, 4.87; N, 13.03. Phenyl acetate was an Eastman Kodak Co. product. The material was either distilled directly at atmospheric pressure followed by distillation at reduced pressure, or alternatively was first treated with 5% ice-cold potassium carbonate solution followed by distillation at reduced pressure. The latter procedure gave a product which contained less than 0.6% phenol.

Diethyl *p*-nitrophenyl phosphate (Paraoxon) was obtained from the American Cyanamid Corp.[33] and used without further purification. Its ultraviolet and infrared spectra agreed well with those in the literature[34] although it had a faint yellow color. Its nmr spectrum agreed with the designated structure, and it liberated 99 ± 1% of the theoretical amount of *p*-nitrophenoxide ion in 0.05 *M* KOH. The compound appeared to contain less than 0.3% *p*-nitrophenol impurity.

N-*trans*-Cinnamoylimidazole[22] was recrystallized from dry (CaH_2-distilled) *n*-hexane, mp 133–134°. N-Acetyl-DL-tryptophan *p*-nitrophenyl ester[35] has been described previously.

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p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, mp 155.0–156.4°, was a gift of Dr. C. J. Martin. α-N-Benzoyl-L-arginine ethyl ester hydrochloride was a Mann Research Labs product (lot M2024), mp 130–133°. Soybean trypsin inhibitor was a Mann Research Labs product (lot M1573), five times crystallized. *p*-Nitrophenyl N-benzyloxycarbonyl-L-valinate was purchased from the Cyclo Chemical Co. and was recrystallized from ether–petroleum ether, mp 63.5–64° (lit.[36] 63°). Sephadex G-25 and G-50 were obtained from Pharmacia Fine Chemicals, Inc. *p*-Nitrophenyl N-benzyloxcarbonyl-L-isoleucinate was a Cyclo Chemical Co. product, mp 57–58° (lit.[37] 60–62°). *p*-Nitrophenyl N-benzyloxycarbonyl-L-leucinate was a Cyclo Chemical Co. product, mp 92.5–93.5° (lit.[37] 95°). *p*-Nitrophenyl N-benzyloxycarbonylglycinate was a Cyclo Chemical Co. product, mp 128.5–130° (lit.[38] 128). *p*-Nitrophenyl furoate was prepared from the acid chloride as described above, mp 164–165°. *Anal.* Calcd for $C_{11}H_7NO_5$: C, 56.65; H, 3.03; N, 6.01. Found: C, 56.77; H, 2.79; N 5.97.

p-Nitrophenyl N-acetyl-L-leucinate was synthesized from N-acetyl-L-leucine and *p*-nitrophenol using ethyl chloroformate according to the method of Martin, Golubow, and Axelrod.[39,40] The product was recrystallized from hexane–methylene chloride, mp 116–117°. *Anal.* Calcd for $C_{14}H_{18}N_2O_5$: C, 57.17; H, 6.15; N, 9.26. Found: C, 57.14; H, 6.17; N, 9.52. Spectrophotometric analysis of the *p*-nitrophenol liberated on treatment with base indicated 100 ± 2% of the expected amount. Spectrophotometric analysis of the *p*-nitrophenol liberated on treatment with α-chymotrypsin indicated that the material contained about 25% of the D compound.

p-Nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride was prepared by selective removal of the *t*-butyloxycarbonyl group from *p*-nitrophenyl N^2-benzyloxycarbonyl-N^6-*t*-butyloxycarbonyl-L-lysinate.[41] The latter compound was prepared by the carbodiimide procedure.[37] The *t*-butyloxycarbonyl group was removed by treatment with excess anhydrous hydrogen chloride in ethyl acetate at room temperature for 20 min. After one recrystallization from acetonitrile, the hydrochloride melted at 150–152°, $[\alpha]^{20}D$ 22° (*c* 2.2, dimethylformamide). *Anal.* Calcd for $C_{20}H_{24}O_6N_3Cl$: C, 54.86; H, 5.52; N, 9.60. Found: C, 55.15; H, 5.74; N, 9.64. Hydrolysis of the titrant in 0.1 *N* sodium hydroxide produced 95–97% of the calculated amount of *p*-nitrophenol. Enzymatic hydrolysis at pH 6.83 gave 100 ± 0.5% of the amount of *p*-nitrophenol produced by nonenzymatic (alkaline) hydrolysis at the same pH, indicating the optical purity of the titrant. *p*-Nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride was also purchased from the Cyclo Chemical Co. (lot M-2135), recrystallized from acetonitrile containing 3% ethanol, and dried over P_2O_5, mp 150–151°.

All buffers and salts were of reagent grade. The water was doubly distilled water, using either a Corning all-Pyrex glass still or an alkaline permanganate still utilizing Pyrex. Buffer solutions were filtered before use if the 0.0–0.2 slide wire of the Cary spectrophotometer was to be used. Eastman Kodak Co. spectral grade acetonitrile was usually distilled repeatedly from phosphorus pentoxide. pH was measured with a Radiometer 4C pH meter, standardized against pH 4.01 phthalate buffer, and equipped with either a G 200B or a G 220B glass electrode. pH was determined at the conclusion of all experiments except as noted.

Enzymes. α-Chymotrypsin was a Worthington three times crystallized, salt-free product. In a few specified instances Worthington chymotrypsin purified by gel filtration through Sephadex G-50 was used. Enzyme solutions were made up in the appropriate buffer, and centrifuged for 30 min at 15,000 rpm.

Bovine trypsin was a Worthingtion twice crystallized, lyophilized product, lots TRL 6253 and TRL 6256. Porcine trypsin was a gift from the Armour Pharmaceutical Co., lot K172185A.[42] In a few instances, Worthington trypsin purified by gel filtration through Sephadex G-25 was used.

There are strong indications that the dissolution of lyophilized bovine trypsin in aqueous solution does not instantaneously yield a stable, well-defined trypsin solution. Previously it was observed that upon dissolution of this substance, the enzymatic activity of the solution, as measured at pH 7.8 toward α-N-benzoyl-L-arginine ethyl ester, shows an initial sharp decrease of the order of 25%, followed at higher pH's, *e.g.*, 7, by an irreversible denaturation at a much slower rate in a manner similar to the denaturation of α-chymotrypsin.[43] The rate of this initial, partial loss of activity appears to be pH dependent, and is accompanied by an observable decrease in the sedimentation rate of the protein.[43] The decrease in activity and the change in the apparent molecular weight thus seem to be different facets of the same fundamental change in the state of the protein.

For kinetic and titration studies, a stable and enzymatically well-defined trypsin solution is necessary. In search of the conditions necessary for stability, we have carried out experiments which have confirmed and extended the above observations.

Our observations yield the following points. (1) The initial, partial loss of activity, as measured by using α-N-benzoyl-L-arginine ethyl ester as substrate at pH 8.0, 0.05 *M* Tris buffer, 25.0°, is first order in trypsin; the rate constant is independent of the protein concentration from 0.5 to 2.0 mg/ml. (2) The initial loss of activity is first order in hydrogen ion concentration since the reaction has a half-life of approximately 120 min at pH 4 and approximately 10 min at pH 3. (3) The extent of the initial loss of activity (about 25% of the total activity) is independent of the pH of incubation of the enzyme between pH 2 and 5. (4) The amount and rate of initial loss of activity are independent of the substrate used to assay the system (α-N-benzoyl-L-arginine ethyl ester or *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate), or the presence or absence of calcium ion. (5) The loss of activity cannot be reversed by changing the pH of the solution. (6) At the end of the initial loss of activity the enzyme solution is stable for several hours at room temperature at pH 3 ($[E]_0$ = 0.5–2.0 mg/ml), whereas at higher pH's the enzyme slowly loses its activity, the rate of inactivation being dependent on the pH and on the total enzyme concentration. (7) When the rate assays are determined at pH's lower or equal to 6, no initial loss of activity is observed; hence, the initial loss of activity mentioned above must involve a change in the pK_i of the catalytic base of the enzyme. (8) Finally, none of these phenomena are observable with porcine trypsin.

Subtilisin (Bacterial Proteinase Novo) was purchased from the Novo Pharmaceutical Co., Copenhagen, Denmark. Enzyme solutions were prepared in pH 5.0, 0.05 *M* acetate buffer. A refrigerated enzyme solution containing approximately 80 mg/ml of subtilisin in this buffer loses about 15% of its activity in a week as measured by the N-*trans*-cinnamoylimidazole titration.

Porcine elastase, electrophoretically purified, lots ESFF 5691/923, 6507B, and 6501, was obtained from the Worthington Biochemical Corp. Crystalline elastase was also a Worthington product. Solutions of the enzyme were prepared in pH 4.5 or 4.7 acetate buffer, I = 0.05. The turbid solution was centrifuged at 9200 rpm for 20 min in the cold. Using electrophoretically purified enzyme, the clear supernatant contained 96% of the original weight of protein as estimated by the optical factor (milligrams of protein per milliliter = OD_{280} (0.54)).[44]

A solution of elastase (lot ESFF 6501) corresponding to 90 mg of elastase in ammonium acetate buffer (I = 0.05) was centrifuged at 14,000 rpm to give a clear supernatant and was chromatographed. The chromatography was carried out in a cold room at 3–4° on a carboxymethylcellulose column previously washed with distilled water, decanted 20 times, washed thoroughly in pH 4.5 ammonium acetate buffer (I = 0.05), and stored in the same buffer. The column[45] had a capacity of 200 mg of protein. A Gilson ultraviolet absorption meter and a Texas instrument strip-chart recorder were used to determine the protein content of the effluent at 280 mμ. An approximately linear sodium chloride gradient was used. The chromatography procedure followed in general that of Naughton and Sanger.[46]

A similar chromatography was carried out with Worthington crystalline elastase which had been previously purified by a modification of the procedure of Smillie and Hartley,[47] which consisted of treatment of a solution of 400 mg of crystalline elastase in Tris

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buffer (adjusted to pH 9.0) with 50 ml of a settled suspension of DEAE–Sephadex A-50 in Tris buffer. The resulting suspension was stirred at 4° for 4 hr and filtered. The filtrate was lyophilized, and the resulting solid was dissolved in 25 ml of 0.1 *M* ammonia and dialyzed against two 1-l. portions of 0.1 *M* ammonia and then against one 1-l. portion of 0.05 *M* ammonia. The contents of the dialysis sacs were lyophilized, giving 117 mg of a fluffy while solid, soluble in ammonium acetate buffer.

One sample of elastase used in this research (JRW-2) was a preparation of crystalline elastase chromatographed on carboxymethylcellulose in pH 8.0, 0.02 *M* Tris–HCl buffer. A sodium chloride gradient was used.[48]

The acetylcholinesterase was a commerical preparation of the Sigma Chemical Co. The enzyme had been isolated from the electric eel and purified by the method of Lawler.[49] This enzyme preparation, designated type 3 by the supplier, was reported to have 16.7 mg of protein/ml and 5 mg of ammonium sulfate/mg of protein. The preparation was offered in lots of 0.4 ml in the form of a frozen soolution and was stored in this form before use. The solution had a light yellow color which did not disappear on dialysis. The enzyme solutions were always dialyzed to remove either the ammonium sulfate present in the original preparation or the products of reaction if the enzyme had been previously used. The dialysis was ordinarily carried out with 0.2 *M* sodium chloride and 10^{-4} *M* phosphate buffer, pH 6.8–7.0. On several occasions it was found desirable to concentrate the enzyme solution. This was most readily accomplished by dialysis against 29% ammonium sulfate solution which precipitated the enzyme. The precipitated enzyme was centrifuged and then redissolved in the buffer mentioned above. An attempt at concentrating an enzyme solution using Sephadex G-25 was unsuccessful. The presence of small particles (probably denatured protein) in the enzyme solution which scattered light created a considerable spectrophotometric problem. An attempt was made without complete success to remove these particles by centrifugation. However, the use of a Millipore filtration apparatus was successful. This apparatus consisted of a Swinny hypodermic adapter (Millipore) no. XX 30-012-00 equipped with a 22-μ filter. After this filtration, stirring the solution resulted only in a 0.0003 absorbance increase which returned to its original value.

Papain was purchased as a suspension of twice-crystallized material (lot No. 5588) from the Worthington Biochemical Corp. and converted to mercuripapain by a modification[50] of the method of Kimmel and Smith.[51] The mercuripapain ($1–2 \times 10^{-3}$ *M* in 0.05 *M* acetate buffer, pH 5.2) was shaken with a toluene solution of 4-methylbenzenethiol;[52] the aqueous layer was filtered through filter paper and chromatographed on a 12 × 1.6 cm column of Sephadex G-25. The active enzyme which was eluted between 8 and 14 ml eluate volume was stored in a stoppered volumetric flask at 2°. When the solution was in use, the flask was kept in ice. After being used, the flask was flushed with nitrogen before it was returned to 2°. The normality of the stock solution varied from 1.5 to 4.0×10^{-4} *M*.

Kinetic and Titration Measurements. Kinetic and titration experiments were conducted using a Cary Model 14PM or CM recording spectrophotometer equipped with a thermostated cell compartment at 25.0 ± 0.2° and 0.0–2.0 and 0.0–0.2 slide wires. Ordinarily a 1-cm quartz cuvette containing 3.0 ml of buffer solution was thermostated for an appropriate period of time. The reference cell contained the corresponding buffer. Then an aliquot of substrate solution in purified acetonitrile (or other organic solvent) was added, and the spontaneous hydrolysis of the substrate was observed spectrophotometrically for 100–200 sec. The enzymatic reaction was then initiated by introduction of an aliquot (20–100 μl) of enzyme solution.

For some "burst" experiments, a Cary cell compartment cover with a 1-in. diameter hole directly above the cell position was used in order to provide the fastest initiation and thus the closest extrapolation to zero time for fast hydrolyses such as the reaction of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate with papain. Experiments showed that if no direct light from overhead fell on the hole, the recorded absorbance did not change when the hole was uncovered. With the spectrophotometer operating, 50 μl of the enzyme solution was added on the end of a flat-tipped stirring rod through the hole in the cover. The rapid rise in absorbance caused by the interruption of the light beam by the stirring rod signaled the precise time of addition. This method of initiation permitted the observation of the reaction as soon as 2.5 sec after the addition of enzyme.

For most substrates, the steady-state phase of the reaction was determined by aligning a straight edge with the trace on the Cary chart, and the "burst" of *p*-nitrophenol was measured by extrapolating the steady-state line back to the time of enzyme addition. A correction was applied to the steady-state velocity by subtracting the velocity of the spontaneous reaction (the zero-order spontaneous reaction was assumed to remain unchanged after the usually small decrease in substrate concentration resulting from the burst).

Absorbance values for *p*-nitrophenol were calculated from the experimental values, ϵ_{400} 18,320 for phenolate ion and $pK_a = 7.04$.[25] The absorption of 2,4-dinitrophenol was measured in pH 5.92 buffer, by completely hydrolyzing 2,4-dinitrophenyl acetate; ϵ_{360} was found to be 1.46×10^4.

The molar absorptivities of N-*trans*-cinnamoylimidazole used in this study are: ϵ_{345} 2.89×10^3 and ϵ_{335} 9.04×10^3 (pH 7.00, 0.025 *M* phosphate buffer, $I = 0.1$ (NaCl), 3.2% (v/v) acetonitrile–water). These values were obtained by extrapolating the absorption of slowly hydrolyzing solutions to zero time. Since the spontaneous hydrolysis of N-*trans*-cinnamoylimidazole is a minimum at pH 7.0, the extrapolation is not great. The values are believed accurate to ±2%. Since the pK_a of this titrant is 3.65,[29] the ϵ's are not very pH dependent near pH 7. The absorption of N-*trans*-cinnamoyl-subtilisin *vs.* subtilisin at 345 mμ is negligible, while it is very small at 335 mμ ($\Delta\epsilon$ 0.26×10^3) and is not pH dependent in the range pH 5–7.

For the trypsin titration using *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride, $\Delta\epsilon_{340}$ 6150 in pH 3.0 citrate buffer (0.05 *M*) and $\Delta\epsilon_{320}$ 8171 in pH 3.71 formate buffer (0.04*M*). The value of $\Delta\epsilon_{340}$ for the hydrolysis of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, at pH 3.2, the pH of the papain titrations, was 6220 ± 30, the average of ten determinations. Phenyl acetate was followed at 275.5 mμ close to the isosbestic point of phenol phenoxide ion: $\Delta\epsilon_{272.5}$ 1.32×10^3 at pH 7.82 and 1.31×10^3 at pH 8.52.

The "burst" of *p*-nitrophenol in an enzyme-catalyzed hydrolysis of a *p*-nitrophenyl ester or decrease in absorbance in an enzyme-catalyzed hydrolysis of an acylimidazole substrate, when an aliquot of enzyme is added to a solution of the substrate, may be calculated from eq 18. The factor $A_0 - A_s$ is the measured burst since A_0 and A_s are the absorbancies extrapolated to time 0 from the enzymatic steady-state turnover and from the spontaneous hydrolysis of the substrate, respectively. Two correction factors must be applied to the measured burst in order to determine the true burst, π: (1) a factor accounting for the dilution of the substrate, $(v_1/v_2)A_s$, where v_1/v_2 is the ratio of volumes of the enzyme aliquot and the final volume of the reaction mixture; and (2) a factor accounting for the absorbance of the enzyme, A_e (which is determined separately). The difference in extinction coefficients (products minus starting species) is $\Delta\epsilon$. The correction factors involving dilution of the

$$\pi = \frac{A_0 - A_s + (v_1/v_2)A_s - A_e}{\Delta\epsilon} \qquad (18)$$

substrate and absorbance of the enzyme tend to cancel one another. Ordinarily their composite correction to the measured burst does not exceed 10%. When the final absorbance of the burst is less than the initial absorbance, all the signs of eq 18 are reversed, as in the acylimidazole titrations.

When an aliquot of substrate is added to a solution of the enzyme, the "burst" of *p*-nitrophenol is given by eq 19, where A_0 and A_2 are again the absorbancy extrapolated to time 0 from the enzymatic steady-state turnover and the absorbance of the substrate, respectively.

$$\pi = (A_0 - A_s)/\Delta\epsilon \qquad (19)$$

The titration of α-chymotrypsin by N-*trans*-cinnamoylimidazole has been described previously.[22] A variant of this procedure[53] which has proved useful is the direct titration of 3 ml of enzyme at pH 7 *via* a method B titration (addition of a microliter quantity

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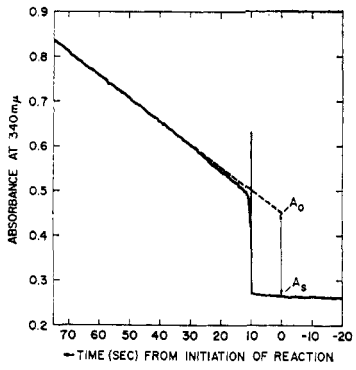

Figure 1. The reaction of *p*-nitrophenyl N^{2}-benzyloxycarbonyl-L-lysinate hydrochloride with bovine trypsin: $[E]_0 = 3 \times 10^{-5}$ *M*, $[S]_0 = 1.05 \times 10^{-3}$ *M*, pH 3.0, citrate buffer (0.05 *M*), 1.3% (v/v) acetonitrile–water, 25°.

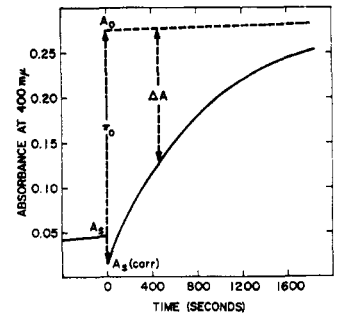

Figure 2. Titration of elastase with diethyl *p*-nitrophenyl phosphate: pH 7.74 Tris–HCl buffer, 7.6% (v/v) acetonitrile–water, 25.0 ± 0.2°, $[E]_0 = 1.71 \times 10^{-5}$ *M*, $[S]_0 = 7.48 \times 10^{-3}$ *M*. The dashed line is extrapolated from the spontaneous hydrolysis of the phosphate after more than eight half-lives of the reaction. Time zero is the time of addition of the enzyme. At negative times the spontaneous hydrolysis of the phosphate alone is seen. The noise level is 0.002 absorbancy unit.

of titrant solution to the enzyme solution in the cuvette). This procedure has two advantages: (1) smaller concentrations of enzyme may be titrated; and (2) the molar absorptivity of N-*trans*-cinnamoylimidazole is not sensitive to pH as it is in the region around pH 5.05, previously suggested for the titration.[22]

The titration of the active sites of a trypsin solution is optimally carried out using *p*-nitrophenyl N^{2}-benzyloxycarbonyl-L-lysinate as titrant, using the two methods described for α-chymotrypsin.[22] **Method A.** A 6×10^{-2} *M* solution (50 μl) of the lysine ester in 20% (v/v) water–acetonitrile[54] was added to 3.0 ml of pH 3.0 citrate buffer (0.05 *M*). The slow spontaneous hydrolysis was observed and then 50 μl of trypsin solution in pH 3.0 citrate buffer (0.05 *M*) was added. A typical burst using this method is shown in Figure 1. First, the slow spontaneous hydrolysis of the ester may be observed, then upon the addition of enzyme, the initial "burst" together with the end of the presteady state, and finally the linear (turnover) portion of the reaction. Burst titrations with chymotrypsin and papain show similar behavior. **Method B.** Trypsin–buffer solution (3 ml) was added to a 1.0-cm silica cuvette. Then 50 μl of titrant solution was added. Since the ester absorbs in the region of 340 mμ, it was necessary to determine its absorbance under the same conditions.

Trypsin solutions were assayed by measuring the initial rate of hydrolysis of α-N-benzoyl-L-arginine ethyl ester spectrophotometrically at 255 mμ.[55] The reaction was carried out in pH 8.0 Tris buffer (0.1 *M*) using 50 μl. of an 8.65×10^{-2} *M* stock substrate solution. This reaction is accelerated by the presence of calcium ions. Care must therefore be taken to prepare the enzyme in a citrate buffer so that any calcium ions present are chelated by citrate ion.

The concentration of a porcine trypsin solution was also measured by the liberation of *p*-nitrophenol from a reaction with excess diethyl *p*-nitrophenyl phosphate. In addition, the pseudo-first-order rate constant for this reaction was determined in two ways: (1) directly from the rate of liberation of *p*-nitrophenol, and (2) by rate assay of the remaining trypsin activity during phosphorylation using *p*-nitrophenyl N^{2}-benzyloxycarbonyl-L-lysinate as a monitor substrate. An aliquot of the substrate was added to 3.0 ml of pH 7.8 Tris buffer (0.1 *M*). The spontaneous hydrolysis was followed at 400 mμ. Then an aliquot of porcine trypsin solution was added and the reaction recorded until the liberation of *p*-nitrophenol was again linear. The pH of the solution was determined at the end of the reaction.

The optimal procedure for the titration of subtilisin by N-*trans*-cinnamoylimidazole involved the addition of an aliquot of the enzyme to a solution of the titrant in the spectrophotometric cuvette, analogous to that described as the "method A" titration of α-chymotrypsin by N-*trans*-cinnamoylimidazole.[22] The alternative procedure involving the addition of an aliquot of the titrant to the solution of the enzyme, described as "method B" in the titration of chymotrypsin, is less accurate in this case and is not recommended. With care the "method A" burst is reproducible to ±1%.

In titrations of elastase, diethyl *p*-nitrophenyl phosphate and then buffer were placed in the cuvette, and the spontaneous hydrolysis of the phosphate ester was observed. An aliquot of a stock solution of elastase in buffer was added on the end of a flattened stirring rod, and the cell was covered to prevent evaporation. Recording was recommenced within 10 sec and continued for ~20 half-lives to facilitate extrapolation to the time of enzyme addition. A typical reaction of diethyl *p*-nitrophenyl phosphate with elastase is shown in Figure 2.

The elastase-catalyzed hydrolyses of *p*-nitrophenyl isobutyrate and trimethylacetate exhibit a presteady-state "burst" followed by a steady-state liberation of *p*-nitrophenol. When the effect of diethyl *p*-nitrophenyl phosphate on the enzymatic activity was measured, an aliquot (100 μl) of the elastase–phosphate reaction mixture was added to an assay cuvette containing 3.0 ml of substrate solution. The 30-fold dilution of the reaction mixture in the assay cuvette was sufficient to quench the phosphate reaction.

For papain titrations using *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, an aliquot of enzyme solution was added to the substrate solution in the cuvette using the technique of addition through a hole in the cell compartment cover. Before and after a given series of titration experiments, the activity toward α-N-benzoyl-L-arginine ethyl ester of the stock enzyme solution was determined. Although the enzyme flask was opened repeatedly during this time interval (2–4 hr), the enzymatic activity measured in this manner decreased by less than 2%.

In order to conserve acetylcholinesterase, quartz cells of the following inner dimensions were used in its titration: 10-mm light path with a 4-mm path width and a 40-mm height (Precision Cell Co.), and 10-mm light path with a 4.5-mm path width and 45-mm height (Pyrocell Manufacturing Co.). It was ascertained that the light path of the spectrophotometer is totally transmitted through the solution in the cells. The most critical problem of this kind involved the Pyrocell cells used with a volume of 500 μl. A procedure was developed which demonstrates that the light beam was completely transmitted during the titration experiments with this cell.[56] An aliquot of acetylcholinesterase solution was added to the cell. After the base line was determined, a 25-μl aliquot of the buffer solution (or water) was added, and the small decrease in absorbance, needed to correct for the decrease when the titrant was added, was recorded. A 25-μl aliquot of the nitrophenyl dimethylcarbamate solution was added and stirred, and the liberation of nitrophenol was recorded. At the end of the steady-state turnover reaction a 25-μl aliquot of nitrophenol in water was added to the enzyme solution, and the absorbance change was recorded on the 1.0 absorbance slide wire in order to obtain the molar absorptivity of the nitrophenol under the reaction conditions. Finally the pH of the solution was determined.

Figure 3 shows a "burst" titration of acetylcholinesterase with *o*-nitrophenyl dimethylcarbamate. The observed burst, π, was

(54) A mixed aqueous solvent is necessary for dissolution of the substrate. This composition minimizes the rate of hydrolysis of the substrate.

(55) G. W. Schwert and Y. Takenaka, *Biochim. Biophys. Acta*, **16**, 570 (1955).

(56) J. K. Stoops, Doctoral Dissertation, Northwestern University, 1966.

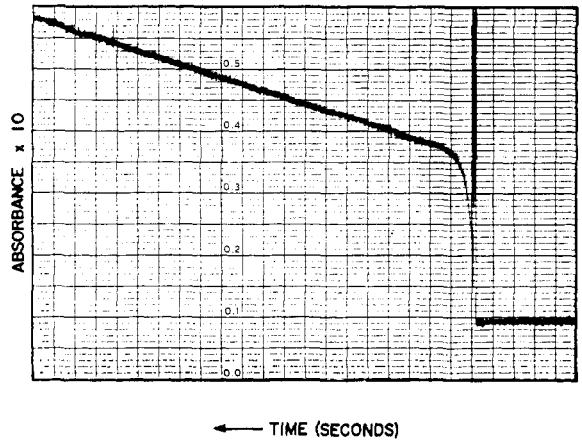

Figure 3. Titration 6 of acetylcholinesterase with *o*-nitrophenyl dimethylcarbamate at pH 7.70. The extrapolation of the absorbance to time zero, which is represented by the initial rise in absorbance, corresponds to an enzyme concentration of 7.45×10^{-6} *M*. The initial presteady-state reaction of approximately 1 min is followed by a steady-state (zero-order) reaction. The abscissa is 60 sec/division.

calculated according to eq 20[57,58]

$$\pi = \frac{A_3 - A_2 + F(A_1 - A_2) - A_4}{\Delta\epsilon} \qquad (20)$$

where A_1 is the initial absorbance of the enzyme solution, A_2 is the absorbance after the addition of an aliquot of water (or of the buffer solution), A_3 is the absorbance measured from the extrapolation of the absorbance to zero time, and A_4 is the absorbance of the carbamate. The difference of A_1 and A_2 is corrected for dilution by the factor F. The difference in extinction coefficients, $\Delta\epsilon$, is ϵ(phenol) − ϵ(ester). The factor F is given by eq 21[59]

$$F = \frac{1 - V_2/V_3}{V_2/V_1 - 1} \qquad (21)$$

where V_n corresponds to the volume of the solution at which the absorbance was measured. The corrections $F(A_1 - A_2)$ and A_4 *in toto* generally decrease the uncorrected burst, $A_3 - A_2$, by 5–20%.

Results for Chymotrypsin

α-Chymotrypsin (chymotrypsin A, EC 3.4.4.5)[3] has been titrated previously using a number of substrates, both specific and nonspecific, in this laboratory and elsewhere; however, several additional titrations are reported here. These titrations involve the substrates, *p*-nitrophenyl acetate, and 2,4-dinitrophenyl acetate using three methods of determining concentration: (1) a comparison of the slopes of the Lineweaver–Burk plots of the steady-state and presteady-state portions of the reaction (method 2); (2) second-order kinetics (method 3); and (3) measurements of the burst of *p*-nitrophenol in the presteady state (method 4). The three methods give results which are in essential agreement with one another.

Comparison of the Slopes of the Lineweaver–Burk Plots of the Steady-State and Presteady-State Portions of the Reaction. Previously results were presented concerning the turnover rates of the α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate at substrate concentrations sufficiently low that complications arising from extraneous reactions were avoided.[25] From the slopes of (turnover) plots of $1/V_0$ *vs.* $1/[S]_0$, values of $[E]_0k_2/K_S$ were determined, following eq 5. In addition, the kinetics of the presteady state were determined, giving from eq 7 an independent value of k_2/K_s. Using these two sets of data, $[E]_0$ was determined as shown in Table I.

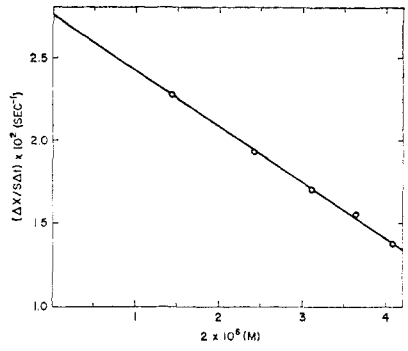

Figure 4. The titration of α-chymotrypsin by *p*-nitrophenyl acetate using second-order conditions: 25.0°, pH 7.8, 0.067 *M* phosphate buffer, $[S]_0 = 6.72 \times 10^{-6}$ *M*, $[E]_0 = 8.17 \times 10^{-6}$ *M*.

Table I. Determination of the Concentration of an α-Chymotrypsin Solution using the Steady-State and Presteady-State Portions of the Hydrolysis of *p*-Nitrophenyl Acetate[a]

Concn of enzyme from wt, g/l.	pH	$k_2[E]_0/K_s$, sec^{-1}	k_2/K_s, M^{-1} sec^{-1}	$[E]_0$, *M*	$[E]_0$,[b] *M*
1.61×10^{-2}	7.8	0.0017	3530	4.8×10^{-7}	5.0×10^{-7}
1.03×10^{-2}	7.7	0.00093	3410	2.7×10^{-7}	3.1×10^{-7}

[a] 1.6% acetonitrile–water at 25°. [b] Titration of the enzyme solution with N-*trans*-cinnamoylimidazole.

Second-Order Kinetics. The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was carried out under second-order conditions (K_m(app) > $[S]_0 \cong [E]_0 < K_s$). The data were treated according to eq 9. Figure 4 shows such a plot. Values of V were obtained by calculating $V \simeq \Delta[P_1]/\Delta t$ from experimental results with Δt approximately $0.25t_{1/2}$. From eq 9, it is seen that the slope of this plot gives k_2/K_s ($= 3380\ M^{-1}\ sec^{-1}$ while a conventional second-order plot gives $k_2/K_S = 3630\ M^{-1}\ sec^{-1}$ for the same reaction), while the intercept of this plot gives $k_2[E]_0/K_S$. The quotient of the intercept/slope = $[E]_0 = 8.17 \times 10^{-6}$ *M* while an N-*trans*-cinnamoylimidazole titration of the same solution gave $[E]_0 = 7.85 \times 10^{-6}$ *M*.

Measurement of the Burst of *p*-Nitrophenol in the Presteady State. The α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenyl acetate was carried out under turnover conditions ($[S]_0 \gg [E]_0$). Plots of eq 13 ($1/\sqrt{\pi}$ *vs.* $1/[S]_0$) are shown in Figure 5 for two different enzyme concentrations. The experimental points lie on fairly good straight lines, whose slopes are in good agreement with the calculated slopes based on values of the various constants of eq 13 given previously.[25] The extrapolated π value at $[S]_0 = \infty$ ($1/[S]_0 = 0$) is in good agreement with the N-*trans*-cinnamoylimidazole titration values, as shown in Table II. In addition the α-chymotrypsin-catalyzed hydrolysis of 2,4-dinitrophenyl acetate was investigated under turnover conditions.

(57) This equation follows that for a method B titration of α-chymotrypsin with N-*trans*-cinnamoylimidazole.[22]

(58) This equation was incorrectly given in a preliminary communication of this work: M. L. Bender and J. K. Stoops, *J. Am. Chem. Soc.*, 87, 1622 (1965).

(59) Derivation of this formula is given in ref 56.

Table II. Titration of Some α-Chymotrypsin Solutions Calculated from the Burst of *p*-Nitrophenol from a *p*-Nitrophenyl Ester Substrate

Substrate	$[S]_0 \times 10^5$, *M*	$[E]_0$, g/l.	$[E]_0 \times 10^6$,[a] *M*	$[E]_0 \times 10^6$,[b] *M*	$[E]_0 \times 10^6$,[c] *M*	% purity
p-Nitrophenyl[d] acetate	3.4–67.7	0.694	27.98	20.42	21.08	75.3
p-Nitrophenyl[e] acetate	6.8–67.7	0.277	11.17	7.82	8.18	73.2
2,4-Dinitrophenyl acetate	13.94			2.82	2.86	
2,4-Dinitrophenyl acetate	6.97			2.82	2.82	
2,4-Dinitrophenyl acetate	14.02			1.14	1.15	

[a] Theoretical using weight of enzyme and a molecular weight of 24,800. [b] Titration with N-*trans*-cinnamoylimidazole. [c] This titration [d] pH 7.8, 1.6% acetonitrile–water, 25°. [e] pH 5.92, 1.6% acetonitrile–water, 25°.

Table III. Titrants for α-Chymotrypsin Solutions[a]

Titrant	Species measured	$[S]_0$[d]/ K_m (app)	k_2/k_3	Titration value × 100% using N-*trans*-cinnamoyl-imidazole	Ref
p-Nitrophenyl N-benzyloxycarbonyl-L-tyrosinate	*p*-Nitrophenol	100	1000 (?)	97–101	*h*
p-Nitrophenyl N-acetyl-DL-tryptophanate	*p*-Nitrophenol	10–100	1000 (?)	91–99	*h*
p-Nitrophenyl acetate	*p*-Nitrophenol	1–10[b]	600	88–104	19, 25, *b, f, i, n*
p-Nitrophenyl isobutyrate	*p*-Nitrophenol	...	...	~100 ± 10	*f*
p-Nitrophenyl trimethylacetate	*p*-Nitrophenol	10	2800	~100 ± 10	*f*
2,4-Dinitrophenyl acetate	*p*-Nitrophenol	30	600	101	*b*
N-*trans*-Cinnamoylimidazole		100	4000	100	22
Phenylmethanesulfonyl fluoride[c]	Protons	∞	∞	103	*g*
	^{14}C labeling of enzyme	∞	∞	103	*g*
3-Nitro-4-carboxyphenyl-N,N-diphenylcarbamate[f]	3-Nitro-4-hydroxybenzoic acid	∞	∞	100 ± 10[e]	23
Diisopropylphosphorofluoridate[c]	Isopropyl groups of enzyme	∞	∞	100 ± 10[e]	6
	P of enzyme	∞	∞	100 ± 10[e]	6
	Protons	∞	∞	100 ± 10[e]	6
Diethyl *p*-nitrophenyl phosphate[c]	*p*-Nitrophenol	∞	∞	100 ± 10[e]	*l, m*

[a] All titrations were carried out using method 4 (measurement of the burst of P_i or ES′ in the presteady state) except for *p*-nitrophenyl acetate where methods 2 + 3 were used. [b] Present investigation. [c] The rigorous alternative treatment using eq 17 gives the same result. [d] These ratios are taken from the references in the table together with references *j*, *k*, and *l*. [e] Estimate on basis of purity or derived molecular weight. [f] C. E. McDonald and A. K. Balls, *J. Biol. Chem.*, **227**, 727 (1957). [g] A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 787 (1964). [h] F. J. Kézdy, G. E. Clement, and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3690 (1964). [i] M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962). [j] G. A. Hamilton and M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2570 (1962). [k] G. E. Clement and M. L. Bender, *Biochemistry*, **2**, 886 (1963). [l] B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952). [m] B. A. Hammond and H. Gutfreund, *ibid.*, **73**, 526 (1959). [n] J. R. Knowles, *ibid.*, **95**, 180 (1965).

Discussion. Solutions of α-chymotrypsin have been titrated many ways. Most of the titrants are listed in Table III together with the relationship of the titration value resulting from each titrant to that found with N-*trans*-cinnamoylimidazole, not because the latter is inherently the best titrant, but because it has been available for comparative purposes as an accessible standard for a longer period of time than any other titrant. All of the titrations in Table III meet the requirements set forth above for measurement of the stoichiometric conversion of enzyme to acyl-enzyme, namely that $[S]_0 \gg K_m(app)$ and that $k_2 \gg k_3$. Furthermore, [S] > [E] in all titrations, although in some titrations, *e.g.*, with N-*trans*-cinnamoylimidazole, the [S]/[E] ratio is low (*vide infra*). Finally, all the titrations including those with the nonspecific substrates such as *p*-nitrophenyl trimethylacetate, those with specific substrates such as *p*-nitrophenyl benzyloxycarbonyl-L-tyrosinate, and those with inhibitors such as 3-nitro-4-carboxyphenyl-N,N-diphenylcarbamate and diisopropylphosphorofluoridate give titrations which are essentially equivalent with one another (some of the comparisons are indirect ones, such as a calculation of molecular weight of the enzyme on the basis of the stoichiometry). Since the titrations are independent of substrate, a strong argument may be made for stating that in all titrations, one and the same active site

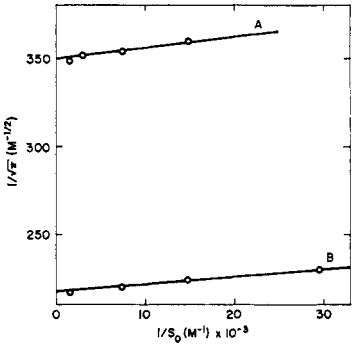

Figure 5. The titration of α-chymotrypsin by *p*-nitrophenyl acetate at 25.0°, pH 7.8, 0.067 *M* phosphate buffer, 1.6% acetonitrile-water (v/v); A, $[E]_0 = 8.2 \times 10^{-6}$ *M*; B, $[E]_0 = 21.1 \times 10^{-6}$ *M*.

Table IV. Titration of Trypsin with Some *p*-Nitrophenyl Esters[a]

p-Nitrophenyl ester of	$[S]_0 \times 10^5$, *M*	*K*m(app) $\times 10^5$, *M*	k_2/k_3	pH	μ	CH_3CN, % (v/v)	Titrated $[E]_0 \times 10^6$, *M*	Enzyme[b] % purity
			Porcine trypsin[c]					
Acetic acid	81–271	15.6	6	6.24		15	15.8	83
N-Acetyl-L-leucine	28–97	32	*d*	4.46	0.2	2.4	32.4	80
	16–42	25	*d*	4.55	0.2	1.6	32.4	84
N-Acetyl-DL-tryptophan	5.3–11.2	32	*d*	3.46	0.05	1.6	3.20	81
	5.3–11.2	7.1	*d*	3.32	0.5	1.6	5.50	80
N^2-Benzyloxycarbonyl-L-lysine	105	*e*	*d*	3.0	0.05	1.3	438	75
Diethylphosphoric acid[f]	305			7.8	0.05	6.1	381	65
			Bovine trypsin					
	10–124	7.7	15.2	7.16	0.5	1.6	22.4	69
Acetic acid	15–124	7.7	15.2	7.59	0.5	1.6	20.6	63
	15–124	7.7	15.2	8.00	0.5	1.6	16.9	64
	15–124	7.7	15.2	8.61	0.5	1.6	15.4	59
N-Acetyl-L-leucine	28–73	23		4.49	0.02	2.4		51
N-Acetyl-DL-tryptophan		131[g,h]					181	50
		104[g,h]					229	64
	5.9–29.3	7.9	*d*	3.46	0.05	1.6	1.95	49
N^2-Benzyloxycarbonyl-L-lysine	7–80	1.0	*d*	3.71	0.02	2.0	18.9	57
	105	1.27	27.6[i]	3.0	0.05	1.3	48	57.5

[a] 25°. [b] Calculated as the per cent of the titrated $[E]_0$ of the $[E]_0$ determined on a weight basis assuming a molecular weight of 24,000. [c] Armour Pharmaceutical Co. [d] Unknown; assumed to be ∞ in calculation of $[E]_0$. [e] Unknown; assumed to be 0 in calculation of $[E]_0$. [f] See text. [g] Using second-order conditions. [h] k_2/K_s, M^{-1} sec^{-1}. [i] *Cf.* ref 11.

is being titrated stoichiometrically, and further that no extraneous reaction is being observed. Titrations of the active site of α-chymotrypsin are also independent of pH, some of the titrations being carried out from pH 9.5 (*p*-nitrophenyl acetate) down to pH 2.5 (*p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate).

The most specific titrant for α-chymotrypsin is *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate; the least specific titrant is diethyl *p*-nitrophenyl phosphate. As an additional check to determine whether these two compounds, and by inference the other titrants of Table III, reflect the same site of the enzyme, the rate constant for the liberation of *p*-nitrophenol from the reaction of diethyl *p*-nitrophenyl phosphate with α-chymotrypsin was compared to the rate constant for the loss of enzymatic activity toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate during this reaction. The two rate constants were found to be identical,[60] within experimental error, confirming the coherence of the titrants of Table III.

Results for Trypsin

The stoichiometric basis of a titration procedure for the determination of the active sites of trypsin (trypsin, EC 3.4.4.4)[3] in terms of eq 1 is predicted on the basis of kinetic studies and isolation experiments.[11] However, unlike α-chymotrypsin, no stoichiometric procedures, other than the early phosphorylation experiments, have been developed for trypsin. On the other hand, as with many enzymes, a host of rate assay procedures have been developed, involving denatured hemoglobin, denatured casein, α-N-benzoyl-L-arginine ethyl ester, α-N-benzoyl-L-arginine amide, and α-N-benzoyl-L-lysine amide as substrates.[61,62] These rate assays suffer from the inadequacies of rate assays as discussed previously. Therefore, a search was made for a titrant for the active sites of trypsin, preferably based on a specific substrate of the enzyme.

Solutions of bovine and porcine trypsin have been titrated using five titrants and two methods of titration. The titrants include diethyl *p*-nitrophenyl phosphate, *p*-nitrophenyl acetate, *p*-nitrophenyl N-acetyl-L-leucinate, *p*-nitrophenyl N-acetyl-DL-tryptophanate, and *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride. The methods of titration involve measurement of the "burst" of *p*-nitrophenol in the presteady state of the reaction and the use of second-order kinetics.

The Burst of *p*-Nitrophenol. The most extensive experiments determined the initial "burst" of *p*-nitrophenol liberated in the presteady state of the trypsin-catalyzed hydrolysis of the *p*-nitrophenyl esters. Representative results of the "burst" titrations of bovine trypsin with *p*-nitrophenyl N^2-benzyloxycarbonyl-L-lysinate are given in Figure 6, showing the dependence of the burst on the substrate concentration. The "bursts" in the reaction of trypsin with the other titrants (except diethyl *p*-nitrophenyl phosphate) are more heavily dependent upon substrate concentration than the lysine "burst." A summary of the titration results for both bovine and porcine trypsin is given in Table IV. Using accessible concentrations of the titrants, *p*-nitrophenyl acetate, N-acetyl-L-leucinate, and N-acetyl-DL-tryptophanate, the burst of *p*-nitrophenol is far from the limiting burst because the ratios $[S]_0/K_m$(app) and/or k_2/k_3 are not sufficiently high.

The burst produced in the reaction of porcine trypsin and diethyl *p*-nitrophenyl phosphate can be shown to be stoichiometric. The kinetics show that $b = k_2[S]_0/K_s = 1.65 \times 10^{-4}$ sec^{-1} for reaction with 3.5×10^{-3} *M* phosphate and $k_3 = 10^{-5}$ sec^{-1}.[30] Thus from eq 17 the enzyme concentration does not differ from the burst by more than 5%.

The optimal titrant of trypsin on the basis of its near-

(60) This result is similar to the result of Hartley and Kilby[30a] who demonstrated the identity of the inhibition of amino acid esterase, amidase, and proteolytic activities with that of *p*-nitrophenol release in the reaction of α-chymotrypsin and diethyl *p*-nitrophenyl phosphate.
(61) See ref 4.
(62) See ref 5.

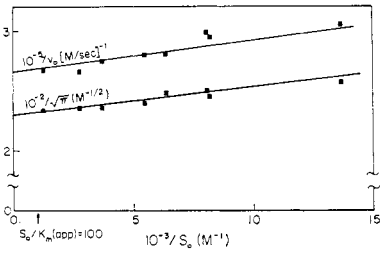

Figure 6. The effect of substrate concentration on the burst titration ($1/\sqrt{\pi}$) and the turnover ($1/v$) of bovine trypsin with *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate, hydrochloride, pH 3.71, 0.04 *M* total formate buffer, I = 0.02, 25°, 2% acetonitrile–water (v/v).

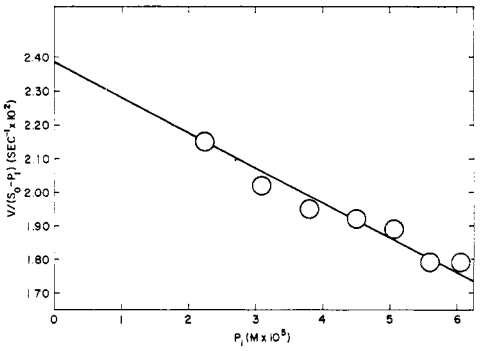

Figure 7. The trypsin-catalyzed hydrolysis of N-acetyl-DL-tryptophan *p*-nitrophenyl ester under second-order conditions: $[S]_0$ = 2.71–7.43 × 10^{-5} *M*, $[E]_0$ = 1.81 × 10^{-4} *M*, pH 3.11, 25°, 0.05 *M* citrate buffer, 1.6% acetonitrile–water (v/v).

stoichiometric burst and its specificity is *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate hydrochloride. The titration of trypsin with this substance has been investigated in detail. Figure 1 shows a typical titration of trypsin with this substance. Figure 6 shows that the burst of *p*-nitrophenol in the presteady state of the titration reaction is not greatly affected by substrate concentration in the region of substrate concentration ordinarily used. Thus, using a substrate concentration of 1.05 × 10^{-3} *M*, the usual concentration for the titration, and the data of Table IV, eq 12 indicates that the ratio $\pi/[E]_0$ = 0.909. That is, under these conditions, the burst corresponds to 91% of the true enzyme concentration.[63]

Since, as indicated in the Experimental Section, solutions of bovine trypsin at pH 3 undergo an initial loss of activity, as measured by rate assay with α-N-benzoyl-L-arginine ethyl ester at pH 8, titrations of trypsin solutions after variable amounts of time were carried out at pH 3, using *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate as titrant. From 4-min incubation to 3-hr incubation at room temperature, no change in the burst was observed.

The rate of a trypsin-catalyzed hydrolysis of α-N-benzoyl-L-arginine ethyl ester is considerably increased in the presence of calcium ion, 10^{-2} *M* calcium ion giving nearly 100% increase in rate (partially an ionic strength effect). Therefore, the dependence of the titration on calcium ion was investigated. Bovine trypsin was prepared both in pH 2.92 citrate buffer and in pH 3.0 0.06 *M* calcium ion. The per cent of active trypsin determined in two titrations of the same trypsin stock using these two solutions were 57 and 60, respectively. Thus the effect of calcium ion on the burst is at best to increase the value 5%.

Second-Order Conditions. Several experiments were performed to determine trypsin concentration in this manner, using N-acetyl-DL-tryptophan *p*-nitrophenyl ester and *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate hydrochloride as substrates. These determinations were limited to the bovine enzyme since a large amount of enzyme is consumed in each experiment. Figure 7 shows a plot of $V/([S] - [P_1])$ for the tryptophan substrate. Although these experiments are subject to error from both spontaneous hydrolysis and deacylation of the acyl-enzyme, the data lead to results which are reasonably consistent with those of the burst titrations (see Table IV).

(63) This correction was not made in the preliminary communication of this work: M. L. Bender, J. V. Killheffer, Jr., and R. W. Roeske, *Biochem. Biophys. Res. Commun.*, **19**, 161 (1965).

Discussion. All titrations of Table IV indicate that the bovine trypsin preparation used is 49–60% active enzyme. Those titrations of Table IV where both the k_2/k_3 and $[S]_0/K_m$(app) (or k_b/k_3) ratios are known indicate that the bovine trypsin preparation contains 59–69% active enzyme and that the porcine trypsin preparation contains 65–83% active enzyme. The purity of porcine trypsin may be appreciably improved by gel filtration using Sephadex G-25 (*vide infra*).

Although the agreement in Table IV between different titrants is not precise, it is certainly reasonable considering the uncertainties of the experiments and different conditions used. The question may still be asked as to whether the various titrants of Table IV reflect the same active site. Two approaches have been used to investigate this problem. The rate constant for the phosphorylation of porcine trypsin with diethyl *p*-nitrophenyl phosphate, as reflected by the release of *p*-nitrophenol, was compared to the rate constant for the inactivation of the enzyme, as reflected by the turnover of *p*-nitrophenyl N²-benzoyloxycarbonyl-L-lysinate at pH 6.55. The first-order rate constants of these two reactions were 1.77 × 10^{-4} and 1.65 × 10^{-4} sec^{-1}, respectively, indicating that the most specific titrant, the lysine ester, and the least specific titrant, the phosphate, react at the same site on the enzyme. Presumably the other intermediate titrants do also. At pH 6.28, the turnover rate of *p*-nitrophenyl N²-benzyloxycarbonyl-L-lysinate with porcine trypsin is inhibited 92% by 3.26 × 10^{-6} *M* soybean trypsin inhibitor. This experiment indicates that at least 92% of the burst of *p*-nitrophenol using the lysine substrate as titrant must arise from the active site as defined by soybean trypsin inhibitor.[64] Thus, the titrations of Table IV appear to be independent both of pH and substrate.

For trypsin solutions, a convenient secondary standard is the rate assay based on α-N-benzoyl-L-arginine ethyl ester as substrate. This reaction has been investigated intensively, and found to obey Michaelis–Menten kinetics.[65] The K_m(app) for this reaction at pH 8 is 2 × 10^{-5} *M* so that at a concentration of 10^{-3}

(64) Since the binding of soybean trypsin inhibitor diminishes greatly at lower pH, it is not possible to carry out this experiment at the pH of the titration: M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947); R. F. Steiner, *Arch. Biochem. Biophys.*, **49**, 71 (1954).

(65) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955); G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.*, **179**, 665 (1949); T. Inagami and J. M. Sturtevant, *Biochim. Biophys. Acta*, **38**, 64 (1960).

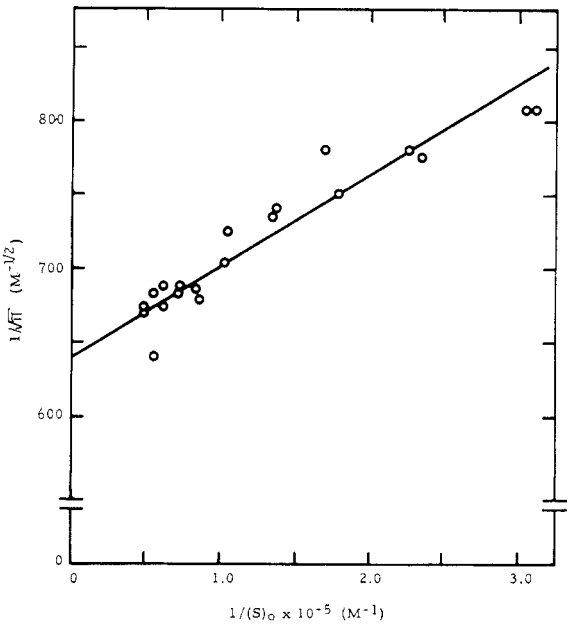

Figure 8. The dependence of the *p*-nitrophenol "burst" on the initial substrate concentration in the titration of papain by *p*-nitrophenyl α-N-benzyloxycarbonyl-L-tyrosinate: 1.6% (v/v) acetonitrile–water, pH 3.18, 0.05 *M* potassium hydrogen phthalate, 25.0°, $[E]_0 = 2.45 \times 10^{-6}$ *M*.

M, saturation conditions hold and the velocity may be expressed by $V_{max} = k_3[E]_0$. Using a trypsin titrant it is possible to determine $[E]_0$ and using the rate assay it is possible to determine V_{max}. Thus, the coefficient k_3 may be determined. The known k_3 may then be used in conjunction with V_{max} to determine the $[E]_0$ of an unknown solution. Using a trypsin solution incubated in 0.05 *M* citrate buffer, pH 3, for at least 20 min, and assay conditions of pH 8.0, 25.0°, and 10^{-3} *M* α-N-benzoyl-L-arginine ethyl ester, k_3 is found to be 1.32×10^4 absorbance units M^{-1} sec^{-1} at 253 mμ.

p-Nitrophenyl N^2-benzyloxycarbonyl-L-lysinate hydrochloride is the most specific substrate of trypsin known today, using as criterion that it has the highest rate constant (k_3(lim) = 170 sec^{-1}). It is interesting that this compound serves as the most convenient titrant of the active sites of trypsin.

Results for Papain

Papain (papain, EC 3.4.4.10)[3] is an enzyme which is distinguished from the enzymes discussed so far by the fact that it contains a sulfhydryl group in its active site, which acts as a nucleophile during the catalytic process converting the substrate first into an intermediate acyl-enzyme having the characteristics of a thiol ester; the acyl-enzyme then reacts with water giving product carboxylic acid, and regenerating the enzyme.[14] Although the important nucleophile of the active site is different from that of α-chymotrypsin and trypsin, the over-all pathway of the papain reaction appears to follow eq 1, which serves as the stoichiometric basis of the titration of the enzyme.

Burst Titration. The papain-catalyzed hydrolysis of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate shows an initial "burst" of *p*-nitrophenol when the zero-order portion of the plot of absorbance of *p*-nitrophenol *vs.* time is extrapolated to time zero. This observation is excellent confirmatory evidence for the mechanism of eq 1.

A typical set of data for "bursts" at various substrate concentrations is shown in Figure 8 plotted according to eq 13. Such plots were analyzed by a weighted least-squares method using $[S]_0$ as the weighting factor. The results of three sets of experiments are listed in Table V.

Several semiquantitative arguments are presented that k_2/k_3 is much greater than unity in this reaction. (1) A survey of reported K_m (K_s) data for papain substrates shows values ranging from 10^{-3} *M* upward. The extremely low K_m(app) ($=k_3/(k_2 + k_3)K_s$) for *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, 0.63 μ*M*, suggests that the "normal" value of K_s ($\geq 10^{-3}$ *M*) is multiplied by a very small ratio of $k_3/(k_2 + k_3)$; *i.e.*, that the ratio k_2/k_3 is very large. (2) In the presence of 30% methanol, the turnover rate increases twofold, possibly because methanol is acting as a nucleophile in deacylation as it is known to do in the deacylation of *trans*-cinnamoyl-papain. The turnover rate constant is predominantly k_3 since a burst is observed and since there is independent evidence for only one active site per molecule.[14] Hence 30% methanol must increase k_3 by a factor of two. Yet the burst was found to be undiminished within the experimental error of 4%, indicating that the ratio $k_2/(k_2 + k_3)$ is sufficiently close to unity so that a twofold increase in k_3 does not affect π more than 4%, *i.e.*, $k_2/k_3 \geq 50$. (3) The substrate *p*-nitrophenyl N-acetyl-DL-tryptophanate shows a burst with papain, the L species only reacting. Although this substrate is more soluble than the tyrosine substrate, the former has a much less favorable K_m(app), resulting in a much steeper extrapolation of the $(\pi)^{-1/2}$ *vs.* $[S]_0^{-1}$ plot and thus in a less precise titration. The results with the tryptophan substrate agree with those using the tyrosine substrate to within 9%, which is considered fair agreement in view of the imprecision associated with the tryptophan substrate. These results indicate that both reactions must have k_2/k_3 ratios much greater than unity, since the two substrates would be expected to have different k_2/k_3 ratios. (4) A lower limiting value of the k_2/k_3 ratio can be calculated from the experimental observation that the presteady state is complete in the time that elapsed between the addition of enzyme and the beginning of observation of the appearance of *p*-nitrophenol. The presteady-state, first-order rate constant, *b*, is given by[25]

$$b = \frac{(k_2 + k_3)[S]_0 + k_3K_s}{K_s + [S]_0} \qquad (22)$$

Since $K_s = ((k_2 + k_3)/k_3)K_m$(app), eq 4 may be transformed into eq 23. The K_m(app) values

$$k_2/k_3 = \frac{b}{k_3 - \frac{K_m(app)}{[S]_0}(b - k_3)} - 1 \qquad (23)$$

were obtained from a Lineweaver-Burk plot (see Table V); k_{cat} ($\sim k_3$, *vide supra*) was calculated directly from the slope of the zero-order appearance of *p*-nitrophenol following the burst. Since the presteady state in the burst titration was "complete" before the first spectrophotometric observation after the addition of enzyme, at least four to six half-lives

Table V. The Titration of Papain Using *p*-Nitrophenyl N-Benzyloxycarbonyl-L-tyrosinate[a]

$[S]_0 \times 10^6$, M	$K_m \times 10^6$, M	k_2/k_3[d]	$[E]_0^{expt} \times 10^6$,[b] M	$[E]_0^{calcd} \times 10^6$,[b,c] M	Enzyme % purity	$([E]_0/k_{BAEE}) \times 10^4$,[f] M sec
4.0–21.5	0.63	100 ± 60	2.45 ± 0.03	6.22	39.4	8.78
3.4–21.8	0.63		1.67 ± 0.02	3.98	42.0	8.83
4.2–19.8[e]	0.39	190 ± 110	2.49 ± 0.03	6.22	39.9	8.92
						8.84 av

[a] 1.6% acetonitrile–water (v/v), $I = 0.05$, pH 3.18, 0.05 M potassium hydrogen phthalate; 25.0° unless otherwise noted. [b] Cell concentration. [c] Determined spectrophotometrically at 280 mμ; see text. [d] Calculated lower limit; see text. [e] 11°. [f] The α-N-benzoyl-L-arginine ethyl ester assay was carried out in 0.05 M acetate, pH 5.17, $\mu = 0.038$, 10^{-5} M ethylenediaminetetraacetic acid, 25.0°; $[S]_0 = 5 \times 10^{-4}$ M.

must have elapsed, depending on the magnitude of the burst and the accuracy with which one can read the absorbances. Hence a lower limit can be calculated for b, and consequently for k_2/k_3. Table V shows lower limiting values (with the attendent experimental uncertainties) for the k_2/k_3 ratio thus calculated. The data at 11° show the tail end of the presteady state at the lowest substrate concentrations, and thus the entry $k_2/k_3 = 190$ in Table V is based on the estimated actual value of b, rather than its lower limit. Although the upper limit of these (lower limiting) k_2/k_3 estimates are quite uncertain, the lower limit of this ratio is believed to be quite certain. This lower limit is, of course, the point of importance here.

Special consideration must be given to the substrate concentrations used in these experiments. The lowest titrant concentrations in Table V are only some 60% greater than the enzyme normality in the cell, in violation of the assumption implicit in the derivation of eq 13 that $[S]_0 \gg [E]_0$. Actually to be more correct, the necessary assumption is that the rate of change of the acyl-enzyme ($d[ES']/dt$) is much less than the rate of change of the substrate ($d[S]/dt$).[66] In the present experiments, since the presteady state is essentially instantaneous with respect to the steady state ($b \gg k_3$), the substrate concentration rapidly diminishes by an amount equal to the concentration of acyl-enzyme formed, and then decreases in the steady-state reaction while the acyl-enzyme concentration remains sensibly constant.

The substrate concentrations reported in Table V and used throughout the calculations are in reality ($[S]_0 - \pi/2$). This corrected substrate concentration gives the closest approximation to the true substrate concentration during the burst, by taking an average of the substrate concentration during this period. The value of π so obtained differs by only ±2% from that obtained when ($[S]_0 - \pi$) or $[S]_0$ are used, respectively. Thus, this correction is not a major consideration. The reason for the insensitivity of the burst to this correction is that the highest values of $[S]_0$ are about 35 times the value of K_m(app), these experiments giving values of π very near the maximum value.

Relationship between the Titration and Rate Assay. The rate of the hydrolysis of α-N-benzoyl-L-arginine ethyl ester, a specific substrate for papain, was carried out simultaneously with the titration experiments. Thus since $K_m(app) \gg [S]_0$, a pseudo-first-order reaction is observed whose rate constant is $k_{BAEE} = (k_{cat}/K_m(app))[E]_0$. This pseudo-first-order rate constant was shown to be proportional to $[E]_0$ and independent of the substrate concentration provided that 10^{-5} M ethylenediamine tetraacetic acid was present. In the absence of the chelating agent, there is a small decrease in k_{BAEE} as the initial substrate concentration increases, possibly due to a small metal ion impurity in the substrate solution. Using $[E]_0$ from the titrations, the ratio $(K_m(app)/k_{cat})_{BAEE}$ (equal to $[E]_0/k_{BAEE}$) was determined as 8.84×10^{-4} M sec^{-1} at pH 5.17 and 25°. Thus the enzyme concentration in the cell is given by $[E]_0 = k_{BAEE} \times 8.84 \times 10^{-4}$ M sec under the conditions specified in footnote *f* of Table V. By this means the normality of any papain solution can be readily determined.

Identification of the Site of Titration as the Active Site. To demonstrate that the titration arises from reaction only at the active site, the following experiments were carried out. Papain can be converted in approximately 90% yield to give the acyl-enzyme, *trans*-cinnamoyl-papain, and then freed of excess substrate by Sephadex filtration.[14] Under conditions where ethyl α-N-benzoyl-L-argininate is hydrolyzed by papain in a first-order reaction ($(BAEE)_0 \ll K_m(app)$), a plot of log $[(BAEE)_0/(BAEE)]$ *vs.* time will, of course, give a straight line. If part of the enzyme is cinnamoylated, a similar first-order plot exhibits initial curvature until the *trans*-cinnamoyl-papain has completely decinnamoylated as indicated by eq 24

$$\log \frac{(BAEE)_0}{(BAEE)} = \frac{k_{cat}}{2.303K_m(app)}\left[\left([E]_0 + (CP)_0)t - \frac{(CP)_0}{k_3^{CP}}(1 - e^{-k_3^{CP}t})\right)\right] \quad (24)$$

where $(CP)_0$ and k_3^{CP} are the initial concentration and deacylation rate constant, respectively, of *trans*-cinnamoyl-papain, and $[E]_0$ is the initial concentration of free papain. The difference between the experimental curve and the extrapolated straight-line portion of such a plot plotted logarithimically *vs.* time gave a value for k_3^{CP} which agreed within 19% of the independently determined value.[14]

The initial slope of the plot of eq 24 (which can be determined analytically) is proportional to the concentration of free papain at the time of initating the reaction, and the final slope is proportional to the total concentration of papain, $[E]_0 + (CP)_0$. When a *trans*-cinnamoyl-papain solution prepared at pH 3.3 was used in a typical "burst" experiment, the "burst" indicated $0.37 \pm 0.13 \times 10^{-6}$ M free papain. A BAEE assay experiment at pH 5.17 was performed simultaneously. From the initial and final slopes of the first-

(66) The titration of α-chymotrypsin with *trans*-cinnamoylimidazole[22] illustrates this point; it is successful as long as $[S]_0$ is only slightly greater than $[E_0]$. The deacylation rate is relatively so small that this condition is met.

order plot for the total BAEE hydrolysis and the factor 8.84×10^{-4} M sec, it was calculated that the free papain concentration in the absorbance cell of the "burst" experiment was $0.32 \pm 0.14 \times 10^{-6}$ M, in good agreement with that determined from the "burst," while the total concentration of papain plus *trans*-cinnamoyl-papain in the cell was 2.12×10^{-6} M. Taking into account the limits of experimental error, 0.32×10^{-6} M $(=[(0.37 + 0.13) - (0.32-0.14)] \times 10^{-6})$ is the maximum amount of *p*-nitrophenol which could be released by a site on the enzyme other than the one occupied by the *trans*-cinnamoyl group. Thus at least 85% of the burst of *p*-nitrophenol must arise from reaction at the active site.

Discussion. The purity of the papain preparations as shown in Table V is calculated as the ratio of the titration to the spectrophotometric determination of total protein at 280 mμ. The value ϵ_{278} 51,800 (based on anhydrous, ash-free protein) may be calculated[67] assuming a molecular weight of 20,700. Thus an ϵ value of 51,100 was calculated for 280 mμ from a spectrum of papain. Recent sequence studies[68] suggest a molecular weight some 6% higher than 20,700. Such a change would have the effect of raising the purity by the same percentage. The data presented in Table V were obtained using an enzyme stock solution which was 15 (line 2) and 18 (lines 1, 3) days old, respectively. Enzyme stock solutions generally show a decrease in activity of about 2% per day, probably due to autolysis and oxidation. The α-N-benzoyl-L-arginine ethyl ester activity of the enzyme stock solution used for the titrations given in Table V was, however, also determined immediately after activation from mercuripapain; from this determination the freshly prepared enzyme was calculated to have been 58.0% pure. There is some variability among different batches of Worthington papain, and purities of 75% have been obtained regularly with two other Worthington lots, no. 5569-70 and 5573. It should be emphasized that the factor relating the pseudo-first-order rate constant for α-N-benzoyl-L-arginine ethyl ester hydrolysis to the titrated normality of the enzyme solution is independent of both the Worthington lot and the age of the activated enzyme stock solution.

The observation that papain is only 58–75% pure reconciles the data of Smith and Parker[69] with that of Whitaker and Bender.[14] The former workers found a maximal k_{cat} (or k_0) for α-N-benzoyl-L-arginine ethyl ester of 9.0 sec^{-1} assuming a 100% pure enzyme preparation, whereas the latter, who were able to titrate their papain solutions, obtained a value of 16.0 sec^{-1}. These two values are in agreement if Smith's enzyme solutions were, in fact, only 56% pure, a reasonable value in view of the findings presented here.

Presumably any stoichiometric reaction with the single sulfhydryl group of the active site may provide a titration for papain. In early studies of papain, Finkle and Smith[70] noted that papain reacted with less than 1 equiv of *p*-chloromercuribenzoate, a sulfhydryl reagent, when the latter was in twofold excess. The fraction of sulfhydryl groups so titrated was observed to be approximately proportional to the activity of the enzyme preparation; thus, this procedure appears to afford a titration of the active site of papain. However, this titration has the disadvantage that a given enzyme preparation may contain protein molecules, or fragments thereof, which, though enzymatically inactive, may still contain free sulfhydryl groups capable of reacting with *p*-chloromercuribenzoate. In fact, experiments in this laboratory indicate that in the course of heat denaturation, papain loses its activity toward the substrate N-α-benzoyl-L-arginine ethyl ester much more rapidly than it loses its ability to react with *p*-chloromercuribenzoate.[71] Liener[72] found that his best preparations of ficin, an enzyme similar to papain, reacted with 0.90–0.95 equiv of N-ethylmaleimide, based on an enzyme concentration calculated from a nitrogen analysis and a molecular weight of 26,000.[73] The release of iodide ion in the reaction of iodoacetamide with ficin[74] indicated that the preparation used was 77% pure as determined on a dry weight basis and a molecular weight of 26,000. However, these titrations suffer from the same disadvantages as those mentioned above for papain. Furthermore, the reactions of *p*-chloromercuribenzoate, N-ethylmaleimide, and iodoacetamide are undesirable for titrations because these substances are not related to papain substrates.

Results for Subtilisin

The stoichiometric basis of a titration procedure for the determination of the active sites of subtilisin (subtilopeptidase A, EC 3.4.4.16)[3] in terms of eq 1 is predicted on the basis of the stoichiometric reaction of the enzyme with diisopropylphosphorofluoridate[75] and the isolation of the acyl-enzyme, *trans*-cinnamoyl-subtilisin.[13] The latter work[13] mentioned a titrimetric procedure for the active site of subtilisin using N-*trans*-cinnamoylimidazole, although no details were given.

Burst Titration. The subtilisin-catalyzed hydrolysis of N-*trans*-cinnamoylimidazole shows an initial (negative) "burst" in the disappearance of the substrate when the zero-order portion of the plot of absorbance *vs.* time is extrapolated to time zero. This observation is excellent confirmatory evidence for the mechanism of eq 1. A typical set of data for the "burst" at various substrate concentrations and enzyme concentrations is given in Table VI.

No presteady-state reaction was observed in a titration when the substrate concentration greatly exceeded that of the enzyme, if the spectrophotometer was turned on 5 sec after the addition of enzyme to the substrate. One can thus conclude on a qualitative basis that $k_2 \gg k_3$.

An attempt to make a quantitative estimate of the k_2/k_3 ratio by observation of the presteady state using a stopped-flow spectrophotometer was of only limited success because the highest substrate concentration possible was only one-tenth that of the apparent K_s. With these limitations, k_2 was found to be $\sim$100 sec^{-1} at pH 7 indicating that $k_2 \gg k_3$, since k_{cat} at pH 7 (presumably k_3) = 1.45×10^{-2} sec^{-1}. An estimate of

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(75) M. Ottesen and C. G. Schellman, *Compt. Rend. Trav. Lab. Carlsberg*, **30**, 157 (1957).

Table VI. The Titration of Subtilisin Using N-*trans*-Cinnamoylimidazole[i]

Titration	Enzyme soln	$[S]_0 \times 10^4$, M[e]	π, absb unit	$[E]_{cell} \times 10^5$, M[f]	$[E]_{stock} \times 10^3$, M	% purity[g]	$k_3 \times 10^2$ sec^{-1}[h]
I[a]	1	1.89	0.507	6.02	1.93	57.1	1.37
II[b]	1	1.98	0.263	3.10	1.95	57.7	1.51
III	2	2.02	0.371	4.38	1.40	57.6	1.44
IV	3	2.02	0.285	3.36	1.08	55.7	1.50
V[c,d]	4	5.81	0.131	4.58	1.47 ± 0.07	...	1.47
VI[c,d]	4	2.87	0.131 ± 0.006	4.64	1.48 ± 0.07	...	1.45
VII	4	2.09	0.383 ± 0.006	4.51	1.44 ± 0.02	...	1.53

[a] 100 μl of enzyme solution 1. [b] 50 μl of enzyme solution 1. [c] 345 mμ, all others 335. [d] $\pm$ based on uncertainty of ± 0.003 absorbance unit in π. [e] Concentration at termination of "burst." [f] Calculated using eq 18. [g] Based on the weight of enzyme used and a molecular weight of 27,400. [h] $(V^{obsd} - V^{spont})/[E]_{total}$ (V^{spont} usually $< 1\%$ V^{obsd}). [i] pH 7.00 $\pm$ 0.05, 3.2% (v/v) acetonitrile, 0.025 M phosphate buffer, $I = 0.1$ (NaCl).

the k_2/k_3 ratio can also be obtained from the pre-steady-state reaction using eq 22. Using stopped-flow spectrophotometry, b at pH 7 was found to be 2.7 ± 0.3 sec^{-1} at $[S]_0 = 4.93 \times 10^{-4}$ M. Since it was not possible to observe a saturation in the presteady state, we know that $K_s \gg [S]_0$. Thus eq 22 reduces to $b = (k_2[S]_0/K_s) + k_3$. Using the known values of k_{cat}/K_m(app) $= k_2/K_s = 5.05 \times 10^3$ M^{-1} sec^{-1} and $k_{cat} = k_3 = 1.45 \times 10^{-2}$ sec^{-1}, the above equation is seen to reduce to $b = k_2[S]_0/K_s$. Once again since we know that $K_s \gg [S]_0$, then $k_2 \gg 2.7$ sec^{-1}. On the basis that $k_2 = 2.7 \pm 0.3$ sec^{-1}, the minimal k_2/k_3 ratio $= 186 \pm 20$, clearly satisfying the requirement that $k_2 \gg k_3$.

The value of K_m(app) determined from a Lineweaver–Burk plot of the steady-state reaction under the conditions of the titration experiment was 2.87×10^{-6} M. The substrate concentrations ordinarily used in titration experiments are 100-fold greater than this value. The calculated values of the enzyme concentration reported in Table VI have utilized eq 12 with the known K_m(app)/$[S]_0$ ratios for each experiment. The use of this ratio usually amounted to a factor of about 3.2% and in no case more than 4.2% in the over-all calculation.

The derivation of eq 12 requires that $[S]_0 \gg [E]_0$. Under the titration conditions $[S]_0/[E]_0$ was usually 5–10, which does not meet this requirement completely. Since the substrate concentration does change appreciably during the period of the burst, the $[S]_0$ listed in Table VI and used in eq 12 is the concentration of substrate after the burst. Since the k_2/k_3 ratio is so high in this titration, there is no theoretical ambiguity connected with the fact that the $[S]_0/[E]_0$ ratio is not very high.[76]

N-*trans*-Cinnamoylimidazole is certainly not a "natural" substrate for subtilisin. In addition, it is a fairly reactive acylating agent and is used in large excess. Therefore it is necessary to determine whether N-*trans*-cinnamoylimidazole reacts with the active site which is operative for other (natural) substrates of subtilisin and with no other groups.

Subtilisin has a broad specificity toward acylamino acid derivatives. When subtilisin is titrated with N-*trans*-cinnamoylimidazole at pH 7, a *trans*-cinnamoyl intermediate is formed.[13] Such a reaction was carried out and immediately afterward the pH of the solution was lowered to pH 4.0 (from 7.0) and the solution was passed through a Sephadex G-25 column. This treatment gave a solution of *trans*-cinnamoyl-subtilisin which had been separated from excess substrate and any hydrolysis products. When an aliquot of this acyl-enzyme is added to a solution containing *p*-nitrophenyl N-benzyloxycarbonyl-L-valinate and the release of *p*-nitrophenol followed, a sigmoid curve reflecting a first-order approach (deacylation of the cinnamoyl-enzyme) to the first-order ester hydrolysis reaction should be observed, if the enzyme is indeed cinnamoylated at the same active site as that responsible for the ester hydrolysis. The rate constant for the approach to the ester hydrolysis turnover should equal k_3, the deacylation rate constant for *trans*-cinnamoyl-subtilisin, according to eq 24. This phenomenon was observed and the rate constant so obtained was $1.52 \pm 0.15 \times 10^{-2}$ sec^{-1}, which agrees within experimental error with the value of k_3 determined directly (1.45×10^{-2} sec^{-1}). Thus the observed "burst" in the reaction of subtilisin with N-*trans*-cinnamoylimidazole represents acylation of the same active site as that involved in the hydrolysis of a substrate containing a naturally occurring amino acid.

In the above discussion, the applicability of eq 1 has been assumed. There is, however, one piece of information which may be interpreted as being inconsistent with eq 1; namely only a questionable saturation was observed in the presteady-state reaction. Thus, the experimental observations on this titration reaction may require that eq 15–17 be utilized. However, the above data also satisfy the requirements of eq 17 for a rigorous titration.

The Use of Rate Assay as Secondary Standard. Only stock solutions of rather high enzyme concentration ($\sim 1 \times 10^{-3}$ M) can be directly titrated by the procedure given above. Since more dilute stock solutions are frequently desired, it is convenient to have a secondary standard that can establish the concentrations in more dilute solutions. The reaction of N-*trans*-cinnamoylimidazole with subtilisin is, in fact, both a titration and a rate assay. The turnover constant k_{cat} ($= k_3$ in this instance since $k_2 \gg k_3$) has been determined under the conditions of the titration by dividing the velocity of the steady-state reaction by the enzyme concentration determined from the burst, as shown in Table VI. Using $k_3 = 1.45 \times 10^{-2}$ sec^{-1}, the enzyme concentration can then be determined from the equation $V_{max} = k_3[E]_0$. If the activity of the enzyme is not affected by dilution, the enzyme concentration calculated from the rate assay should equal the enzyme concentration calculated by dilution of the titrated stock enzyme solution. Such an identity does, in fact, hold over at least a 40-fold range in enzyme concentration from 1.7×10^{-6} to 4.8×10^{-5} M.

(76) See the discussion in the papain section.

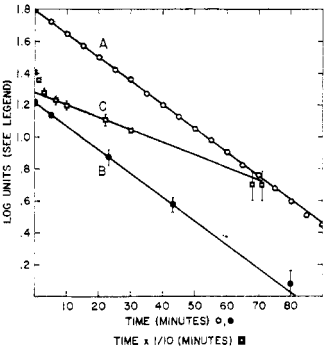

Figure 9. The reaction of diethyl *p*-nitrophenyl phosphate with elastase ESFF 6501 (fraction 29) chromatographed on carboxymethylcellulose and dialyzed against pH 7.8 ± 0.03 phosphate buffer (I = 0.1), $[E]_0 = 4 \times 10^{-5}$ M, $[S]_0 = 2.2 \times 10^{-3}$ M, 3.85% (v/v) acetonitrile–water: A, release of *p*-nitrophenol, ordinate, log $(10^4 \Delta A)$; B, loss of activity of enzyme toward *p*-nitrophenyl isobutyrate, ordinate log $(10^2 V_{net})$ when 100 μl of phosphate solution was assayed; C, loss of activity of enzyme toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, ordinate, log $(10^3 V_{net})$ when 200 μl of phosphate solution was assayed.

Discussion. Throughout this discussion the assumption has been made that only one enzyme exists in the test solutions. The possibility that more than one enzyme is being titrated seems unlikely since the two enzymes would have to be very similar indeed to give consistent first-order acylation kinetics and first-order deacylation kinetics and produce no effects on dilution. The titration indicates the presence of approximately 57% active enzyme and 43% (w/w) inert material in a typical sample of crystalline subtilisin. The possibility that other enzymatically active components are present in this material has not been excluded. The pronounced tendency of subtilisin to autolyze during purification has been noted by several workers[77] and it is possible that the inert material could consist largely of autodegradation products.

Results for Elastase

The stoichiometric basis of a titration procedure for the determination of the active sites of elastase (pancreatopeptidase E, EC 3.4.47)[3] is predicted on the basis of the stoichiometric inhibition of elastase by diisopropylphosphorofluoridate[78] and the observation of an initial burst of *p*-nitrophenol in the elastase-catalyzed hydrolysis of *p*-nitrophenyl isobutyrate and trimethylacetate (*vide infra*).

Burst Titration. With the other enzymes reported in this series, stoichiometric titrations, corresponding to step k_2 of eq 1, have been observed with specific and/or nonspecific substrates of the enzyme. With elastase, we have not as yet found a carboxylic ester that is satisfactory as a titrant. However, *p*-nitrophenyl diethyl phosphate (Paraoxon), which was previously shown to react with chymotrypsin and trypsin resulting in a completely inhibited enzyme and the liberation of approximately 1 mole of *p*-nitrophenol per mole of enzyme,[30a,79] has been used here as a titrant of the active sites of elastase. Elastase reacts with excess diethyl *p*-nitrophenyl phosphate at pH 7.8 in a reaction which is characterized by a first-order liberation of *p*-nitrophenoxide ion (Figure 2). Before the reaction takes place and after the reaction has reached five half-lives, a slow spontaneous zero-order hydrolysis of diethyl *p*-nitrophenyl phosphate is evident; the rates of the spontaneous hydrolysis before and after reaction are identical within the experimental error of 4%. That the reaction is essentially irreversible was shown by inhibition of elastase with excess diethyl *p*-nitrophenyl phosphate, separation of the inhibited enzyme from the excess reactant by gel filtration using a Sephadex G-25 column, and observation of the activity of the enzyme toward *p*-nitrophenyl trimethylacetate. At the conclusion of the gel filtration, the enzyme had no activity (less than 1% of the original activity). After 65 hr at pH 7.8, the inhibited enzyme had regained no activity (less than 5%) while a blank, uninhibited enzyme still retained 35% of its activity toward *p*-nitrophenyl trimethylacetate. These results indicate that there is probably no "turnover" of diethyl *p*-nitrophenyl phosphate during the course of a titration experiment.

By plotting the logarithm of ΔA, the difference between the absorbance at any time and the infinite absorbance extrapolated from the spontaneous hydrolysis of the phosphate after the reaction is complete (see Figure 2), *vs.* time, the pseudo-first-order rate constant, b, of the raction may be obtained (Figure 9). In addition, by extrapolating this plot to zero time, π, a measure of the *p*-nitrophenol liberated in this reaction, and thus a measure of the enzyme consumed in this reaction, may be obtained, using eq 18 where A_0 is defined as the extrapolation to time zero of the spontaneous hydrolysis reaction, whereas previously it was defined as the extrapolation of the steady-state turnover.

The *p*-nitrophenol produced from the reaction of diethyl *p*-nitrophenyl phosphate with elastase is proportional to the concentration of enzyme protein present over a tenfold range, while the pseudo-first-order rate constant of the reaction is independent of the enzyme protein concentration over this range. On the other hand, the pseudo-first-order rate constant of the reaction is directly proportional to the phosphate concentration. Both of these results are presented in Table VII.

Table VII. Effect of Enzyme and Substrate Concentrations on the Reaction of Elastase with Diethyl *p*-Nitrophenyl Phosphate[a]

Enzyme stock soln, μl	pH	Phosphate × 10^3, M	π, absb unit	[Enzyme stock][b] × 10^4, M	$b \times 10^3$, sec^{-1}
50	7.80	7.48	0.0554	2.11	1.19
250	7.74	7.48	0.250	2.02	1.21
200	7.79	7.48	0.215	2.06	1.24
25	7.87	7.48	0.0253	1.88	1.14
50	7.92	2.99	0.0522	1.99	0.501

[a] 25.0° 7.6% (v/v) acetonitrile–water, Tris–HCl buffer, enzyme ESFF 5691 dissolved in pH 4.6 acetate buffer, total volume 2.975 ml. [b] Calculated from observed burst of *p*-nitrophenol.

Since the diethyl *p*-nitrophenyl phosphate–elastase reaction is stoichiometric within experimental error, π is a direct measure of the normality of active sites of elastase in the solution. Table VII records the results of a number of such reactions. These experiments

(77) G. Johansen and M. Ottesen, *Compt. Rend. Trav. Lab., Carlsberg*, 34 199 (1965).
(78) M. A. Naughton and F. Sanger, *Biochem. J.*, 70, 4P (1958).
(79) B. A. Kilby and G. Youatt, *ibid.*, 57, 303 (1954).

Table VIII. The Reaction of Elastase with Diethyl *p*-Nitrophenyl Phosphate[a]

Enzyme	[CH_3CN], % (v/v)	[Phosphate] $\times 10^3$, *M*	pH	π, absb unit	[E] $\times 10^5$,[e] *M*	$\frac{[E]_0(exptl)^d}{[E]_0(cald)}$	$b \times 10^4$, sec^{-1}
ESFF 6501[b]	3.85	2.2	~7.8[f]	0.65	5.2	0.79	5.75
Crystalline[c]	3.85	2.2	7.77	0.72	5.6	0.83	5.56
ESFF 5691	7.6	7.48	7.80 ± 0.1	0.025[g]	20.2	0.78	12.0
				0.250			

[a] 25.0 ± 0.2°. [b] Purified by carboxymethylcellulose chromatography. The fraction used corresponds to the peak of the main protein absorption of the effluent. [c] Material which had been purified with DEAE-Sephadex A-50 (see Experimental Section) and then subjected to carboxymethylcellulose chromatography. This fraction corresponds to the peak of the main protein absorption of the effluent. [d] $[E]_0$(exptl) is that determined in the reaction with diethyl *p*-nitrophenyl phosphate. $[E]_0$(calcd) is that calculated from the known protein absorbance, the optical factor, and the assumption that the equivalent weight of the enzyme is 25,000, which probably has an uncertainty of 10%.[80] [e] Concentration of enzyme stock solution. For the first two entries, this also corresponds to the concentration in the cuvette. [f] pH was not determined at the conclusion of this titration. [g] These titrations include the five experiments of Table VII.

(which were carried out with three different enzyme preparations, two different phosphate concentrations, and three different enzyme concentrations) give results which are quite consistent with one another. The purity of the three enzyme preparations ($[E]_0$(exptl)/$[E]_0$(calcd)) (defined in Table VIII) is the same within experimental error (±10% maximum deviation). The pseudo-first-order rate constants for the three reactions are reasonably consistent considering the unknown effects of different acetonitrile concentrations and phosphate concentrations.

The titration of elastase with diethyl *p*-nitrophenyl phosphate follows eq 15–17. The condition that $[S]_0 \gg [E]_0$ is easily met by the design of the experiments. The condition that $k_{obsd} \gg k_3$ is met since k_{obsd} is as high as 1.2×10^{-3} sec^{-1} (Table VII) whereas k_3 must be less than 3×10^{-6} sec^{-1} since the half-life of the attempted dephosphorylation of the inhibited enzyme must be greater than 65 hr.

The Relationship of the Reaction of Elastase with Diethyl *p*-Nitrophenyl Phosphate to Other Reactions of Elastase. The question may be asked as to whether the titration occurred at the active site of the enzyme and only at the active site of the enzyme. To this end, two sets of experiments were carried out. In the first experiments a determination was made of the rate of abolition of enzymatic activity toward different substrates during the phosphate inhibition. Three substrates were used: *p*-nitrophenyl trimethylacetate, isobutyrate, and N-benzyloxycarbonyl-L-tyrosinate. The results of these experiments are shown in Table IX and partially illustrated in Figure 9.

The rate constant of the liberation of *p*-nitrophenol in the reaction of elastase with diethyl *p*-nitrophenyl phosphate is equivalent to the rate constant of the loss of activity of elastase toward *p*-nitrophenyl trimethylacetate and isobutyrate. (Table IX). However, the rate constant of the loss of activity of elastase toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate is 23-fold slower than the rate constant of the titration reaction with elastase ESFF 5691 while the activity loss of elastase ESFF 6501 shows two rate constants, one of which agrees roughly with the rate constant of the titration reaction, and the other of which is 15-fold slower. In contrast, the rate constant of the loss of activity of α-chymotrypsin and trypsin toward *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate equals the rate constant of the reaction of these enzymes with diethyl *p*-nitrophenyl phosphate.

Table IX. Kinetics of the Loss of Activity of Elastase during Reaction with Diethyl *p*-Nitrophenyl Phosphate[a]

Enzyme	ESFF 5691[b]	ESFF 6501[c]
k(release of *p*-nitrophenol from diethyl *p*-nitrophenyl phosphate), sec^{-1}	1.22×10^{-3}	5.7×10^{-4}
k(loss of activity toward *p*-nitrophenyl trimethylacetate), sec^{-1}	1.3×10^{-3} [d]	
k(loss of activity toward *p*-nitrophenyl isobutyrate), sec^{-1}		5.5×10^{-4}
k(loss of activity toward N-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester), sec^{-1}	5.6×10^{-5}	6 ± 2[e] $\times 10^{-4}$ 3.67×10^{-5}

[a] 25.0°. [b] 7.6% (v/v) acetonitrile–water, 7.48×10^{-3} *M* diethyl *p*-nitrophenyl phosphate, pH 7.8 Tris–HCl or phosphate buffer. [c] Fraction 29 of chromatography; 3.85% (v/v) acetonitrile–water, 2.2×10^{-3} *M* diethyl *p*-nitrophenyl phosphate, pH 7.8 phosphate buffer ($I = 0.1$). [d] The actual pseudo-first-order rate constant was 5-fold less using a concentration of diethyl *p*-nitrophenyl phosphate which was 5-fold less. [e] This rate constant is an approximate value based on one point (see Figure 9).

In the second set of experiments, the second-order rate constant for the elastase-catalyzed hydrolysis of a considerable number of substrates of elastase was determined, using two enzyme preparations which had been purified in entirely different ways. Values of $k_{cat}[E]_0/K_m$(app) were determined either as observed pseudo-first-order rate constants or from Lineweaver–Burk plots. Since $[E]_0$ is known by titration, k_{cat}/K_m(app) is readily evaluated. As seen in Table X, the k_{cat}/K_m(app)'s determined for a given substrate with the two enzyme preparations were quite similar except for *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate. This result indicates that the titration of the active sites of elastase with diethyl *p*-nitrophenyl phosphate gives a consistent measure of the activity of the enzyme toward seven substrates of elastase, including both acylamino acid and carboxylic acid derivatives.

The Use of a Rate Assay as a Secondary Standard. Any of the reactions shown in Table X, which have been shown to give concordant results of k_{cat} or k_{cat}/K_m(app) with two different enzyme preparations (with the exception of *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate), can be used as a rate assay to determine enzyme concentration. Using the equations $V_{max} = k_{cat}[E]_0$ or $k_{obsd} = (k_{cat}/K_m)[E]_0$ and the values of k_{cat} or k_{cat}/K_m(app) for a particular substrate under the

(80) U. J. Lewis, D. E. Williams and N. G. Brink, *J. Biol. Chem.*, 222, 705 (1956); Naughton and Sanger[78] report 28,500.

Table X. The Kinetics of Some Elastase-Catalyzed Reactions Using Two Elastase Preparations[a]

p-Nitrophenyl ester	pH	$[E]_0 \times 10^6$, *M*	$[S]_0 \times 10^5$, *M*	Kinetics	k_{cat}/K_m(app) $\times 10^{-3}$, M^{-1} sec^{-1} Enzyme 1[c]	k_{cat}/K_m(app) $\times 10^{-3}$, M^{-1} sec^{-1} Enzyme 2[d]	$k_{cat} \times 10^3$ sec^{-1} Enzyme 1	$k_{cat} \times 10^3$ sec^{-1} Enzyme 2
Trimethylacetate	7.33	5	5.5	MM[i]	0.099[b]	0.123[b]	1.75	1.77[b]
Acetate	7.43	7.0	16	MM[i]	0.47	0.41[h]	20.2	21[h]
Isobutyrate	7.68	0.7–2.6	0.7–15	MM[i]	1.21[b]	1.62	43[b]	34
Fourate	7.69	0.5–2.6	0.1–2.0	MM[i]	2.35[b]	1.69	19.5[b]	22.4
N-Benzyloxycarbonyl-L-isoleucinate	7.70	0.5–2.9	0.53	First order	0.296	0.250[e]		
N-Benzyloxycarbonyl-L-leucinate	7.79	0.1–0.5	0.5	First order	28.7	30.4		
N-Benzyloxycarbonyl-glycinate	7.85	9.6	1.0	First order	15.2	13.3[b,f]		
N-Benzyloxycarbonyl-L-tyrosinate	7.79	0.53–5.6	0.95	First order	19.3	0.41[g]		

[a] 25.0 ± 0.2°, phosphate buffer, $I = 0.05$. [b] Average of two or more determinations. [c] Enzyme 1 was Worthington electrophoretically purified elastase ESFF 6507B. [d] Enzyme 2 (JRW-2) was a Worthington crystalline elastase purified by chromatography on carboxymethylcellulose at pH 8.0 (see Experimental Section). [e] pH 7.52. [f] pH 7.72. [g] pH 7.39. [h] pH 7.26. [i] Michaelis–Menten.

conditions of reaction shown in Table X, $[E]_0$ may be calculated directly from V_{max} or k_{obsd}.

Discussion. Elastase appears to contain one active site per mole since the molecular weight determined by sedimentation (25,000)[80] agrees roughly with the equivalent weight determined by diisopropylphosphorofluoridate inactivation (28,500).[46] On this basis, titrations of both the electrophoretically purified and chromatographically purified elastases discussed here with diethyl *p*-nitrophenyl phosphate indicate that 62–80% of the protein in these solutions is active enzyme. The rate of loss of activity of the enzyme toward *p*-nitrophenyl trimethylacetate and isobutyrate (and N-benzyloxycarbonylglycinate) is essentially the same as the rate of the titration reaction, indicating a common active site for all processes. Furthermore, the second-order rate constants for seven substrates of elastase are essentially the same for two different elastase preparations, using the titrated value of the enzyme concentration, again indicating that the titration gives a consistent measure of the elastase active sites. Finally, one of these enzymes (ESFF 6507A has been shown to have high elastolytic activity.[81] Thus, it appears that the titration occurs at the active site of the enzyme.

However, with the substrate *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate, the two tests indicated above gave inconsistent results. Chromatography of several elastase samples reveals that whereas the *p*-nitrophenyl isobutyrate activity parallels the ultraviolet protein absorption in the effluent, the *p*-nitrophenyl N-benzyloxycarbonyl-L-tyrosinate activity has a peak several fractions prior to (and overlapping with) the main protein peak. Since no protein peak is detected at the maximum tyrosine activity, it may be concluded that an extraneous enzyme, highly active toward tyrosine ester, is present in low concentration and is imperfectly separated from elastase under the purification conditions. The actual amount of this extraneous enzyme is unknown, although it is problably present to an extent less than 1–7%, and the actual amount may be much lower. An impurity has been identified by electrophoresis in the present preparation as elastomucase,[83] which is known to have a substantial activity toward tyrosine esters.[82] Another impurity possessing unusually high activity toward tyrosine substrates has been found in elastase and designated elastase 1.[84] It is inactivated by diisopropylphosphorofluoridate. While these other enzymes would presumably react with diethyl *p*-nitrophenyl phosphate, the titration of elastase is still valid when the other enzymes are present in trace amounts.

(81) Personal communication from Dr. W. A. Loeven, The Netherlands Institute for Preventive Medicine, Lieden, The Netherlands. The activity corresponded to 3.4 eu/mg of protein[82] when tested against alkali-treated elastin of pH 8.7. This enzyme is free of the α_2 globulin fraction which contains elastase inhibitor.

(82) W. A. Loeven, *Acta Physiol. Pharmacol. Neerl.*, **12**, 57 (1963).

Results for Acetylcholinesterase

The enzyme acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7)[3] serves physiologically as a catalyst for the hydrolysis of acetylcholine. However, acetylcholinesterase is similar to the proteolytic enzymes in this series in that its kinetic pathway also follows eq 1.[85]

The following evidence supports this pathway. The enzyme is inhibited by certain organophosphorus compounds (for example, tetraethyl pyrophosphate and diisopropylphosphorofluoridate)[86] by forming a phosphoryl enzyme.[87] Like the other enzymes of this series, the phosphoryl moiety is isolated on a serine hydroxyl group after the degradation of the protein.[88] The phosphoryl enzyme can be reactivated by nucleophilic compounds.[89] Dimethylcarbamoylcholine and chloride also inhibit the enzyme.[90] The enzyme inhibited by two carbamates regains activity in water at the same rate and is reactivated by hydroxylamine again at the same rate.[90] These results indicate the formation of acyl-enzyme in the enzyme-catalyzed hydrolysis of these substrates. Finally, noncompetitive inhibition[91] and pH dependency[92] studies using acetate esters are consistent with this three-step kinetic equation

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(92) R. M. Krupka, *Biochem. Biophys. Res. Commun.*, **19**, 531 (1965).

Table XI. The Titration of Acetylcholinesterase Solutions with *o*-Nitrophenyl Dimethylcarbamate (Numbers 1–4 and 6–8) and *p*-Nitrophenyl Dimethylcarbamate (Number 5)[i,o]

No.	[Titrant] $\times 10^4$, M	pH	Dilution factor[a]	$\pi = [E]_0 \times 10^6$ [b]	$V \times 10^{10}$, M sec^{-1} [b]	$k_3 \times 10^4$, [c] sec^{-1}	Buffer soln[d]
1[l,j]	6.64	8.20	3.750	2.0 ± 0.1	9.2 ± 0.28	4.8 ± 0.4	0.0525 M Tris,[e] 0.024 M HCl, 0.16 M NaCl, 1.2% (v/v) acetonitrile–water
2[l,j]	6.87	8.20	4.833	1.5 ± 0.1	6.8 ± 0.14	4.6 ± 0.4	0.0569 M Tris,[e] 0.025 M HCl, 0.15 M NaCl, 1.2% (v/v) acetonitrile–water
3[m]	10.03	7.73	3.750	3.6 ± 0.15	19.9 ± 0.6	5.5 ± 0.4	4.22×10^{-3} M KH_2PO_4, 4.51×10^{-2} M Na_2HPO_4, 5.33×10^{-2} M NaCl, 1.7% (v/v) acetonitrile–water
4[m,k]	5.02	7.72	3.750	3.6 ± 0.15	22.5 ± 0.3	6.2 ± 0.3	4.22×10^{-3} M KH_2PO_4, 4.51×10^{-2} M Na_2HPO_4, 5.33×10^{-2} M NaCl, 1.7% (v/v) acetonitrile–water
5[m,k]	10.33	7.78	7.500	1.8 ± 0.08[f]	5.15 ± 0.13	[g]	5.02×10^{-3} M KH_2PO_4, 5.37×10^{-2} M Na_2HPO_4, 2.67×10^{-2} M NaCl, 1.7% (v/v) acetonitrile–water
6[m]	5.94	7.70	1.100	7.45 ± 0.13	48.9 ± 0.4	6.57 ± 0.17	6.78×10^{-2} M Na_2HPO_4, 6.41×10^{-3} M KH_2PO_4
7[n]	6.01	9.00	1.375	3.46 ± 0.09	21.0 ± 0.2	6.08 ± 0.2	6.19×10^{-3} M Na_2CO_3, 3.18×10^{-2} M $NaHCO_3$ 0.3 M NaCl
8[n]	5.89	7.01	1.100	1.4 ± 0.4	6.77 ± 0.07	3.9 ± 0.2[h]	3.14×10^{-2} M KH_2PO_4 and 5.28×10^{-2} M Na_2HPO_4

[a] The number of times the enzyme was diluted in the titration. [b] The uncertainty has been estimated from the uncertainty in the absorbance values. [c] Calculated from the expression, $V = k_3[E]_0$. The uncertainty for these values represents the sum of the uncertainty estimated for V and $[E]_0$. [d] Buffers solutions were at ionic strength 0.2 except for no. 7 which was 0.35. [e] Tris(hydroxymethyl)aminomethane. [f] Enzyme concentration was calculated from concentration determined in no. 3 and 4. [g] It was not possible to calculate this value using the expression $V = k_3[E]_0$ since [S] was not much larger than K_m and k_2 is not much larger than k_3 (see later). [h] The uncertainty in this titration was large so that the concentration determined by rate assay 8 (1.74×10^{-6} N) was used in the calculation of k_3. The uncertainty given here is the sum of the uncertainties in V_m for rate assay 8, k_{cat} of 1.76×10^4 sec^{-1}, and the V in column six. [i] *o*-Nitrophenoxide ion was followed at 415 mμ and *p*-nitrophenoxide ion was followed at 400 mμ. [j] Dust introduced into titrations 1 and 2 produced the large uncertainty in these experiments. [k] A linear steady-state reaction was found in titrations 4 and 5 for at least 84 min. [l] Enzyme solution dialyzed and concentrated as described in the Experimental Section. [m] Enzyme solution dialyzed as described in the Experimental Section. [n] Enzyme solution dialyzed and filtered as described in the Experimental Section. [o] More details on the treatment of enzyme solutions may be found in ref 56.

involving the formation of an acyl-enzyme intermediate in the reaction pathway.

Three titrations of acetylcholinesterase have been made on the basis of the stoichiometry of eq 1. The enzyme has been phosphorylated with ^{32}P-labeled diisopropylphosphorofluoridate leading to a labeled enzyme from which the concentration of active sites could be calculated.[87] Similar titrations were carried out using diethoxyphosphorylthiolcholine and tetraethyl pyrophosphate.[93] In both these instances possible nonspecific phosphorylation was taken into account. A different approach utilized dimethylcarbamyl fluoride as substrate. The ratio of the activity of the carbamylated enzyme solution to the original activity of the enzyme was followed nearly to completion of the carbamylation and decarbamylation reactions; from this determination and the decarbamylation rate constant, the normality of the enzyme solution could be evaluated.[94]

Here we report the direct spectrophotometric observation of a stoichiometric reaction in the eel acetylcholinesterase-catalyzed hydrolysis of a carboxylic ester and its use in the development of a procedure for the determination of a concentration (normality) of the active site of this enzyme. *o*-Nitrophenyl dimethylcarbamate was used as the substrate.[95]

Titrations. The titrations of eel cholinesterase by *o*-nitrophenyl and *p*-nitrophenyl dimethylcarbamate are shown in Table XI. All the titrations except no. 5 are characterized by an initial rapid release of *o*-nitrophenol followed by a slow steady-state release of the phenol. The initial release of nitrophenol was usually of the order of 20×10^{-4} absorbance unit. In titration 6 shown in Figure 3 the initial release of nitrophenol was considerably greater than this value; in titrations 1, 2, 5, and 8 it was considerably less.

For titrations 1–4, k_2 was determined by using *p*-nitrophenyl acetate to assay the enzyme-catalyzed hydrolysis of *o*-nitrophenyl dimethylcarbamate under conditions similar to those in the titrations.[96] The k_2 was 0.5–0.7 sec^{-1} at pH 7.78 in 1.6% (v/v) acetonitrile–water, KH_2PO_4–Na_2HPO_4 buffer at $I = 0.2$. Values of k_3 determined in titrations 1–4 are in the range $4.6–6.2 \times 10^{-4}$ sec^{-1} (Table XI). Thus for titrations 1–4, k_2/k_3 is 800–1500. For titrations 6–8 carried out in the absence of acetonitrile, the value of k_2 has not been determined directly. However, the carbamylation rate constant, b, was determined for titration 6 (Figure 3) by plotting log ΔA *vs.* time for the presteady-state reaction. The rate constant, b, is given by eq 22. From eq 22, the value of k_2 can be approximated using the experimental

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Table XII. The Values of K_m[a] for the Acetylcholinesterase-Catalyzed Hydrolysis of *o*-Nitrophenyl Dimethylcarbamate and a Comparison of the Values of k_3 for the Deacylation of the Dimethylcarbamyl-Enzyme Determined by the Rate Assay Method[b] with the Values Determined by the Titrations

From the rate assay method			From the titrations in Table XI		
pH	$k_3 \times 10^4$,[c] sec^{-1}	K_m(app) $\times 10^6$, M	$k_3 \times 10^4$, sec^{-1}	pH	Buffer soln[d]
7.85	6.7 ± 0.3		6.2 ± 0.3	7.72	5.92×10^{-3} M KH_2PO_4, 6.29×10^{-2} M Na_2HPO_4, 1.2% (v/v) acetonitrile–water
7.98	7.3–7.6		4.8 ± 0.4	8.20	3.88×10^{-3} M KH_2PO_4, 6.55×10^{-2} M Na_2HPO_4
9.24	6.0 ± 0.1		6.1 ± 0.2	9.00	1.23×10^{-1} M $NaHCO_3$, 2.36×10^{-2} M Na_2CO_3, 1.7% (v/v) acetonitrile–water
7.03	3.5 ± 0.0		3.9 ± 0.2	7.01	3.21×10^{-2} M KH_2PO_4, 5.42×10^{-2} M Na_2HPO_4, 1.2% (v/v) acetonitrile–water
7.00	4.3,[e] 3.2[f]				0.1 M NaCl, 0.01 M $MgCl_2$, 0.02 M sodium phosphate[e,f]
7.04		2.9 ± 0.1			3.51×10^{-2} M KH_2PO_4, 5.55×10^{-2} M Na_2HPO_4
7.82		5.4 ± 0.2			5.46×10^{-3} M KH_2PO_4, 5.82×10^{-2} M Na_2HPO_4
7.82		3.0 ± 0.1			5.46×10^{-3} M KH_2PO_4, 5.82×10^{-2} M Na_2HPO_4, 1.7% (v/v) acetonitrile–water
9.16		4.5 ± 0.1			3.17×10^{-2} M $NaHCO_3$, 6.13×10^{-3} M Na_2CO_3, 1.50×10^{-1} M NaCl

[a] *o*-Nitroacetanilide was used to determine the K_m for the enzyme-catalyzed hydrolysis of *o*-nitrophenyl dimethylcarbamate.[96] [b] *o*-Nitroacetanilide was used to assay the enzyme-catalyzed hydrolysis of *o*-nitrophenyl dimethyl carbamate.[96] [c] Reproducibility of two or more determinations is given after these values. [d] Buffer solutions were at ionic strength 0.2. These buffer solutions pertain to the rate assays. [e] See ref 99. [f] I. B. Wilson, M. A. Harrison, and S. Ginsburg, *J. Biol. Chem.*, **236**, 1498 (1961).

value of b, a value of 2×10^{-3} M for K_s,[96] and 6.6×10^{-4} sec^{-1} for k_3 (Table XI). This calculation yields $k_2 \sim 0.3$ sec^{-1} and thus $k_2/k_3 \sim 400$. It is of interest that the value of k_2 in the absence of acetonitrile is approximately one-half the value in the presence of 1.7% (v/v) acetonitrile, indicating a peculiar solvent effect. In conclusion, for all titrations using *o*-nitrophenyl dimethylcarbamate, $k_2/k_3 \geqq 400$.

The K_m(app) for the acetylcholinesterase-catalyzed hydrolysis of *o*-nitrophenyl dimethylcarbamate, determined under conditions similar to the titrations, are listed in Table XII. By comparing the titrant concentrations in Table XI and the K_m(app)'s of Table XII it is seen that the $[S]_0/K_m$(app) ratio was greater than 100 for all titrations.

As seen in Table XI, the ratio $[S]_0/[E]_0 > 100$ for all the titrations and therefore the requirement that $[S]_0 < [E]_0$ is satisfied.

In the steady-state turnover of the titration experiments, $k_2 \gg k_3$ and $[S]_0 \gg K_m$(app) (except for titration 5). Thus $V = k_3[E]_0$. The values of k_3 so determined are shown in Tables XI and XII.

The enzyme preparations used in these titrations consisted of approximately 7% active enzyme based on a comparison of the activity per milligram of protein determined by Kremzner and Wilson[97] on the purest preparation to date and the activity per milligram of protein of the present lot 23B-7587.[98] Thus 7% is an upper limit. The corrections made in this comparison[98] are all small and therefore the value of 7% is probably correct within ±3%. Since the enzyme preparations consist of approximately 93% inactive protein,[99] it is important to demonstrate that the titrant does not react with this protein. Even if the enzyme preparations were 100% pure, the reliability of the titration depends on the demonstration that the carbamate reacts only at the active site. One argument against reaction by an impurity is the pure first-order nature of the carbamylation reaction. Furthermore, if the titrant were to react unspecifically with the enzyme or protein, k_3 determined from the titration would be lower than k_3 determined by the rate assay. The comparison shown in Table XII indicates that all values of k_3 except those determined in titrations 1–3 are within 10% of each other, which is within experimental error. The low value of k_3 in titrations 1–3 are probably due to the incursion of substrate inhibition (titration 1) or noncompetitive inhibition by the Tris buffer (titrations 1 and 2). Since the values of k_{cat}(lim) determined for phenyl acetate in all these titrations are in good agreement (Table XIII), the low values calculated in titrations 1–3 cannot be the result of some nonspecific reaction of the titrant.

An unlikely possibility exists that the titrant reacts unspecifically and decarbamylates at approximately

(97) L. T. Kremzner and I. B. Wilson, *J. Biol. Chem.*, **238**, 1714 (1963).

(98) The activity reported by Kremzner and Wilson was multiplied by 1.124[94] to correct for substrate inhibition and was multiplied by 1.25 to calculate a pH-independent activity.[92] The corrected activity is 0.26 mmole of acetylcholine hydrolyzed per second per milligram of protein. The activity of lot 23B-7587, sample 1, determined at pH 7.62 with phenyl acetate was multiplied by 1.05 to calculate the pH-independent value, and multiplied by 0.90 to account for the difference between phenyl acetate and acetylcholine activities.[92]

(99) This inactive protein apparently has no effect on the kinetics of the acetylcholinesterase-catalyzed hydrolysis of substrates. The value of k_3 for the decarbamylation of the dimethylcarbamyl–enzyme measured in the range of 10^{-6} M (by titration) and 10^{-8} M (by rate assay) (Table XII) is independent of the protein concentration. Furthermore, the k_{cat} and K_m(app) for the enzyme-catalyzed hydrolysis of phenyl acetate (Table XIII) are also independent of the protein concentration.

Table XIII. Kinetics of the Acetylcholinesterase-Catalyzed Hydrolysis of Phenyl Acetate

Rate assay[i]	$V_m \times 10^7$,[a] M sec^{-1}	$[E]_0 \times 10^6$,[b] before dilution	Dilution factor $\times 10^{-3}$	$[E]_0 \times 10^{11}$,[c] after dilution	$k_{cat} \times 10^{-4}$,[d] sec^{-1}	K_m(app) $\times 10^3$ M	pH	k_{cat}(lim)[e] $\times 10^{-4}$ sec^{-1}
1–2	10.0 ± 0.3	7.4[f]	122	6.07 ± 0.3	1.65 ± 0.14	1.9	7.62	1.77 ± 0.15
3–5	9.0 ± 0.2	13.5	244	5.53 ± 0.23	1.63 ± 0.10	1.8	7.82	1.71 ± 0.11
6	140 ± 2	8.21	10.32	79.4 ± 1.4	1.76 ± 0.06	2.1	8.57	1.76 ± 0.06
7	168 ± 2	4.76	5.16	92.2 ± 2.4	1.82 ± 0.07	2.2	8.52	1.82 ± 0.07
8	65.1 ± 1	1.91[g]	5.16	37.0 ± 0.18[h]		1.9	8.52	

[a] Determined from the intercepts of Eadie plots; uncertainty was estimated from the intercepts. [b] These values were calculated by multiplying the normality values in Table XI by the number of times the enzyme was diluted in the titrations. [c] The enzyme solution was diluted as shown in the preceding column for assay. This column then is the value in column three divided by the corresponding value in column four. The uncertainty in these values represents the uncertainty in the titrations, column five, Table XI. [d] Calculated using the expression $V_m = k_{cat}[E_0]$ and the values in columns two and five. The uncertainty given here is the sum of the uncertainty of the values in columns two and five. [e] Calculated using $k_{cat} = k_{cat}(lim)/(1 + (H^+/K_a))$, the values in column six and eight, and a pK_a of 6.5.[56] The uncertainty given here is the uncertainty in the values in column six. [f] This value is the average value in titration one and two (Table XI). [g] This value was calculated from the expression $[E]_0 = V_m/k_{cat}$ and the value in column two and a value of 1.76×10^4 sec^{-1} for k_{cat}. [h] See footnote *g*. The uncertainty given here is the sum of the uncertainty in k_{cat}(lim) for rate assay 6 and the V_m in rate assay 8. [i] In rate assays 1–5, the phenyl acetate was introduced in acetonitrile while in rate assays 6–8, it was dissolved in aqueous solution (see Experimental Section).

the same rate as it does at the active site, thus perturbing both V and $[E]_0$ in the same way and leading to an unperturbed k_3. Using a different titrant and a different titration method, Wilson and Harrison[94] were able to determine the k_{cat} for the acetylcholinesterase-catalyzed hydrolysis of acetylcholine which when extrapolated to the pH-independent value (k_{cat}(lim)) is 1.7×10^4 sec^{-1}.[100] This value is within experimental error of the values reported for k_{cat}(lim) for phenyl acetate, using the titration of the enzyme with *o*-nitrophenyl dimethylcarbamate and the phenyl acetate rate assay, and is another check on the specificity of this titrant.

In conclusion, the titration of the active sites of eel acetylcholinesterase by *o*-nitrophenyl dimethylcarbamate meets all the criteria for a valid titration.

The results using *p*-nitrophenyl dimethylcarbamate as titrant were disappointing. Although the spectral properties of this reaction are favorable, the reaction has a low k_2/k_3 ratio (approximately 5) and a high K_m(app) (4×10^{-4} M).[96] Although it is still possible to utilize this substrate as a titrant under these conditions, the composite uncertainty which would be introduced in the calculation of $[E]_0$ using the reduction in the size of the burst ($\pi < [E]_0$, eq 12) and the very long pre-steady-state reaction of ~50 min indicate that *p*-nitrophenyl dimethylcarbamate is not a suitable titrant for acetylcholinesterase.

The titration with *o*-nitrophenyl dimethylcarbamate can be carried out from approximately pH 5 to 10, spanning the entire range where the enzyme is stable.[101] The lower limit of enzyme concentration usable in this titration is about 10^{-6} M, where the uncertainty would be about 20% if the titration was carried out above pH 8 in order to obtain the maximal spectral change.

The hydrolyses of *o*-nitrophenyl and *p*-nitrophenyl dimethylcarbamates are not catalyzed by chymotrypsin, subtilisin, trypsin, elastase, or papain, using a 10^{-5} M enzyme solution at pH 7–8 for 15–30 min. On the other hand, pig liver[102] and ox liver[103] carboxylesterases catalyze the hydrolyses of *o*- and *p*-nitrophenyl dimethylcarbamates. In fact, titrations of these enzymes using these substrates have been developed.[102,103]

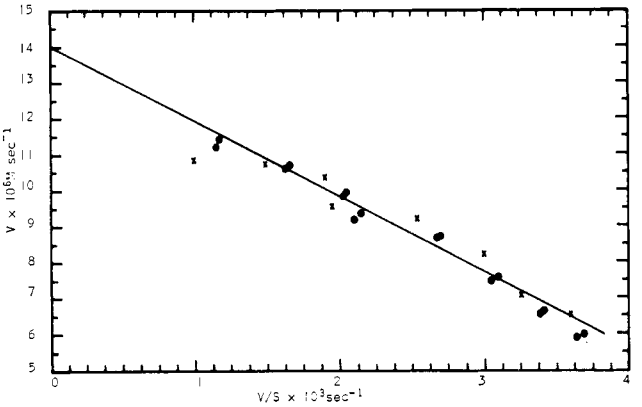

Figure 10. Eadie plots for the acetylcholinesterase-catalyzed hydrolysis of phenyl acetate for rate assay 6: x, first determination of the initial rates, ●, second determinations carried out 1 week later.

Rate Assay Using Phenyl Acetate. A spectrophotometric rate assay has been developed for determining enzyme concentration down to at least 5.5×10^{-11} M, using a value of k_{cat} based on the titration reported here. An aliquot of enzyme, which had been previously titrated, was diluted and the initial rate of the enzyme-catalyzed hydrolysis of phenyl acetate was determined over the range of concentration of the ester. These data were plotted in the form of Eadie plots in order to determine V_m, the maximal velocity. An example is given in Figure 10. The rate assay was carried out for every titrated enzyme sample. The acetylcholinesterase-catalyzed hydrolysis of phenyl acetate follows Briggs–Haldane kinetics up to 10^{-2} M where substrate inhibition becomes apparent. Thus $k_{cat} = V_m/[E]_0$. Using this relationship and the values for $[E]_0$ and V_m, k_{cat} can be calculated, as shown in Table XIII. The values of k_{cat}(lim) calculated from the various titrations agree well with those calculated from their corresponding rate assays, the greatest difference being about 6%. The rate assays were determined on enzyme solutions

(100) At pH 7.0, a k_{cat} of 1.4×10^4 sec^{-1} was reported;[94] k_{cat}(lim) was calculated using the equation in footnote *e* of Table XIII, this value of k_{cat}, and a pK_a of 6.4.[92]

(101) The k_{OH^-} of *o*-nitrophenyl dimethylcarbamate is quite low (8.7×10^{-5} M sec^{-1} at 25.0°), leading to negligible spontaneous hydrolysis in these experiments.

(102) D. J. Horgan, E. C. Webb, and B. Zerner, *Biochem. Biophys. Res. Commun.*, 23, 18 (1966).

(103) M. Runnegar and B. Zerner, personal communication.

from 5.5×10^{-11} N to 9.2×10^{-10} N, and on enzyme solutions which had been diluted from 5160 to 244,000 times from solutions used in the titrations. The agreement among the k_{cat}(lim) values indicates that the enzyme can be diluted quantitatively to this low concentration range, and indicates that in this range any possible association–dissociation phenomena of the enzymes do not affect k_{cat}(lim).

Since k_{cat}(lim) for the acetylcholinesterase-catalyzed hydrolysis of phenyl acetate is found to be 1.76×10^4 sec^{-1}, the concentration of an enzyme solution can be determined by this rate assay, using the relationship, $[E]_0 = V_m/k_{cat}$(lim). The rate assay may be carried out over the pH range 6.4–9.0 where the pH dependency of k_{cat} is given by the equation in footnote *e* of Table XIII. Rate assays have been reported for the acetylcholinesterase-catalyzed hydrolysis of acetylcholine, leading to a k_{cat} of 1.4×10^4 sec^{-1} at pH 7, 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.02 M sodium phosphate at 25.0°,[94] and a k_{cat} of 1.2×10^4 sec^{-1} at pH 7.4, 38°, 3.4×10^{-2} M $MgCl_2$, 2.5×10^{-2} M $NaHCO_3$, and 1.5×10^{-2} M substrate.[87] Since the acetylcholinesterase-catalyzed hydrolysis of acetylcholine and of phenyl acetate are reported to give k_{cat}'s within 10% of one another,[92] they may be compared. The first of the acetylcholine rate constants gives a k_{cat}(lim) of 1.7×10^4 sec^{-1}, which is in good agreement with the rate constant of phenyl acetate reported here.

Discussion

Titrations. The titrations described above are based on the mechanistic pathways of eq 1 or eq 15. If these equations do not describe the titration reactions completely, for example, if another intermediate exists in finite amount, then, of course, the titration is no longer quantitatively valid. However, all the burst titrations described here may be quantitatively described by eq. 1 or 15, and are considered to be valid. Furthermore, the titrations meet the criteria for a rigorous determination of the concentration of active sites in terms of eq 12 and 17. These criteria require the determination of k_2 or k_{obsd}, k_3, and K_m(app), as well as the knowledge of $[S]_0$ and $[E]_0$. The former constants have been determined in a variety of ways. The constant k_2 was determined by: (1) measurement of the rate of loss of enzyme activity by an external assay (acetylcholinesterase); (2) the determination of b and from it an estimate of k_2 according to eq 22 (acetylcholinesterase and subtilisin); (3) an analysis of the presteady state at different substrate concentrations (chymotrypsin);[19,25] (4) an analysis of both the presteady state and steady state at different substrate concentrations (trypsin).[11] The constant k_3 was ordinarily determined from the steady-state reaction (k_{cat}), knowing that $k_2 \gg k_3$ and thus that $k_{cat} = k_3$ (acetylcholinesterase, elastase, subtilisin, and chymotrypsin).[19,25] For trypsin, a complete analysis of both the presteady state and steady state was used.[11] The constant b was determined directly. For papain, the k_2/k_3 ratio was considered directly by: (1) the determination of b and the use of eq 23, (2) the absolute magnitude of K_m(app), (3) the effect of methanol on the kinetics, and (4) the agreement of burst from two titrants of different k_2/k_3 ratio.

The titrations of α-chymotrypsin, trypsin, and papain utilize specific substrates of these enzymes, while the titrations of elastase, subtilisin, and acetylcholinesterase do not. However, the titrations of each of the latter enzymes has been shown to occur at the active site of the enzyme, as defined by a specific substrate. The titrations of α-chymotrypsin and tryspin appear to be independent of both titrant, over a wide range of specificity, and of pH of the titration. The titration of papain gives identical results with two substrates. The titration of elastase gives the same results with *p*-nitrophenyl trimethylacetate[104] as with the diethyl *p*-nitrophenylphosphate described here. All of these identities indicate the determination of a fundamental quantity, namely the intact active site.

The optimal titrant should have the following characteristics: (1) be a specific substrate, (2) give a titration in a few minutes in order to obviate denaturation, and for convenience, (3) give a titration around neutrality in order to reproduce best physiological conditions, (4 be a stable, available, and soluble reagent whose stoichiometric reaction is easily detectable, and (5) give a titration over a wide range of enzyme concentrations. Not one of the titrants described fulfills all of the requirements listed above. However, each fits these requirements most closely for a given enzyme.

Impurities. It is the thesis of this paper that the concentration of active enzyme may be determined by a titration procedure in the presence of impurities and furthermore that once this concentration is known it may be used for subsequent kinetic studies where its value is needed. The presence of impurities in an enzyme preparation could affect the titration procedure by interfering with the active site and/or the substrate. Impurities which reversibly block the active site would not be expected to influence a titration since it is independent of such problems. A competitive inhibitor such as a peptide impurity may raise the K_m(app) to some apparent higher value, K_m(app)′, but it is likely under such a perturbation that $[S]_0$ will still be much greater than K_m(app)′ for many titrants, and thus the titration will remain stoichiometric. Since the presence of a considerable amount of competitive inhibitor would not be expected to affect a titration, the possibility exists that it may lie undetected in the titration and later may affect the kinetics. However, if an enzyme is used in the determination of the kinetics of a specific substrate, the concentration of enzyme, and thus the concentration of any competitive inhibitor associated with it, will be at such a low level that the latter will probably be in too low concentration to affect the former. Irreversible inibitors will, of course, affect both the titration and the kinetics in the same way. Finally, an impurity which is finitely but slowly reversible may complicate both the titration and the kinetics.

In the titrations reported above, the enzyme purity has varied from a low of 7% with acetylcholinesterase to a high of 83% with porcine trypsin.[105] Even Worthington three-times-crystallized α-chymotrypsin titrates

(104) T. H. Marshall, Ph.D. Thesis, Northwestern University, 1966.

(105) The percentages of purity listed in this paper can be expressed in a different manner. The titrations can be used to determine the number of sites per milligram of protein, which also expresses the purity. The chemist (and the authors) may prefer the former, the biochemist the latter. The two ways of expressing purity are in fact identical.

Table XIV. α-Chymotrypsin- and Trypsin-Catalyzed Hydrolysis of Specific Substrates

Enzyme	Batch no.	Substrate	% purity by wt	$[E]_0$ soln, M	$[S]_0$ soln, M	k_{cat}, sec^{-1}	K_m(app), M[c]
Chymotrypsin	CDI-6114-5 untreated	Methyl N-acetyl-L-tryptophanate	87.1[a]	1.411×10^{-7}[b]	2.041×10^{-3}	40.1[c-e]	13.0×10^{-5}[c]
	CDI-6114-5 Sephadexed through G-25 (fine)[i]	Methyl N-acetyl-L-tryptophanate	86.6[a]	2.117×10^{-7}[b]	2.041×10^{-3}	39.0[c-e]	9.3×10^{-5}[c]
	CDI-6110-1 untreated	Methyl N-acetyl-L-tryptophanate	66.1[a]	0.9869×10^{-7}[b]	2.041×10^{-3}	40.5[c,d]	13.8×10^{-5}[c]
Trypsin	TRL-6261 untreated	Ethyl α-N-benzoyl-L-argininate	57[f]	2.504×10^{-7}[g]	1.623×10^{-4}	16.2[h]	1.2×10^{-5}[h]
Trypsin	TRL-6261 Sephadexed using G-50	Ethyl α-N-benzoyl-L-argininate	77[f]	1.65×10^{-7}[g]	1.584×10^{-4}	13.7[h]	1.06×10^{-5}[h]

[a] Assuming mol wt 24,800. [b] Determined by an N-*trans*-cinnamoylimidazole titration using $\Delta\epsilon$ 8950 at 335 mμ and CI $\geq 2[E]_0$. [c] pH 8.34, Tris buffer, 0.05 M, 3.16% CH_3CN, 25.0°, 300 mμ, $\Delta\epsilon$ 251, methyl N-acetyl-L-tryptophanate, H and M Co., used as received. Enzyme used 10 min after dissolution. For experimental procedure, see ref 10. [d] These values are slightly lower than others reported in the literature[107-110] because 3.6% acetonitrile was used here. [e] Reference 106 reports k_{cat} of 45 ± 0.9 and 28.0 ± 1.05 for untreated and Sephadexed CDI 6114-5 at pH 8.00 in aqueous solution. While the former number is compatible with the present results because of the solvent effect, the latter number is not. [f] Assuming mol wt 24,000. [g] Determined by titration with *p*-nitrophenyl α-N-benzyloxycarbonyl-L-lysinate at pH 3.5. [h] pH 9.88, 0.10 M carbonate buffer, 25°, 260 mμ, $I = 0.3$. [i] Gift of Dr. C. J. Martin.

at 90% purity only in certain instances (Table XIV). These numbers raise substantial doubts about either the purity of the various enzyme preparations or the titrations themselves. The validity of each titration has been discussed above. Therefore, let us look at the impurities.

The purities mentioned above have been calculated from the relationship of the titrated value to that calculated on the basis of the weight and molecular weight of the enzyme. A fraction of the impurities calculated on this basis can be accounted for by the moisture content of these preparations, perhaps 5%. The hygroscopic nature of the lyophilized proteins can possibly increase this percentage. Another fraction of the impurities in α-chymotrypsin[106] and trypsin[107] (and probably other proteolytic enzymes) consists of ninhydrin-sensitive materials, which may be identified most easily as degradation products of the enzyme such as amino acids and peptides. These materials can be separated from α-chymotrypsin or trypsin using Sephadex G-25 or G-50. An additional impurity has been separated from α-chymotrypsin using Sephadex G-25 and must be a small molecule since it is eluted from the column considerably after the enzyme and peptide fractions.[106] This material has not yet been identified.

Recently different batches of α-chymotrypsin have been found to give different results in experiments involving proton transfer from an indicator to the imidazole group of the active site of α-chymotrypsin.[106] As a consequence of these findings the gel filtration purification using Spehadex G-25 mentioned above was developed.[106] In order to test the effect of batch difference and Sephadex treatment on both the titration of active sites and the kinetics of enzymatic catalysis, the experiments in Table XIV were carried out. For one batch of α-chymotrypsin which gave disparate results in temperature-jump experiments,[106] Sephadex filtration changed neither the purity of the enzyme preparation, as determined by N-*trans*-cinnamoylimidazole titration, nor the kinetic constants in the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester. Furthermore, two batches of α-chymotrypsin, different in purity, gave identical kinetic constants when their differences in purity were taken into account by determination of the concentration of active enzyme *via* titration. Finally, Sephadex filtration improved the purity of a bovine trypsin preparation, but its kinetic constants remained unchanged. This has been the history of the titration usage so far. For example, the k_{cat}(lim) for the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester in *aqueous* solution carried out by four different workers over a period of 4 years using four different preparations of α-chymotrypsin were: (1) 57 sec^{-1},[108] (2) 55 sec^{-1},[109] (3) 53 sec^{-1},[110] and (4) 50 sec^{-1}.[111,112]

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(106) A. Yapel, M. Han, R. Lumry, and A. Rosenberg, *J. Am. Chem. Soc.*, **88**, 2573 (1966).
(107) S. P. Jindal, unpublished experiments in this laboratory.
(108) G. E. Clement and M. L. Bender, *Biochemistry*, **2**, 836 (1963).
(109) M. L. Bender and G. A. Hamilton, *J. Am. Chem. Soc.*, **84**, 2574 (1962).
(110) Extrapolated from pH 7.9: J. R. Knowles, *Biochem. J.*, **95**, 780 (1965).
(111) M. J. Gibian, unpublished observations in this laboratory using Worthington chymotrypsin CDI 6084-5.
(112) See exception in footnote *e* of Table XIV.