Fluorescent Probes for Conformational States of Proteins. III. The Activation of Chymotrypsinogen*

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ABSTRACT: The conversion of chymotrypsinogen to chymotrypsin has been studied by the use of the fluorescent probe, 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS). The fluorescence intensity of TNS in solutions of chymotrypsinogen increases dramatically when the zymogen is activated by trypsin and the changes in fluorescence are proportional to the formation of active chymotrypsin. Using this phenomenon as an assay, the kinetics of activation have been examined. The initial rates of activation exhibit a maximum at pH 7.4, and are inhibited by increasing ionic strength. The variation in reaction rates with both chymotrypsinogen and trypsin concentrations follows the Michaelis–Menten kinetics.

2-*p*-Toluidinylnaphthalene-6-sulfonate[1] is a fluorescent probe of the hydrophobic areas of protein surfaces (Weber and Laurence, 1954; McClure and Edelman, 1966). A marked enhancement of fluorescence of the probe occurs when it is bound by noncovalent interaction with the protein. TNS has proven useful in the study of biological systems, such as the action of polymyxin on cells of *Pseudomonas aeruginosa* (Newton, 1954). This fluorophore or related compounds have also been used to investigate protein systems, including Bence–Jones protein (Gally and Edelman, 1965), apomyoglobin (Stryer, 1965), and ribonuclease and chymotrypsin (Alexander and Edelman, 1965; McClure and Edelman, 1967).

To extend these studies, we wished to examine the fluorescence of TNS in the presence of a protein undergoing a well-characterized change in conformation. Selection of the conversion of chymotrypsinogen to chymotrypsin was based upon several considerations. The quantum yield of TNS fluorescence in solutions of α-chymotrypsin is larger than the quantum yield observed in solutions of chymotrypsinogen (McClure and Edelman, 1966). Both proteins possess easily assayed biological activities and have been extensively characterized. In addition, the activation of the zymogen has been studied in some detail. The first step of the activation is the scission of one internal peptide bond (Bettelheim and Neurath, 1955; Rovery *et al.*, 1955). Cleavage of this bond is followed by conformational changes that eventually lead to formation of active enzyme, δ-chymotrypsin. The conformational changes that accompany the appearance of enzymic activity are reflected by variations in physical properties of the protein, including the optical rotation at 589 mμ (Neurath *et al.*, 1956) and the absorbance at 280 mμ (Chervenka, 1959).

In the present study, TNS fluorescence in solutions of chymotrypsinogen was measured during the trypsin-catalyzed activation of the zymogen. The fluorescence intensity was proportional to the appearance of chymotrypsin activity. Using this property as an assay, the effects of ionic strength, pH, and concentration of reactants upon the rate of activation have been evaluated. The results demonstrate the usefulness of fluorescent probes in studies of changes in protein conformation.

Materials and Methods

Unless otherwise noted, the procedures and experimental conditions used were described by McClure and Edelman (1967). δ-Chymotrypsin (lot COD 6032) and trypsin (lot TRL 6242, three times recrystallized, lyophilized, and salt free) were obtained from the Worthington Biochemical Corp., Freehold, N. J., and used without further purification. Stock solutions of trypsin (about 10^{-4} M) were prepared in 10^{-3} M HCl. Concentrations of trypsin were determined from absorbance measurements at 280 mμ, using an extinction coefficient of 1.44 (1 mg/ml, 1-cm light path) and a molecular weight of 23,800 (Davie and Neurath, 1955). Chymotrypsin activity was measured using ATEE as a substrate following the procedure of Schwert and Takenaka (1955). Only initial rates of formation of chymotrypsin were measured; therefore, Ca^{2+} was omitted from the reaction mixtures (Chervenka, 1959).

Theory

The mechanism of Figure 1 describes the activation of chymotrypsinogen by trypsin in the presence of TNS. In this figure, F represents the free fluorophore, E represents free trypsin, and the other symbols used

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[1] Abbreviations used: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ATEE, acetyl-L-tyrosine ethyl ester.

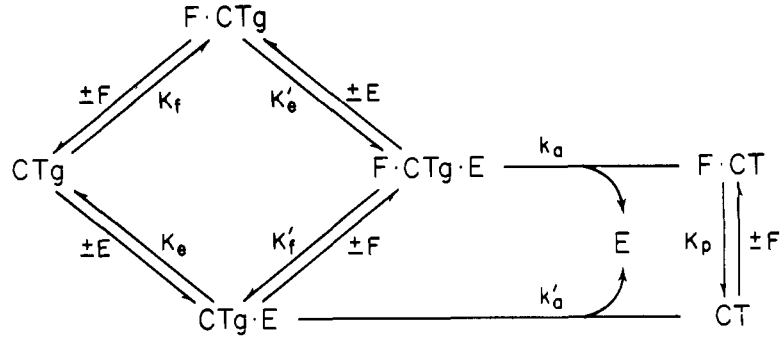

FIGURE 1: The mechanism of activation of chymotrypsinogen by trypsin in the presence of TNS. For details see Theory.

refer to complexes containing chymotrypsin (CT) and chymotrypsinogen (CTg). The various K values are defined as dissociation constants, and the rate at which the equilibria are established is assumed to be fast with respect to the rate of formation of products.

If $F_0 \gg S_0 \gg E_0$

$$\begin{aligned} F_0 &= [\mathrm{F}] \\ S_0 &= S_t + P_t \\ S_t &= [\mathrm{CTg}] + [\mathrm{F{\cdot}CTg}] \\ P_t &= [\mathrm{CT}] + [\mathrm{F{\cdot}CT}] \end{aligned} \tag{1}$$

where the subscript zero refers to the total concentration of the species and S_t and P_t are defined in eq 1.

If the intensity of fluorescence (I) is due entirely to fluorophore bound to protein

$$I = \psi_f[\mathrm{F{\cdot}CTg}] + \psi_p[\mathrm{F{\cdot}CT}] \tag{2}$$

where ψ_f and ψ_p are proportionality constants relating I and the appropriate complex.

Using the equilibria of Figure 1, eq 1 and 2 may be solved to yield

$$\Delta I = \left[\psi_p \frac{F_0}{F_0 + K_p} - \psi_f \frac{F_0}{F_0 + K_f}\right] P_t \tag{3}$$

in which ΔI is the increase in fluorescence intensity above the zero time value.

The reaction velocity (v) is measured by the enzymic activity of the product after a several hundredfold dilution; therefore, $v = dP_t/dt$. Furthermore, all the terms in the bracket of eq 3 are independent of the time of observation; hence

$$\frac{d\Delta I}{dt} = \frac{dI}{dt} = \left[\psi_p \frac{F_0}{F_0 + K_p} - \psi_f \frac{F_0}{F_0 + K_f}\right] v \tag{4}$$

Under these conditions ΔI should be proportional to the extent of the reaction. More important, the rate of change of the fluorescence intensity should be proportional to the reaction velocity. Although no restriction has been placed upon the relative values of F_0 and K_f or K_p, it should be recognized that the relationship between ΔI and P_t (or $d\Delta I/dt$ and v) depends upon F_0 unless $F_0 \gg K_f$ and $F_0 \gg K_p$. If F_0 is maintained constant over a series of experiments, directly comparable data will be obtained in any case.

If $F_0 \simeq S_0$, it can be shown that the fluorescence intensity and P_t will no longer be proportional unless $K_f = K_p = K$. If these two constants happen to be equal, however, and we assume $F_0 \simeq S_0 \gg E_0$, we can treat the system in the following manner. From the equilibria defined by K_f and K_p and the conservation of mass, $[\mathrm{F{\cdot}CTg}]/[\mathrm{F{\cdot}CT}] = S_t/P_t$. Introducing this result into the expression $F_0 = [\mathrm{F}] + [\mathrm{F{\cdot}CTg}] + [\mathrm{F{\cdot}CT}]$ and simplifying, we find

$$[\mathrm{F{\cdot}CTg}] = \left(\frac{F_0}{F_0 + S_0 + K}\right) S_t + R_f \tag{5}$$

in which R_f is the residual of a binomial series. In this case, as in a previous use of this technique (McClure and Edelman, 1967), the residuals are vanishingly small and their explicit form is immaterial.

[F·CT] may be obtained in a similar fashion and introduced together with eq 5 into eq 2. After simplifying, we find

$$\Delta I = \left[(\psi_p - \psi_f) \frac{F_0}{F_0 + S_0 + K}\right] P_t + \psi_f R_f + \psi_p R_p \tag{6}$$

Since the residuals are negligible, the change in fluorescence intensity will be proportional to the appearance of products. Furthermore, the terms in brackets in eq 6 are independent of the time of observation and we may write

$$\frac{d\Delta I}{dt} = \frac{dI}{dt} = \left[(\psi_p - \psi_f) \frac{F_0}{F_0 + S_0 + R}\right] v \tag{7}$$

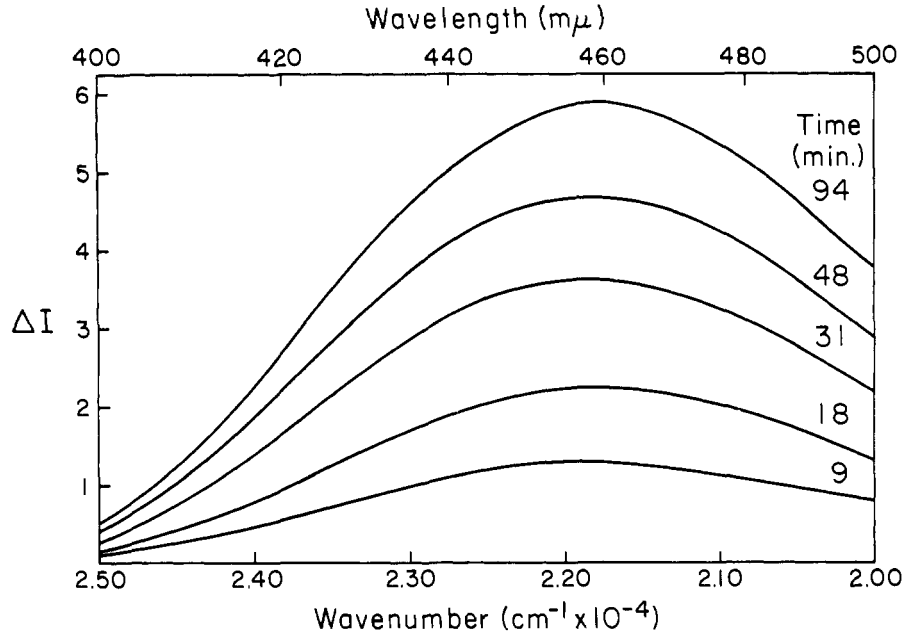

FIGURE 2: Fluorescence spectra of TNS obtained during activation of chymotrypsinogen by trypsin. Portions of the spectra at wavelengths longer than 500 mμ were omitted. ΔI, increase in fluorescence intensity. The numbers above each spectrum represent the time in minutes after beginning the reaction. pH 7.6 in 0.1 M KPO_4, 25.0°. Concentrations: chymotrypsinogen, 8.8×10^{-5} M; trypsin, 4.4×10^{-7} M; TNS, 1.2×10^{-5} M. Exciting wavelength, 366 mμ.

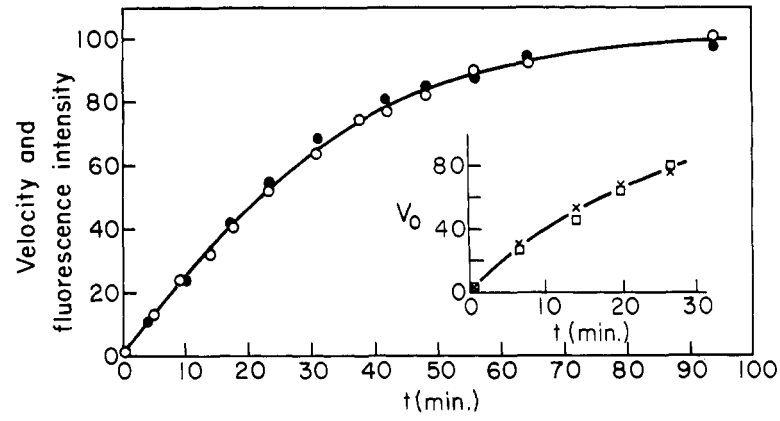

FIGURE 3: The increase in TNS fluorescence (○) and ATEEase activity (●) in the activation of chymotrypsinogen by trypsin in the presence of TNS (main figure) and the activation of chymotrypsinogen in the presence and absence of TNS (inset). In the inset the data represent two activation mixtures, one lacking TNS (□) and one containing 2.9×10^{-5} M TNS (×). V_0, initial velocity of ATEE hydrolysis, expressed as the percentage of maximal activity. Conditions as given in Figure 2.

Therefore, the rate of change of the fluorescence intensity will be proportional to the reaction velocity in this case, independent of the relative values of F_0 and S_0.

Results

The fluorescence intensity of TNS dissolved in solutions of chymotrypsin is much greater than that observed in solutions of chymotrypsinogen (McClure and Edelman, 1966). When solutions of chymotrypsinogen containing TNS are activated by the addition of trypsin ("fast" activation; Jacobsen, 1947), the fluorescence intensity of TNS increases (Figure 2). There was very little change in the shape or wavelength maximum of the emission spectrum during activation, even though the intensity of fluorescence increased nearly 20-fold. As shown in the main portion of Figure 3, the increase in fluorescence intensity parallels the formation of chymotrypsin activity. Using ATEE as an assay sub-

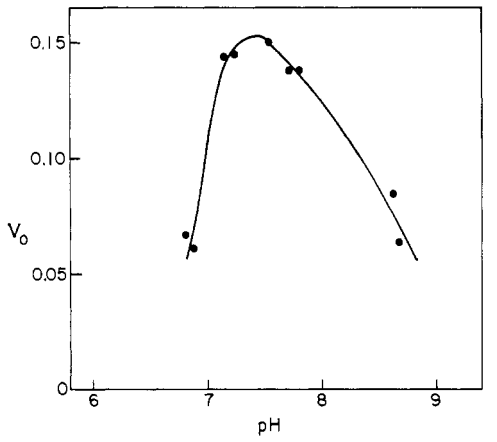

FIGURE 4: The effect of pH on the initial rate of the trypsin-catalyzed activation of chymotrypsinogen. Data were obtained by measuring the increase in fluorescence intensity of TNS in the reaction mixtures. Concentrations: chymotrypsinogen, 4.0×10^{-5} M; trypsin, 5.4×10^{-7} M; TNS, 1.5×10^{-3} M. The reactions were carried out in unbuffered aqueous solutions adjusted to the desired pH with NaOH. The pH (glass electrode) changed less than 0.01 unit during measurement of the rates. Temperature, 25.0°; exciting wavelength, 366 mμ; analyzing wavelength, 460 mμ.

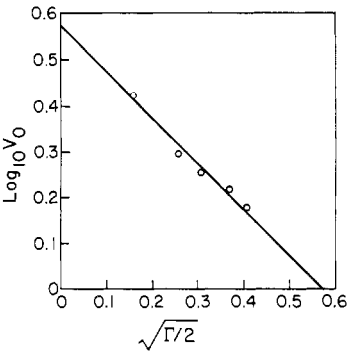

FIGURE 5: The effect of ionic strength upon the initial rate of the trypsin-catalyzed activation of chymotrypsinogen. Data were obtained by measuring the increase in fluorescence intensity of TNS in the reaction mixtures. Concentrations: chymotrypsinogen, 5.4×10^{-5} M; trypsin, 5.5×10^{-7} M; TNS, 1.1×10^{-3} M. pH 7.6 in 0.02 M KPO_4, 25.0°. NaCl was used as the inert electrolyte. Exciting wavelength, 366 mμ; analyzing wavelength, 460 mμ. V_0, initial velocity in arbitrary units. Γ/2, ionic strength in moles per liter.

strate, the specific activity of the activation mixture after 95 min was equal to the specific activity of commercial δ-chymotrypsin. Apparently the fluorescence of TNS in chymotrypsinogen solutions changes in response to variations in protein conformation accompanying the activation process.

TNS has no effect upon the rate of activation of chymotrypsinogen by trypsin. The appearance of ATEEase activity was measured in two duplicate activation mixtures, only one of which contained TNS (Figure 3, inset). No effect of TNS could be demonstrated at the concentrations used in this experiment. Similar experiments showed that TNS had no effect on the rate of activation at concentrations below 10^{-3} M. The results given in Figures 2 and 3 were obtained using approximately equimolar concentrations of TNS and chymotrypsinogen. Under these conditions (*cf.* Theory), the increases in fluorescence intensity and chymotrypsin activity will be proportional only if TNS is bound with approximately equal affinity to chymotrypsin and chymotrypsinogen. TNS is bound to α-chymotrypsin about twice as firmly as it is bound to chymotrypsinogen (McClure and Edelman, 1967). In order to render negligible any variations in affinity of TNS for δ-chymotrypsin and chymotrypsinogen, the remaining activation studies were carried out at TNS concentrations (about 10^{-3} M) which were much larger than the chymotrypsinogen concentrations employed (about 10^{-5} M). In this case, the intensity of fluorescence must parallel the appearance of chymotrypsin regardless of the binding constants involved (*cf.* Theory).

The kinetics of the trypsin-catalyzed conversion of chymotrypsinogen to chymotrypsin have been studied using the fluorescence of TNS as an assay system under the conditions discussed above. The dependence of the initial rates upon the pH of the reaction mixture is presented in Figure 4. A maximum in the pH–activity profile occurred near pH 7.4.

Sodium chloride incorporated in activation mixtures diminished the initial rates. These data are plotted in Figure 5, using equations developed in Laidler (1958). A line with a slope of -1.00 ± 0.04 was obtained.

Data presented in Figure 6 give the dependence of the initial velocity of activation upon the concentrations of reactants. It was found that the reaction rates were first order in trypsin concentration. In contrast, when the chymotrypsinogen concentration was varied, the reaction deviated from first-order kinetics at concentrations greater than 8.0×10^{-5} M (2 mg/ml). The data at concentrations above this value were plotted according to Eadie (1942) and gave a straight line (Figure 7), implying that the data fit the Michaelis–Menten equation (Michaelis and Menten, 1913). The observed value of K_m was 5.6×10^{-4} M (13.9 mg/ml).

Discussion

The appearance of enzymic activity during the trypsin-catalyzed conversion of chymotrypsinogen to chymotrypsin is accompanied by changes in the physical characteristics of the enzyme. Neurath *et al.* (1956) found that the rate of formation of activity was equal to the rate of change of optical rotation at 589 mμ. Chervenka (1959) obtained similar data relating the activation process and a change in absorbance at 280

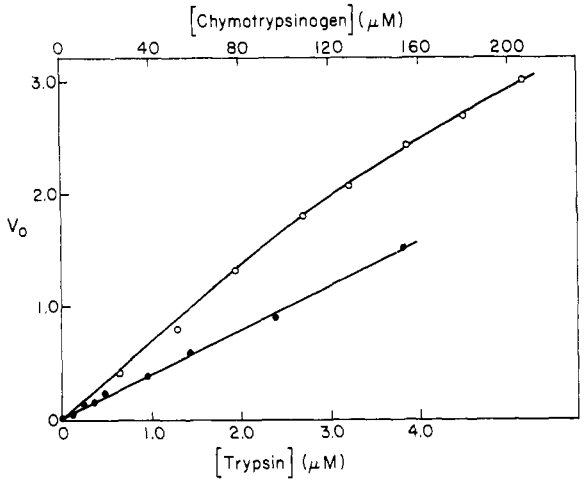

FIGURE 6: The effect of reactant concentrations upon the initial rate of the trypsin-catalyzed activation of chymotrypsinogen. Data were obtained by measuring the increase in fluorescence intensity of TNS in the reaction mixtures. ○, variation of chymotrypsinogen concentration at concentrations of: trypsin, 2.9×10^{-7} M; TNS, 1.0×10^{-3} M. ●, variation of trypsin concentration at concentrations of: chymotrypsinogen, 4.0×10^{-5} M; TNS, 1.0×10^{-3} M. pH 7.6 in 0.02 M KPO_4, 25.0°. Exciting wavelength, 366 mμ; analyzing wavelength, 460 mμ. V_0, initial velocity in arbitrary units. The velocities in the two experiments are not directly comparable.

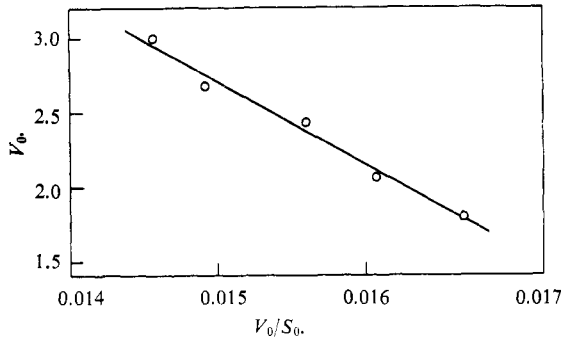

FIGURE 7: The effect of substrate concentration upon the initial rate of the trypsin-catalyzed activation of chymotrypsinogen. Data of Figure 6 plotted according to Eadie (1942).

mμ. Both experiments suggest that conformational changes in the zymogen accompany the formation of active enzyme. Furthermore, several studies have demonstrated differences in the conformation of isolated chymotrypsinogen and chymotrypsin (Vaslow and Doherty, 1953; Neurath *et al.*, 1956; Massey *et al.*, 1955; Imahori *et al.*, 1960; Raval and Schellman, 1965; Biltonen *et al.*, 1965; Deranleau and Neurath, 1966).

The present studies indicate that the fluorescence of TNS bound to chymotrypsinogen is a particularly convenient and accurate measure of the changes in conformation accompanying activation. The increase in the fluorescence intensity of TNS is accurately proportional to the appearance of chymotrypsin activity. The extent of rearrangement in protein structure required to produce the changes in TNS fluorescence is not known. Very small changes would suffice, if they effectively alter the local environment of the fluorophore. Preliminary studies indicate that fluorescent probes may be used to follow the activation of other zymogens, such as pepsinogen.

TNS has no effect on the rates of the trypsin-catalyzed activation of chymotrypsinogen. In conjunction with previous data (Bettelheim and Neurath, 1955), this implies that chymotrypsinogen possesses three sites capable of binding other molecules: the preformed binding site for substrates, which becomes part of the active site upon conversion to chymotrypsin: a site to which trypsin is bound during the process of activation; and a site of unknown function which binds TNS (McClure and Edelman, 1967).

In carrying out a study of the activation of chymotrypsinogen in the presence of TNS, the choice of reactant and probe concentrations is extremely important (*cf.* Theory). If the concentrations of TNS and protein are about equal, the interconversion of two forms of the protein will, in general, result in a redistribution of bound and free forms of TNS. This redistribution occurs when the two proteins bind TNS with different affinities, and will become negligible only if the two proteins happen to bind the probe with equal affinity. If any appreciable redistribution occurs, the fluorescence intensity and the appearance of product will not be proportional.

Conditions can be obtained which overcome this difficulty. If the concentration of TNS is raised until it is much greater than the total concentration of protein, the fluorescence intensity and the extent of the reaction will again be proportional. The proportionality is independent of the magnitude of, and the differences in, the binding constants (*cf.* Theory). In order to assure proportionality, the data to be discussed below were obtained using concentrations of TNS which were large with respect to the total chymotrypsinogen concentrations.[2]

Activation of chymotrypsinogen by trypsin is markedly affected by varying the pH of the solution. The rate of zymogen activation possesses a maximum at slightly less than pH 7.5 and decreases at higher and lower pH values. Similar pH–activity profiles have been obtained in several other trypsin-catalyzed reactions (Bergmann *et al.*, 1939). This suggests that the same ionized groups on trypsin may be involved in the hydrolysis of small substrates and the activation of

[2] It should also be recognized that a proportionality between the fluorescence intensity and the appearance of product will be assured if the concentration of TNS is much less than either K_f or K_p, irrespective of the values of the constants.

chymotrypsinogen.

In agreement with earlier results of Butler (1941), initial rates of activation are reduced by the presence of sodium chloride. The decrease in initial rate can be related to the ionic strength as described in Laidler (1958). The slope of the line in Figure 5 is approximately equal to $z_a z_b$, where z_a and z_b represent the charges brought into juxtaposition during the formation of an activated complex from two reactants, a and b. At the low concentrations of chymotrypsinogen used in this experiment, the reaction was second order; therefore, the two species corresponding to a and b must be chymotrypsinogen and trypsin. The observed value of $z_a z_b(-1.00)$ is consistent with the presence of a positive charge in the binding region of one of the reactants and a negative charge in the binding region of the second. This observation may be related to the presence of a guanidinium ion at the site of cleavage in chymotrypsinogen (Dreyer and Neurath, 1955; Rovery *et al.*, 1955), and a negative charge which must exist in the active site of trypsin.

The activation of chymotrypsinogen followed Michaelis–Menten kinetics. The reaction rates were directly proportional to the concentration of trypsin. As shown by Kunitz and Northrop (1935), the rate of reaction is first order up to substrate concentrations of about 8×10^{-5} M (2 mg/ml). At the concentrations of chymotrypsinogen used in our experiments, the observed rates deviated from a first-order reaction in a manner which agrees with the Michaelis–Menten formulation. A least-squares analysis of the Eadie plot used to obtain the kinetic parameters indicated that $K_m = 5.56 \pm 0.20 \times 10^{-4}$ M (13.9 ± 0.5 mg/ml). It should be pointed out, however, that the substrate concentrations we have used ($\lesssim 2 \times 10^{-4}$ M) are considerably less than this value, and for this reason we feel that the estimate of K_m is only provisional.

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