Alpha-chymotrypsin is one of many crystalline enzymes whose properties can be investigated on a molecular basis. Its physiological role is the catalysis of the hydrolysis of protein; thus it is a catalyst for the cleavage of peptide or amide bonds. In addition to the cleavage of polymeric amides, it also catalyzes the cleavage of amides of individual amino acids \( (1) \). Furthermore, it catalyzes the hydrolysis of all other carboxylic acid derivatives, in addition to that of amides. For experimental reasons, the hydrolysis of esters is the most convenient reaction for the investigation of \( \alpha \)-chymotrypsin catalysis.

\( \alpha \)-Chymotrypsin is a protein of molecular weight 24,800 \( (2) \) which has one “active site” per molecule, defined by the fact that it reacts stoichiometrically with the nerve gas, diisopropylphosphorofluoridate, to give a monophosphorylated enzyme which is completely inactive \( (3) \). The phosphorus atom of diisopropylphosphorylchymotrypsin was found to be bonded to the hydroxyl group of a serine amino acid of the enzyme by hydrolysis of the phosphoryl derivative and identification of serine phosphate \( (4) \). This particular serine was later identified as amino acid 195 in the sequence of 246 amino acids \( (5) \). On the basis of this stoichiometric reaction, \( \alpha \)-chymotrypsin has been called a serine proteinase \( (6) \). Other members of this family of enzymes include trypsin, elastase, thrombin, plasmin, and subtilisin. Of this family of related enzymes, \( \alpha \)-chymotrypsin is the enzyme most carefully studied because of the ease of its isolation and purification and the straightforwardness of its study.

In common with essentially all enzymes, the reactions catalyzed by \( \alpha \)-chymotrypsin are characterized by a saturation phenomenon. That is, when a plot is made of reaction velocity versus substrate concentration, as shown in Figure 1, linear behavior is observed at low substrate concentrations; but at very high substrate concentrations, the velocity eventually becomes independent of the substrate concentration. This phenomenon may be easily reconciled with a situation involving a very low concentration of enzyme (catalyst), a very high concentration of substrate, and the requirement that the enzyme bind the substrate before the catalytic reaction takes place. This situation is defined by eqn. (1), where:

\[
E + S \rightleftharpoons ES \rightarrow E + P \quad (1)
\]

\( E \) is enzyme, \( S \) is substrate, \( K_m \) is the (dissociation) constant of binding, and \( k_{cat} \) is the catalytic rate constant.

When \([S]_0\) is greater than \([E]_0\), it may be readily shown that:

\[
V = \frac{V_{max}[E]_0[S]_0}{[S]_0 + K_m} \quad (2)
\]

where \( V \) is the velocity and \( V_{max} \) the maximal velocity of the reaction graphed in Figure 1. Equation (2), usually described as Michaelis-Menten kinetics \( (7) \) based on some original observations of Henri, \( (8) \) adequately describes the behavior in Figure 1.

Spectrophotometric methods have played a large role in the elucidation of the kinetics of \( \alpha \)-chymotrypsin catalyses. In particular, chromophoric substrates have been of great importance since they permit the observation of low concentrations of substrate, and thus permit the observation of corresponding low enzyme concentrations in stoichiometric reactions between enzyme and substrate. One of the first such chromophoric substrates of \( \alpha \)-chymotrypsin was \( p \)-nitrophenyl acetate \( (9) \). \( \alpha \)-Chymotrypsin was shown to catalyze the hydrolysis of this substrate, but the reaction proceeded in a very peculiar fashion \( (10) \). When the reaction was carried out using a much greater substrate than enzyme concentration, the reaction, as monitored by the appearance of \( p \)-nitrophenol, appeared to be biphasic. Initially a concentration of \( p \)-nitrophenol approaching the enzyme concentration was rapidly liberated, followed by a slow (zero-order) release of \( p \)-nitrophenol, \( P_1 \), as shown schematically in Figure 2. This behavior implied two rate steps in the reaction, one of which was controlled by a stoichiometric process between substrate and enzyme. Thus, two distinctly different rate...
The compound was acetyl-α-chymotrypsin, in which the acetyl group was bound covalently to the serine of the active site. This hypothesis was confirmed when radioactive p-nitrophenol acetate was used. The enzyme-substrate compound isolated from this reaction was radioactive; furthermore, on degradation of this enzyme derivative, the radioactivity was found associated with the same serine 195 as in the reaction with diisopropylphosphorofluoridate (12). Thus, eqn. (3) may be described chemically as:

\[
\text{En-OH} + \text{RCOR'} \rightleftharpoons (\text{EnOHR} - \text{RCOR'})
\]

Using a stopped-flow spectrophotometric technique that permitted observations on a millisecond time scale, it was found that the reaction in Figure 2 could more accurately be described as shown in Figure 3 (13). That is, the initial “burst” of p-nitrophenol was not infinitely fast but was, in fact, measurable on a faster time scale. The α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl trimethylacetate was shown to exhibit the same characteristics as the hydrolysis of p-nitrophenyl acetate. However, since the former ester is sterically hindered by the bulky trimethylacetate group, all of its reactions are slowed down, and the behavior shown in Figure 3 is seen on a “seconds” time scale (14). In fact, the rate constants of its reaction are so small that it was possible by careful technique to crystalize the compound trimethylacetyl-α-chymotrypsin at low pH (15). Thus, the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl trimethylacetate is a particularly convenient reaction to use to investigate chymotrypsin reactions.

**Theory of Kinetics:**

The observations from Figure 3 allow the calculation of the following quantities: (1) the absolute concentration of enzyme in the solution; (2) the binding constant \( K_S \); (3) the rate constant of acylation of the enzyme, \( k_2 \), and (4) the rate constant of deacylation of the acyl-enzyme, \( k_3 \). It is the object of this experiment to determine some of these quantities in the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl trimethylacetate.

A complete kinetic description of reactions following eqns. 3 and 4 has been given (13, 16, 17). The following derivation is based on the work of Kézdy and Bender (17).

The following conservation equation and differential equations follow from eqn. (3), assuming \([S]_0 \gg [E]_0\).

\[
[E]_0 = [E] + [ES] + [ES']
\]

\[
d[P_1]/dt = k_2[ES]
\]

\[
d[P_2]/dt = k_3[ES']
\]

\[
d[ES']/dt = k_1[ES'] - k_2[ES]
\]

Since we have five unknowns and only four equations, we need one further equation. The simplest fifth equation is produced if we assume that the primary adsorptive time, \( E + S \rightleftharpoons ES \), is a fast pre-equilibrium:

\[
K_S = \frac{[E][S]}{[ES]}
\]
Since \([S] \equiv [S]_0\), eqn. (9) may be transformed to:
\[
(E) = [ES]K_s/[S]_0
\]
(10)

Using eqn. (10), eqn. (5) may be transformed to:
\[
[ES] = \frac{[E]_0 - [ES']}{1 + K_s/[S]_0}
\]
(11)

Then eqn. (8) may be transformed to:
\[
d[ES']/dt = \frac{k_3[E]_0}{K_s} - \frac{k_2[ES']}{1 + K_s/[S]_0} - k_2[ES']
\]
(12)

or
\[
=k_2[ES']_0 - \left(\frac{k_3}{1 + K_s/[S]_0}\right)[ES']
\]

For a given experiment, the first term and the coefficient of the second term are constants, and thus eqn. (12) may be transformed to eqn. (13) where
\[
a = \frac{k_2[E]_0}{1 + (K_s/[S]_0)}
\]
and
\[
b = k_3 + \frac{k_2}{1 + (K_s/[S]_0)}
\]

Equation (13) is integrable, as shown in eqn. (14).
\[
-\frac{1}{b} \ln \left((a - b[ES'])\right) = -t + c
\]
(14)

When \(t = 0\) and \([ES'] = 0\), \(c = (1/b) \ln a\). Substitution of this value of \(c\) into eqn. (14) gives:
\[
b' = \ln \frac{a}{a - b[ES']}
\]
(15)

or
\[
[ES'] = \frac{a}{b} (1 - e^{-at})
\]
(16)

Substituting eqn. (16) into eqn. (11) gives:
\[
[ES] = \frac{[E]_0 - (a/b)(1 - e^{-at})}{1 + K_s/[S]_0}
\]
(17)

Then substituting eqn. (17) into eqn. (6) gives:
\[
d[P_1]/dt = \frac{k_3[E]_0 - k_2[a/b](1 - e^{-at})}{1 + K_s/[S]_0}
\]
(18)

Equation (18) may be integrated to yield an equation which predicts the observable curve in Figure 3, \([P_1]\) versus \(t\):
\[
[P_1] = \frac{k_3[E]_0 - k_2[a/b]}{1 + K_s/[S]_0} (1 - e^{-at})
\]
(19)

Equation (19) has the form of:
\[
[P_1] = At + B(1 - e^{-at})
\]
(20)

which is seen to be the form of the curve in Figure 3. From eqn. (20), the following relations may be seen. At \(t\) approaching infinity, \([P_1] = At + B\), a straight line relationship. On the other hand, at very low \(t\), \([P_1] = At + B = Be^{-bt}\). Thus the extrapolated straight line from large \(t\), \([P_1] = At + B = Be^{-bt}\), will yield \(Be^{-bt}\). In practice all that need be done to obtain \(b\) is to subtract the experimental \([P_1]\) curve from the extrapolated straight line and plot the differences according to a first-order equation.

Thus, we have as primary experimental observables the slope and intercept of the straight line portion of Figure 3-A and \(B\) respectively—and the first-order rate constant, \(b\), of the initial exponential part of the curve. What can we do with these values?

Let us consider \(A\) first. Substituting values of \(a\) and \(b\) (eqns. (12) and (13)) into the value of \(A\) (eqn. (19)) gives
\[
A = \frac{k_3k_2}{k_3 + k_2} [E]_0 [S]_0
\]
(21)

Equation (21) has the form of an expression for the rate of a reaction conforming to eqn. (2)—that is, Michaelis-Menten kinetics. This expression can be transformed to
\[
A = \frac{k_3k_2}{K_m(apparent) + [E]_0}\]
(22)

by using the transformations \(k_{cat} = k_3k_2/(k_2 + k_3)\) and \(K_m\) (apparent) = \(K_sK_3/(k_2 + k_3)\) which follow directly from the relationship between eqns. (21) and (22). The reciprocal of eqn. (22) gives:
\[
\frac{1}{A} = \frac{1}{k_3k_2} \left(\frac{[E]_0}{[S]_0}\right) + \frac{K_m(apparent)}{k_3k_2 [E]_0 [S]_0}
\]
(23)

Thus by determining \(A\) at several \([S]_0\)'s, and by plotting \(1/A\) versus \(1/[S]_0\), \(k_{cat}\) \([E]_0\) and \(K_m\) (apparent) can be determined from the intercept and slope/intercept ratio respectively. Alternatively from eqn. (22), if \([S]_0 >> K_m\) (apparent), then \(A = k_{cat}\) \([E]_0\).

The value of \(B\) gives a direct measure of \([E]_0\). From eqns. (19) and (12):
\[
B = \frac{(k_2 + k_3)}{[S]_0 (1 + K_m(apparent)+ k_{cat})}
\]
(24)

Thus if \(k_3 \ll k_2\) and \(K_m\) (apparent) \(\ll [S]_0\), both of which conditions are usually met in the \(\alpha\)-chymotrypsin-catalyzed hydrolysis of \(p\)-nitrophenyl trimethylacetate, \(B = [E]_0\). Thus the intercept \(B\) is a direct measure of the stoichiometric, active enzyme concentration. In the general case, eqn. (24) can be transformed to an inverted form:
\[
\frac{1}{\sqrt{B}} = \frac{k_3 + k_3}{k_2} \cdot \frac{1}{\sqrt{[E]_0}} + \frac{K_m(apparent)}{k_3k_2 [E]_0}
\]
(25)

Then by determining \(B\) at several \([S]_0\)'s, \([E]_0\) \(k_2/(k_2 + k_3)^2\) can be determined from the intercept of a plot of \(1/\sqrt{B}\) versus \(1/[S]_0\). If \(k_2 \gg k_3\), this determination will also lead to \([E]_0\).

According to eqn. (12), the constant \(b\) is given as follows:
\[
b = \frac{(k_3 + k_2)[S]_0 + k_3K_s}{K_s + [S]_0}
\]
(26)

In the \(\alpha\)-chymotrypsin-catalyzed hydrolysis of \(p\)-nitrophenyl trimethylacetate, \(k_3 K_s \ll (k_2 + k_3) [S]_0\) (see table) and thus:
\[
b \approx \frac{(k_3 + k_2)[S]_0}{[S]_0 + K_s}
\]
(27)
When eqn. (27) is transformed to reciprocal form:

$$\frac{1}{b} = \frac{1}{k_e + k_a} + \frac{K_s}{k_e + k_a} \frac{1}{[S]_0}$$  \(\text{(28)}\)

\((k_e + k_a)\) and \(K_s\) may be determined by measuring \(b\) at several \([S]_0\)/s, from the intercept and slope-intercept of a plot of \(1/b\) versus \(1/[S]_0\).

At pH 8.2, the values of the various constants for the \(\alpha\)-chymotrypsin-catalyzed hydrolysis of \(p\)-nitrophenyl trimethylacetate are those shown in the table (18).

### Kinetic Constants of the \(\alpha\)-Chymotrypsin-Catalyzed Hydrolyses of \(p\)-Nitrophenyl Trimethylacetate at pH 8.2* (18)

<table>
<thead>
<tr>
<th></th>
<th>(k_s)</th>
<th>(k_e)</th>
<th>(K_s)</th>
<th>(k_{cat}) (apparent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.37 ± 0.11 sec(^{-1})</td>
<td>1.3 ± 0.03 (\times 10^{11}) sec(^{-1})</td>
<td>1.6 ± 0.5 (\times 10^{-4})mol(^{-1})sec(^{-1})</td>
<td>1.3 (\times 10^{-5}) sec(^{-1})</td>
</tr>
</tbody>
</table>

* 0.01 M tris-HCl buffer, ionic strength 0.06, 25.6 ± 0.1°C, 18% (v/v) acetonitrile-water.

It can be seen that \(k_2/(k_2 + k_3)\) is nearly unity and at \(10^{-4}\) M \([S]_0\), the ratio of \(K_m\) (approx.)/\([S]_0\) will be vanishingly small; thus from the equation (24) the intercept \(B\) of Figure 3 will be equal to the enzyme concentration within a few percent. By comparing the value of the observed enzyme concentration to that calculated on the basis of the amount of enzyme weighed out and a molecular weight of 24,800, it is possible to calculate the purity of the enzyme preparation.

Ordinarily samples of \(\alpha\)-chymotrypsin crystallized three times have a purity of 75-90%, a good part of the difference being due to water of hydration.

From the table the ratio \(k_2/k_3\) is seen to be very large, approximately 3000. Thus, \(k_{cat} = k_3k_2/(k_2 + k_3)\) is essentially equal to \(k_3\). Furthermore, at a substrate concentration of approximately \(10^{-4}\) M, it may be seen from the table that \([S]_0 \gg K_m\) (apparent) and thus from eqn. (22), the slope, \(A\), of the zero-order portion of Figure 3, will be directly equal to \(k_{cat}[E]_0\). Since \([E]_0\) may be determined independently from the intercept, \(B\), \(k_{cat}\) may be determined. Finally since \(k_{cat} = k_3\), the value of the latter is also known.

Since \(k_3\) is much greater than \(k_2\), eqn. (27) may be simplified to eqn. (29). Equation (29) indicated that \(b = k_2\) only when \([S]_0 \gg K_s\). However, because of solubility difficulties which limit the concentration of \([S]_0\), to \(1.5 \times 10^{-4}\) M, it is not possible for \([S]_0\) even to approach \(K_s\). Under these conditions, one cannot utilize eqn. (28) for the calculation of \(k_2\) without incurring huge experimental errors; thus under these conditions, eqn. (29) reduces to \(b = k_2[S]_0/K_s\). This is the observable quantity that may be calculated from the presteady state of this reaction.

### The Experiment:

**Problems.** For the \(\alpha\)-chymotrypsin-catalyzed hydrolysis of \(p\)-nitrophenyl trimethylacetate determine the values of \(A\), \(B\), and \(b\). From these values determine \([E]_0\) and the purity of the enzyme, and determine the values of the kinetic constants, \(k_2[S]_0/K_s\), \(k_3\), and \(k_{cat}\) at pH 8.5. (Extra credit): Determine the effect of \(pH\) on \(k_2[S]_0/K_s\) and \(k_3\), and determine the effect of substrate and enzyme concentrations on the rate constants and the initial burst.

**Apparatus.** Any spectrophotometer operative at 400 mp may be used. The cell compartment must be thermostatted. A timer is necessary if the spectrophotometer is not a recording device. A thermostatted water bath is helpful for preequilibration of solutions, but is not necessary. The following pipets are useful: 3.0 ml, 10, 25, 50, and 100 ml. A glass stirring rod with a flattened tip that will hold 50 ml of solution and be able to introduce this into the spectrophotometric cuvet and stir the mixture may be made by heating the end of soft glass rod to a fluid bead and then pressing the end against a flat stone surface, giving a circular flattened end twice the diameter of the original rod.

**Reagents.** A pH 8.5 tris(hydroxymethyl)-aminoethane buffer (0.01 M) is suggested since at pH 8.5 \(p\)-nitrophenol is more completely ionized than at pH 8.2, while the enzymatic rates are almost constant in this region.

Weigh 50 mg of \(\alpha\)-chymotrypsin (2.0 mmole) (Worthington Biochemical Corp., Freehold, N. J. — thrice crystallized) and dissolve in 1.0 ml of a pH 4.6 acetate buffer. In this acidic solution and in the cold, \(\alpha\)-chymotrypsin is stable for a considerable period of time (usually as long as 1 week with not more than a few percent decomposition).

Weigh out 7.8 mg (3.5 mmole) of \(p\)-nitrophenyl trimethylacetate (M.W. 223) (Aldrich Chemical Co., Milwaukee, Wis.) and dissolve in 10.0 ml of pure acetonitrile (preferably distilled from PO\(_4\) to remove all H\(_2\)O). The ester may be prepared from trimethylacetyl chloride and \(p\)-nitrophenol in pyridine solution. Recrystallization from 95% ethanol gives needles, m.p. 94-95° (14).

**Determination of the Extinction Coefficient of \(p\)-Nitrophenol.** Determine the extinction coefficient of \(p\)-nitrophenoxide ion at 400 mp (since the experiments are being carried out at pH 8.5 and since the \(pK_a\) of \(p\)-nitrophenol is 7.0, all the \(p\)-nitrophenol will be in the form of the anion) by dissolving an aliquot of the stock ester solution in acetonitrile (50 \(\mu\)l) in 3.0 ml of 0.1 M sodium hydroxide and determining the absorbance. Check to see if Beer’s law is obeyed.2 The extinction coefficient of \(p\)-nitrophenol ion at 400 mp is about \(1.8 \times 10^{-4}\) M\(^{-1}\) cm\(^{-1}\).

**Observation of the Enzymatic Reaction.** Three ml of the buffer solution is placed in a cuvet in the thermostatted cell compartment of the spectrophotometer and the instrument is balanced. Then 10-100 \(\mu\)l of the acetonitrile solution of \(p\)-nitrophenyl trimethylacetate is added (100 \(\mu\)l corresponds approximately to \([S]_0 = 1.1 \times 10^{-4}\) M).3 Both the absolute absorbance

2 A more foolproof method of obtaining the extinction coefficient is to dissolve an aliquot of the ester solution in the buffer to be utilized (but time must be taken to be sure that the ester has completely hydrolyzed). If the enzymatic reaction is to be carried out at a lower \(pH\), be sure to take into account the fact that the extinction coefficient of \(p\)-nitrophenol is dependent on its \(pK_a\), that is, it is an indicator.

3 The limit of solubility of the substrate in the buffer solution is approximately \(1.1 \times 10^{-4}\) M—be aware of spectral artifacts due to precipitation.
of this solution (to detect any impurity) and the spontaneous hydrolysis of the substrate are then measured spectrophotometrically, over the length of time expected for the reaction course. Although the (linear) spontaneous hydrolysis is small, it must be subtracted from the observed \([\text{P}]\) at a given time, before any calculations are made. Then 10–50 \(\mu\)l of the enzyme stock solution is introduced, with stirring for about 5 sec (20 \(\mu\)l should give \([\text{E}]_0 = 1 \times 10^{-4} \text{M}\)). Then spectrophotometric readings are again taken up to 2000 sec.

Optimal concentrations that conform to the requirement\(^4\) of \([\text{S}]_0 \gg [\text{E}]_0\) and also to the requirement that \([\text{E}]_0\) is large enough so that the “initial burst” of \(p\)-nitrophenolate may be seen are \([\text{S}]_0 = 1 \times 10^{-4} \text{M}\) and \([\text{E}]_0 = 1 \times 10^{-4} \text{M}\), as described above. Data obtained from an experiment conducted with a Beckman DU spectrophotometer under conditions approximately these are shown in Figure 4. From the slope, intercept, and exponential of a plot such as Figure 4, \(A\), \(B\), and \(b\), defined above, may be obtained. The value of \(B\) gives the enzyme concentration directly.

\(^4\) A ratio of \([\text{S}]_0/[\text{E}]_0\) = 10 conforms to the requirement of \([\text{S}]_0 \gg [\text{E}]_0\) reasonably well. Because of the very high \(k_2/k_1\) ratio even values below 10 deviate only slightly (see Fig. 4).

From the pseudo first-order rate constant \(b\), the value of \(k_2[S]_0/K_S\) may be obtained directly. Using both the slope \(A\) and the intercept \(B\), the value of \(k_{\text{cat}} = k_3\) may be calculated.

Both \(k_2[S]_0/K_S\) and \(k_3\) depend on a basic catalytic group on the enzyme of \(pK_a\) approximately 7. Thus, if the above reaction is conducted at \(pH\) 7, these rate constants will be only 50% as large as at \(pH\) 8.5.

Doubling the enzyme concentration at constant \([\text{S}]_0\) should double both the intercept \(B\) and the slope \(A\) but should not affect the rate constant \(b\). Doubling the substrate concentration at constant \([\text{E}]_0\) should not affect either the intercept \(B\) or the slope \(A\) but should double the rate constant \(b\).

**Literature Cited**