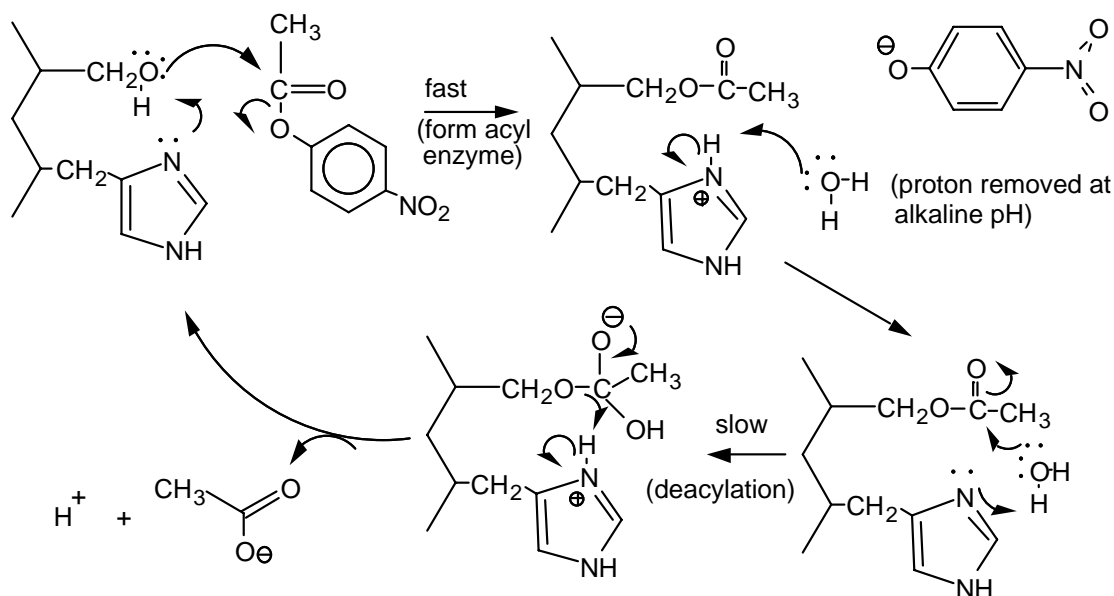


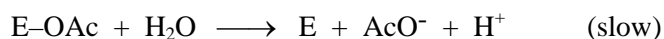
CHYMOTRYPSIN CATALYSIS

Chymotrypsin is a proteolytic enzyme of about 25,000 daltons found in secretions from the pancreas. Its function is the hydrolysis of peptides present in the chyme which is introduced into the duodenum from the stomach. The enzyme preferentially cleaves peptide bonds in which the carbonyl function is donated by an aromatic amino acid (phe, tyr, or trp). However, it is also able to hydrolyze synthetic substrates. One such substrate is p-nitrophenyl acetate (NPA), which is cleaved relatively slowly to produce p-nitrophenol, acetate ions, and H^+ .

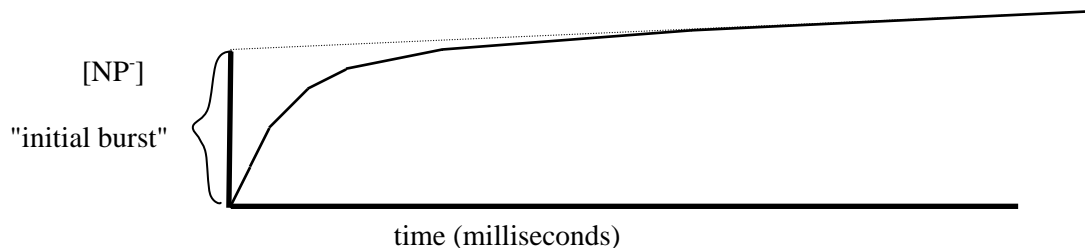
The mechanism of NPA cleavage involves a "catalytic triad" of **ser**, **his** and **asp**. **Ser** and **his** cooperate as shown below; **asp** assists his in manipulating protons:



The net result is the following:

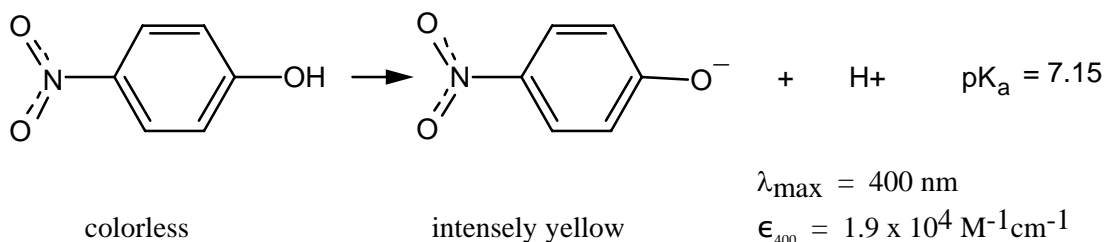


If the experiment is arranged so that all of the enzyme (chymotrypsin) molecules begin their catalytic process at the same instant, then one sees a rapid "burst" of p-nitrophenol production, followed by a much slower steady-state rate of hydrolysis. The concentration of NP^- released during the first cycle or turnover corresponds exactly to the concentration of chymotrypsin active sites: each active site cleaves one molecule of NPA and releases one molecule of NP^- . The slow step (deacylation) is the "bottleneck" which creates the burst phenomenon. Since it is a random process, some enzyme molecules will lose their acyl groups sooner than others and immediately begin a new round of cleavage. This results in a rapid loss of synchrony, so that after about one turnover the enzyme molecules are cleaving NPA at random times with respect to each other. Thus, after the initial burst, the rate of NP^- production decreases dramatically to a very much slower steady-state rate which is the rate at which the enzyme molecules deacylate. The time course of NP^- production would appear as on the next page if one had the means to observe very fast processes:



The objectives are to see whether enzyme concentration affects the steady-state rate of the reaction, to see how rate and concentration can be used to find a turnover number, and to explore how pH affects rate. The latter can be used as a test of the validity of the proposed hydrolysis mechanism. In this experiment the initial burst is used as a way to estimate the concentration of chymotrypsin active sites.

The product used to monitor reaction progress (NP^-) is a weak acid whose absorption spectrum depends on its state of ionization:



We are interested in the total amount of p-nitrophenol released by hydrolysis, but can observe only that fraction which happens to be ionized at the given pH. A little algebra reveals that the total amount of p-nitrophenol can be calculated from the observed amount using the relation

$$[\text{NP}]_{\text{total}} = [\text{NP}^-](1 + \{[\text{H}^+]/K_a\})$$

$[\text{NP}^-]$ can be obtained from its absorbance using the Beer-Lambert law (assuming a 1 cm cell):

$$[\text{NP}^-] = A_{400}/\epsilon = A_{400}/(1.9 \times 10^4)$$

Combination of these two equations permits us to compute $[\text{NP}]_t$ at any pH, given A_{400} :

$$[\text{NP}]_t = \frac{(A_{400})(1 + \{[\text{H}^+]/K_a\})}{1.9 \cdot 10^4}$$

Similarly, the steady-state rate of the reaction is simply the slope of the line given by the plot of $[\text{NP}]_t$ vs time, corrected for pH and ϵ , or

$$\text{Rate} = \frac{\Delta(A_{400}) \cdot (1 + \{[\text{H}^+]/K_a\})}{\Delta(\text{time}) \cdot (1.9 \cdot 10^4)}$$

EXPERIMENTAL

Get 6 plastic cuvetts and label them on the side with a grease pencil. Four are needed for part 1 and five will be needed in part 2 of the experiment. The sixth, which should be the "best" cuvet, will contain a water blank. Use it to zero the colorimeter before starting assays (below). *Pay attention to the rationale for preparing proper controls.*

1. Dependence of rate on enzyme concentration

Read this entire procedure before starting. Prepare all samples in cuvetts, not test tubes. This prevents losses caused by transferring solutions. *Complete your measurements on a given sample before adding chymotrypsin to the next.* Do you understand the purpose of sample #1? Be sure you do before you proceed.

Prepare samples (can do one at a time):	<u>Sample #</u> (additions in ml)			
<u>Contents</u>	1	2	3	4
0.1 M phosphate, pH 7.0	2.1	2.1	2.1	2.1
10 ⁻³ M HCl	0.8	0.6	0.4	---
* Chymotrypsin in 10 ⁻³ M HCl	---	0.2	0.4	0.8

*Add **just before starting** run - chymotrypsin self-digests at this pH.

(Stock solution contains *about* 3.8 mg of purified chymotrypsin per mL of 10⁻³ M HCl)

To start a run, add 0.1 mL of 0.03 M NPA to the center of the surface of the solution in the cuvet (*ethanol, the solvent for NPA, damages the plastic cuvet*). **START TIMING AS YOU ADD THE NPA.** Mix **immediately** by inverting the cuvet twice (let the bubble to reach the bottom each time), then insert into the colorimeter and record absorbance as a function of time elapsed since mixing the NPA.

(Technically, to get a baseline reading, one should measure the A400 after adding chymotrypsin but before adding NPA. This reading would be subtracted from the "y-intercept" for that sample to be estimated later. We will assume that this baseline reading is close to zero.)

2. Dependence of rate on pH

Prepare the following set of samples (but see *italics* below) to determine the pH dependence of the catalytic process. Before starting please read the whole section (which continues to the next page) so that you are aware of the strategy involved in timing these reactions. The seemingly strange volumes of enzyme and 1 mM HCl are necessary to make this set comparable to the previous set.

<u>Contents</u>	<u>Sample #</u> (additions in ml)							
	5a	5b	6a	6b	7a	7b	8a	8b
0.1 M phosphate , pH 6.0	2.1	2.1	----	----	----	----	----	----
" , pH 6.5	----	----	2.1	2.1	----	----	----	----
" , pH 7.5	----	----	----	----	2.1	2.1	----	----
" , pH 8.0	----	----	----	----	----	----	2.1	2.1
10 ⁻³ M HCl	0.4	0.8	0.4	0.8	0.4	0.8	0.4	0.8
Chymotrypsin in 10 ⁻³ M HCl	0.4	----	0.4	----	0.4*	----	0.4*	----

***** *Do not add enzyme to 7a & 8a until ready to run them because of the "cannibalism" problem.******

Strategy: Samples at the more alkaline pH values react very rapidly, so be prepared for some hasty data collection. The reactions at low pH go excruciatingly slowly, so some planning is wise if you want dinner. Start 5a, 5b, 6a and 6b first (conveniently spaced, of course; record the time of day that each is started). Read

these at convenient (5 - 10 minute?) intervals. You can "sandwich" readings for these samples between the runs for the faster ones. After the slow ones are going, use your remaining cuvet for 7b and get 5-8 readings (enough for a good line). Then do the same for 8b, then 7a and finally 8a.

Execution: We are not interested in the initial burst for this set - we care only about the net rates of reaction. To start a run, add 0.1 ml of NPA stock solution, begin timing, and mix as before (by inverting twice). Monitor the rate of each reaction as suggested above.

REPORT: do not divide it artificially into parts 1 and 2. Keep in mind the objectives of the experiment, and make the report show how those objectives were met. Use appropriate subheadings to organize each section to reflect the objectives. At minimum the following items need to appear at appropriate places, but not necessarily in the order shown. Remember to write from the point of view of scientific significance, not process.

Data from part 1 (*don't call it "part 1"*) should be plotted on a single graph as A_{400} vs. elapsed time (4 lines).

(a) Use these data to determine the **molar concentration** of chymotrypsin active sites in each sample (#2 - #4). *The 3.8 mg/mL "concentration" in the stock solution is useless information for this purpose!* You will need the "y-intercept" for each sample. Remember the purpose of sample #1.

(b) Use the same data to determine the steady-state rate of the **enzyme-catalyzed** reaction in each sample. (This is the **net** rate due to action of the enzyme. Compute the net slopes for chymotrypsin-containing tubes by subtracting the slope of sample #1 from each of the other slopes. Then apply ϵ and the pH correction to each net slope.) *In what units should your values be?*

(c) (*Report in Appendix, not body of report*) Determine an average value for the molar concentration of active sites in the chymotrypsin stock solution. To do this, use the active site concentrations you just computed for each of samples 2-4, account for dilution of enzyme stock solution in each sample, and compute 3 estimates of the concentration of chymotrypsin active sites in the stock solution. Find the average of these values (which should be close to one another).

Assuming that the reaction is first order in chymotrypsin, the rate law is

$$\text{rate} = k_{\text{cat}} \cdot [\text{E}] \quad \text{where } \text{rate} \text{ is the net enzyme-catalyzed rate in a given sample, } k_{\text{cat}} \text{ is the turnover number for the enzyme, and } [\text{E}] \text{ is the active site concentration in that sample.}$$

Use a **graphical method** that plots information from part 1 to estimate the turnover number for the enzyme. (Caution: What are $[\text{E}]$ and **rate** for sample #1? These values comprise one of the four data points that you should plot.) The value of k_{cat} *must be reported with proper units*.

Analyze data from part 2 as follows: Determine the enzymatic rate of cleavage at each of the five pH tested (including pH 7.0 – where do you have the data for this?). Do this as before: subtract the "raw" nonenzymatic rate (slope) from that of the corresponding enzyme-containing sample to get the *uncorrected enzymatic rate*. Then account for ϵ and the effect of pH on ionization of p-nitrophenol. These corrections are critical!!!

Plot the corrected enzyme-catalyzed rates as a function of pH.

Comment on the shape of the pH-rate profile. Estimate the pK_a of the catalytic group involved in the slow step of the reaction and tell how you used your data to make this estimate (get help if unsure!). Do your results support or conflict with the mechanism proposed at the beginning of this protocol? Why?