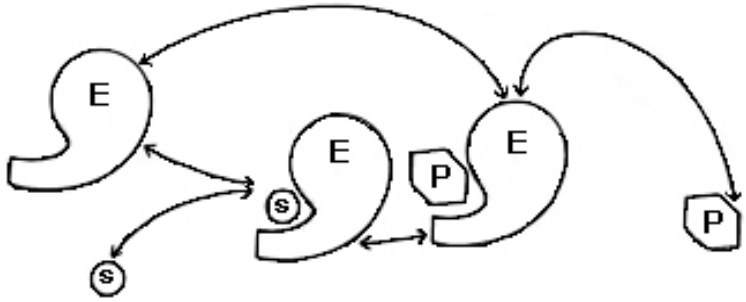
COMPUTER SIMULATION OF ENZYME KINETICS

I. Introduction.

Enzymes are biological catalysts. A catalyst alters the speed at which a chemical reaction reaches its completion or equilibrium point. It does not affect the proportions of the reactants at equilibrium and it is found unchanged at the end of the reaction. Enzymes are responsible for the many chemical processes on which life depends, but each enzyme catalyses only one specific reaction. An enzyme works by combining temporarily with the reactants or substrates. A chemical change then occurs, converting the enzyme-substrate complex into an enzyme-product complex and this allows the products to be released.



As enzymes are proteins they are composed of long chains of amino acids. Each protein has a precisely defined composition with a complicated structure. This structure can be rapidly destroyed at high temperatures, when the protein is said to be denatured. Enzyme molecules are usually very large compared to their substrates and products. Only part of the enzyme surface, called the active site is directly involved in catalysis. It includes a binding site which binds the substrate to the enzyme and a catalytic site at which the chemical reaction occurs.

Hundreds of different enzymes are known, but the same general approach may be used to study them all. The enzyme and substrate are mixed under controlled conditions and the reaction is followed by measuring either the disappearance of the substrate or the appearance of the product over time. Two particular enzymatic characteristics can be examined in studying a reaction. One is the extent of the reaction – how much product is made altogether and the second is the rate at which the reaction occurs – how much product is made per unit time. An analogy can be made with automobile travel. Say you drove from Cortland to your home near New York City. It took you 3 hr to cover the 210 miles (discounting the time it took to get the ticket). The 210 miles is the extent of the trip and the rate is, of course, 70 miles per hour.

There are a variety of factors which can affect the extent and rate of reaction. There are some "environmental" conditions such as incubation time, temperature and pH. Also important are the concentrations of the enzyme and the substrate themselves. In this computer lab we will examine the effects of all of these parameters on the enzymatic reaction.

II. The Computer Program.

The Enzkin computer program allows you to simulate actual experiments as if you were in the lab. Since they take just seconds to set up and the results are achieved instantaneously, you will be able to do many "experiments" quite rapidly. You will start by picking one of six enzymes

with which to work. Each enzyme has different characteristics such as pH optimum and K_m. With your enzyme you will run some experiments using the different parameters mentioned above: pH, temperature, incubation time, enzyme concentration and substrate concentration. In each case, all of these are kept constant except for one that is varied. For instance, you can determine how much product is made when 50 mM substrate is incubated with 100 µM enzyme at 37ºC for 5 min over a range of pH's from 0 to 14. In other words, it is like setting up an experiment with a series of test tubes, each tube has the same amount of substrate and enzyme, each is held at the same temperature and incubated for the same time. However, each tube has a different pH. The results are given in terms of how much product is made at each pH. After you see the results of one experiment, another can be done either changing the pH range or varying another parameter, such as incubation time while holding the pH and everything else constant.

As you can see in the table below each parameter has limits – minimum and maximum values that you can not exceed. In addition, how finely you can divide the values for the parameters is limited. So, for example, you can see that incubation time can be varied only from 0 to 60 minutes and while you can use a value of say, 34 min, you can not use 34.5 min.

Parameter	Minimum Value	Maximum Value	Minimum Interval
1. pH	0	14	0.1
2. Substrate volume (ml)	0	9.5	0.1
3. Enzyme volume (ml)	0	9.5	0.1
4. Incubation time (min)	0	60	1.0
5. Temperature (ºC)	0	100	1.0

So how do you actually "run" an experiment? The computer will ask you a series of questions as you can see on the last sheet of this handout. These questions set up the conditions of the experiment. Starting with what enzyme you want to use, it goes on to which of the 5 parameters you want to vary. (Each is numbered as in the table above.) After telling it what minimum and maximum values you want, you then need to tell it what the other, constant values are. When you finish answering the questions the computer gives you the results. Note that it converts the substrate volume you used to substrate concentration. Then it lists the varying parameter (time in the example) with the product concentration. Finally, it reminds you of the set-up conditions you started with.

There are several important points to keep in mind when running these simulations.

1) In the table above the amounts of enzyme and substrate are expressed in volumes (ml). This is done because it convenient (see point #2 below), but, of course, it is often more useful to express these amounts in terms of concentrations – millimolar (mM) or micromolar (µM). As seen in the example above, the computer will sometimes convert the volumes to concentrations. At other times you will do perform some of these conversions also.

2) In the experiments you are simulating imagine that you are doing them in test tubes with a total volume of 10 ml of solution. This volume never varies. So, even if you only add say, 2 ml of substrate and 2 ml of enzyme, the

computer will make up the difference by adding 6 ml of buffer. Finally, the computer will always add at least 0.5 ml buffer. Thus you are limited to adding any combination of enzyme and substrate up to only 9.5 ml. And, for instance, if you want to use 8 ml of substrate, the greatest amount of enzyme that you can add will be 1.5 ml.

3) The computer "wants" to give you 11 values for results. If you ask it to vary pH from 7 to 8 it will be happy because it can give you the results for pH 7.0, 7.1, 7.2 · · · 8.0 which is a total of 11 values. But if you ask it to vary pH from 7 to 7.5, it will ask you to try again because the results at 7.0, 7.1, 7.2, 7.3, 7.4, 7.5 only make up 6 values.

Now let's turn to the individual parameters and discuss the background for each of those in a little more detail. The order of these is different than that presented in the program. **In lab, it would be best if you proceed through the parameters in the order that follows rather than that in the program.**

III. Experimental Parameters

– Background Information.

pH Optimum. The existence of an optimum pH is a consequence of the acidic and basic groups present in enzymes. These groups change their charge as pH varies (remember the pH Lab). If such a group should be located in an enzyme's active site, its charge will affect enzyme activity. If there are two such groups, one may permit activity only below a particular pH and the other only above some other pH. Therefore the enzyme will be active over only a narrow range of pH. This situation is the rule, although there are exceptions. The substrate may also vary in charge according to the pH. An enzyme is so specific that it normally accepts its substrate in only one of the substrate's charge configurations.

In addition to finding the pH optimum, your instructor may ask you to determine the Specific Activity at the optimum pH. Specific activity is defined here as micromoles of product per minute per ml of enzyme. To determine this value you must first convert the micromolar concentration the simulation gives you at the optimum pH to the number of micromoles in the solution (10 ml). Say, for example, you determine that pH 7.6 is the optimum and in your simulation, you used a 4 min incubation and 2 ml of enzyme, which gave you a product concentration of 800 µM.

To determine the number of micromoles in the solution you must realize that 800 µM is equal to 800 µmoles/liter. This, of course, converts to 8 µmoles/10 ml. Since the 8 µmoles were produced with a 4 min incubation and 2 ml of enzyme, the enzyme activity is 1.0 µmole per min per ml enzyme.

Enzyme Concentration. From the previous lab exercise on succinate dehydrogenase, you know the effect of enzyme concentration. When the substrate concentration is kept high, the reaction rate is proportional to enzyme concentration.

Incubation Time. When the rate of reaction (appearance of product, or Product Concentration) is plotted against time, these graphs are called "progress curves". Initially, it should be evident that the incubation time will allow more substrate to be converted to product. Further, when different substrate concentrations or different enzyme concentrations are used, you can change both the rate of the reaction (how fast product is made) and the extent of the reaction (how much product is made). The rates and the extents of the reactions will be seen respectively as the initial slopes and the heights of the progress curves.

Temperature. Under typical conditions for enzyme assays there is an "optimum temperature" for an enzyme. This is a temperature at which the enzyme functions maximally with minimal denaturation. We want to focus on something a little different and that is the

optimum temperature of the reaction. This peak of activity represents an optimum temperature for particular reaction conditions.

Consider for a moment that very low temperatures slow the rate of an enzymatic reaction. As the temperature rises, the rate of the reaction increases, reaches a maximum and then declines. Why does it decline? Is the rate of catalysis decreasing? No, the rate declines because the enzyme will be altered by temperature: at too high a temperature it becomes denatured and loses ability to function. Thus, as the temperature increases, the rate of catalysis increases AND the rate of denaturation increases. This combination leads to a peak of product produced. Do you think if a reaction was allowed to proceed for only a short time, an increased rate of catalysis or an increased rate of denaturation would have greater effect? If the reaction was allowed to proceed for a long time, which would have the greater effect?

Substrate Concentration. Whenever an event depends on a fixed number of operators that work at a fixed rate, such as carrier molecules in a membrane or enzymes, you will see a hyperbolic curve when the rate of the operation is plotted against the concentration of the material being operated upon. In short, if the rate of the reaction versus substrate concentration is examined, a linear relationship at low substrate concentration would be expected and at higher substrate concentrations the rate would progressively slow and reach some constant value. In studying this portion of the lab, you must, again, make certain that at the various substrate concentrations you use, the concentration does not change significantly over the time course of the simulation. You must also beware that certain conditions, or sets of parameters, may only reveal one part or another of the hyperbola and you must choose the correct parameters to see the entire curve in one simulation.

In this portion of the lab exercise, you will also determine the V_{max} and the K_m of an enzyme. V_{max} is simply the maximum velocity, or rate, at which an enzyme functions. We will estimate V_{max} by determining the highest rate achieved by an enzyme. The height of the hyperbola will be taken as V_{max}. K_m is the Michaelis constant, the value of which is characteristic of each enzyme. You will determine the K_m of your enzyme. It is the substrate concentration which corresponds to one half the maximum velocity of the reaction. So, in practice, you will determine the V_{max}, then $\frac{1}{2}V_{max}$ and then read off your graph the K_m.

The only tricky part of this section of the lab is to determine the substrate concentration in the 10 ml of the reaction mixture. This is done using the substrate volume you pick and the fact that the concentration of the substrate in the substrate stock solution is 20 mM. To calculate the final substrate concentration, determine the relative volume of substrate in the reaction mix (sub. vol./10 ml total volume), and multiply this by 20 mM.

IV. Procedures.

Again, please note that it would be best if you examined each parameter in the order that follows rather than in order specified by the program. The number in parentheses is the one the program uses and the one you will need when working on the computer.

A. pH Optimum. **(1)**

You want to find the pH optimum for your particular enzyme. This is the pH at which the reaction occurs most rapidly, making the greatest amount of product. Start with a large range of pH values and then plug in reasonable values for the "non-varying" parameters. Narrow the range in subsequent simulations to get the optimum pH for your enzyme down to a tenth of a pH unit. You should then do several more simulations adjusting some of the other parameters. This will show if the pH optimum you tentatively determined is real and will demonstrate if it changes under various conditions. Be sure to tell the instructor the value of your pH optimum before proceeding with the rest of the lab since you should use the optimum in the rest of your simulations.

In addition to determining the pH optimum, you can determine the specific activity of the enzyme at the optimum pH. The methods for this are described in the background information above.

B. Enzyme volume. **(3)**

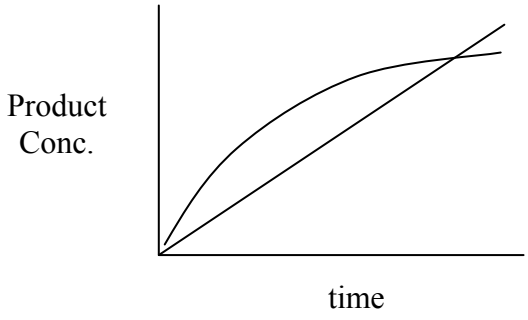
(For our purposes, enzyme volume can be taken as enzyme concentration.) Make your simulations using the optimum pH, optimum temperature and reasonable values for enzyme volume, substrate volume and incubation time. Remember to set substrate volume high enough so that substrate concentration does not become limiting at high enzyme volumes. Graph your results (Product Concentration vs. Enzyme Volume).

C. Incubation time. **(4)**

Use this parameter to investigate the effects of various substrate volumes and various enzyme volumes.

First, vary the time between 0 and 60 minutes and use your pH optimum and a reasonable temperature. Then choose different values for substrate volume and enzyme volume in a series of simulations. Keep all other parameters constant. Obtain data from a progress curve that demonstrates substrate exhaustion (see graph below). Next, still varying the time from 0 to 60 min, obtain data to demonstrate continued product formation. Try to do this by altering just one parameter – either enzyme volume or substrate volume. If it turns out to be impossible to get a straight line by altering just one of the parameters, then go ahead and change the other a little to get the straight line.

Graph the two progress curves produced by these parameters. It may be possible to get both curves on the same graph as shown below, but the values may be so different that two graphs may be necessary. Indicate on the graph all non-varying parameters and the initial velocities of the reactions.



D. Temperature. **(5)**

Acquire data from simulations to show that the optimum temperature of a reaction can change depending on the length of time the experiment is run. Start by picking a short period of time for an experiment. Then vary the temperature over a wide range to obtain the approximate temperature that gives the peak of activity. Next run the experiment again with a narrow range of temperatures around the approximate one to determine the precise temperature for maximum activity. To determine if the there is a different temperature optimum if you run the experiment for a different length of time, repeat the above procedures using longer incubation times. Report data of at least three simulations (short, medium and long times), each showing an optimum temperature of the reaction. Note: It is important that the substrate concentration be kept high, because the results due to low substrate concentration are similar to the effect of increased temperature.

E. Substrate volume. **(2)**

You will need to use all your recently acquired skills for this last part. Think about what conditions you need to determine K_m. You need conditions that first reveal V_{max}, so you want the product concentration to change very little at high substrate volumes. One hint that seems to help is to keep your maximum product concentration around 500μM. Try various combinations of conditions to achieve this. When you feel you have done the best you can you can, roughly determine $\frac{1}{2}V_{max}$ and K_m even before you graph the results (Product Concentration vs. Substrate Volume). Discuss your results with the instructor. Finally, after graphing your results, do a Lineweaver-Burk or "double-reciprocal" plot. Determine the K_m from this graph and note which type of graph most easily gives the most accurate results.

Note: A large portion of this material was adapted from both the "Students' Notes" and the "Teachers Guide" of Enzkin: Unit on Enzyme Kinetics by M.T. Heydeman. (1977). Edward Arnold (Publishers) Ltd.