Lab #4: Enzymes

Background

Most of the chemical reactions that take place within a cell involve protein catalysts called *enzymes*. Enzymes, like other catalysts, speed up the rates of chemical reactions by lowering the activation energy of that reaction (i.e., the amount of energy needed to start a chemical reaction). They do so by binding reactants (hereafter referred to as *substrates*) and holding them in a particular orientation that maximizes the chances that a particular chemical reaction will occur, converting the substrates into *products*. Like other catalysts, enzymes themselves are not permanently altered in the chemical reaction they catalyze—enzymes return to their original form at the end of the reaction. Also, like other catalysts, the enzyme does not provide the free energy necessary to drive otherwise energetically unfavorable reactions, but simply facilitates energetically favorable ones.

The ability of enzymes to function as catalysts depends on the three dimensional shape of the protein. Recall that all proteins have a particular shape which is due to various types of chemical interactions that occur among amino acid side chains (e.g., ionic interactions among charged side chains, hydrogen bonds, polar/nonpolar interactions, disulfide bonds, etc.) and between amino acid side-chains and the surrounding environment. In the case of enzymes, some of the amino acids of the polypeptide are arranged in such a way that they form a pocket-like structure called an *active site* (Fig 4.1). The amino acids in the active site are arranged in such a way that they can *a)* form a number of non-covalent bonds with the substrate(s), thus temporarily binding the substrate(s) and *b)* help to destabilize certain chemical bonds within the substrate(s), increasing the chances that a particular chemical reaction will take place.

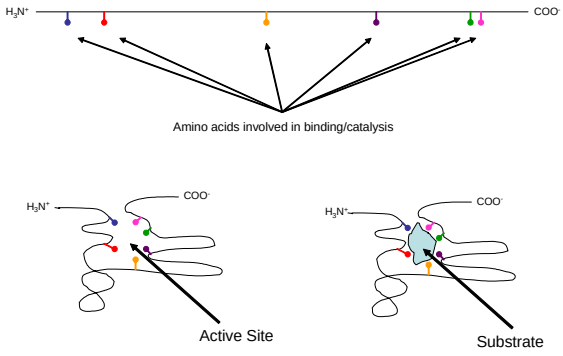

Fig 4.1. Folding of a polypeptide chain into a 3-D structure with an active site. Notice that the amino acids involves in binding and catalysis may be from distant locations in the primary sequence of the polypeptide chain, but are brought into close proximity with one another in the tertiary structure.

There are many different types of enzymes, which perform a variety of different chemical interactions. However, the process of enzyme catalysis is similar among different enzymes (Fig 4.2). First, the substrate(s) binds to the active site to form an *enzyme-substrate complex*. Secondly, the reaction occurs converting the substrate(s) into product(s), forming an *enzyme-product complex*. Finally, the products are released from the active site, leaving the enzyme in its original, unaltered form.

Because the active sites have a particular orientation of specific amino acid side chains (and their respective chemical properties) there is usually only one molecule or at most a

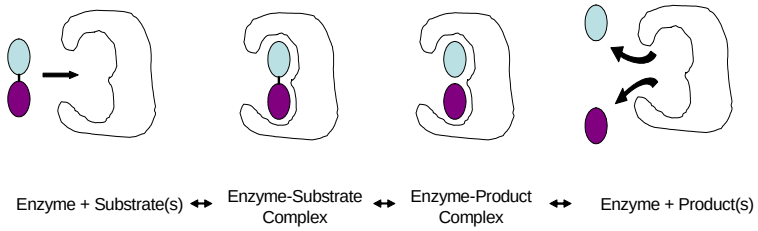

Fig 4.2 An example of an enzyme catalyzed reaction. In this case, the substrate binds to the active site of the enzyme. The reaction takes place, cleaving the substrate into two products. The products are then subsequently released.

few types of molecules that can bind to the active site for a long enough period of time for a chemical reaction to take place. Thus most enzymes show a very high degree of *specificity*—they bind specific substrates, catalyze specific reactions involving those substrates, and thus produce specific products. For example, enzymes that use D-glucose as a substrate often will not perform the same reactions on fructose, galactose, or other isomers of D-glucose, because the shapes of these isomers are different to the point that they cannot bind properly to the active site of the enzyme. This high degree of specificity of enzymes enables precise control over the chemical processes taking place inside the cell.

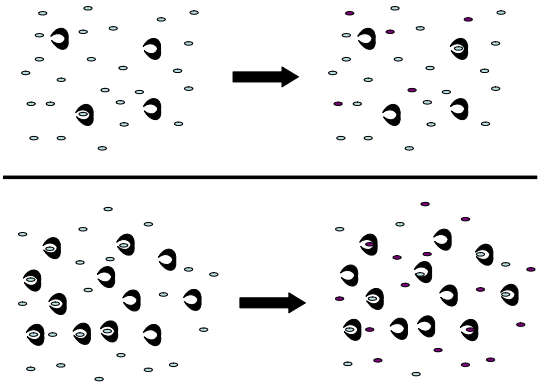

Fig 4.3. An example of the effect of enzyme concentration on reaction rate. In the top panel, four enzyme molecules are able to convert three molecules of substrate (light blue) into product (dark purple) in x time, whereas in the lower panel twelve enzyme molecules are able to produce 15 molecules of product in the same amount of time.

Factors influencing enzyme activity

Enzymes are among the fastest catalysts known. It is not atypical of an enzyme to increase a reaction rates by 10,000x, 100,000x or even 1,000,000x. Indeed, some enzymes increase reaction rates to 100,000,000x the rate they would occur spontaneously. Clearly, the catalytic function of enzymes is essential to homeostasis, as without these catalysts many of the chemical processes needed for homeostasis simply would not occur quickly enough.

The ability of an enzyme to convert substrate into product is referred to as *enzyme activity*, and is often used as a synonym for reaction rate (since as enzyme activity increases, more substrate is converted into product per unit time). Enzyme activity is not necessarily constant—there can be a number of factors that influence how quickly substrate can be converted into product. Here we address several (although not all) factors that can influence enzyme activity.

1. *Effect of Enzyme Concentration*

An enzyme molecule is analogous to a worker on an assembly line in a factory. The worker picks up the raw materials, does something with them, releases the altered materials, then picks up the next set of raw materials. Likewise an enzyme molecule binds its substrate(s), catalyzes a reaction, and releases the product(s). Each step in this process requires time—time to receive the raw materials, do what needs to be done to them, and release the product. So each enzyme molecule requires *x* amount of time to produce one unit of product. The more enzyme molecules that are available, however, the more product can be produced in *x* time: two enzyme molecules would produce two units in that time period, three enzyme molecules would produce three units of product, etc. Thus the more enzyme is available, the more quickly substrate can be converted into product (Fig 4.3). In general, then, (and assuming all other factors are constant) as enzyme concentration increases, there is a proportional increase in reaction rate (Fig 4.4).

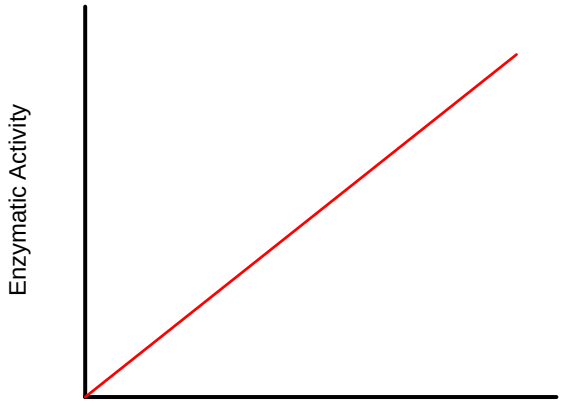

Fig 4.4. Plot illustrating the mathematical relationship between enzyme concentration and enzyme activity

2. *Effect of Substrate Concentration*

In order for an enzyme to convert substrate into product, the substrate must first bind with the enzyme. This is usually achieved simply by random collisions between enzyme and substrate as these particles diffuse around in solution. The frequency with which these collisions occur can be influenced by a number of factors. One of them is simply the amount of each substance that is present in solution. For example, the more concentrated the substrate is within a solution, the more frequently substrate molecules will randomly collide with the enzyme in the proper orientation for the active site to bind to the substrate, and thus the more often substrate will be converted into product. So in general (again, assuming that all other factors, such as enzyme concentration, are constant), one would expect the rate of an enzyme catalyzed reaction to increase as substrate concentration increases (Fig 4.5).

Note, however, that this is not an indefinite increase. That is because that although the rate at which substrate can bind to the enzyme increases with increasing substrate concentration, once that enzyme has bound the substrate the enzyme must still catalyze the reaction and release the product. Since there is a minimum amount of time needed to process each substrate molecule once bound, there is a maximum rate at which a given number of enzyme molecules can convert substrate into product. Thus, enzymes can demonstrate *saturation* in response to increasing substrate concentration (Figs 4.5, 4.6). As substrate

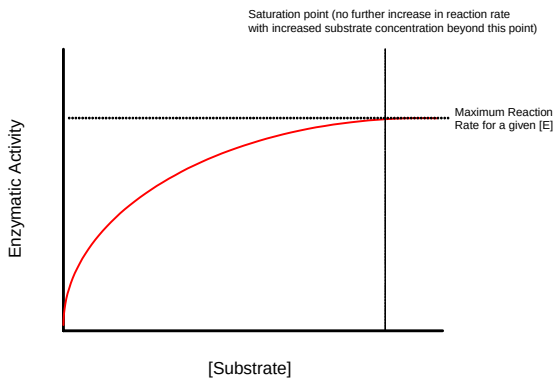

Fig 4.5. Plot illustrating the relationship between substrate concentration and reaction rate. Note that as substrate concentration increases, rate increases, but the change in rate becomes progressively less until a maximum rate is reached when the enzyme becomes saturated with substrate.

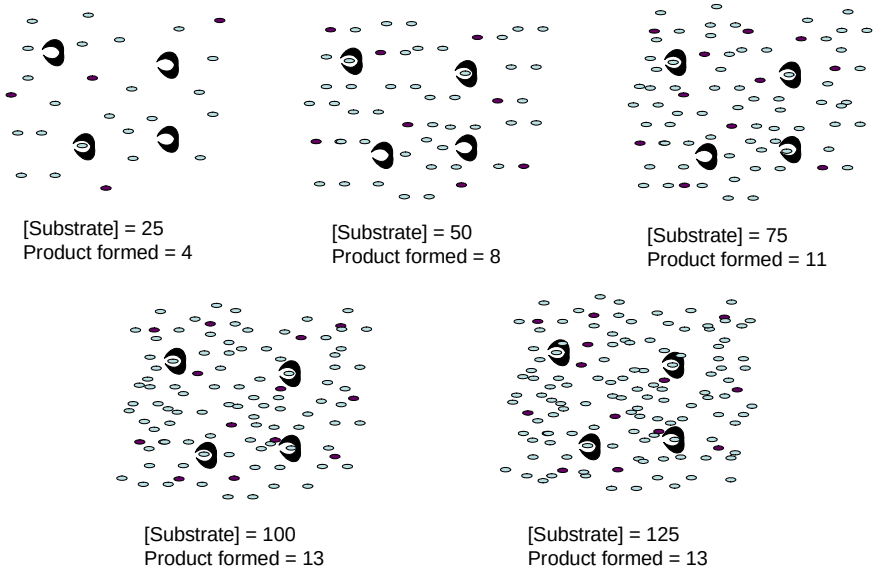

Fig 4.6. Illustration of the effect of substrate concentration on reaction rate. Sets of four enzyme molecules each are exposed to different concentrations of substrate for *x* time. Notice that as substrate concentration increases from low levels the rate of the reaction increases, but the degree to which the rate increases become progressively less and less as more enzymes are occupied at any given time in enzyme substrate complexes. Eventually, saturation of the enzyme is achieved when all of the enzyme molecules are bound to substrate at any given time. At this point, the reaction has reached its maximum rate, and no further increase will occur with increasing substrate concentration

Fig 4.7. Example of a cofactor (red) used to activate an enzyme by altering the shape of its active site to the configuration needed to bond and catalyze substrates

concentration increases, more and more enzyme molecules are locked up in enzyme-substrate complexes at any given time. At a particular substrate concentration the enzyme will become completely saturated with substrate—at any given time virtually all of the enzyme molecules will be occupied in enzyme-substrate complexes, and thus no further increase in reaction rate will accompany further increases in concentration.

3. Effect of Cofactor / Coenzyme Concentration

Many (but not all) enzymes require certain additional substances to be bound to them in order to function as catalysts. These substances are often referred to as *cofactors* and *coenzymes*. These auxiliary substances may need to be bound to the enzyme in order for the enzyme to have the proper shape to its active site (Fig. 4.7) or may be the actual catalytic agent used to facilitate the reaction taking place, whereas the

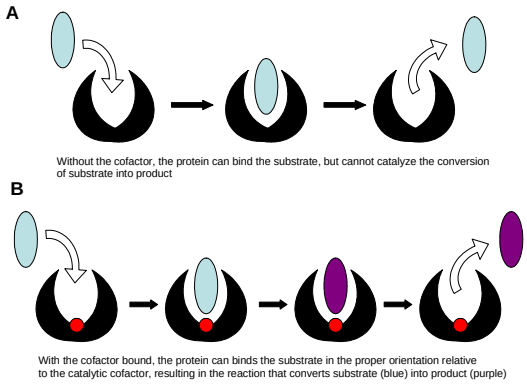

Fig 4.8. Example of an enzyme requiring a cofactor as a catalytic agent. In the top panel, the enzyme is able to bind the substrate (light blue), but without the cofactor present, it cannot convert the substrate into product. In the lower panel, the cofactor (red) acts as the catalytic agent for converting the substrate into product (dark purple)

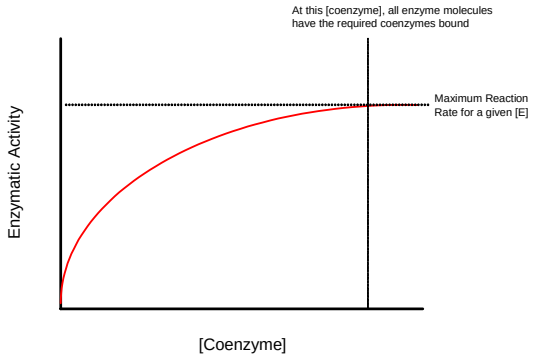

Fig 4.9 Plot of the relationship between cofactor/coenzyme concentration and reaction rate. Note that the effect of cofactor concentration on reaction is minimal once the concentration is sufficient to enable all enzyme molecules to function properly.

enzyme merely binds the substrate and holds it in the proper orientation (Fig 4.8).

For those enzymes that require a cofactor or coenzyme, enzyme activity is dependent upon the concentration of that cofactor (Fig 4.9). If the cofactor is at very low concentrations, few enzyme molecules will have the necessary cofactor bound, thus few will be able to catalyze the reaction, and reaction rates will be low. As cofactor concentration increases, more and more enzyme molecules will have bound cofactor and thus be catalytically active. However, as cofactor concentration increases, there will be a progressively smaller and smaller increase in reaction rate as the majority of enzyme molecules will already have the cofactor they need. Indeed, above a certain point, virtually all enzyme molecules will have the cofactor they need, and thus increasing cofactor concentration will have no further influence on reaction rate.

4. Effect of Temperature

Temperature is the average kinetic energy of a system. Kinetic energy, in turn is the energy in motion. This means that at higher temperatures particles tend to be moving more quickly than they are at slower temperatures. In solids, molecules remain in roughly the same position in space but vibrate more. In liquids and gases, where particles are free to move from one location to another, these particles tend to do so at greater speeds (Fig 4.10). Since particles are moving more quickly, they also tend to collide

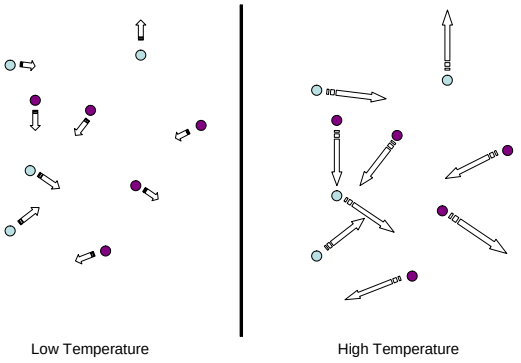

Fig. 4.10. Effect of temperature on motion of particles. Increased temperature increases movement of particles in solution

with one another more frequently and with greater energy. Therefore, the rates of chemical reactions (both catalyzed and non-catalyzed) tend to increase as temperature increases. Hypothetically, this should be an indefinite relationship, meaning that an increase in reaction should accompany an increase in temperature regardless of how high that temperature is.

Many enzymes show an unusual relationship between reaction rate and temperature (Fig 4.11). Although over much of the range of temperatures biological organisms experience there is an increase in enzyme activity with increased temperature there is often a *decrease* in reaction rates at very high temperatures (e.g., above 70 °C). Why does this occur? There could be a number of reasons. For example, the increase in temperature may weaken and destabilize the bonds that link enzymes with necessary cofactors, thus the rate of spontaneous

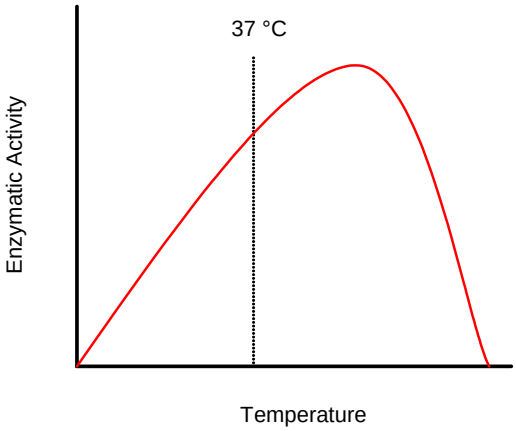

Fig. 4.11. Plot illustrating the relationship between temperature and the rate of enzyme catalyzed reactions.

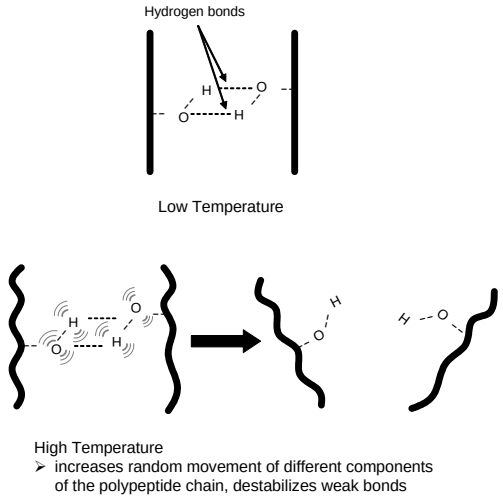

Fig 4.12. Illustration of heat denaturization between two polar amino acid side-chains. As temperature increases, random movements of the polypeptide chain pull against the hydrogen bonds linking the two side chains, causing them to destabilize and break.

deactivation of enzymes increases. However, perhaps the most important factor to consider is that the shape of the enzyme can be influenced by temperature. The secondary, tertiary, and quaternary structures of proteins all rely on relatively weak non-covalent bonds (e.g., hydrogen bonds, van der Waals forces, and ionic bonds) to link different regions of the protein together. Increasing temperature causes increased random movement in different regions of the protein, thus destabilizing these weak bonds (Fig 4.12) and causing a change in the shape of the protein (*denaturization*). If enough of these weak bonds are broken, the shape of the active site will begin to distort, and the enzyme will lose its ability to bind substrate and catalyze the reaction. Thus the decrease in reaction rate is due to the inability of the enzyme to function as a catalyst when it is denatured by heat.

5. Effect of pH

Recall that pH is an index of hydrogen ion (H^+ concentration). The H^+ concentration of a water-based solution can vary due to the presence of particular solutes. Some solutes, called acids, are normally weakly bound to one or more hydrogen ions, so that if dissolved in releasing one or more H^+ ions into solution. If the undissociated acid had a neutral charge, the

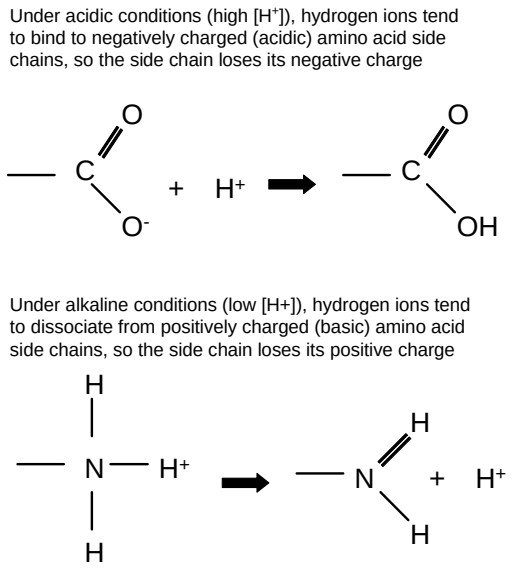

Fig 14.13. Examples of dissociation and binding of H^+ by acidic and basic amino acids under non-neutral pHs.

water the acid tends to dissociate (break apart) dissociated acid, once losing a H^+ without the shared electron, would become negatively charged (Fig 4.13). Conversely, other solutes called bases lead to a reduction in H^+ concentrations in aqueous solutions by binding H^+ ions. If the base had a neutral charge before being mixed with water, it would have a positive charge upon binding a H^+ ion.

Some amino acids have side chains that can act as weak acids or weak bases. At a pH of 7, acidic side chains will tend to be dissociated (will have a negative charge) whereas basic side chains will have bound a H^+ ion (will have a net positive charge). Since these side chains have net charges, they can form ionic interactions with one another, where like charged side chains repel one another and opposite charged side chains attract one another (Fig 4.14). These types of interactions contribute to the tertiary and quaternary structure of proteins. But what if the pH surrounding the protein is altered? The tendency for an acid or a base to be bound to a H^+ or to release a H^+ is influenced largely by $[H^+]$ in the surrounding environment. If the $[H^+]$ is high (low pH), then the side chain will tend to be bound to H^+ so acidic side chains would be neutrally charged and basic side chains would be positively charged. Conversely, if $[H^+]$ is low, then side chains will tend to release a bound H^+, so the acidic side chains would be negatively charged, and the basic side chains neutrally charged. Note that this would dramatically alter the ionic interactions that could otherwise occur among acidic and basic amino acids—ionic bonds that would exist at pH 7 would no longer exist at higher or lower pHs. This in turn, could radically alter the tertiary and quaternary structure of the protein (Fig 4.14). Since the catalytic ability of an enzyme is so tightly linked to the specific shape and chemical properties of its active site, alteration of normal ionic bonding patterns within the protein tends to reduce catalytic function. For any enzyme, then, there is an optimal pH where the right degree of H^+

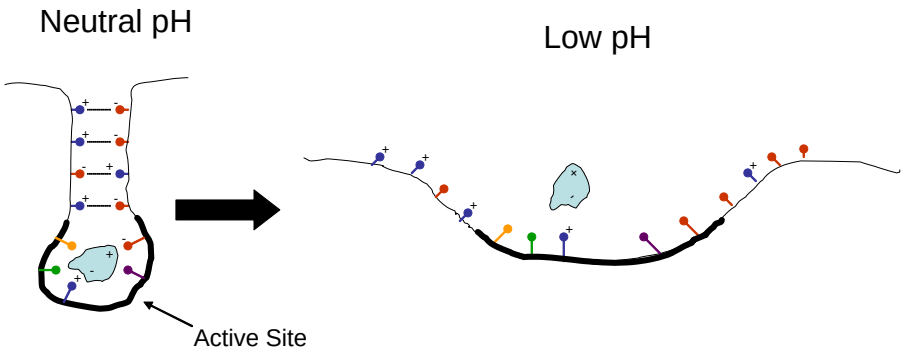

Fig 14.14. Denaturization of an enzyme when exposed to acidic conditions. Note that because of the high $[H^+]$ both basic (blue) and acidic (red) side chains are bound to H^+ when exposed to low pH. As a result, the ionic bonds that normally would hold the shape of the active site in its proper confirmation at neutral pH (left) cannot form at low pH (right). The enzyme thus is unable to bind substrate and catalyze its conversion into product.

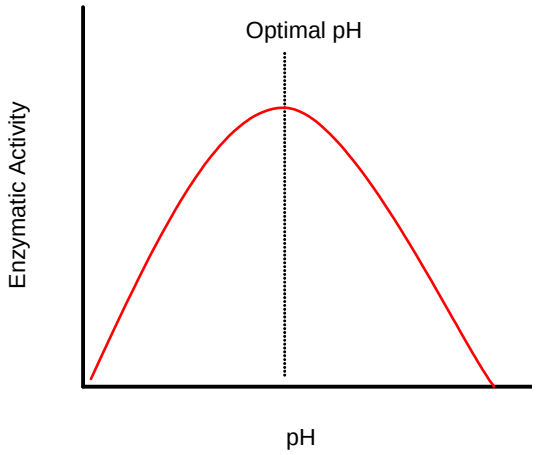

Fig 14.15. Plot illustrating variation in the rate of enzyme-catalyzed reactions with pH.

binding and dissociation of various acidic and basic amino acids exists such that the active site of the protein has the shape for maximum catalytic activity (Fig 14.15). Deviations of pH from this optimal level, to lesser or greater degrees, tend to reduce the ability of the enzyme to catalyze its reaction. The optimal pH can vary considerably among enzymes—whereas most enzymes in the human body function optimally at a pH that is roughly neutral (cytoplasm and most extracellular fluids tend to have a pH between 7 and 8), some enzymes, such as those of the digestive system, may have optimal pHs at very acidic or very alkaline levels.

Measurement of Enzyme Concentration

Measurement of enzyme concentration in body fluids is a powerful tool in the diagnosis of various ailments. Many enzymes are almost exclusively intracellular and may be produced only by specific cell types. Elevated levels in body fluids, therefore, could be the result of damage to particular tissues.

How does one measure the concentration of an enzyme? The measurement of concentrations of specific proteins in body fluids is much more complicated than for other types of substances. Remember, most proteins are made up of the same 20 amino acids—so simple chemical reactivity methods like the one we used for glucose last week probably wouldn't allow you to discriminate among the many different proteins you would find in a sample of body fluid. There are rather complex methods for directly measuring some proteins (e.g. radioimmunoassay), but there is a convenient method that can be used to measure enzyme concentration. Recall that if all else is constant (substrate and coenzyme concentrations, temperature, and pH), the rate of an enzyme catalyzed reaction is proportional to the concentration of enzyme in the solution. To put it another way, the rate at which substrate is converted into product is proportional to enzyme concentration. So if you ran an experiment where you allowed solutions of different enzyme concentrations to react with substrate for a fixed amount of time, the amount of increase in the concentration of product present in the solution at the end of the experiment should be proportional to the concentration of the enzyme (Fig 14.16). So we could use changes in the concentration of product formed over time as an indirect means of measuring enzyme concentration.

In many cases we can measure changes in product concentration in a solution using spectrophotometry. Remember that according to Beer's Law the concentration of a solute in solution (in this case, the product of the reaction) is proportional to the absorbance of light by the solution (Fig 14.16). Therefore, the rate at which the absorbance changes for a solution undergoing an enzyme catalyzed reaction is proportional to the rate that product is being formed. And since rate of product formation is proportional to enzyme concentration, the rate that absorbance changes during the reaction is proportional to enzyme concentration (Fig 14.16).

We now have a way of measuring enzyme concentration indirectly (via rate of product formation). Can we use this to get a direct estimate of how much enzyme is present? Not really. The reason why is that different tissues may produce different variants of the same enzyme that catalyze the same reaction but at different rates. Also, given the sheer number of factors that can influence the rate of a reaction, it is exceedingly difficult to tell precisely how

many enzyme molecules are present in a given volume of solution. Yet for many applications, measuring the number of enzyme molecules present is not as important as measuring how quickly an enzyme catalyzed reaction proceeds (i.e., what that amount enzyme *does*). So rather than expressing enzyme concentration in conventional terms (e.g., number of particles per unit volume), the concentration of enzymes is often expressed in terms of enzymatic activity (how much substrate can be converted into product in a given amount of time). The standard unit used to quantify the amount of enzyme (based on enzyme activity) is called the *international unit of enzyme activity* (U), and is equal to the amount of enzyme needed to convert 1 µmole[a] of substrate into product in 1 min. Enzyme concentration, then, would be the measure of enzyme activity divided by volume (e.g., U/L).

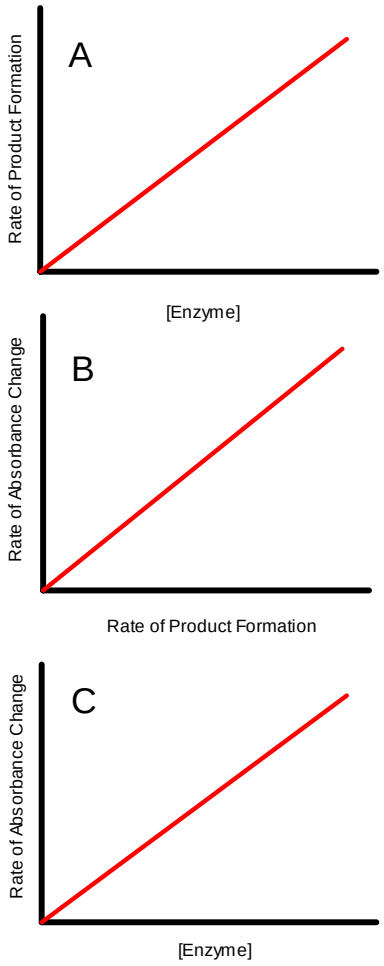

Fig 14.16 Correlations that enable us to measure enzyme concentration using spectrophotometry. Since the rate of product formation during an enzyme-catalyzed reaction correlates with enzyme concentration, and since the rate that absorbance changes during the reaction is proportional to the rate of product formation, then the rate that absorbance changes is proportional to enzyme concentration.

[a] A *mole* is a term given to a specific quantity (similar to the way the term "dozen" is used to describe a quantity of twelve), in this case equal to 6.02×10^{23}. One µmole (micromole), therefore, is a millionth of a mole, or 6.02×10^{17}

Demonstration: "Catalytic Championship Match" – Catalase vs. Ferrous Oxide

Your instructor will run a brief demonstration comparing the ability of two catalytic agents in accelerating break down peroxides[b]. In this case we will expose hydrogen peroxide (H_2O_2) to two different catalysts. One is the fairly nonspecific inorganic catalyst ferrous oxide (rust). The other is *catalase*, a peroxidase enzyme found at high concentrations in liver. The reaction catalyzed by both is as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

O_2 is a gas, and will bubble out of solution as it is formed. Describe any differences in the rate these bubbles are formed between the two solutions.

Experiment: Determining of Alkaline Phosphatase (ALP) Concentration in Blood Plasma

We will be conducting an assay for plasma alkaline phosphatase[c] (ALP). In this experiment, we will expose samples of blood plasma (containing ALP) to a solution containing the substrate *p*-nitrophenyl phosphate (PNPP). ALP will catalyze a hydrolysis reaction cleaving off the phosphate group, resulting in the products *p*-nitrophenol (PNP) and hydrogen phosphate (Fig 4.17). *p*-nitrophenol absorbs light optimally at ~405nm (at the violet end of the visible spectrum), so we can use a spectrophotometer to measure changes in *p*-nitrophenol as ALP converts PNPP into PNP and hydrogen phosphate.

Note of Caution!

The PNPP reagent solution we will be using contains a fairly strong organic base. Wear eye protection and gloves, and do not wear open-toed shoes.

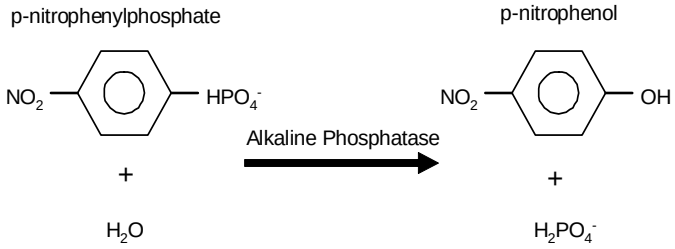

Figure 14.17. Conversion of PNPP into PNP and hydrogen phosphate.

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[b] A *peroxide* is any molecule that has within its structure two oxygen atoms that are linked together by a single covalent bond. This particular bond is quite unstable and thus peroxides in general are very reactive substances. Since peroxides could easily react with a vast array of different substances within a cell and disrupt cellular metabolism or damage cell structures, most cells try to quickly degrade any peroxides that form within them.

[c] A *phosphatase* is an enzyme that cleaves phosphate (PO_4) groups off of other organic molecules through hydrolysis reactions. This particular phosphatase is called "alkaline phosphatase" because its optimal pH is ~10. *Kinases*, a second family of enzymes, do the opposite of phosphatases—they add phosphate groups onto other molecules through dehydration synthesis reactions. Because phosphate groups typically have strong negative charges, their presence or absence can greatly alter the shape of proteins by affecting the various noncovalent interactions that determine tertiary and quaternary structure. Thus, phosphatases and kinases are used to activate or deactivate other enzymes, motor proteins, and a host of other components of a variety of cellular mechanisms.

In humans, the normal range of concentration for circulating ALP is 9-35 U/L. Elevated levels of alkaline phosphatase activity are often associated with liver and bone disease.

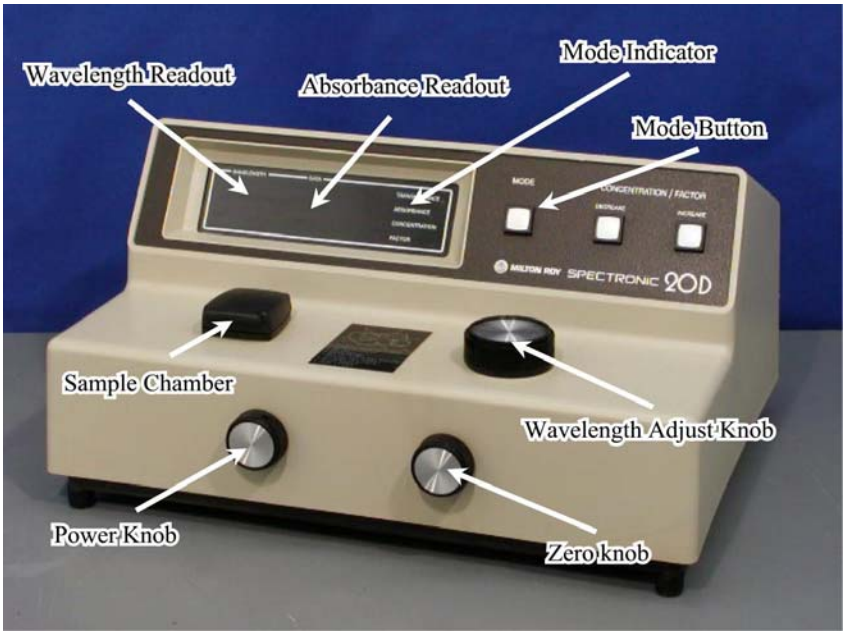

Fig 4.18. The Spectronic Spec 20 spectrophotometer.

Procedures

READ THIS BEFORE YOU START!!!

You are measuring reaction rates (change in product concentration per unit time). Therefore, it is *absolutely critical* that you take your absorbance measurements at the precise time intervals specified. In addition, you must keep in mind that even when you are taking your absorbance measurement the reaction will still be proceeding, and more product will be forming. So when you place your tube into the spectrophotometer, wait a few seconds, then record whatever absorbance value it gives you for a split second. Do not wait for the numbers to stop going up—they won't! This also means that you should not "pause" your timing of the reaction when you are taking your readings.

1. Your lab station should already have a prepared blank solution sealed in a spectrophotometer cuvette. You will need to calibrate the spectrophotometer first. See Fig 4.18 for a reminder of where the various controls are located on the instrument.

    - If not already on, turn on the machine with the Power Knob (front left)
    - Set wavelength to 405nm with the Wavelength Adjustment Knob (top right)
    - The instrument should have nothing in the sample chamber, the sample chamber lid should be closed, and the mode should be set to "Transmittance". If it is not set to transmittance, push the Mode Button until the mode indicator light is next to "Transmittance".
    - Set the transmittance to read "0.0" with front left knob.
    - Transfer the blank solution (Tube #1) into a clean cuvette. Place the cuvette in the sample chamber and close the lid. The readout on the instrument should read "100.0". If it does not, use the Zero Knob (front right) to adjust the value to 100.0.

- Push the Mode Button to switch the mode to "Absorbance". The readout should change to ".000". The instrument has now been calibrated.

Again, be sure that your instrument is set to ***absorbance* before you begin the experiment. Your instrument should read ".000", not "0.0" or "100.0" with the blank solution in the sample chamber.**

2. Add 3.0 ml of ALP reagent (the PNPP solution) to an empty cuvette with an automatic pipetter

3. Add 0.1 ml blood serum of blood serum, and immediately start timing your reaction. Quickly place your tube into a 30°C water bath.

NOTE: be *very* careful to remove *only* plasma from the sample. Avoid contaminating the plasma by disturbing the packed cells in the bottom of the microtube. Do not add contaminated (red tinted) plasma to your tube.

4. At EXACTLY 1 min after you have begun timing, remove the cuvette from the bath, quickly return to your station, wipe the outer surface of the cuvette dry with a Kimwipe, place the tube in the sample chamber, close the lid, and read the absorbance. Quickly remove the tube and return it to the 30°C bath.

NOTE: Do not "stop the clock" when you remove the tube from the bath. Your next reading should be timed in reference to the start of the reaction, not from the time you return the tube to the bath.

5. At EXACTLY 2 min after you have begun timing, take a second reading.

6. Repeat at EXACTLY 3, 4, and 5 min after you have begun timing.

Calculation of ALP concentration (U/L)

Since we are estimating enzyme concentration based on reaction rate, we need to calculate a rate value based on the data we have collected (absorbance and time). We can do so by dividing how much absorbance changed between the first and last measurement with how much time elapsed between the first and last measurement, as in the following equation:

$$\Delta A/min = \frac{A_{5min} - A_{1min}}{5\text{ min} - 1\text{ min}} = \frac{A_{5min} - A_{1min}}{4\text{ min}}$$

where $\Delta A/min$ is the average change in absorbance per minute, and A_{5min} and A_{1min} are the absorbance readings at 5 min and 1 min respectively.

Now that we have a measurement of change in absorbance per minute, we need to use this value to calculate enzyme activity. Normally, we would convert our rate of absorbance change into rate of product formation by determining the concentration of product needed to generate a particular absorbance (e.g., Beer's Law). In this experiment (and ONLY in this experiment), though, we can take a shortcut. The amount of light absorbed at 405 nm by precise concentrations of PNP is very well documented. As a result, we do not need to create a series of standard solutions of PNP to generate a standard curve so we can translate absorbance into product concentration. Rather, we can directly calculate *enzyme concentration* based on the following equation:

$$ALP\ Activity\ (\text{U/L}) = \frac{\Delta A/min \times total\ volume \times 1000}{18.45 \times path\ length \times sample\ volume}$$

The constant 18.45 is the millimolar absorptivity of PNP (basically, how much light would be absorbed at 405 nm by a solution of 0.001 mole PNP per liter). For our experiment, the total volume (ALP reagent + plasma) is 3.1 ml, which is subsequently multiplied by 1000 to convert ml measurements to L measurements. The path length (how far light travels through the reaction solution) is 1 cm, and the sample volume (plasma) is 0.1 ml. Thus our equation would look like this:

$$\textit{ALP Activity}\ (\text{U/L}) = \frac{\Delta A/min \times 3.1 \times 1000}{18.45 \times 1 \times 0.1}$$

and indeed we can simplify the equation to:

$$\textit{ALP Activity}\ (\text{U/L}) = \Delta A/min \times 1680.2$$