

Enzymes - Exercise 3 - Rockville

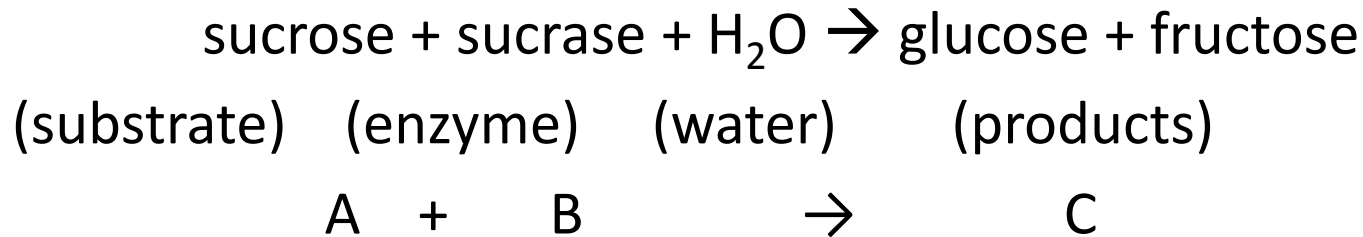
Objectives

- Understand the function of an enzyme.
- Know what the substrate, enzyme, and the product of the reaction for this lab.
- Understand how at various environments (e.g. temperature and pH) how it effects the function of an enzyme.
- Understand oxidation-reduction reaction(redox reaction)
OIL RIG (Oxidation is loss; reducing is gain).
- Understand: Increased amylase activity → increase maltose
→ increased redox reaction → increase reduced DNS →
→ increased color change → more abs.**

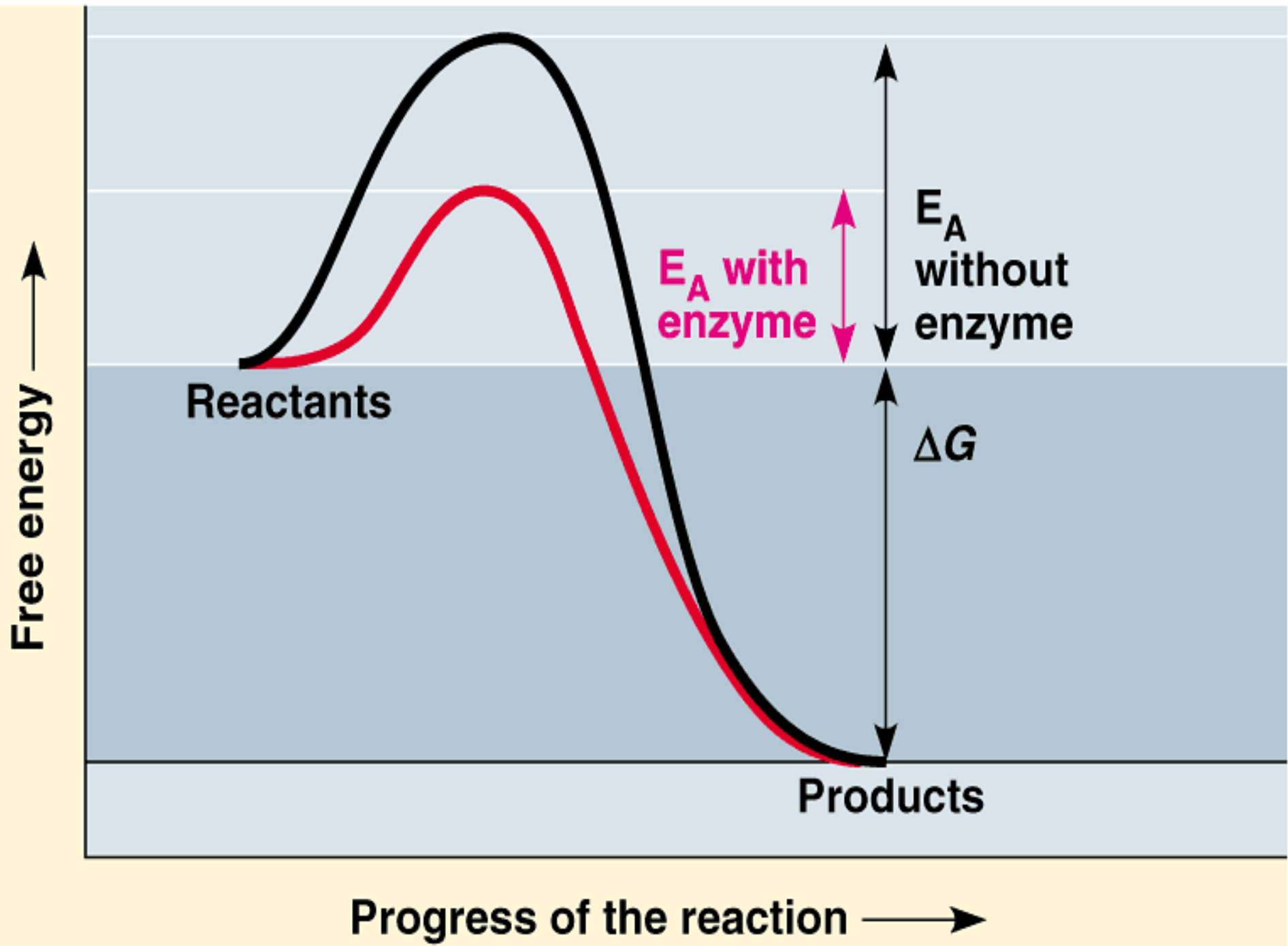
- Enzymes are proteins that act as catalysts. Catalysts increase the rate of a chemical reaction without being consumed by the reaction.
- An enzyme speeds up a reaction by lowering the reaction's activation energy. All reactions require some energy to get started (activation energy). Enzymes lower the activation energy they do not provide activation energy. All chemical reactions require activation energy (E_a). Without enzymes reaction in cells would be slow and take a very long time.
- Enzymes are not altered during a reaction and therefore they can be reused again and again.
- Enzymes are specific for certain reactions. Their three dimensional structure (tertiary structure) determines their specificity (FORM FITS FUNCTION). Temperature, pH, and salt concentration can alter the enzyme's tertiary structure, thereby altering the enzyme's function.
- In an enzymatic reaction, substrates bind to an enzyme's active site, creating an enzyme-substrate complex (ES). The enzyme-substrate complex then turns into an enzyme-product complex (EP). Finally, the enzymes detach from the product (P) and the reaction is complete.
- The function of the enzyme is dependent on the shape of its active site.

Enzymes

- Speed up chemical reactions (catalysts).
- Work by lowering the activation energy.
- Reactants the enzyme acts upon are called substrates.



- Chemical reactions change reactants into products by rearranging the chemical bonds between atoms. In the example below, substances A and B are the reactants while substance C is the product.
- Very specific for reactions
- Three dimensional shape determines function (tertiary, quaternary structure)
- Active site is region where the substrate binds
- Enzymes are not altered in a reaction and can be used again



Substrate Specificity of Enzymes

- The substrate
 - Is the reactant an enzyme acts on
- The enzyme
 - Binds to its substrate, forming an enzyme-substrate complex
- The active site
 - Is the region on the enzyme where the substrate binds

In an enzymatic reaction the substrate binds to the active site

- The catalytic cycle of an enzyme

1 Substrates enter active site; enzyme changes shape so its active site embraces the substrates (induced fit).

2 Substrates held in active site by weak interactions, such as hydrogen bonds and ionic bonds.

3 Active site (and R groups of its amino acids) can lower E_A and speed up a reaction by

- acting as a template for substrate orientation,
- stressing the substrates and stabilizing the transition state,
- providing a favorable microenvironment,
- participating directly in the catalytic reaction.

4 Substrates are converted into Products.

5 Products are Released.

6 Active site is available for two new substrate Mole.

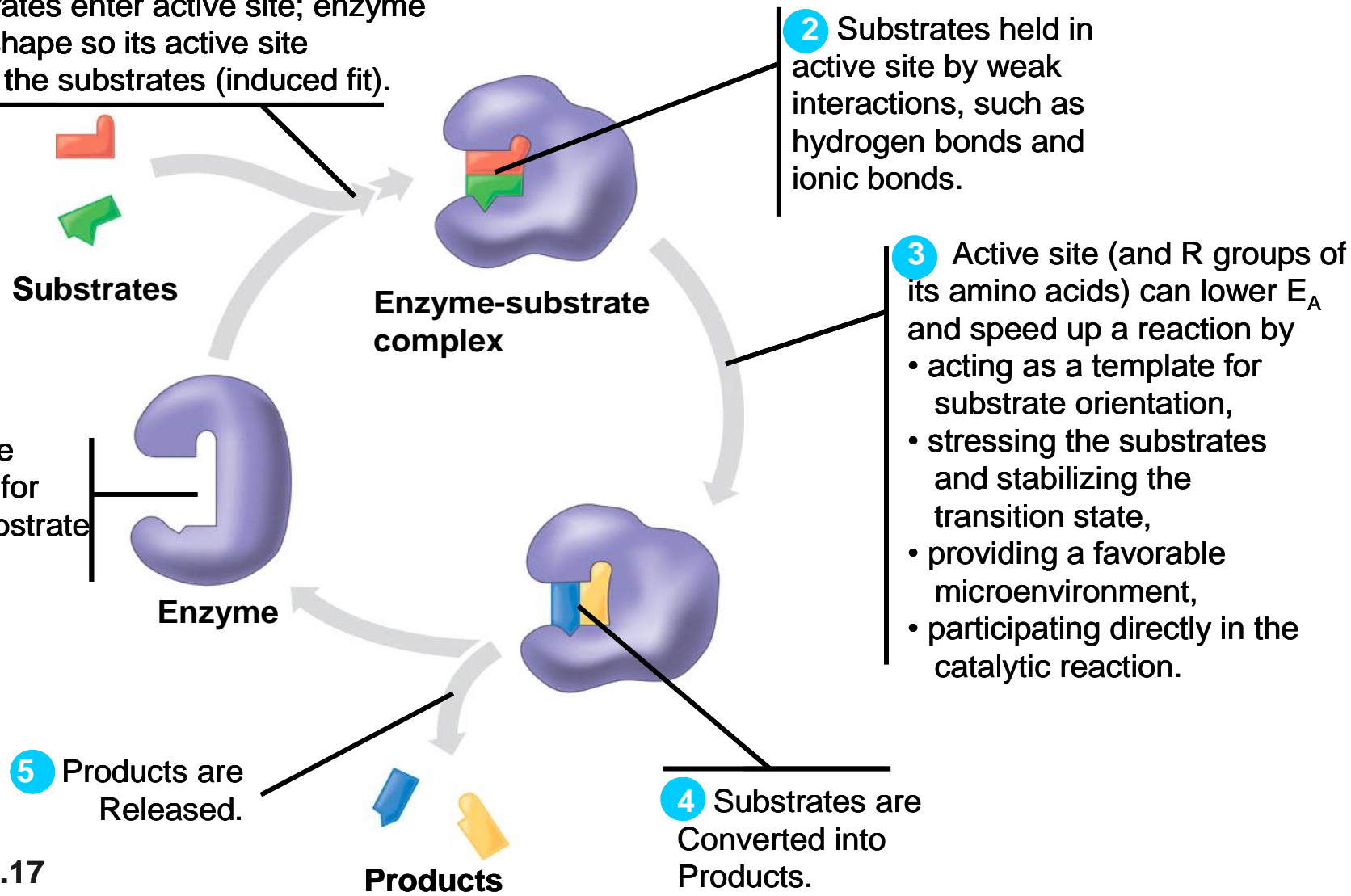


Figure 8.17

Chemical reactions change reactants into products by rearranging the chemical bonds between atoms. In the example below, substances A and B are the reactants while substance C is the product.



The functioning of an enzyme is dependent on the proper shape of its active site. Since all enzymes are globular proteins, a change in the tertiary structure of an enzyme may change the shape of the active site and either slows down or stops the reaction. **An enzyme's physical and chemical environment plays very important roles on its tertiary structure, thus on its activity. Physical factors like hydrogen ion concentration (pH), temperature (the average kinetic energy of molecules), and salt concentration can affect an enzyme's activity by altering its tertiary structure. Chemical factors like inhibitors and cofactors also influence an enzymes activity.** Inhibitors are chemicals that selectively inhibit the action of specific enzymes. An inhibitor can inhibit the function of a specific enzyme by competing the substrate for the enzymes. An inhibitor can inhibit the function of a specific enzyme by competing the substrate for the enzyme's active site (competitive inhibitor). Cofactors are non-protein helper molecules required for many enzymes' catalytic activities. Not all enzymes require cofactors. A cofactor for a specific enzyme can be an inorganic ion or molecule (prosthetic group) or an organic molecule (coenzyme). Mg^{++} , Ca^{++} , Fe^{++} , NADH, $FADH_2$ and NADPH are some examples of cofactors. **Note: Most enzymes end in -ase.**

- **Today you worked with amylase which catalyzes the digestion (hydrolysis) of the polysaccharide starch to the disaccharide maltose.** Amylase is produced by a wide range of organisms, including humans. The amylase used in this exercise has been derived from a microorganism.

amylase

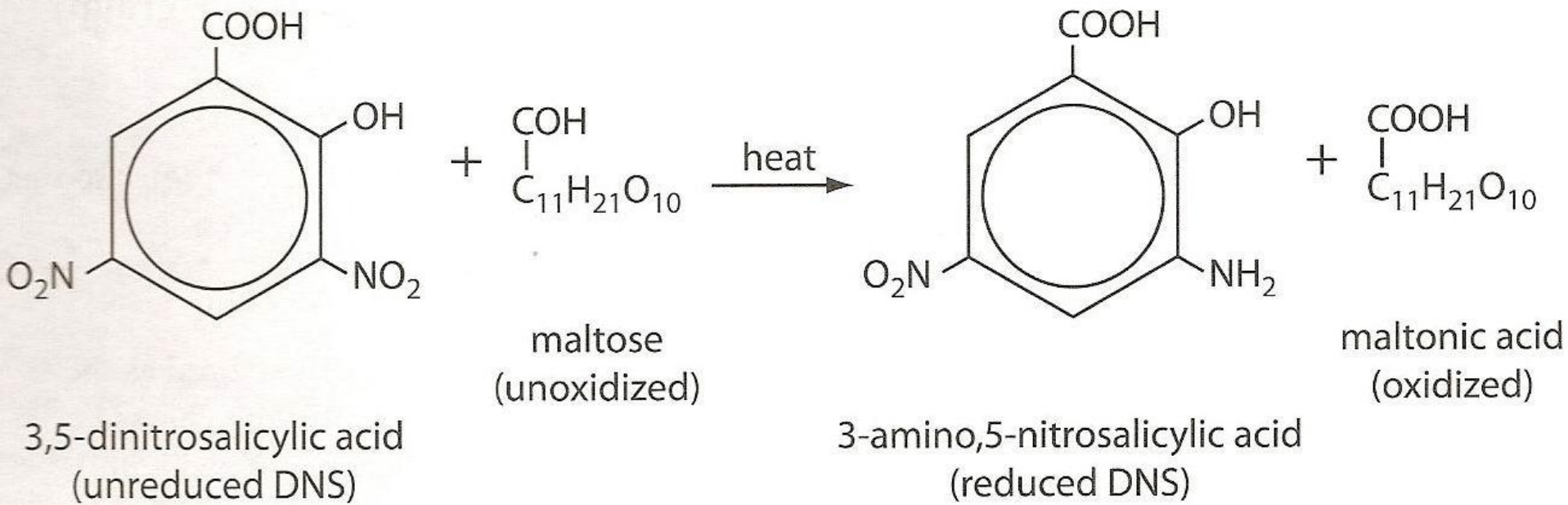
Reaction: STARCH + Water -----→ MALTOSE

- **In the above enzymatic reaction, starch and water are the substrate molecules, amylase is the enzyme, and maltose is the product. Notice that the enzyme amylase is not consumed or changed in the reaction.**
- Most substrate and products of enzymatic reactions are colorless. This is true for the reaction conducted in lab. In the laboratory, colorimetric procedures are performed to determine the disappearance of substrate (s) or the appearance of the product(s) in the reaction mixture. These kinds of chemical identifications of enzyme substrate and products are called enzyme assays.

- To determine positive amylase activity by a simple assay, you can add iodine to the reaction mixture after incubation of amylase with starch for certain time intervals. Normally a starch solution turns a blue-black when iodine is added. If amylase works on starch, the complex structure of starch is destroyed, and the intensity of the dark blue color fades away. Complete hydrolysis of starch is indicated by a yellowish color when combined with iodine.
- **Starch + no amylase + incubation + iodine = dark blue-black.**
- **Starch + amylase + incubation + iodine = light blue to brown to yellow.**

- Another kind of assay (which you will be using today) can also be performed to determine positive amylase activity. **In this assay, the presence of maltose, a reducing sugar, is detected by performing an oxidation-reduction reaction.** Here, the reaction mixture (water, amylase, undigested starch, and maltose) is mixed with an alkaline solution of 3, 5-dinitrosalicylic acid (DNS) and boiled for 5-10 minutes. **Due to the presence of a carbonyl group (C=O), maltose participates in an oxidation-reduction reaction with DNS. The carbonyl group of maltose gets oxidized and changed into a carboxyl group and the 3, 5-dinitrosalicylic acid is reduced and changed into 3-amino, 5-nitrosalicylic acid (simply can be termed as reduced DNS). This reaction cause a change in color form yellow to orange or red, depending the concentration of maltose produced.** As you might expect, there will be no color change on a control without the enzyme, Using the controls as the “blank” for the absorbance in a colorimeter, **the value of absorbance is measured on the “samples” where the enzyme was present and is interpreted as the amount of maltose produced, thus showing the extent of amylase activity.**

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OXIDATION-REDUCTION REACTION BETWEEN MALTOSE AND DNS

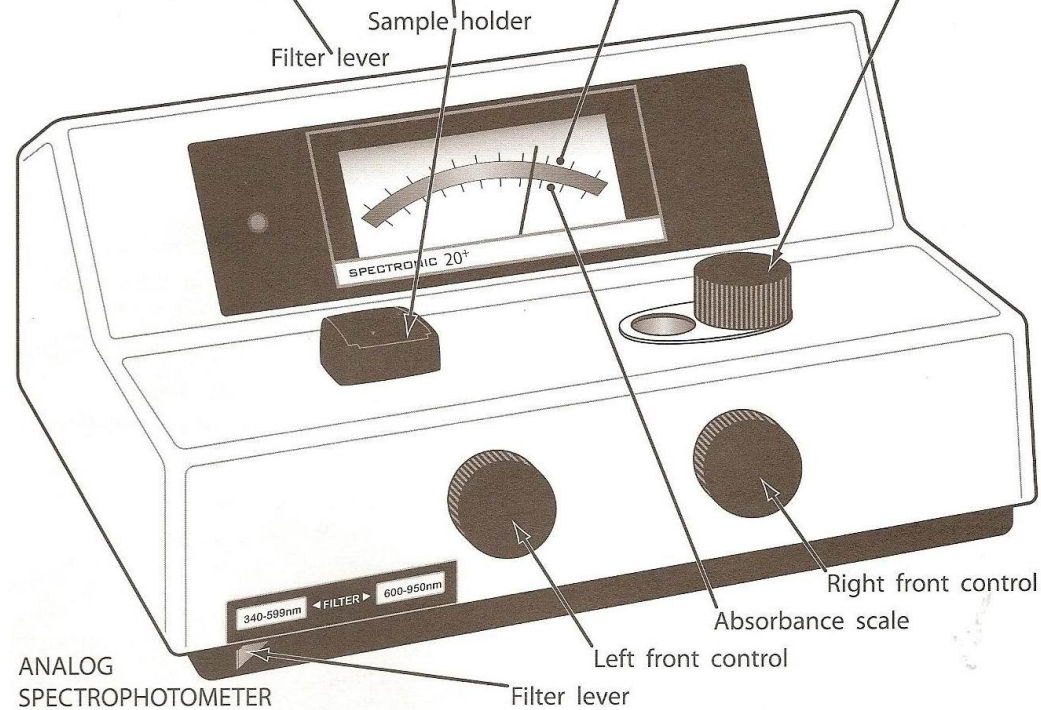
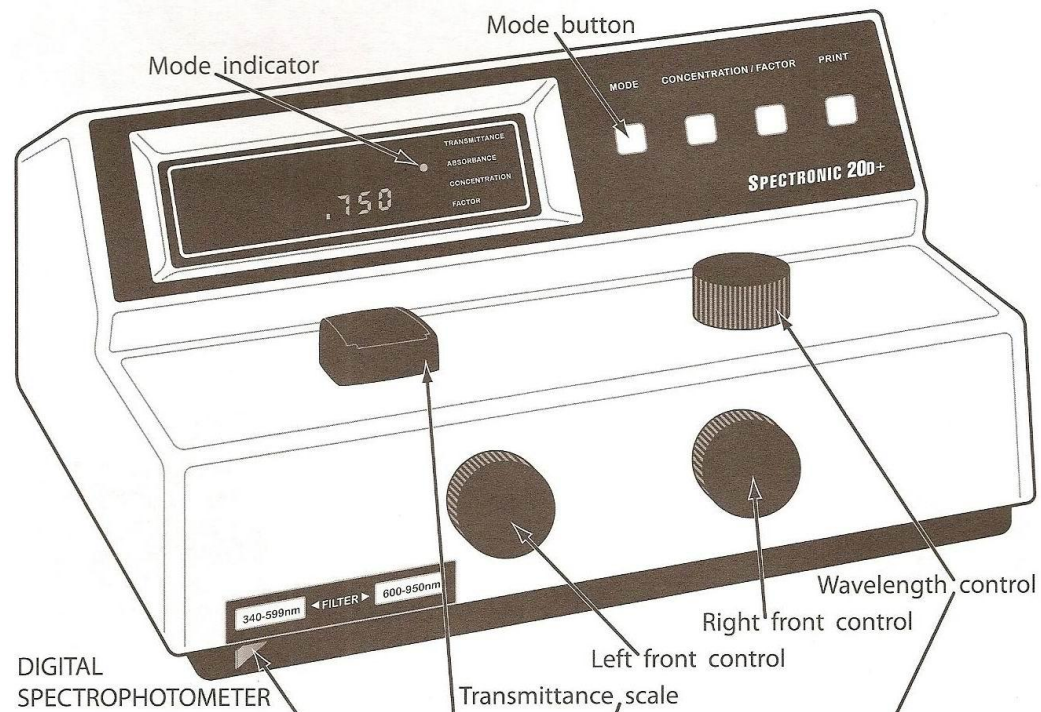
DNS reagent (yellow solution)

Effect of Varying Temperature

- Equipment and materials required:
- Spectrophotometer with a pair of cuvettes.
- 2. Buffered starch solution (2%) for temperature experiment.
Please do not use this starch for pH experiment.
- 3. Frozen α -amylase solution, frozen enzyme solution in microfuge tube(s) will be given to you.
- 4. Water baths fixed at 45°C, 65°C, and 85°C.
- 5. Ice and a beaker to make a 3°C ice bath.
- 8. **DNS reagent (yellow solution).**

- Procedure:
- 4. Label the tubes as follows: “B/t,” “3°,” “25 °,” “45 °,” “65 °,” and “85 °.” The ‘B/t’ tube will be used as the control or ‘blank’ and will be kept at room temperature. Add an identifying character after each labeled to enable you to recognize your tube if they become mixed with the tubes of other students.
- 5. Ask your instructor and obtain 2% buffered starch solution. ***(Please be careful to avoid the 3% regular starch that is made for pH experiment. Using the wrong tube of starch will completely jeopardize your experiment.)***
- 8. Leave the “B/t” tube along with the tube marked at 25 ° C at room temperature. Keep the tubes away from the Bunsen burner. Temperature may not be uniform near the burner.
- 9. Place the remaining tubes into their appropriate temperature-controlled environments. Tube labeled “3°” goes into ice bath, tube “45 °” into 45 ° water bath, tube “65 °” into 65 ° water bath, and tube “85 °” into 85° water bath.
- 10. Incubate in their environments for 10 minutes to allow the starch to equilibrate to the temperature. ***Do not remove the tubes from their respective environments until you finish step 14, the addition of DNS to the tubes.***

- 12. Dispense 100uL of water to the “B/t” tube and left slightly out to quickly agitate the tube and replace back into its environment. This tube will serve as the control; do not add enzyme to this tube.
 - 13. Using a fresh tip, add 100uL of enzyme to each of the remaining tubes, letting the tip touch the side of the rest tube as your dispense.
 - 14. Collect DNS reagent from your instructor if you have not done so. Set to appropriate 1,000uL (1mL), and add a fresh tip. While the tubes are in their environmental temperatures, pipette the DNS reagent (orange) into each tube (including the B/t tube). This will stop the enzymatic reaction.
 - 15. Agitate each tube well, take all the tubes from their respective environments, and place the tubes into the beaker of boiling water for 5 minutes to speed up the oxidation-reduction reaction. You should see a color change in several tubes.
 - 18. Use the spectrophotometer to get your reading.
 - 19. Measure the absorbance of colored solutions.
- Spectrophotometer was set at 450nm wavelength.*

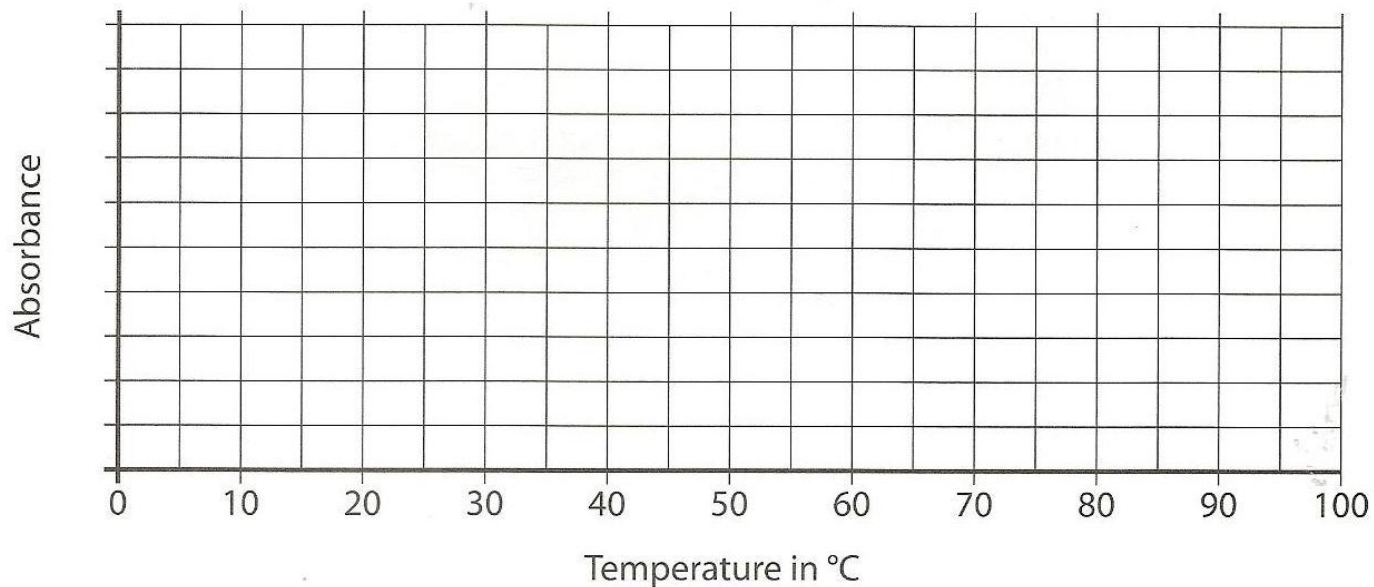


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Data for Effect of Varying Temperature (Heat)

| Tube/temperature | Initial absorbance reading | Final absorbance value (absorbance \times dilution factor) |
|------------------|----------------------------|---|
| B/t | | |
| 3°C | | |
| 25°C | | |
| 45°C | | |
| 65°C | | |
| 85°C | | |

20. Plot a graph with your data.



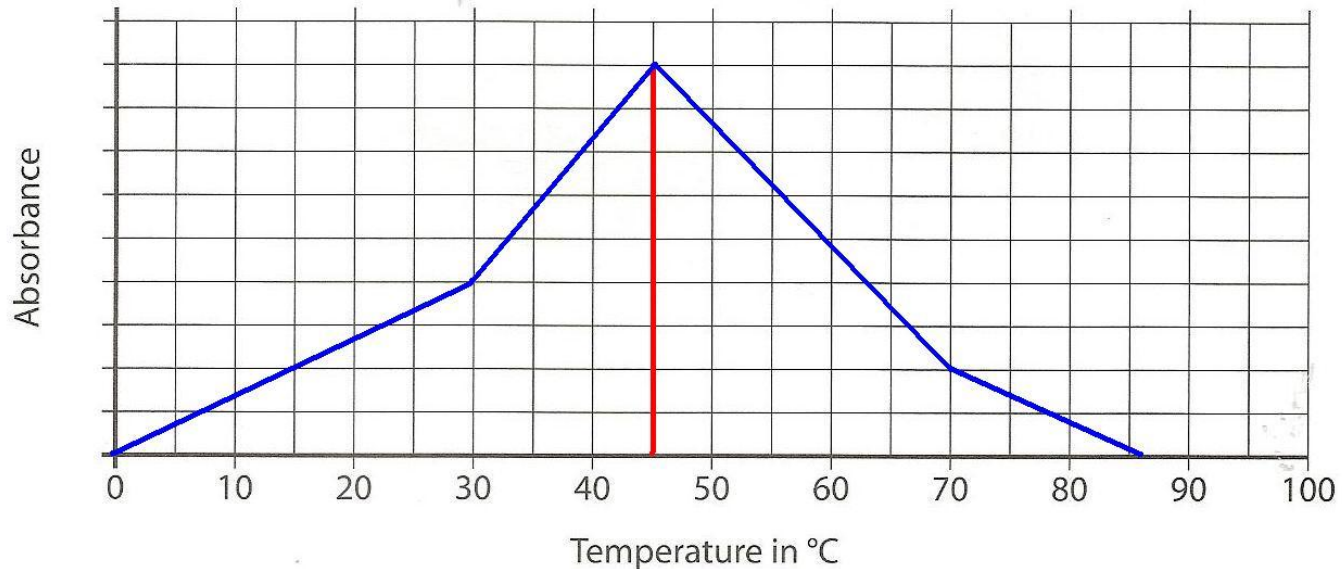
Your graph should look something like this

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Data for Effect of Varying Temperature (Heat)

| Tube/temperature | Initial absorbance reading | Final absorbance value (absorbance \times dilution factor) |
|------------------|----------------------------|---|
| B/t | | |
| 3°C | | |
| 25°C | | |
| 45°C | | |
| 65°C | | |
| 85°C | | |

20. Plot a graph with your data.

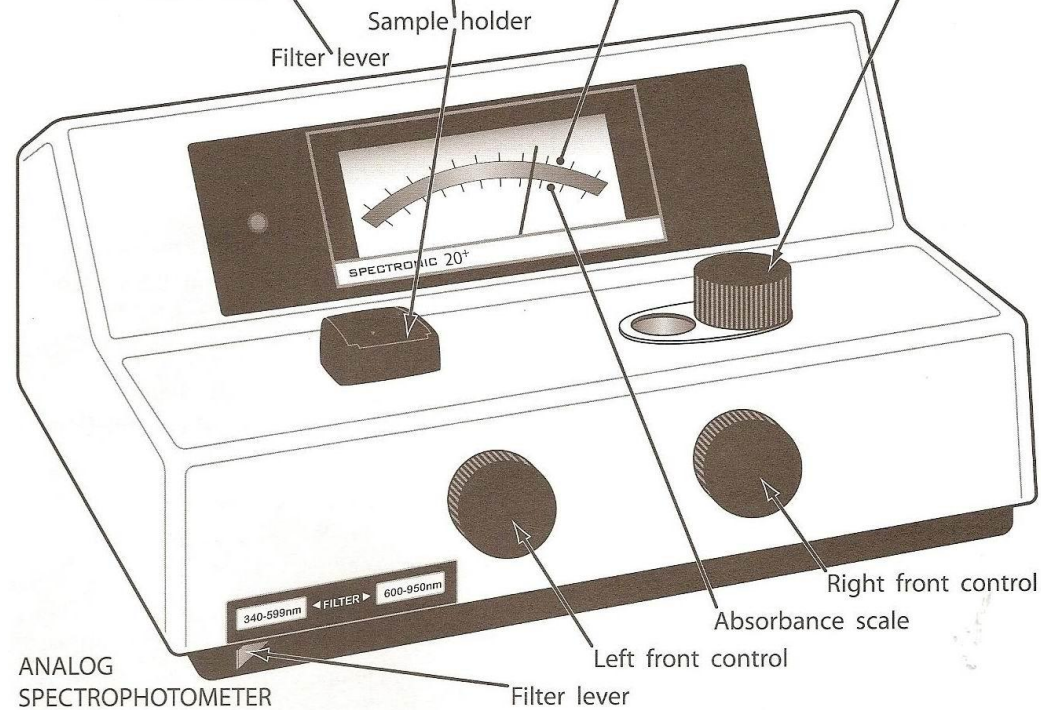
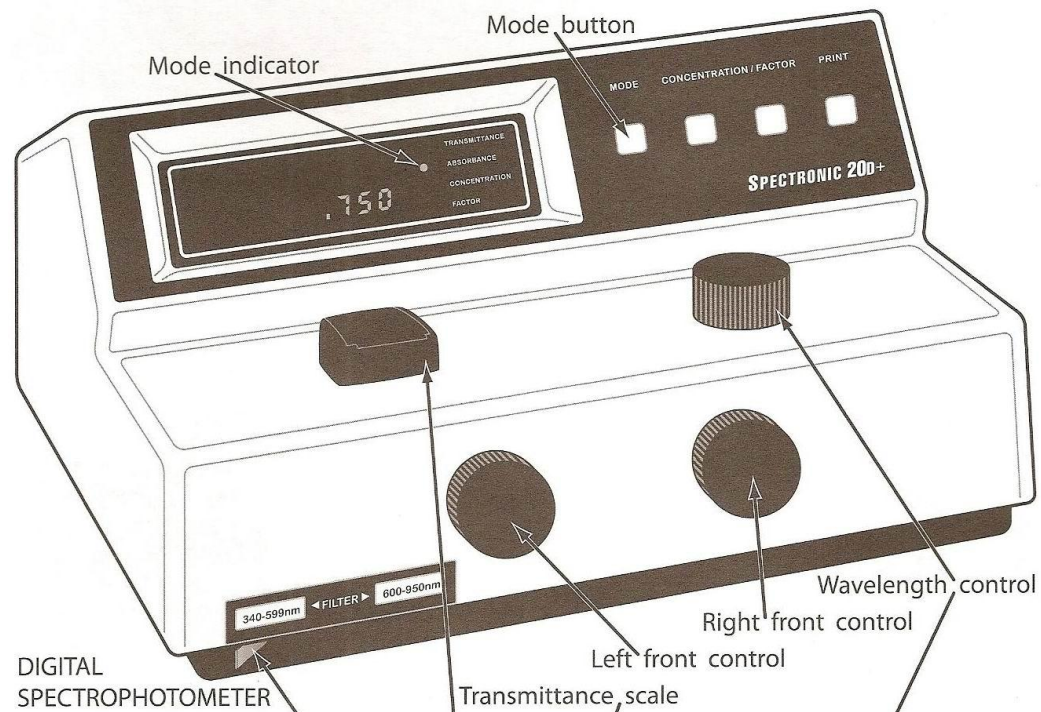


Effect of Varying pH (acidity)

- Equipment and materials required:
- 1. Spectrophotometer with 1 cuvette.
- 2. Starch solution (3%) for pH experiment.
- 3. Frozen α -amylase solution. Frozen enzyme solution in microfuge tube(s) will be given to you.
- 4. Buffers for pH 1 (0.1M HCl), pH 3, pH 5, pH 7, and pH 12 (0.1M NaOH)
- 5. Six test tubes.
- 6. DNS reagent (yellow solution). Obtain it from your instructor when you require it.

- Procedure:
- 3. Label the tubes as follows: “B/p,” “1,” “3,” “5,” “7,” and “12.” The “B/p” tube will be used as the control or blank. Add an identifying character after each label to enable you to recognize your tubes if they become mixed with the tubes of other students.
- 4. Collect the 3% regular starch solution from your instructor.
- 5. Set up the micropipette to 450uL, add a fresh tip, and dispense 450uL of starch into each tube.
- 6. Using fresh tips for each buffer, add 450uL of pH 1 buffer to the tube “1”, pH 3 buffer to tube “3”, pH 5 buffer to tube “5”, pH 7 buffer to tube “7”, and pH 12 buffer to tube “12”. To the tube labeled “B/p”, add 450uL of buffer pH 5. Keep the tubes at room temperature.
- Set up the micropipette to 100uL and add a fresh tip. Dispense 100uL of water to the “B/p” tube and agitate the tube, and let it sit. This tube will serve as control; do not add enzyme to this tube.
- 7. Using a fresh tip, add 100uL of enzyme to each of the remaining tubes, letting the tip touch the side of the test tube as you dispense. Immediately check the time. Agitate the mixture of ensure that it is well mixed and let it remain in room temperature for 7 min.

- 8. Set the appropriate micropipette to 1,000uL (1mL) and a fresh tip, then pipette the DNS reagent (orange) into each tube (including the B/p tube). This will stop the enzymatic reaction.
 - 9. Agitate each tube well and place the tubes into the boiling water bath for 5 minutes. You should see a color change in some of the tubes.
 - 10. Carefully remove the tubes and put them into the test tube rack.
 - 12. Mix the content in each tube by agitating the tube. You are now ready to use the spectrophotometer to get your reading.
 - 13. Measure the absorbance of the colored solutions.
- Spectrophotometer was set at 450nm wavelength.*

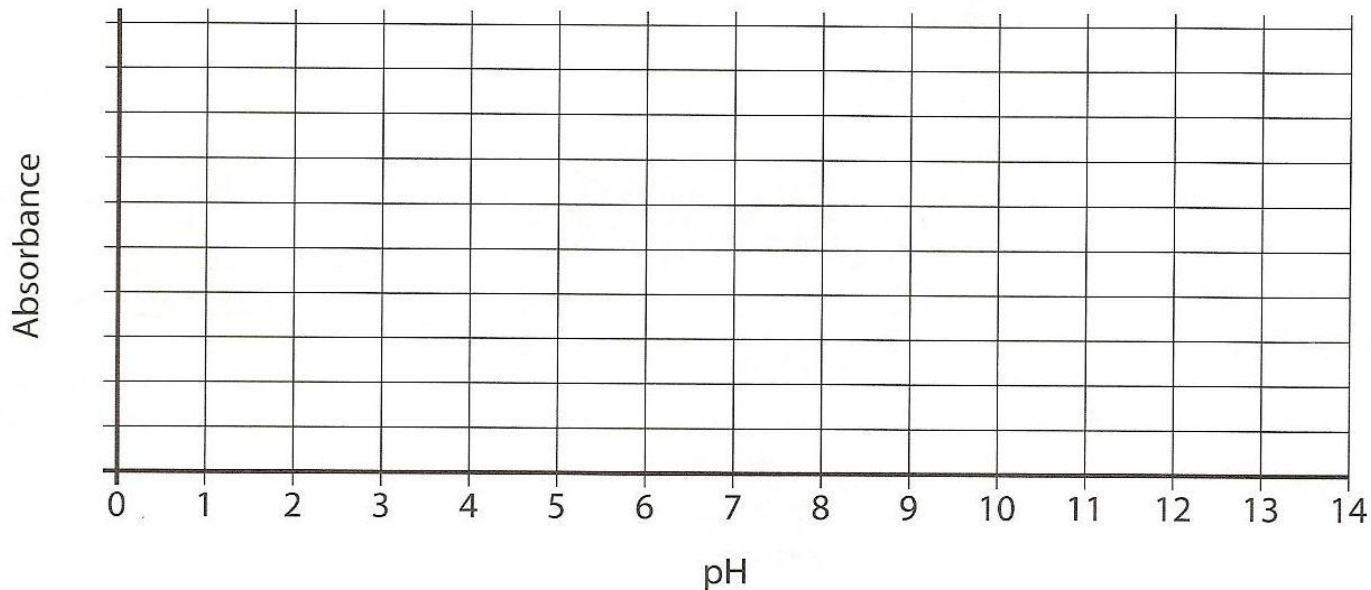


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Data for Effect of Varying pH (Acidity)

| Tube/pH | Initial absorbance reading | Final absorbance value (absorbance \times dilution factor) |
|---------|----------------------------|---|
| B/p | | |
| pH 1 | | |
| pH 3 | | |
| pH 5 | | |
| pH 7 | | |
| pH 12 | | |

15. Plot a graph with your data.



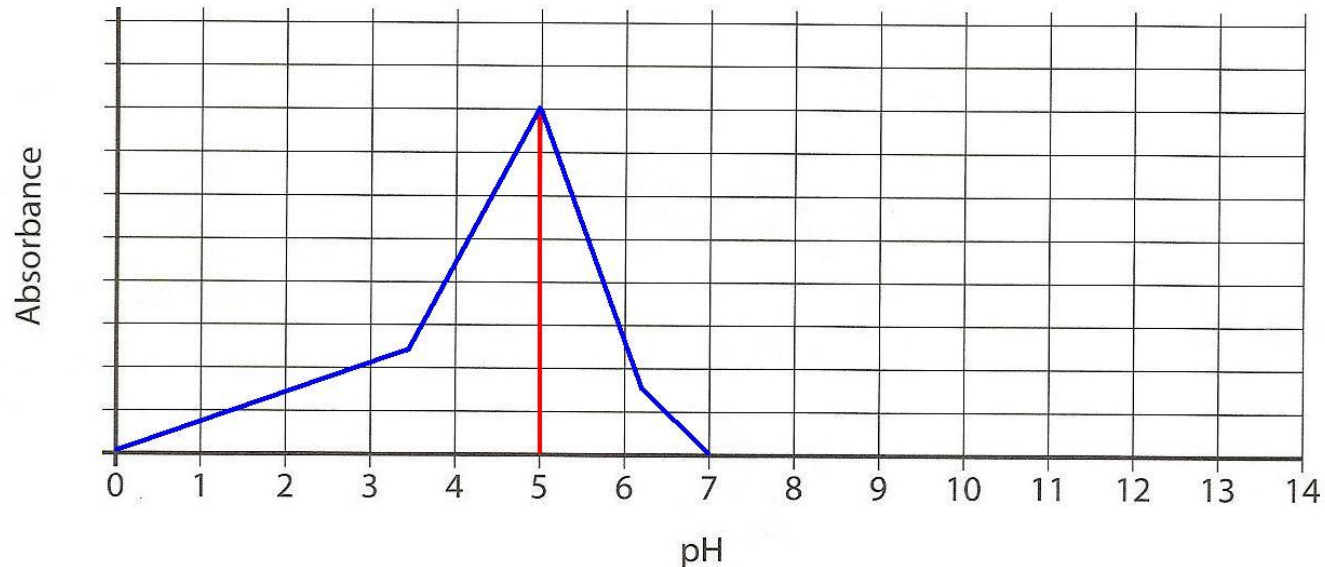
Your graph should look something like this

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Data for Effect of Varying pH (Acidity)

| Tube/pH | Initial absorbance reading | Final absorbance value (absorbance × dilution factor) |
|---------|----------------------------|--|
| B/p | | |
| pH 1 | | |
| pH 3 | | |
| pH 5 | | |
| pH 7 | | |
| pH 12 | | |

15. Plot a graph with your data.



- **Purpose: Looking at different environment on enzyme function. This reaction is to determine how well this enzyme works based on different environments.**
- **Amylase** is the enzyme that catalyzes the **hydrolysis** of the **polysaccharide starch** in to the **disaccharide maltose**:

Hydrolysis

Enzyme (amylase)

- **Substrate (Starch) + water -----> Product (Maltose)**
- **Maltose donates e- (reducing sugar – gets oxidize) -----> DNS (Becomes reduced DNS).**
- **This changes color to from yellow to orange to red.**

- Look at amylase activity by performing an **oxidation-reduction reaction**. An oxidation-reduction reaction (redox reaction) is a chemical reaction involving **the transfer of one or more electrons** from one reactant to another.
- OIL RIG (Oxidation is loss; reducing is gain).
- **Oxidation** - the loss of electrons by a substance.
- **Oxidizing agent** - the electron acceptor
- **Reducing agent** - The electron donor
- This is a generalized redox reaction where (e^-) depicts an electron
- $X^{e^-} + U \rightarrow X + Y^{e^-}$
- X is the reducing agent; Y gets reduced
- Y is the oxidizing agent; X gets oxidized

- You will use the redox reactions between maltose and DNS to study the extent of the amylase activity.
- DNS is the oxidizing agent (it is the electron acceptor).
- Maltose is the reducing agent (it is the electron donor) because it contains the carbonyl (C=O) functional group.
- It produces reduced DNS, which cause a color change from yellow to orange to red (depending on the concentration of maltose).
- The color of your reaction indicates the extent of amylase activity.

Examine absorbing of reduced of DNS.

- If you have a high absorbance of reduce then more maltose produced because higher amylase activity.
- Increased amylase activity → increase maltose → increased redox reaction → increase reduced DNS → increased color change → more abs
- In the optimal pH or temperature, the most rapid hydrolysis of starch to maltose occurs.

Effects of Temperature

- Each enzyme
 - Has an optimal temperature in which it can function

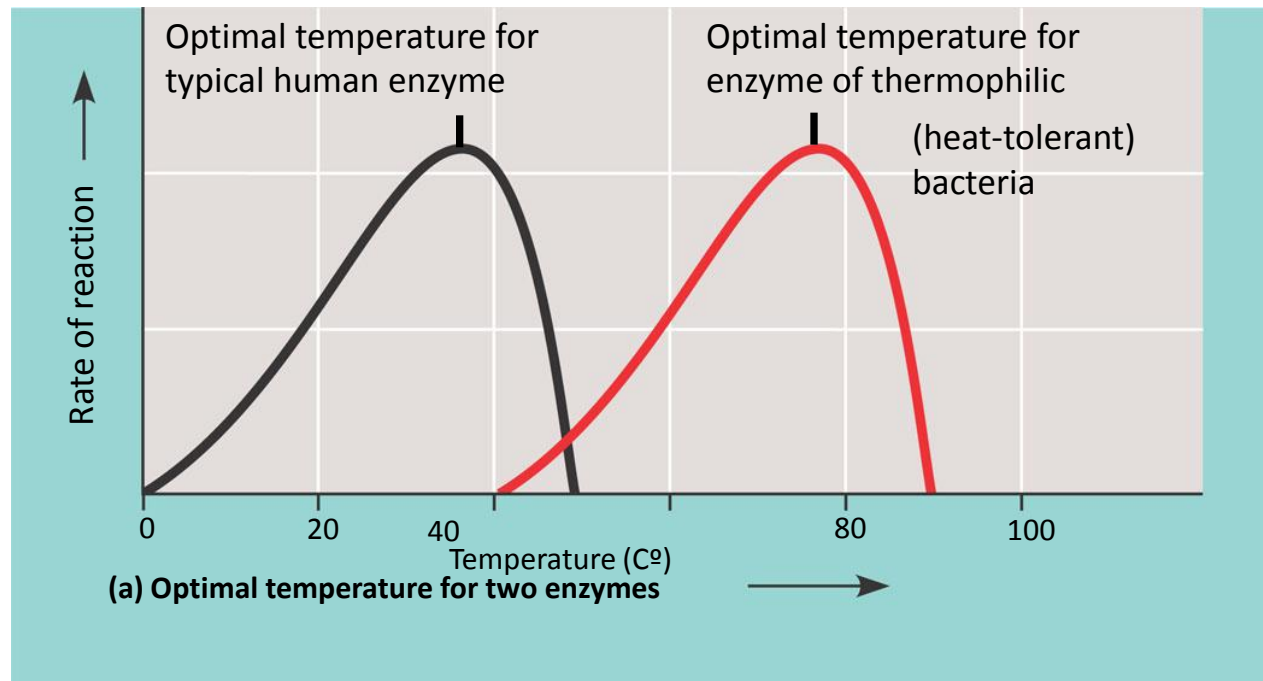
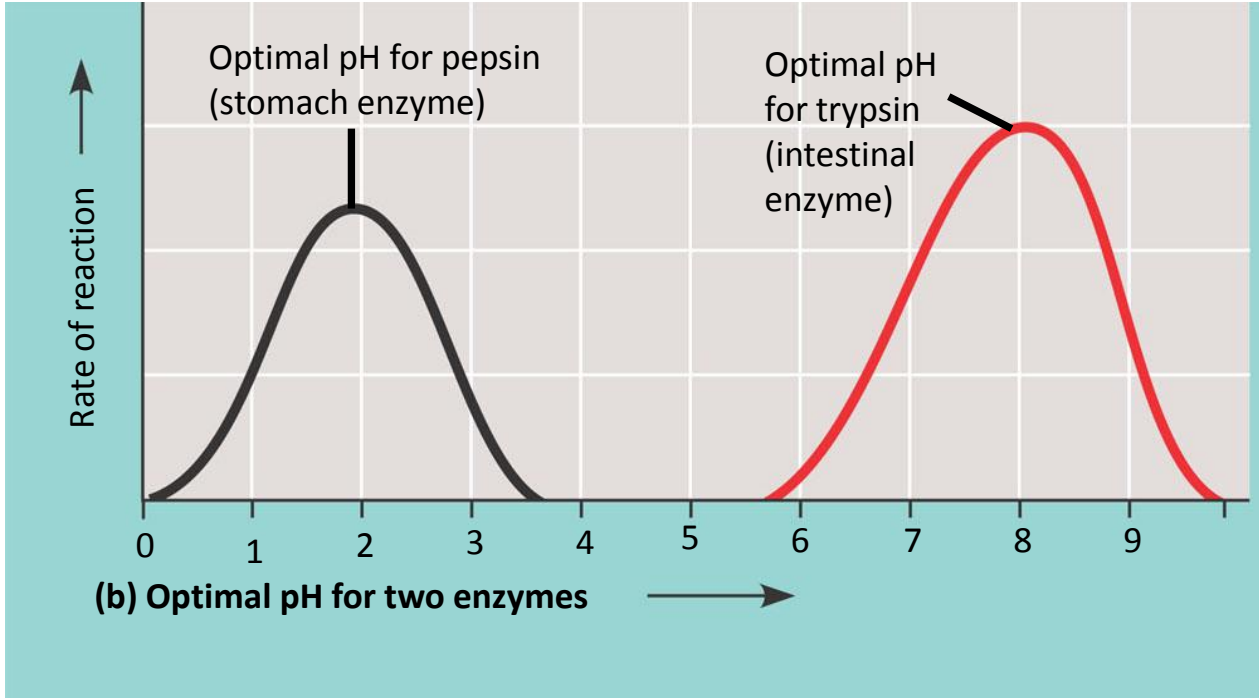
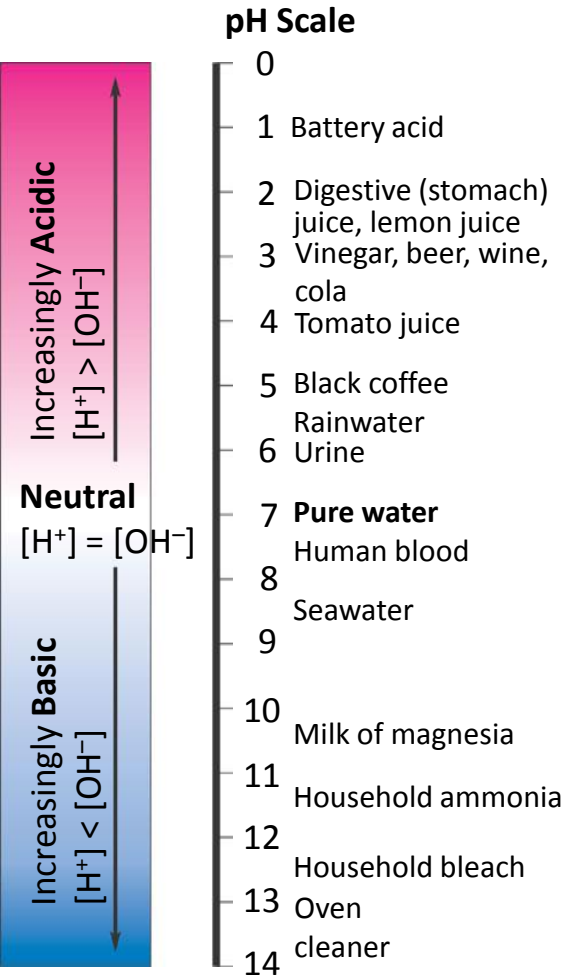


Figure 8.18

Enzymes have an optimal pH in which it can function

The pH of a solution

- Is determined by the relative concentration of hydrogen ions (H⁺)
- Is low in an acid
- Is high in a base



Questions

- 1. What is an Enzyme?
- 2. Enzymes increase the rate of a reaction by.
 - A. Decreasing the requirement of activation energy.
 - B. Providing the activation energy to the reaction.
 - C. Both A & B
 - D. None of the above.
- 3. Do you expect an enzyme to be active at 0 degrees C. Why?
- 4. Name three physical and chemical factors that affect enzyme's activity (denature a protein/enzyme)?
- 5. All enzymes function optimally at PH 7.4
True or False

Questions

- 1. What is an Enzyme: **A protein catalyst that speeds up a reaction without getting consume in a chemical reaction. It lowers the activation energy barrier too.**
- 2. Enzymes increase the rate of a reaction by.
A. Decreasing the requirement of activation energy.
B. Providing the activation energy to the reaction.
C. Both A & B
D. None of the above.
- 3. Do you expect an enzyme to be active at 0 degrees C. Why? **Yes, the enzyme is still active but is working at slower rates.**
- 4. Name three physical and chemical factors that affect enzyme's activity (denature a protein/enzyme)?
Temperature, pH, Concentration (salt), Cofactors, & Inhibitors
- 5. All enzymes function optimally at PH 7.4
True or **False**

Questions

- 6. What is the importance of enzyme in living systems?
- 7. The optimum temperature of our enzyme was?
- 8. In general, as temperature increases, the rate of enzyme activity (increases/decreases) up to a certain point called the optimum temperature, at which activity is at a maximum. After this point, activity (increasing/decreases). At very high temperatures we see (high/low/no) activity because the enzyme is denatured.

Questions

- 6. What is the importance of enzyme in living systems?

Enzymes speed up reaction

- 7. The optimum temperature of our enzyme was **35 degrees Celsius.**

- 8. In general, as temperature increases, the rate of enzyme activity (**increases**/decreases) up to a certain point called the **optimum** temperature, at which activity is at a maximum. After this point, activity (increasing/**decreases**). At very high temperatures we see (high/low/**no**) activity because the enzyme is **denatured.**

Questions

- 9. Why hydrolysis of starch by amylase, in your enzyme lab experiment, was very slow or failed to happen at 85°C?
- 10. What happens to the active site of amylase at very high temperatures?

Questions

- 9. Why hydrolysis of starch by amylase, in your enzyme lab experiment, was very slow or failed to happen at 85°C? **The enzyme (amylase) at this point was denatured, so there was no enzyme to work on the starch, so less product was produced.**
- 10. What happens to the active site of amylase at very high temperatures? **It's not active anymore; therefore, the reaction doesn't occur.**

Questions

11. Suppose you were given amylase solution and starch solution in two separate containers. You labeled three test tubes (A, B, & C) and took 1 ml of amylase solution in each tube. Then you put (incubate) the tube A at 0° C, tube B at 45° C, and tube C at 100° C for 30 minutes. At this stage assume that the enzyme incubated at 100° C will be completely denatured. After taking the tube out of the incubator, you added 2 ml of 2% starch solution to each tubes and kept the tubes (A, B, and C) at 45 for an hour. Then you took small amount of the solutions and performed DNS assay, Iodine assay, Benedict's assay and Biuret assay. Fill in the following table based on the expected results of your experiments

| Test Done | Solution in Tube A (+ or -) | Solution in Tube B (+ or -) | Solution in Tube C (+ or -) |
|----------------|-----------------------------|-----------------------------|-----------------------------|
| DNS Assay | | | |
| Iodine Assay | | | |
| Benedict Assay | | | |
| Biuret Assay | | | |

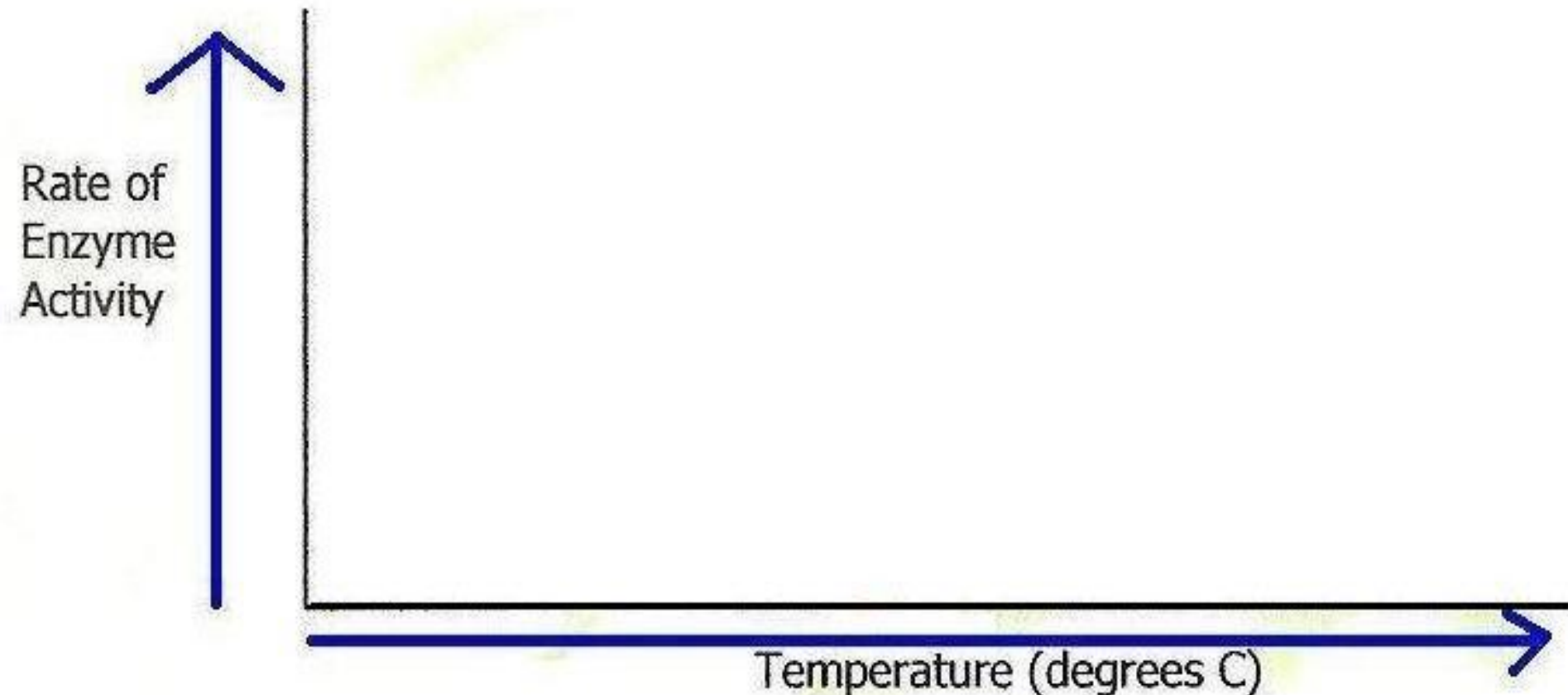
Questions

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| Test Done | Solution in Tube A (+ or -) | Solution in Tube B (+ or -) | Solution in Tube C (+ or -) |
|----------------|-----------------------------|-----------------------------|-----------------------------|
| DNS Assay | + | + | - |
| Iodine Assay | - | - | + |
| Benedict Assay | + | - | - |
| Biuret Assay | + | + | + |

Questions

- Draw a curve representing enzyme activity as it changes with temperature. Label the point of the optimum temperature.



Questions

- Draw a curve representing enzyme activity as it changes with temperature. Label the point of the optimum temperature.

