

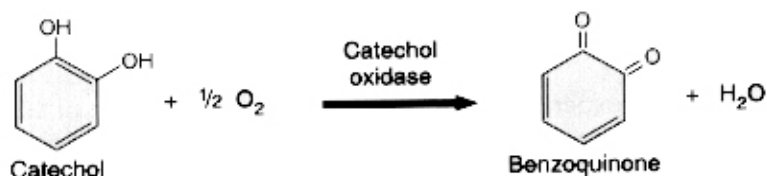
Enzyme Activity

Try to imagine a life in the absence of enzymes. Even reading this line would take years if it could be read at all! Enzymes act as catalysts for the chemical reactions that occur in living organisms, allowing reactions to occur in the milliseconds necessary to maintain life.

Chemically, enzymes are proteins. Each specific enzyme has a unique physical structure that is essential for its function. The shape of each specific enzyme "fits" the shape of the reacting molecule(s) for which the enzyme serves as a catalyst. Because of the enzyme "fit", the reacting molecules are brought together at the appropriate bonding sites. The enzyme, therefore, "lowers" the activation energy of the chemical reaction.

In a reaction catalyzed by an enzyme, the reacting molecules are called the **substrate**. The substrate molecules combine with the active site of the enzyme forming a temporary complex called the **enzyme-substrate complex**. As the chemical reaction takes place and the **products** are formed, the enzyme is released, unchanged from its original structure. Since the enzyme is not consumed or changed by the chemical reaction, it can be used over and over to catalyze additional substrate molecules.

In this laboratory you will observe the activity of the enzyme, catechol oxidase, an enzyme that catalyzes the conversion of the chemical catechol to a brown pigmented substance, benzoquinone. Catechol oxidase is found in the cells of many organisms. You have probably observed this reaction a number of times when you have cut potatoes or many fruits and left them out on the counter. They turn a rusty or brownish color. The potato is a good source for this enzyme.



Since catechol is colorless and the product, benzoquinone, is a rusty-brown color, the chemical reaction is easy to detect. In addition, the intensity of the pigment produced is a reflection of the amount of catechol that is converted. This, in turn, can tell you how effectively the catalyst, catechol oxidase, has worked under a set of experimental conditions.

Materials Needed For the Entire Class:

Spectrophotometers (Turned on at Beginning of Lab)
Spectrophotometer cuvettes
Kimwipes tissues
Hot plates
Hot water bath at 40°C
Hot water bath at 60°C
Stock buffer solutions, with pipette assembly, of:
pH 2
pH 4
pH 6
pH 7
pH 8
pH 10
pH 12
Ice
Bottle of pH7 Buffer – kept cold

Distilled water – kept cold
Test tubes (50 per group)
Test tube racks (3 per group)
Medicine droppers
Test tube clamps
China markers (grease pencil)
600-ml Beakers (3 per group)
Thermometers
Potato juice extract* (the catechol oxidase source)
Dropper Bottles of:
1% Catechol (**Poison!**)
1% Hydroquinone (**Poison!**)
Phenylthiourea (PTU) (**Poison!**)
Large culture dish (1 per group)
50-ml beaker (1 per group)
250-ml beaker (1 per group)

Catechol, hydroquinone, mercuric chloride and phenylthiourea are poisons and catechol, in concentration, is a mutagen. Use caution handling these chemicals. Avoid contact with these solutions. If a spill occurs, immediately wipe up the spill with dry paper towels and then use disinfectant solution on the spill site. Dispose of all towels in a designated contamination location. Be sure to notify your instructor of the spill and your clean-up procedures. Your personal safety and the safety of other students is most important.

Preparation of the Catechol Oxidase Extract (This will be done in advance by the lab staff, your instructor or by student volunteers)

Materials Required

- 2 potatoes, peeled and cut into chunks
- 1 Blender
- 3 Liters cold distilled water
- 1 1000-ml beaker
- 1 Large funnel
- Several layers of cheesecloth

Procedure

- Peel and chunk the potatoes. Put the peeled, chunked potatoes in a blender. Add 700 ml of cold distilled water and blend at high speed for 2 minutes.
- Line a large funnel with several layers of cheesecloth and place the funnel in a 1000 ml beaker, which has been placed in a container of ice. Filter the potato juice through the beaker
- The filtrate is the potato juice-catechol oxidase extract. It is called "catechol oxidase" throughout the exercises.

Potatoes also contain catechol, so you will need to keep the catechol oxidase extract on ice at all times to retard any natural chemical reaction that might occur.

Standardizing the Spectrophotometer

1. ***Review the procedure for operating the spectrophotometer from the Spectrophotometer handout.***
2. Set the wavelength to 540 nm and make any other instrument adjustments that are mentioned in the spectrophotometer handout.
3. Add 1 ml of the potato extract (catechol oxidase) and 5 ml of distilled water to a clean test tube. Agitate the tube thoroughly. Fill a spectrophotometer cuvette about 3/4 full of this mixture. It will be your control for the spectrophotometer.

Why should you use the catechol oxidase mixture to standardize the spectrophotometer rather than distilled water?

4. Standardize the absorbance to "0" using the control cuvette containing the catechol oxidase following the instructions in the handout. Keep this cuvette cool at all times during the exercise, and be sure to remove any condensation from the surface of the cuvette prior to standardizing the spectrophotometer.
5. Remove the blank cuvette. The spectrophotometers should have spaces to measure absorbance for four experimental cuvettes at a time.
6. Each group may have to re-standardize the spectrophotometer for the different exercises and time readings.

Cuvettes will have to be cleaned and dried after each exercise. Be sure that a cuvette that is re-used in an exercise contains the same experimental solution for each reading.

Establishing a Reference Reaction.

Since you are testing conditions that affect the activity of the enzyme, catechol oxidase, you will need to determine qualitatively the reaction that occurs when you perform your experiments. To provide you with an idea of what to expect, you can establish a reference for the catechol oxidase-catechol reaction with no additional experimental conditions. You will also set up a reference for a negative reaction.

1. Obtain 2 test tubes. Label one test tube catechol oxidase and a second test tube water
2. Fill both tubes 1/4 full of distilled water
3. Add 60 drops of catechol to each tube
4. Add 60 drops of catechol oxidase (potato juice) to the catechol oxidase tube only
5. Agitate both test tubes. After 5 minutes, shake both tubes again and observe the results. The catechol oxidase tube will be your reference standard for a positive reaction. The water tube will be your negative control. Some of the other chemicals used in the set of exercises may affect the benzoquinone "color" some of the time. That's OK.

Exercise I: The Effect of Temperature on Enzymatic Activity

Within limits, the rate of a chemical reaction mediated by an enzyme increases as the temperature increases. However, enzymes are proteins subject to denaturation. The maximum enzyme activity occurs at the temperature just below the point where the enzyme is denatured. Once denatured, enzyme activity rapidly declines.

In this exercise you will determine the effect of temperature on the activity of catechol oxidase at 5 different temperatures: ice water, room temperature, 40°C, 60°C and 100°C.

Setting up the experimental temperature conditions

1. There are two hot water bathes set up in the room: one at 40°C and one at 60°C
2. Set up a boiling water bath using a 600-ml beaker and a hot plate **on the side counter** for your 100°C. When the water boils, turn the hot plate down to a "simmer".
3. Fill a 600-ml beaker with crushed ice. Add water. The ice water will be about 5°C.
4. Fill a 600-ml beaker with room temperature water (about 20 –25°C).

Procedure

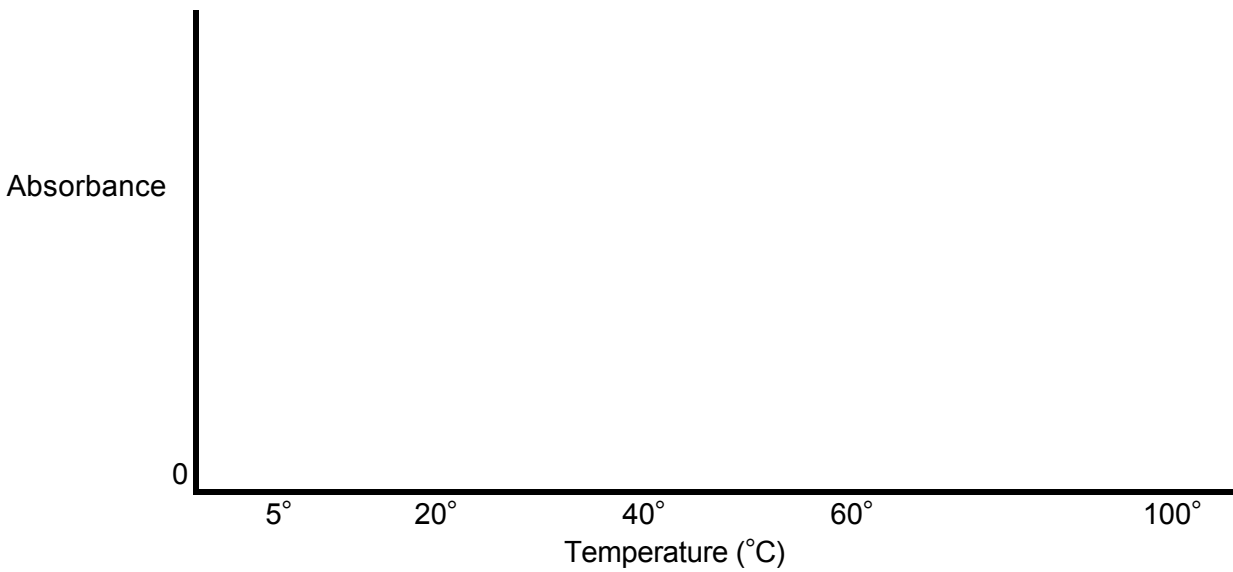
1. Label 5 test tubes 1 through 5.
2. Fill each tube 1/4 full of distilled water
3. Add 10 drops of catechol oxidase (potato juice) to each test tube.
4. Put Tube 1 in the ice water.
Put Tube 2 in the beaker of room temperature water.
Put Tube 3 in the 40°C hot water bath.
Put Tube 4 in the 60°C hot water bath.
Put Tube 5 in the boiling water.
5. Incubate the tubes at the designated temperatures for 10 minutes. This is to bring the tubes to the appropriate temperature. **Do not collect any data yet.**
6. After incubating the tubes for 10 minutes, add 10 drops of catechol to each of the test tubes. Shake each tube vigorously.
7. Transfer contents from each of the 5 test tubes into 5 spectrophotometer cuvettes, filling each cuvette about 3/4 full. Immediately take the time "0" absorption readings for each temperature and record them in the table below. Pour the contents of each cuvette back into the appropriate test tube, shake each test tube thoroughly and return the test tubes to their respective experimental conditions. Step 7 should be done as rapidly as possible to minimize the time the tubes are out of their experimental condition.
8. Continue to incubate the tubes at the designated temperatures. After 5 minutes shake each tube. Repeat step 7 at 5-minute intervals for a total of 10 minutes.

Enzyme Activity at Different Temperatures

Time (min)	Absorbance				
	Ice Water	Room Temp	40°C	60°C	100°C
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
0					
5					
10					

Plot the data for just the 10-minute interval time readings of the effect of temperature on the activity of catechol oxidase on the following graph.

Effect Of Temperature On Enzyme Activity



What, according to your data, is the optimum temperature for the enzyme, catechol oxidase?

How did the 100°C condition affect the enzyme, catechol oxidase?

Exercise II: The Effect of Enzyme Concentration on Reaction Rate

When the substrate (in this case, catechol) is present in sufficient quantity so that it does not limit the reaction, the rate of the chemical reaction is usually directly proportional to the concentration of the enzyme (in this case, catechol oxidase).

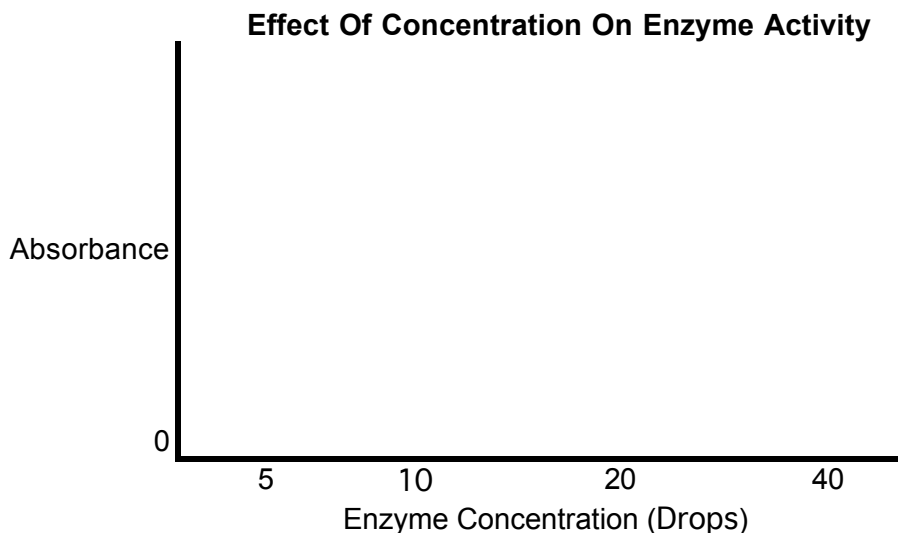
Procedure

1. Label 4 test tubes 1 through 4.
2. Fill each tube 1/4 full of distilled water.
3. Add the following amounts of catechol oxidase to the 4 test tubes.
 - Add 5 drops of catechol oxidase to tubes 1
 - Add 10 drops of catechol oxidase to tubes 2
 - Add 20 drops of catechol oxidase to tubes 3
 - Add 40 drops of catechol oxidase to tubes 4
4. Add 10 drops of catechol to each tube. Agitate all test tubes thoroughly.
5. Transfer contents from each of the 4 test tubes into 4 spectrophotometer cuvettes, filling each cuvette about 3/4 full. Immediately take the time "0" absorption readings for each and record them in the table below. Pour the contents of each cuvette back into the appropriate test tube and shake each test tube thoroughly. Step 5 should be done as rapidly as possible.
6. Repeat step 5 at 5-minute intervals for a total of 10 minutes.

Enzyme Activity at Different Concentration

Time (min)	Absorbance			
	5 drops	10 drops	20 drops	40 drops
	Tube 1	Tube 2	Tube 3	Tube 4
0				
5				
10				

Plot the data for just the 10-minute time interval results of the effect of concentration on the activity of catechol oxidase on the following graph.



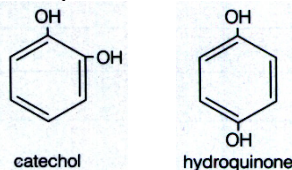
Explain the results you have recorded above, with reference to the effect of concentration on enzyme activity.

Does the concentration of the enzyme affect the overall reaction or does the concentration affect just the rate at which the reaction proceeds? In other words, given enough time would all of your experimental tubes reach the same intensity (the overall reaction)?

Exercise III: Enzyme Specificity

Most enzymes are specific; just one substrate "fits" the shape of the active site of the enzyme to form induced fit of the enzyme-substrate complex. Some enzymes can catalyze a set of structurally similar molecules, the common structure of each binds to the enzyme's active site.

Catechol and hydroquinone are structurally similar molecules as illustrated below.



This exercise is designed to see if catechol oxidase can catalyze hydroquinone as well as its intended substrate, catechol. The results of this exercise may also help you determine the portion of the substrate that "fits" the active site of the enzyme.

Procedure

1. Label 3 test tubes 1 through 3.
2. Fill each tube 1/4 full of distilled water.
3. Add 20 drops of catechol oxidase to each tube.
4. Incubate the tubes in the 40°C hot water bath for 10 minutes.
5. Add 20 drops of catechol to tube 1. Add 20 drops of hydroquinone to tube 2. Add 20 drops of distilled water to tube 3. Agitate all test tubes thoroughly.
6. Transfer contents from each of the 3 test tubes into 3 spectrophotometer cuvettes, filling each cuvette about 3/4 full. Immediately take the time "0" absorption readings for each and record them in the table below. Pour the contents of each cuvette back into the appropriate test tube and shake each test tube thoroughly. Step 6 should be done as rapidly as possible.
7. Return the test tubes to the 40°C hot water bath and continue to incubate for 5 minutes. Repeat step 6 at 5-minute intervals for a total of 10 minutes.

Time (min)	Absorbance		
	Catechol	Hydroquinone	Distilled H ₂ O
	Tube 1	Tube 2	Tube 3
0			
5			
10			

What do the data tell you about the catalytic activity of catechol oxidase with respect to the two substrates: catechol and hydroquinone?

Did the enzyme work faster on one substrate? If so, which substrate?

Comparing the molecular structure of the two substrates (shown above), what part of the molecules would you predict to fit the enzyme's active site?

Exercise IV: Inhibition of Enzyme Activity

Enzyme inhibitors are substances that can prevent an enzyme from catalyzing its chemical reaction. Enzyme inhibitors include those that compete with the substrate for the enzyme's active site and wind up blocking the active site (since they do not undergo a chemical reaction and get released from the enzyme), and those which bind to the enzyme at some other place changing its shape so it can no longer function effectively. Substances that compete for the enzyme's active site are called competitive inhibitors and those that bind to the enzyme inactivating it are called non-competitive inhibitors. A number of poisons block enzyme activity. For example, many pesticides block essential nervous system enzymes.

Some enzymes can only work when they have associate molecules, called coenzymes or cofactors present. Many of our vitamins function as coenzymes, and some of our minerals, notably zinc, copper and iron, function as cofactors. Heavy metals may interfere with enzyme function. Mercury, for example, inhibits the function of many enzymes, partly by blocking the attachment of the needed cofactor. Substances that interfere with coenzymes or cofactors will also inhibit the enzyme.

Copper is a cofactor for catechol oxidase. This exercise will help you to determine if phenylthiourea (PTU), which selectively binds to copper is an inhibitor of catechol oxidase, and if so, whether it is a competitive or non-competitive inhibitor.

Procedure

1. Label 4 test tubes 1 through 4.
2. Fill each tube 1/4 full of distilled water
3. Use the following table to add the appropriate amounts of distilled water, catechol oxidase (the potato juice) and PTU to each of the three tubes in the order listed. **Do not add the catechol at this time!**
4. Incubate the test tubes for 10 minutes in the 40°C hot water bath.

Tube #	Distilled Water	Catechol Oxidase (potato juice)	PTU
1	20 drops	20 drops	20 drops
2	none	20 drops	20 drops
3	40 drops	20 drops	none
4	20 drops	20 drops	none

Catechol Add After 10 Minutes
20
40
20
40

5. Remove the test tubes from the 40° hot water bath.
 - Add 20 drops of catechol to tubes 1 and 3.
 - Add 40 drops of catechol to tubes 2 and 4.
 - Agitate thoroughly all test tubes.
6. Transfer contents from each of the 4 test tubes into 4 spectrophotometer cuvettes, filling each cuvette about 3/4 full. Immediately take the time "0" absorption readings for each and record them in the table below. Pour the contents of each cuvette back into the appropriate test tube and shake each test tube thoroughly. Step 5 should be done as rapidly as possible.
7. Return the test tubes to the 40°C hot water bath and continue to incubate for 5 minutes. Repeat step 6 at 5-minute intervals for a total of 10 minutes.

Inhibition of Enzyme Activity

Time (min)	Absorbance			
	PTU20/Cat20	PTU20/Cat40	Cat20	Cat40
	Tube 1	Tube 2	Tube 3	Tube 4
0				
5				
10				

From your results, determine if phenylthiourea is a competitive or non-competitive inhibitor.

How did using a different concentration of catechol in tubes 2 and 4 compared to tubes 1 and 3 help you determine whether phenylthiourea was a competitive or non-competitive inhibitor?

Exercise V: The Effect of pH on Enzyme Activity

The hydrogen ion concentration (pH) of a solution can affect an enzyme's three-dimensional structure in much the same way as high temperatures can, thereby affecting the ability of an enzyme to function. Most enzymes function best near neutral pH ranges. Some enzymes, like those of the human stomach, require an acid pH to function, while others function in basic pH ranges.

This exercise will help you to determine the optimum pH for the enzyme, catechol oxidase, as well as illustrating the effect that the pH of a solution has on the enzyme.

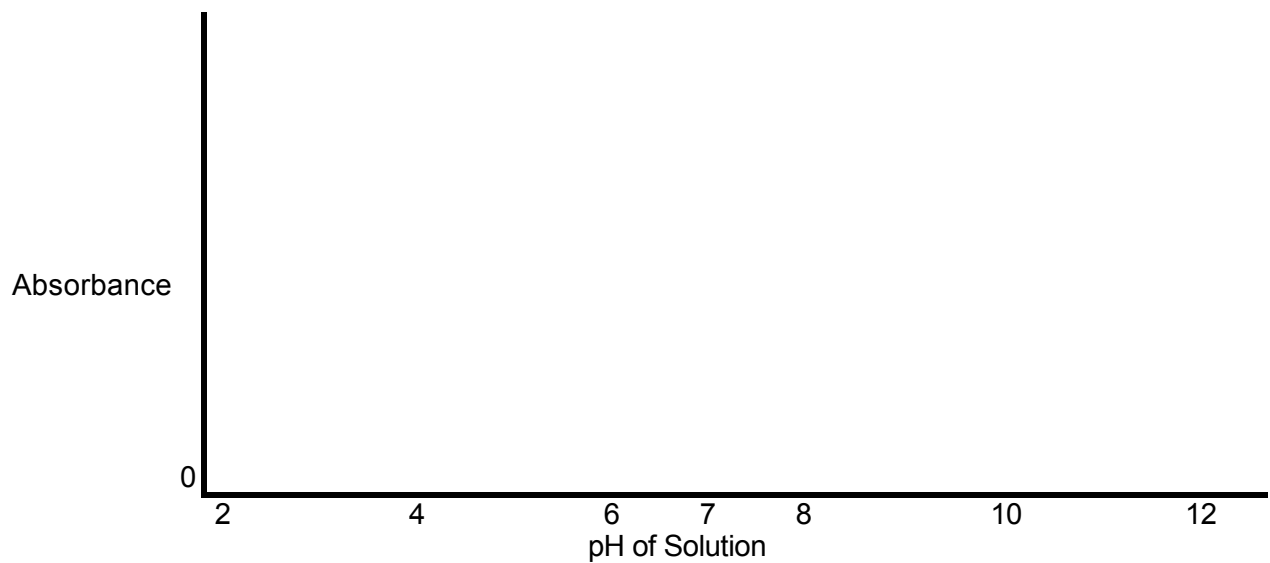
Procedure

1. Label 6 test tubes 1 through 6.
2. Take the numbered test tubes in a test tube rack to the stock table where the buffer solutions are located. Each buffer solution should have a 10-ml pipette with a pipette pump attached; do not mouth pipette. Use only the designated pipette for each of the buffer solutions to avoid contaminating the buffers. Add the buffer solutions to the proper tubes and return to your lab table.
3. Add the buffers in the following way:
 - Fill tube 1 1/4 full with the pH 2 buffer solution.
 - Fill tube 2 1/4 full with the pH 4 buffer solution.
 - Fill tube 3 1/4 full with the pH 6 buffer solution.
 - Fill tube 4 1/4 full with the pH 7 buffer solution.
 - Fill tube 5 1/4 full with the pH 8 buffer solution.
 - Fill tube 6 1/4 full with the pH 10 buffer solution.
 - Fill tube 7 1/4 full with the pH 12 buffer solution.
4. Return to your lab table with the buffer-filled test tubes.
5. Add 10 drops of catechol oxidase to each of the 7 test tubes.
6. Add 10 drops of catechol to each tube. Shake each tube thoroughly.
7. Transfer contents from each of the 7 test tubes into 7 spectrophotometer cuvettes, filling each cuvette about 3/4 full. Immediately take the time "0" absorption readings for each and record them in the table below. Pour the contents of each cuvette back into the appropriate test tube and shake each test tube thoroughly. Step 7 should be done as rapidly as possible.
8. Repeat step 7 at 5-minute intervals for a total of 10 minutes.

Time (min)	Absorbance						
	pH 2	pH 4	pH 6	pH 7	pH 8	pH 10	pH 12
	tube 1	tube 2	tube 3	tube 4	tube 5	tube 6	tube 7
0							
5							
10							

Plot the data for your results for just the 10-minute time interval on the following graph.

Effect Of pH On Enzyme Activity



What is the optimum pH for the enzyme, catechol oxidase?

What do you think the optimum pH would be for pepsin, an enzyme found in your stomach?

When you have completed all of the exercises, rinse out your test tubes and cuvettes and put them in the "used test tube" container. Clean any other glassware and put it in its proper location. Dispose of solutions in the designated waste location(s). Return all of your remaining supplies to the designated locations and clean your table top thoroughly.