

Enzymes

The Protein Standard Curve

Objectives

Two approaches are given here; an investigative approach and a more traditional cookbook approach. The objectives are the same.

1. Observe enzyme activity and specificity by means of a colorimetric enzyme reaction
2. Determine the effect of temperature on enzymatic activity
3. Determine the effect of substrate concentration on enzymatic activity
4. Prepare a protein standard curve
5. Determine the protein concentration of a sample by means of a total protein assay and use of a standard curve

Background

Enzymes are biological catalysts that are characterized by their ability to rapidly carry out cellular reactions that would otherwise occur only very slowly or under extreme conditions. Enzymes are effective in minute amounts, are not used up in the reaction and are very specific as to the reactions they catalyze. In fact, one of the defining characteristics of a reaction catalyzed by an enzyme is the extraordinary degree of specificity that the enzyme displays for a particular substrate. The change in orientation of a hydroxyl group in the structure of the substrate may be enough to prevent an enzyme catalyzed reaction from occurring because it may no longer "fit" into the active site of the enzyme.

Most biochemical reactions proceed slowly at low temperatures because the reaction molecules do not collide with each other with sufficient energy to form an "activated complex" or "transition state complex". It is only when such an activated complex is formed that the reaction can go to completion. By raising the temperature, a large proportion of the molecules will achieve this minimal energy and the rate of the reaction will increase accordingly. If the temperature is increased too much though, enzymes will gain so much kinetic energy (and thus will vibrate with such vigor) that they will lose their characteristic structure and no longer will be able to function as an efficient catalyst. Thus, unlike most uncatalyzed chemical reactions, enzymatic reactions in general display a temperature optimum.

Enzyme activity is usually expressed in terms of the rate of the reaction catalyzed by the enzyme. The rate is defined as the amount of substrate transformed, or the amount of product formed, per unit of time. This rate will change depending on the concentration of substrate available for the reaction and the temperature and pH of the reaction mixture.

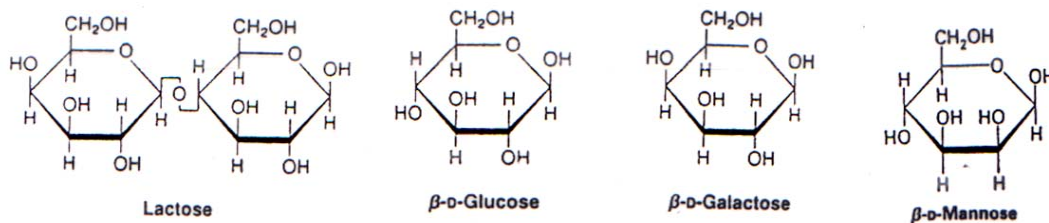
In this lab, you will be looking at how different substrates, different substrate concentrations, and different temperatures affect the rate of the reaction catalyzed by the enzyme glucose oxidase. Since this is a colorimetric reaction (the product is pink), you can measure the amount of product formed by using a spectrophotometer. As more product is produced, the intensity of the color will increase, and thus absorbance will increase.

A. Investigative Approach

Procedure

1. **Measuring the reaction** - The first thing you need to do is find the wavelength at which to read your samples. Since we will determine if the reaction is occurring by the appearance of a pink product, we will need to find the wavelength at which that product absorbs light maximally.
 - a. Do an absorption spectrum of the product of this reaction from 400-700nm. Take a reading every 20nm. Be sure to zero the spectrophotometer every time you change the wavelength. What should you use as a blank?
 - b. The wavelength that gives you the highest reading is the wavelength to use for the rest of the experiment.

2. **Specificity** – Determine which of 4 sugars -galactose, glucose, mannose, or lactose- gives the best reaction with the enzyme glucose oxidase (note structures below). This may be done by adding 1.0ml of enzyme to tubes containing 1.0ml dH₂O and 1.0ml of each of the sugars. Why might you not get a reaction with some of the sugars even though they all have the same chemical formula?



3. **Substrate concentration** – Once you've decided on the best sugar to use as the substrate for the reaction, vary the concentration of that sugar by varying the volume of sugar and water in the reaction mixture. For your reactions, the total

volume of the reaction mixture should always be 3.0ml, and of that, 1.0ml should always be the enzyme. By varying the volumes of water and sugar so they always add up to 2.0ml, your reaction volume (and thus enzyme concentration) will stay constant.

- a. Try the reaction with four different substrate concentrations. The stock substrate concentration is 0.2 mg/ml. If for one of your trials you use 0.5 ml of the substrate stock (0.2 mg/ml) the concentration of substrate in the reaction would be:

$$\frac{(0.2 \text{ mg/ml})(0.5 \text{ ml})}{3 \text{ ml}} = 0.033 \text{ mg/ml}$$

Substitute the volumes you use into the equation to determine the substrate concentration for each of your four reactions. Keep track of what concentrations you use.

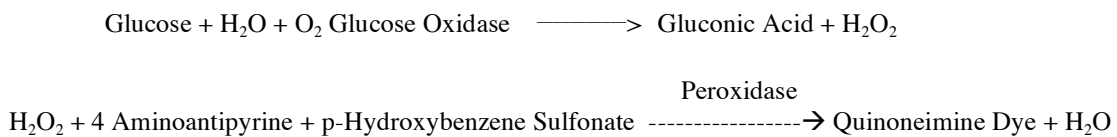
- b. Measure the reactions for 3 minutes each, by taking readings at 15, 30, 45, 60, 120 and 180 seconds after the addition of enzyme to substrate/water. If any of the readings for your reactions go off scale in the 3 minutes you are running the reaction you have used too much substrate and will need to revise the concentrations you plan to use.
4. **Temperature** - pick one of the substrate concentrations used above and try the reaction at different temperatures. You will have already done the reaction at room temperature, now try it at 4°C, 37°C and 70°C.
 - a. Be sure to get all components of the reaction to the temperature being tested before adding them together. You might put the appropriate volume of enzyme into one tube and water and substrate into another tube in a water bath. Let the liquids equilibrate to temperature at least 10 minutes before mixing to start the reaction.
 - b. Between readings you will need to put the tube back into the water bath to maintain the reaction temperature. You might want to change your readings to 1 minute intervals to allow for putting the reaction mixture back into water bath between readings.
 5. **Enzyme concentration** – You have kept the enzyme concentration constant in each of your reactions, but what is that concentration? You will need to do a

protein standard curve and then determine the amount of protein in your enzyme solution by using that curve.

B. Traditional Approach

The particular assay employed in this lab is a quantitative enzymatic determination of glucose used in the diagnosis of disorders associated with abnormal carbohydrate metabolism. The most significant of these diseases is diabetes mellitus, which is characterized by abnormally high concentrations of glucose in physiological fluids. Increased glucose concentration also occurs during hyperactivity of endocrine glands such as the thyroid and adrenals. Hypoglycemia is a condition characterized by low glucose levels that can result from a variety of conditions such as insulin overdose liver diseases, and hypopituitarism.

The actual reaction that you will be following is a composite of two reactions catalyzed by two different enzymes, so in a sense it is a bit complicated, but it is an easily measurable colorimetric assay which is why we use it in an introductory course. The enzymatic reactions involved are as follows:

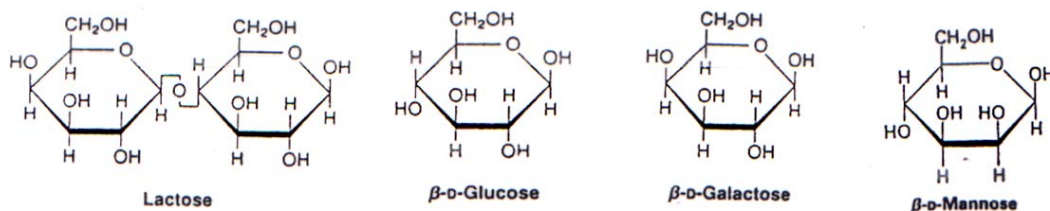


Glucose is first oxidized to gluconic acid and hydrogen peroxide in the reaction catalyzed by glucose oxidase. The hydrogen peroxide formed reacts with 4-aminoantipyrine and p-hydroxybenzene sulfonate to form a quinoneimine dye which is pink in color. The intensity of the color produced (as measured on the spectrophotometer) is directly proportional to the glucose concentration in the sample. Hence the more glucose present, the darker the pink color in the reaction mixture, and the greater the absorbance.



Experimental Procedure

1. **Specificity** - One of the defining characteristics of a reaction catalyzed by an enzyme is the extraordinary degree of specificity the enzyme displays for a particular substrate. As an example of this specificity, consider the following sugars, all of which are physiologically important:



Note that galactose and mannose differ from glucose only in the orientation of a single -OH group with respect to the plane of the ring. Lactose is made up of a glucose and galactose molecule.

Procedure:

- a. Set the wavelength at 510 nm.
- b. Zero the spectrophotometer with a spectrophotometer tube containing 4.0 ml dH₂O and 1.0 ml enzyme. This will be your blank.
- c. To another spec. tube add 3.0 ml dH₂O and 1.0 ml glucose (0.2 mg/ml).

***NOTE: In this next step the addition of the enzyme starts the reaction and is therefore time zero. Be sure to keep track of time.**

- d. Add 1.0 ml enzyme to the spec. tube from step c. Quickly mix in the contents of the tube on the vortex mixer and insert into the spec.
- e. Read and record absorbance at 15 sec., 30 sec., 45 sec., 1 minute, 2 minutes and 3 minutes after the addition of the enzyme.
- f. Repeat steps c-e except use galactose instead of glucose as the substrate. Then repeat using mannose, then lactose.
- g. Plot your data -- Absorbance (y-axis) vs time (x-axis) for all substrates. Plot all lines on one graph, using different symbols or colors to distinguish between them.

2. **Temperature** - Most biochemical reactions proceed slowly at room temperatures because the reaction molecules do not collide with each other with sufficient energy to form an “activated complex”, or “transition state complex”. It is only when such an activated complex is formed that the reaction can go to completion. By raising the temperature, a large proportion of the molecules will achieve this minimal energy, and the rate of the reaction will increase accordingly. However, if the temperature is increased too much, enzymes will gain so much kinetic energy (and thus will vibrate with such vigor) that they will lose their characteristic structure and no longer will be able to function as an efficient catalyst. Thus, unlike most uncatalyzed chemical reactions, enzymatic reactions in general display a temperature optimum.

Procedure:

- a. Set up six test tubes as follows:

Test Tube	ml dH ₂ O	ml glucose (0.2 mg/ml)	ml enzyme
1	--	--	1.0
2	3.0	1.0	--
3	--	--	1.0
4	3.0	1.0	--
5	--	--	1.0
6	3.0	1.0	--

- b. Place test tubes #1 and 2 into the ice bath (4°C), test tubes #3 and 4 into the 37°C water bath, and test tubes # 5 and 6 into the 65°C water bath, for at least 10 minutes. This is to equilibrate the components of the reactions to the appropriate temperatures.
- c. At the end of the equilibration period, mix the components of test tubes #1 and 2 in a spec. tube. This is time zero. Place the spec. tube back into the ice bath. Take readings at 1 minute intervals for 5 minutes. **Be sure to wipe all water off spec. tubes before inserting into the spectrophotometer.**
- d. Mix test tubes #3 and 4 in a spec. tube (time zero) and quickly place back in 37°C water bath. Take readings at 1 minute intervals for 5 minutes.
- e. Mix test tubes #5 and 6 in a spec. tube and place back into the 65°C water bath. Take readings at 1 minute intervals for five minutes.
- e. Plot your data--Absorbance vs time for all four temperatures (4°C, 37°C, 65°C and room temperature, approx. 22°C, data for this one from part 1) on one graph.

3. **Substrate concentration**- In this portion of the experiment you will be looking at the effect of varying substrate concentration on the velocity of the enzyme reaction. Enzyme activity is usually expressed in terms of the rate of the reaction catalyzed by the enzyme. The rate is defined as the amount of substrate transformed, or the amount of product formed, per unit of time. sample data are shown in figure below.

Procedure:

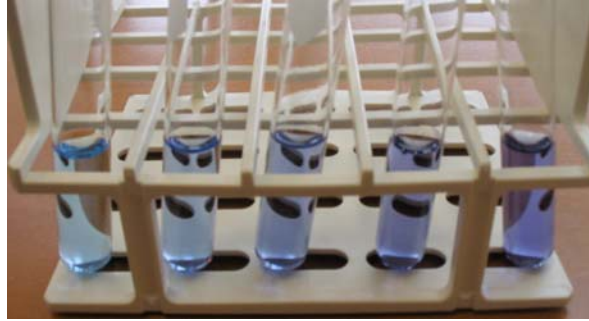
- a. Set up the following in cuvettes:

Sample	ml dH ₂ O	ml glucose (0.2 mg/ml)
1	3.9	0.1
2	3.5	0.5
3	3.0	1.0
4	2.5	1.5
5	2.0	2.0

- b. Add 1.0 ml of enzyme to test tube #1. Quickly mix (this is time zero), and put into the spectrophotometer. Take readings at 15, 30, and 45 seconds, 1, 2, and 3 minutes. Record data.
- c. Repeat step b for samples 2-5.
- d. Plot your data -- Absorbance vs. time for each of the 5 substrate concentrations on one graph.

C. Protein Standard Curve and Assay

In order to determine the amount of protein present in an unknown sample one first needs some way to compare the unknown to known protein values. This is accomplished by setting up a protein standard curve. You will take known amounts of a protein, in this case bovine serum albumin (BSA), and do a standard colorimetric test known as a total protein assay. When the total protein reagent (TPR) is added to a solution containing protein, a chemical reaction occurs that turns the solution blue. The more protein present the deeper blue the resultant product, and thus the higher the absorbance reading in the spectrophotometer. By graphing the relationship between these absorbance readings and the known amounts of protein assayed you will then have a standard curve. To determine the protein content of your enzyme solution you will do the protein assay on it, take an absorbance reading, and plug that number into the line equation of your protein standard curve. By knowing the volume of enzyme you assayed you then can find the concentration of protein in the enzyme solution.



The Standard Curve

1. To obtain data for your standard curve set up six test tubes with the following amounts of protein and deionized water. The protein being used as the standard is bovine serum albumin (BSA) in a concentration of 5 mg/ml.

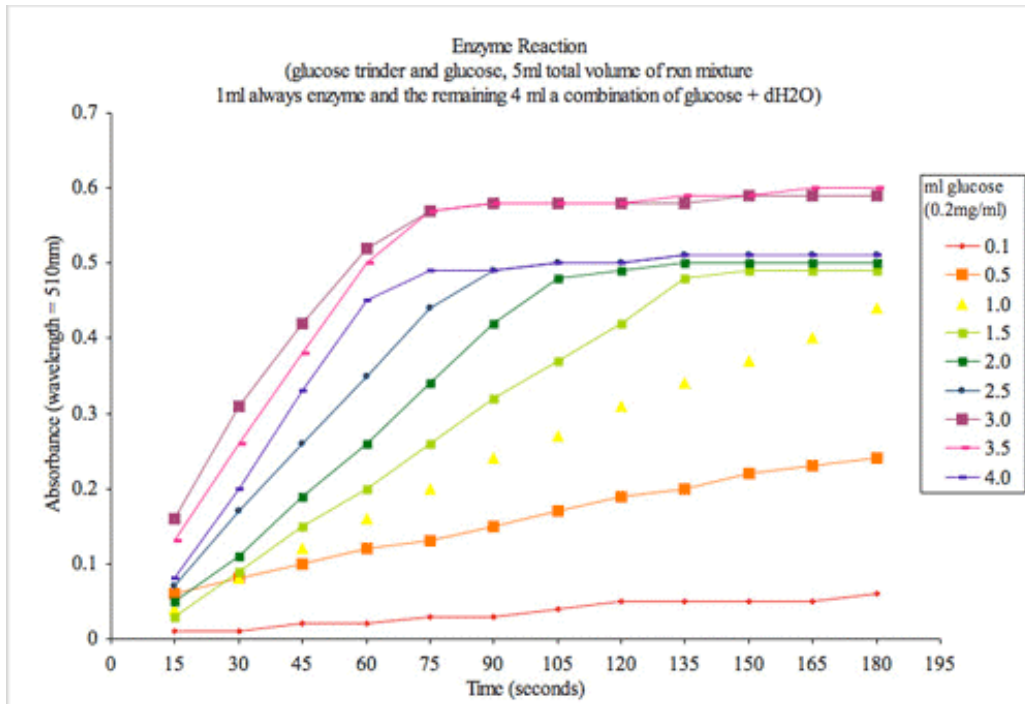
Test Tube	ml BSA (5 mg/ml)	ml dH ₂ O	mg protein in tube
1	0.0	2.0	0
2	0.1	1.9	0.5
3	0.3	1.7	?
4	0.5	1.5	?
5	0.7	1.3	?
6	1.0	1.0	?

2. To determine the protein content of your enzyme set up 2 tubes each with 1.0 ml of Enzyme. This is 5X the concentration used for enzyme reactions. You are doing this in duplicate to check your accuracy.
3. Add 3.0 ml of Total Protein Reagent to each of the test tubes (#1-6 and to the 2 tubes with 1 ml of enzyme); mix and wait 10 minutes.
4. Pour the contents of test tube #1 into a spec tube (this is your blank). Set the spec to 540nm, put the blank into the spectrophotometer and zero (set the needle to zero on the absorbance scale).
5. Take the blank out. Sequentially take the readings for tubes 2-6 and then your enzyme samples making sure you record your data in each case (spec readings must be done in spec tubes).

- Plot your protein standard curve. The absorbance should be plotted on the y-axis and protein content (mg) on the x-axis. Plug your absorbance (y) value for the enzyme into the equation of the line to determine the protein content (x). The concentration of protein in the enzyme solution would be the protein content/volume of enzyme assayed (in this case 1 ml). The concentration of the enzyme used in the enzyme kinetics experiments will be 1/5 of this so divide by 5.

D. Chemical Ordering Information

- Enzyme - Glucose trinder (#220-32, four bottles, each make 100 ml) from Diagnostic Chemicals, Ltd. 1-800-325-2436, www.dclchem.com/
- Total Protein Reagent (TPR) – (#T1949) Sigma Life Science, 1-800-325-3010, sigma-aldrich.com/order
- Bovine Serum Albumin (BSA) – (#A7030) Sigma Life Science, 1-800-325-3010, sigma-aldrich.com/order



Sample substrate concentration data.

Spectrophotometry

Visible light

Visible light (that which can be seen by the naked eye) is composed of different wavelengths (λ) of light ranging from violet (380 nm) to red (760 nm). A wavelength of light is defined as the distance from one peak to the next in the wave and is measured in nanometers (nm). One nm is equivalent to 10^{-9} meters.

Colors

Many kinds of molecules interact with or absorb specific types of radiant energy in a predictable fashion. When white light strikes an object, the color the human eye perceives is determined by the wavelengths of light absorbed and the remaining wavelength(s) that are reflected or transmitted. An object that appears red absorbs wavelengths of all colors but red. The red wavelength of light is what reflects back to the eye. If all wavelengths of light are absorbed by an object, the object appears black, if all are reflected, it appears white.

Spectrophotometry –the Spectrophotometer

The perception of color by the eye, as just described, is qualitative. Spectrophotometers electronically quantify the amount and kinds of light that are absorbed by molecules in solution. **Spectrophotometry** is the measurement of the interaction of radiant energy with matter in the UV and visible portion of the electromagnetic spectrum. In its simplest form a **spectrophotometer** has a **source of white light** (for visible spectrophotometry) that is focused on a prism or **diffraction grating** that separates the white light into individual bands (wavelengths) of radiant energy. Each wavelength (color) is then selectively focused through a narrow slit. The width of the slit is important to the precision of the measurement: the narrower the slit the more closely the absorption is related to a specific wavelength of light. Conversely, the broader the slit, the more light of different wavelengths passes through which reduces the precision of the measurement.

The light that passes through the slit, the **incident beam (I_0)**, then passes through the sample being measured. The **sample**, which is dissolved in a suitable solvent, is contained in an optically selected tube called a **cuvette**. The light that passes through the sample is known as the **transmitted beam (I_t)**. If the substances in the cuvette have absorbed any of the incident light, the transmitted light will be reduced in total energy content. If the substance in the cuvette does not absorb any of the incident beam, the radiant energy of the transmitted beam will be about the same as the incident beam. When the transmitted beam strikes the **photodetector** it generates an electrical current proportional to the intensity of light striking it. The photodetector is connected to a

galvanometer that directly measures the current, thus the intensity of the transmitted beam. In the Bausch & Lomb Spectronic 21 spectrophotometer the galvanometer has **two scales**: one indicates the **% transmittance (%T)** and the other, a logarithmic scale with unequal divisions graduated from 0.0 to 2.0, indicates the **absorbance (A)**. The term optical density (OD) may be used instead of absorbance, especially when experimenting with cell suspensions, however absorbance is the term more commonly used.

Transmittance

As mentioned above, the light transmitted (T) is the ratio of the intensity of the light exiting the sample (I_t) to the intensity of the light entering that entered the sample (I_o).

$$T = \frac{I_t}{I_o}$$

The percentage of light transmitted (%T) is equal to $T \times 100$.

The amount of light transmitted depends on three factors:

1. if the sample will absorb light at the particular wavelength (λ) tested (this is dependent on the color of the sample – pure H_2O transmits all visible wavelengths)
2. the amount of sample the light passes through (cell width)
3. the concentration of the absorbing material

The transmittance of the sample varies logarithmically with the concentration of the absorbing material.

$$T = 10^{-abC}$$

$$\log (1/T) = -\log T = abC$$

where

a= molar absorbtivity

b= path length

c = concentration of the absorbing material

Absorbance, Beer's Law, and Standard Curves

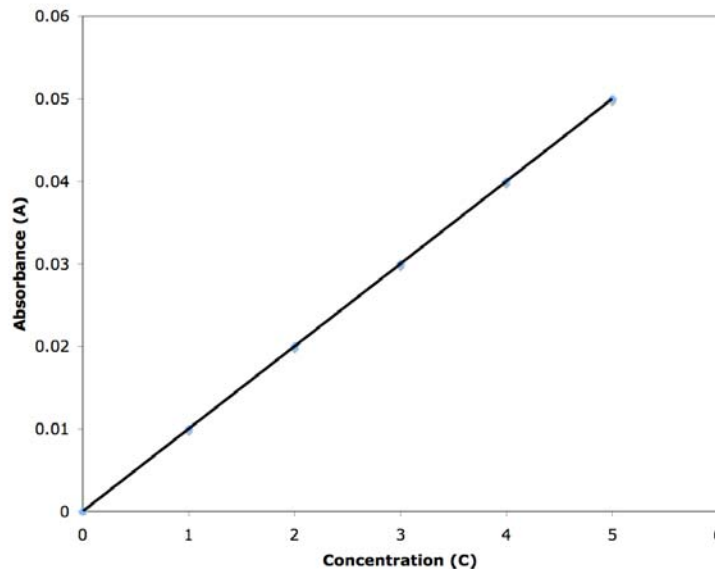
There is an inverse correlation between transmittance and absorbance. The more light that is absorbed by a sample, the less light will be transmitted.

0 absorbance = 100% transmittance

By substituting absorbance (A) for transmittance, absorbance and concentration become directly proportional.

$$A = \log (1/T)$$

A = abC this is known as **Beer's Law**



The relationship between concentration and absorbance

This relationship enables us to set up what is known as a **standard curve**. If we take absorbance readings of at least five dilutions of a standard of known concentration plus a blank we can plot absorbance vs concentration and get a straight line. By then taking an absorbance reading of a sample of the same substance of an unknown concentration and locating where that absorbance reading (Y axis) falls on the standard curve we can find the corresponding concentration on the X axis.

Using the Spectrophotometer

1. Turn the power on by turning the Power Switch (front left side, fig 1). Allow at least a 15-minute warm up period before taking readings.
2. Select either the % transmittance or absorbance mode by pressing the %T/A selector switch (near the display)
3. Select the wavelength to be used by turning the wavelength selector knob located on the top right side of the spec. The wavelength selected shows in the window to the left of the knob.
4. Wipe off fingerprints from the reference blank cuvette with a kimwipe. To assure the spec measurement is due only of the light absorption/transmittance of the molecules being studied a mechanism for “subtracting” the absorbance/transmittance of the solvent is necessary. To achieve this a “blank” of the solvent is read first to calibrate the spec.
5. Insert the cuvette into the sample holder lining up the vertical mark on the cuvette with the notch in the sample compartment. Close the sample compartment cover.
6. Adjust the display to 0 absorbance or 100% transmittance by turning the Transmittance/Absorbance control knob (located front right of the spec)
7. Remove the reference blank cuvette
8. Wipe fingerprints off of cuvette containing sample and insert into sample compartment. Close the cover and read the display.
9. Whenever changing the wavelength be sure to “reblank” the spec with the reference blank before putting the sample in to be read.
10. Whenever operating at a fixed wavelength for an extended period of time it is best to periodically check the absorbance with the blank to be sure it is still zeroed.

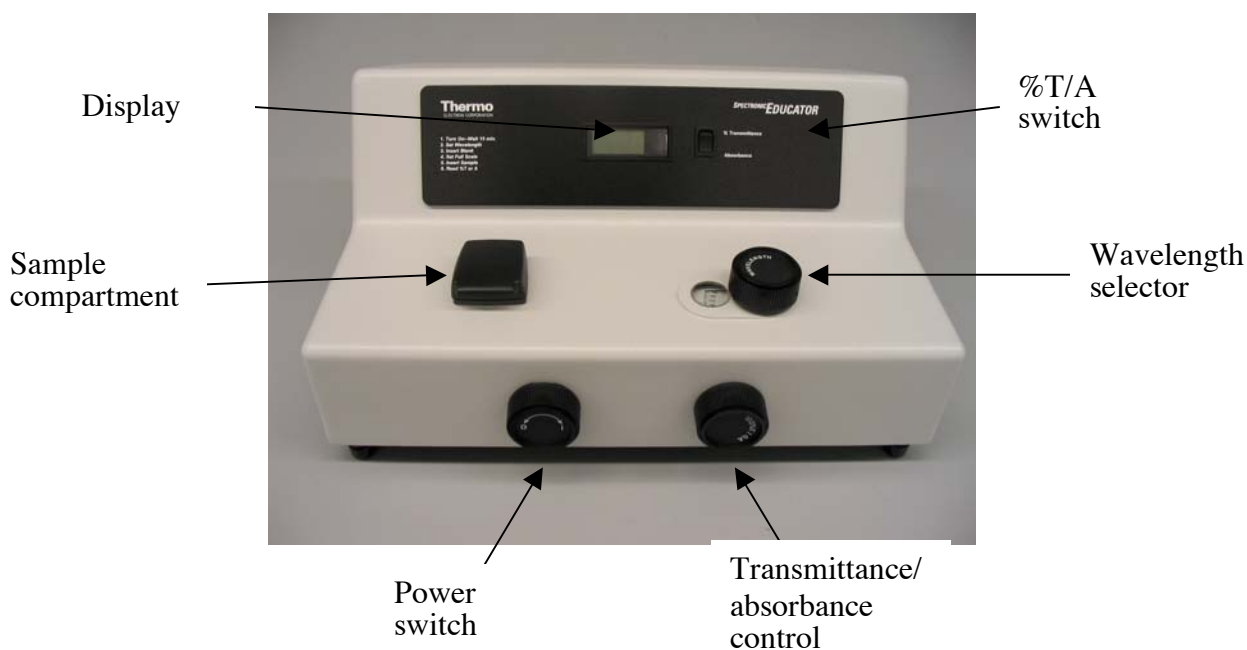


Figure 1. Spectronic Educator