

## EXPERIMENT 9

### Kinetic Methods of Analysis: Enzymatic Determination of Glucose

#### SAFETY WARNING

In this experiment you will be using syringes (glass, breakable) that have sharp pointy needles on them. Work carefully. Never use force on a syringe. Some of them might fit pretty tightly, especially when dry, but they need to slide relatively smoothly with a minimum of force. Please don't spear yourself with the working end of a syringe. I don't want to have to walk you over to the Student Health Service, where, if you weren't sick or injured when you walked in, you will be so after you leave. The phenol and 4-aminoantipyrene in the "Enzyme Stock Solution" that you will be using are mildly toxic, but both are present in such low concentrations that this is not a major hazard problem at all. Nevertheless, you might wish to wear latex gloves when working with the reagents. Just stay alert and work in a controlled and *neat* manner. Keep things clean at all times.

#### UNKNOWN

You do not need to submit a container for your unknown. Because your unknown needs to be prepared (thawed) the day it is to be used, the Teaching Assistant will provide you with your unknown in a 50-mL volumetric flask. However, you *will* need to wash the flask out thoroughly, rinse with distilled water, and return it to the Teaching Assistant at the end of the laboratory period.

#### INTRODUCTION

Literally billions of clinical chemistry tests are performed annually in the United States. The bulk of these analyses are performed by use of spectrophotometric methods. Of these, ordinary absorption spectroscopy in the visible region is the one most commonly used. The instrumentation for visible absorption spectroscopy is simple, stable, relatively inexpensive, and easy to automate, thus it can handle the large numbers and variety of tests to be done. The method has a large number of advantages and very wide applicability. Many substances absorb light, and if the analyte itself does not absorb, it can often be treated with reagents to generate a product with suitable absorbance characteristics.

The vast majority of chemical analyses are performed on systems that are *at equilibrium*, clinical or otherwise. The **chemistry** is "over." This is true whether the analysis method is spectrometric, electrochemical, chromatographic, calorimetric, or even radiochemical in nature. If not technically at complete equilibrium, as is the case in redox electrochemistry and radiochemistry, the analyses performed are under *quasi steady-state* conditions. When chemistry is performed to prepare samples for analysis, even with use of enzymes, the samples are

normally allowed to react or to “incubate” for a sufficient period of time that the system is at or very near equilibrium.

However, *kinetic methods* of analysis, wherein one measures the *rate* of an ongoing chemical reaction to determine the quantity or concentration of the analyte of interest, are becoming increasingly popular. The first, and probably most important, advantage of kinetic methods in general is speed. You don’t have to wait for the reaction to go to completion to make your measurement. Kinetic methods based on reaction-rate measurements are usually done only during the first 3 to 10% of the reaction, before possible back-reactions start to become significant. In this situation, one measures the so-called *initial* reaction-rate with little error.

Kinetic methods are also particularly useful in samples in which some interferent is present, even if in large and variable concentrations from sample to sample. For example, take absorption spectroscopy as the method and a colored sample such as whole blood or bottled/canned soft drinks and juices. If you can develop a kinetic method that reacts only with the analyte, or at least does *not* react with the interferent, modern instruments with microprocessors can often succeed in providing you with a decent or even very good analysis by measuring the *rate of change* in absorbance of a sample undergoing a reaction without having to do complicated and time-consuming chemistry to eliminate the interfering colored background or to resort to some separation method.

The primary objective of this experiment is two-fold: To provide you experience with a kinetic method of analysis and to acquaint you with the use of enzymes as an analytical tool or “reagent”.

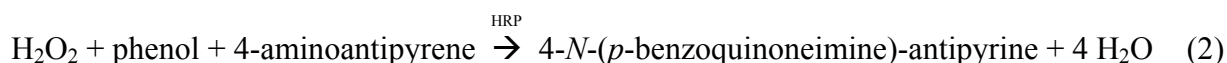
## THE ENZYMATIC REACTION

Glucose is a major component of animal and plant carbohydrates. Quantitative determination of glucose is important in clinical chemistry, biochemistry, and food science. In this experiment you will measure glucose levels using enzymatic reactions and visible absorption spectrophotometry. While there are several variants in enzymatic determinations of glucose, this one will use **glucose oxidase (GOX)** and **horseradish peroxidase (HRP) enzymes**. Essentially the *initial rate of the first enzymatic reaction* will be the basis for quantitation.

Glucose is oxidized to  $\delta$ -D-gluconolactone and hydrogen peroxide in the presence of GOX:



Unfortunately, neither of the products absorbs light in the visible region. Therefore, the production of hydrogen peroxide is coupled to a reaction catalyzed by HRP:



The reaction product absorbs light maximally at  $\lambda_{\max} = 505 \text{ nm}$  with a molar absorptivity  $\epsilon = 1.27 \times 10^4 \text{ L/mol-cm (M}^{-1}\text{cm}^{-1}\text{)}$ .

### Enzyme Concentrations

Enzyme “concentrations” are usually expressed in units of **IU**. IU is an acronym for International Unit, which is essentially an enzymatic “activity” – how well the enzyme does its catalysis. An IU is typically defined as *the amount of an enzyme that will produce 1  $\mu\text{mol}$  of product per minute or that will consume 1  $\mu\text{mol}$  of reactant per minute*. This, of course, is highly dependant on reaction conditions such as temperature, pH, and other solution components. While the total molarity of the enzyme itself would stay constant under different experimental conditions (except for the effect of volume changes with change in temperature), its activity can change markedly. For example, the activities of almost all enzymes are highly dependent on the pH. The rates of *chemical* reactions typically double for a 5 °C increase in temperature, and high enough temperature can denature any enzyme rendering it totally inactive. For example, think about a hardboiled egg. Proteins in their natural form are called “native proteins.” Any significant change in the secondary, tertiary, and quaternary structures of a native protein is called *denaturation*.

For kinetic enzyme assays, enzyme “concentrations” in IUs is more fundamental, useful, and sensible.

The *specific activity* of an enzyme is defined as the units divided by the mg of protein present, IU/mg protein.

There are many ways of measuring the mg of protein present in an enzyme preparation. A quick and convenient estimate can be made by measuring the ultraviolet absorbance of the solution at 280 nm, assuming there are no other absorbing species present. The tyrosine and tryptophan residues in proteins have ultraviolet absorption maxima at 275 and 280 nm, respectively. No other common amino acids absorb. Because the combined content of these two amino acids is roughly constant among many proteins, the concentration of protein is roughly proportional to the absorbance at 280 nm, although the absorbance of individual pure proteins at 280 nm can vary widely. The average absorbance of protein mixtures is about 0.8 absorbance units, AU, at 280 nm in a standard 1-cm cuvette

Nucleic acids are a common interferent in enzyme preparations. Nucleic acids have a  $\lambda_{\max}$  at 260 nm, but absorb significantly at 280 nm. Correction for any nucleic acid present is possible using the  $A_{280}/A_{260}$  ratio of the preparation. Proteins typically have a ratio of about 1.75 (with allowance made for a specific, pure protein), whereas nucleic acids have a ratio of 0.5. Any intermediate ratio would suggest a nucleic acid impurity is present, which can then be corrected for.

The *total activity* of an enzyme is the specific activity times the total mg of protein present in the entire preparation –  $\text{IU/mg} \times \text{mg}_{\text{protein}}$ .

Enzymes are extracted from some natural source such as horseradish, algae, beef heart, human liver, bacteria, etc. The crude enzyme extract must then be separated from other components present and purified a number of times, often by two or more different methods, then concentrated to make the preparation useful. Typically, at least three different purification steps are necessary. During purification the specific activity will increase but the *yield* of total activity in the entire preparation will decrease, owing to removal of protein impurities and loss or denaturation of the enzyme of interest. For example, increases of 100x or more in the specific activity, with yields of 10% or less are not uncommon.

## THEORETICAL BACKGROUND

### Basic Kinetics

The *rate*,  $R$ , of a chemical reaction is the speed or velocity,  $v$ , at which the reaction proceeds. The reaction rate is expressed as the change in concentration of a reactant or product during a given time interval. For a reaction is written as



where  $\mathbf{A}$  and  $\mathbf{B}$  are the reactants and  $\mathbf{P}$  is the product, the rate can be expressed in terms of either the appearance of the product  $\mathbf{P}$  or the disappearance of either of the reactants  $\mathbf{A}$  or  $\mathbf{B}$ . Thus, for any finite increment of time  $\Delta t$ , one can write

$$R = \Delta[\mathbf{P}]/\Delta t = -\Delta[\mathbf{A}]/\Delta t = -\Delta[\mathbf{B}]/\Delta t \quad (4)$$

Note that there is a minus sign in front of the change in reactant concentrations. This indicates that their concentrations are decreasing. If the interval of time considered becomes smaller and smaller, approaching zero, the calculus can be applied and Equation (4) can be written as

$$R = d[\mathbf{P}]/dt = -d[\mathbf{A}]/dt = d[\mathbf{B}]/dt \quad (5)$$

where the **d function** indicates a derivative.

The actual rate of many reactions, however, is proportional to some power of the reactant concentrations other than one. This can be expressed mathematically as

$$R \propto [\mathbf{A}]^a[\mathbf{B}]^b \quad \text{or} \quad R = k[\mathbf{A}]^a[\mathbf{B}]^b \quad (6)$$

where  $k$  is a proportionality constant and  $a$  and  $b$  are numerical exponent powers that must be determined experimentally for the reaction at hand and under the conditions used.

### Reaction Order

The exponents in the rate Equations (6) are usually small whole numbers whose values depend on the number of molecules involved in the rate-determining step in a reaction. They can often

be hypothesized from the stoichiometric factors in the balanced chemical reaction, but their values must be verified experimentally. For example, if a reaction can be expressed as



and can be shown to obey the rate equation

$$R = k [\mathbf{A}]^1 \quad (8)$$

the reaction is said to be **first order** with respect to **A**. Radioactive decay is a very good example of a pure first-order reaction.

Consider the generalized reaction  $\mathbf{A} + \mathbf{B} \rightarrow \mathbf{C} + \mathbf{D}$  in which two reactants produce two products. The stoichiometry of the reaction would suggest that one **A** comes together with one **B** to generate the products **C** and **D**. If it can be shown experimentally that the rate of the reaction follows the rate equation

$$R = k [\mathbf{A}]^1 [\mathbf{B}]^1 = k [\mathbf{A}][\mathbf{B}] \quad (9)$$

then the reaction is said to be first order in **A** and first order in **B**. The whole reaction is therefore called **second order** overall. If the reaction is found experimentally to follow the rate law

$$R = k [\mathbf{A}]^1 [\mathbf{B}]^2 = k [\mathbf{A}][\mathbf{B}]^2 \quad (10)$$

The reaction would be third order overall, first order in **A** and second order in **B**.

Finally, consider the second-order reaction rate in Equation (9). If one of the reactants **B** is present in very large concentration relative to **A**, **[B]** will change only very, very little throughout the whole reaction to completion. Therefore **[B]** is effectively constant,  $[\mathbf{B}]_{const}$ , and the rate law can be written as

$$R = k [\mathbf{A}] [\mathbf{B}]_{const} = k' [\mathbf{A}] \quad (11)$$

where  $k'$  is a constant and equal to  $k[\mathbf{B}]$ . Such a reaction under these conditions would be called **pseudo first order** overall, first order in **A** and zero order in **B**.

### The Michaelis-Menten Model of Enzyme Catalysis

There are several mathematical models for enzymatic reactions. One of the simplest and most useful is that developed by Michaelis and Menten. Consider the following set of reactions for an enzyme-catalyzed process:



where **E** is the *free enzyme*; **S** is the *free substrate*, the substance for which the enzyme serves as a reaction catalyst; **ES** is the *enzyme-substrate complex*; **P** is the *product* of the reaction; and the  $k_n$ 's are the individual forward and backward rate constants.

Note that the equilibrium constant for the first step in the reaction is  $K_{eq} = k_1/k_{-1}$ .

Usually the reaction is studied only in its early stages so that there is no significant buildup of the product **P**. Therefore, very little of the back reaction represented by  $k_{-2}$  occurs. In this case, Equation (12) can be simplified to



where  $k_{cat}$  is the catalytic rate constant for the conversion of the enzyme-substrate complex **ES** to the product **P** and regenerating the free enzyme **E**, which is then able to react with another substrate molecule.

Under these conditions, the reaction rate or reaction velocity  $v$  is linearly proportional to the enzyme concentration  $[\mathbf{E}]$ , assuming it remains at a low, catalytic level. However, a plot of  $v$  as a function of  $[\mathbf{S}]$  shows a linear dependence and first-order kinetics only during the initial stages of the reaction, about the first 10% or so, before the rate of the back reaction becomes significant. The plot then becomes curvilinear downward and approaches an asymptomatic value of  $v$ .

Without going into the details of its derivation, the Michaelis-Menten equation was developed to explain the observed kinetic behavior:

$$v = \frac{V_{max} [\mathbf{S}]}{K_m + [\mathbf{S}]} \quad (13)$$

where  $v$  is the velocity of the reaction,  $V_{max}$  is the maximum (theoretical) velocity, and  $K_m$  is the Michaelis constant,  $(k_{-1} + k_{cat})/k_1$ .

The maximum theoretical velocity,  $V_{max}$ , is the velocity when the substrate binds to all of the active sites on all the enzymes, when it is totally "saturated". This is impossible because there will always be some free **E** available; the reaction to produce product and free **E** is always going on.

The Michaelis constant  $K_m$  turns out to be numerically equal to the substrate concentration  $[\mathbf{S}]$  that produces a velocity  $v = V_{max}/2$ .  $V_{max}$  can be roughly estimated from plots of  $v$  vs.  $[\mathbf{S}]$ , and then  $K_m$  can be obtained from the value of  $[\mathbf{S}]$  at  $V_{max}/2$  on the plot.

$K_m$  and  $V_{max}$  provide very important information about an enzymatic reaction, and are among the very first things that scientists try to determine or verify for an enzyme they are using. One of the reasons that  $K_m$  is important is that it provides an idea of the affinity, the binding strength, of the enzyme for the substrate. With  $V_{max}$  and the actual molar concentration of the enzyme,  $k_{cat}$  can

be calculated. This is also called the *turnover number*, the number of substrate molecules transformed to product per unit time by a single enzyme molecule under maximal conditions. This provides a good measure of the speed and efficiency of an enzyme.

It is highly desirable to understand the foregoing theoretical background on kinetics in order to perform our quantitative experiment well, but you will not actually be determining the kinetic constants of either of the enzymes used. This would be nice to do, especially in a biochemistry or a physical chemistry laboratory, but our focus in the laboratory is quantitative analysis. Basically, we just want to use the enzyme properly.

Both of the enzymes used in this experiment, glucose oxidase (GOX) and horseradish peroxidase (HRP), display Michaelis-Menten kinetics under the proper conditions. If you look at the total assay reaction, Equations (1) and (2) together, we have a two-step sequential process to get to the final product, which can then be monitored spectrophotometrically.

What does all this mean? What will our kinetics be like? How does the second reaction with HRP affect the first one, or vice versa?

In a trivial (and actually incorrect) sense, it shouldn't matter much because we will only be monitoring the early part of the reaction to get a measure of the initial velocities for quantitative purposes. However, when you get to the Preparation of Solutions below, you will see that the "effective" concentration of the enzyme in the second step, HRP, is present in at approximately 40x the concentration of the enzyme in the first step, GOX. Essentially, therefore, the first step becomes the rate-limiting reaction, the second step is simply a very rapid follow-up step and does not affect the overall kinetics of the reaction.

The rate of formation of the final product, which is monitored spectrophotometrically, is effectively identical to the rate of formation of the products of the first reaction, gluconolactone and hydrogen peroxide, within experimental error.

## **INSTRUMENTATION**

Varian Cary 50 UV-Vis Spectrophotometer.

This is a computer-controlled double-beam grating spectrophotometer with a constant 20-nm bandpass.

## **PREPARATION OF SOLUTIONS**

### **Stock Solutions**

This experiment uses two stock solutions: A 0.200 M Glucose Stock Solution and an Enzyme Working Solution. The latter contains 7.50 mM phenol, 2.50 mM 4-aminoantipyrene, 0.5 IU/mL GOX, and 20 IU/mL HRP in 0.10 M pH 7.5 phosphate buffer. Both of these solutions are rather unstable at room temperature and must be stored at 5-8 °C to remain stable for 3

months or more. For this reason, you will be provided containers of these two stock solutions already prepared for you. All you need to do is check to see that they have warmed to room temperature prior to use.

### Glucose Standard Solutions, 2-16 mM (18-288 mg/dL)

Clean and label six 50-mL volumetric flasks 0 (the “blank”), 2, 4, 8, 12, and 16 mM. Using a 5-mL graduated pipet or a 5- or 10-mL burette, carefully pipet 0, 0.5, 1, 2, 3, and 4 mL of the 0.200 M Glucose Stock Solution into the six flasks, respectively. Dilute to volume with deionized water and mix thoroughly. This will result in the blank and standard solutions of 2, 4, 8, 12, and 16 mM glucose.

Carefully dilute the unknown solution to volume with deionized water and mix thoroughly.

## PROCEDURE

### Preparing the Cary 50 Spectrophotometer

1. Turn on the instrument and let it warm up and stabilize for at least 15 minutes prior to use.
2. To measure individual standards and your unknown sample, select **SIMPLE READ** in the Cary WinUV menu.
3. Click **SET UP** and select **READ at WAVELENGTH** and enter the wavelength of maximum absorption – 505 nm.
4. In the **Y** mode select **ABS** and click **OK**.
5. Insert the a cuvette containing only deionized water and click **ZERO**. The instrument should now be ready for use.

### Analyzing the Samples and Standards

1. Line up the 50-mL volumetric flasks in the following order: blank, 2-, 4-, 8-, 12-, and 16 mM glucose, and the unknown solution(s). Place 2 clean, dry plastic cuvettes in front of each flask. Throughout this entire procedure take care NOT to touch the optical surfaces of the cuvettes.
2. Cut enough small pieces of Parafilm so that you have 1 piece for each of the cuvettes.
3. Carefully pipet 2.00 mL of the **Stock Enzyme Solution** into each of the dozen or so cuvettes.
4. Re-zero the instrument with the cuvette containing **deionized water** to see if the zero setting has drifted from before.

5. Now, take the **blank** cuvette (containing the Stock Enzyme Solution), quickly inject 0.400 mL of the “blank” solution in the 50-mL flask into it with a 1-mL syringe, cover the cuvette with a piece of Parafilm, shake the cuvette 2 or 3 times to mix the contents, carefully insert the cuvette into the instrument, and close the cover.
6. **Immediately** click **READ** and start a stopwatch. Write down the initial absorbance value. Continue to click **READ** and record absorbance values every 1.00 minute for about 10 minutes total.
7. Repeat Steps 5-6 for each of the standards and the unknown solutions remaining.

[This assumes that the instrument is not drifting and the zero setting has not changed significantly. Note that there will likely be some offset in the reading at “0 time” in any solution containing the enzyme stock solution. If there is significant *drift* in the 0 setting, you may need to re-zero the instrument with the cuvette containing only deionized water. Why would a drifting zero reading **not** be especially critical in this experiment?]

In 10 minutes, the absorbance readings should increase from near 0 A to about 0.05 A for the 2 mM glucose standard solution, and to about 0.75 A for the 16 mM standard. After you have done a few kinetic runs, you will have a better idea of how frequently to take data in order to obtain a valid rate curve. The nominal measurement frequency of  $1 \text{ min}^{-1}$  is not sacred. Also, 10 measurements per kinetic run can be adjusted for better results or faster completion. What you need *at a minimum* are 5-6 “good linear” points on the initial portion of the kinetic plot.

8. Repeat the entire process with the remaining set of cuvettes so that you obtain duplicate assays for the unknown(s). This time, run the unknown sample(s) between the two standards that are closest to the putative concentration of the unknown(s) – that is when the apparent reaction rate of the unknown is intermediate between those of two of the standards.

**If you are working with a partner, the other person should run the kinetic assays on the second set of solutions.**

## DATA ANALYSIS

Use Excel (or some other suitable software) to analyze your raw kinetic data. Plot the data points for each run to see that each set of data is generating a plot that appears to be roughly linear. If there is significant and clear downward deviation from linearity in the data at longer times, it may be necessary to drop some of the data prior to obtaining the initial rate of the reaction. Now perform a linear-least-squares fit to the data from each kinetic run. Use the LINEST function to obtain the slope, which should be the *initial rate* of the reaction, for each run.

Plot the initial rates as a function of the concentration of glucose and perform a linear-least-squares fit to these data. From this plot, obtain the concentration of your unknown. Calculate

the standard deviation of your unknown concentration using the standard error of the estimate ( $S_r$ ,  $S_e$ , or SEE) from the Excel LINEST function.

After considering the duplicate values for the unknown concentration and the associated standard deviations, report your **best estimate** for the unknown glucose concentration and its associated uncertainty (standard deviation) that you can estimate from the values you have. If you do not simply average the two concentration values from the duplicate runs, discuss why.

Include at least one plot of a kinetic run and of a calibration curve in your report. With a little extra effort, you can prepare a very nice single plot containing the kinetic runs for all the standards.

## CLEANUP

Thoroughly clean up **both** of your work areas when done – at your assigned station and by the instrument you used. Rinse out all the glassware and cuvettes that you used, including a final rinse with distilled or deionized water. Place the cuvettes upside down in a rack so that they will dry out. Although billed as “disposable”, our budget situation requires that we re-use them. Fill the volumetric flasks with distilled or deionized water, cap them, and store them in the locker labeled for this experiment. **CAREFULLY** disassemble the syringes completely and thoroughly rinse all parts with deionized water. The pipettes and other equipment used in the experiment are also to be returned to this locker. Wipe down the counter top and shelves in your work areas. Return your unknown volumetric flask and tell the Teaching Assistant that the instrument and computer are ready to be shut down for the day.

## HAZARDOUS WASTE DISPOSAL

*Although some of the solutions are colored and contain enzymes and some organic compounds (including a very small amount of phenol) the solutions are not classified as toxic or hazardous. All of the solutions and remaining reagents you have can simply be emptied down the sink drain with copious amounts of cold water.*

## REFERENCES

- D. A. Skoog, D. M. West, F. J. Holler, and S. R. Crouch, *Analytical Chemistry: An Introduction*, 7th ed., Chapters 21 and 22, pp. 547-592. [Information on UV-Vis absorption spectroscopy]
- A-M. G. Vasilarou and C.A. Georgiou, *J. Chem. Educ.*, **77**, 1327-1329 (2000)
- R.H. Garrett and C.M. Grisham, *Biochemistry*, 2<sup>nd</sup> Ed., Saunders College Publishing, 1999. Chap. 14, “Enzyme Kinetics”, pp. 426-459. Or any other decent biochemistry textbook. Basic physical chemistry texts always have a chapter or two on kinetics, some include material on enzyme kinetics.

D.A. Skoog, D.M. West, F.J. Holler, and S.R. Crouch, *Fundamentals of Analytical Chemistry*, Thompson Learning - Brooks/Cole, 2004. Chap. 29, "Kinetic Methods of Analysis," pp. 878-905.

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