BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 3
Spectrophotometry

The purpose of this laboratory session is to determine the absorption characteristics of the substrates and products of the L-lactate dehydrogenase reaction, to learn how to use a simple spectrophotometer, and to measure the concentrations of two unknown NADH solutions using both the Beer-Lambert Law and a standard curve. The methods learned during this session will be used throughout the semester. Refer back to the Introduction to L-Lactate Dehydrogenase handout for information about the basic properties of this enzyme.

I. PreLab Preparation

Before the lab, please read Chapter 3 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. Be sure you understand the basic properties of light, the Beer-Lambert Law, and the purpose of constructing a standard curve. Look closely at the calculations shown in Practice Sessions 3.1, 3.2, and 3.3. Notice that there are several ways of preparing standard curves, which are discussed on pages 70-72. We will use **Method 2** throughout this course, in which the response (absorbance) is plotted as a function of the amount of the substance of interest. This method simplifies the calculations. It does require, however, that the final volume in all of the assay tubes be the same. This is normally very easy to do. You will not be determining protein concentrations as described in Experiment 3 this week, although you will do this later in the semester.

II. Laboratory Procedures

A. Spectrophotometry and the LDH Reaction

Recall from Cell Biology that there are four basic methods for detecting a particular type of molecule: 1) spectrophotometric assays, 2) radiochemical assays, 3) activity assays, and 4) immunological assays.

1. **Spectrophotometric assays.** This type of assay involves exposing molecules to light and then measuring either the resulting absorption or fluorescence. Light absorption or fluorescence may result directly from the intrinsic chemical properties of the molecules of interest. Alternatively, they may occur indirectly as a result of treating the molecules of interest with other compounds which react with them to create new chemicals that exhibit absorption or fluorescence.

2. **Radiochemical assays.** This type of assay is based on the incorporation of a radioactive isotope such as ^{3}H, ^{14}C, ^{32}P, or ^{35}S into the molecules of interest. The molecules then can

be detected or traced through the release of energy in the form of beta-particles (high-energy electrons) or gamma-radiation.

3. **Activity assays.** This type of assay involves measuring the activity or effect of the molecules of interest. Most enzymes are detected by their ability to catalyze a specific chemical reaction and most transport proteins are detected by their ability to bind a substrate or to translocate it across a cellular membrane. In these cases, the amount of the molecule of interest (the enzyme or binding protein) is inferred from the amounts of other molecules (for example, an enzyme's substrates or products).

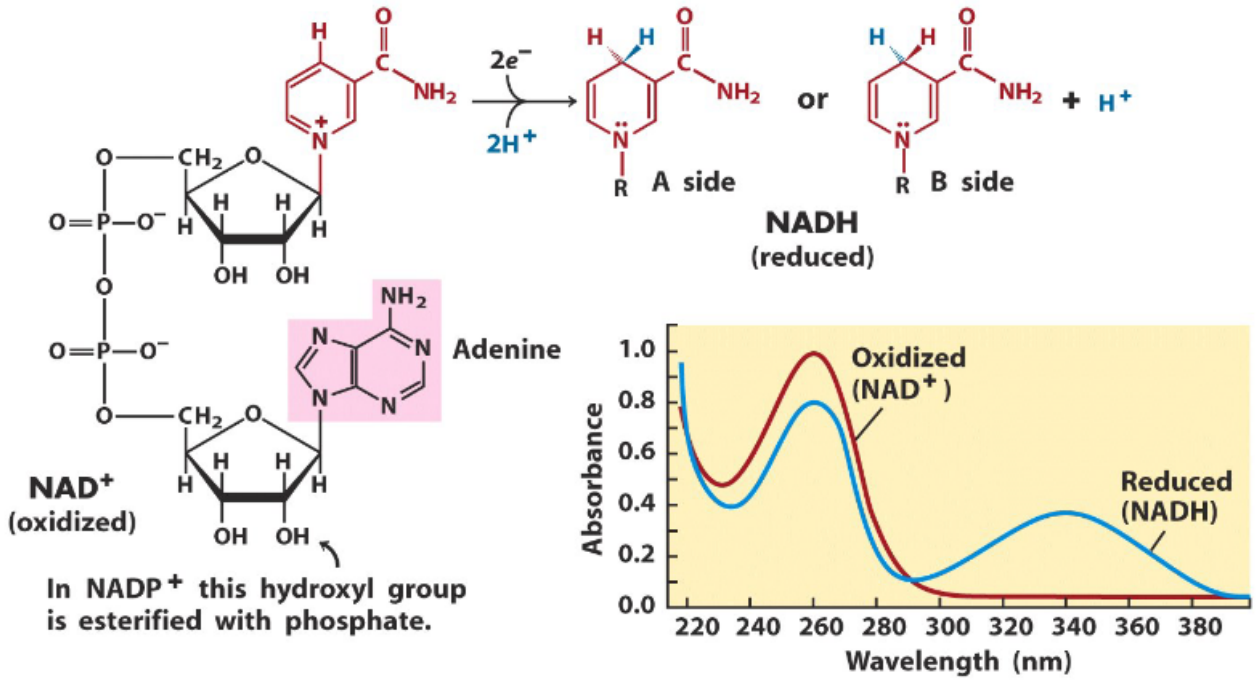
4. **Immunological assays.** This type of assay is based on the use of antibody proteins that bind specifically to the molecules of interest. Binding of antibodies may lead to the formation of a visible precipitate, a radioactive complex, or an enzyme-linked complex that can catalyze a chemical reaction.

Of these assays, spectrophotometric assays are usually the simplest and the most convenient to perform. In the case of an enzyme, the amount of activity is usually inferred from the rate at which one of the substrate(s) disappears or one of the products(s) is formed. This can be inferred from a change in absorbance or fluorescence at a particular wavelength.

Recall from the first lab session and the handout entitled Introduction that the L-lactate dehydrogenase (LDH) reaction is relatively simple and can occur in either direction:

pyruvate + NADH <====> L-lactate + NAD^+

As you will see as part of this lab, pyruvate and L-lactate have little absorbance in the ultraviolet (UV) or the visible region of the electromagnetic spectrum and so cannot be easily measured by spectrophotometry. However, both NAD^+ and NADH have interesting absorption spectra at wavelengths that are easy to work with.



Both compounds show a peak of absorbance at around 260 nm due to the adenine nucleotide component of the molecule. NAD^+ has no additional peaks but NADH does show a prominent broad peak at about 340 nm. The absorbance at 340 nm due to NADH is very useful. You can follow a reaction involving NADH either in terms of the rate at which absorbance at 340 nm increases as NAD^+ is reduced to NADH or in terms of the rate at which absorbance at 340 nm decreases as NADH is oxidized to NAD^+. In this course, we will measure the L-lactate dehydrogenase reaction by following the increase in absorbance at 340 nm.

B. Absorption Spectra of pyruvate, L-lactate, NAD^+, and NADH

The purpose of this part of the experiment is to determine the **absorption spectrum** of each of these four compounds in the LDH reaction over a wide range of wavelengths. For convenience, you will use the Perkin Elmer Lambda 25 UV-visible spectrophotometer located in the instrument area behind the main lab. Note that this is a double-instrument in which the absorbance of sample is continuously compared to a reference solution. Thus, it is not necessary to put in a reference solution or blank, set the instrument to zero, put in a sample, and then take a reading. This instrument also rapidly can scan through a series of wavelengths and store the results.

1. You will be provided with 1.0 mM solutions of Na pyruvate, Na L-lactate, NAD^+, and NADH. Transfer about 3 ml of each of the four solutions to a clean glass test tube. When the instrument is free and you have time, take your solutions to the instrument room. To save time, two groups will carry out the scans at the same time using a single set of samples.

2. Check to see that 1) the Perkin Elmer Lambda 25 UV-visible spectrophotometer has been turned on with the GREEN switch at the top; 2) the HP LaserJet P2035 printer has been turned on with the button on the front; and 3) the Dell Latitude laptop computer has been turned on and the UVWinLab software has been opened.

3. The **BCH Survey Scan Task** program should have been opened. This program is designed to run scans from 1100 nm to 190 nm. The other parameters that were built into this program were the scan speed, the sample ID information box, and the printer output directions. The reporting directions should be set to **Example – Short Scan** output.

4. Under the **Task** Function, move down to **Sample Info** and double click. The sample information from the last scans that were done may be displayed. Highlight each of the old IDs and click on Delete to clear them.

5. Change the number of samples to **5**. Double click on the first **ID box** and change the name of the sample to WATER. In the same way, change the names of the remaining samples to PYRUVATE, L-LACTATE, NAD+, and NADH.

6. Now go back to the **Task** function and move down to **Data Collection** and double click. Click on the GREEN ARROW at the top of the screen and set the starting wavelength to 1100 nm. Now click on the BLUE ARROW at the top of the screen to start collecting data.

7. You should first see a Dialog Box that says remove samples and click OK to perform an auto-zero scan. Open the sample compartment and be sure there are no cuvettes in the cuvette holders. Click OK to start the auto-zero scan.

8. You should then see a new Dialog Box that says to place the first or WATER sample in the instrument. Fill both of the quartz cuvettes with distilled water and place them in the cuvette holders so that clear sides are in the light paths. Click OK to start the scan. You should see the scan as it occurs on the plot of absorbance vs. wavelength and actual values of the wavelengths and absorbances near the bottom of the screen. At the end of the scan, a printout of the scan should be made directly on the HP LaserJet P2035 printer. The printout will show you the scan and the experimental details.

9. You should then see a new Dialog Box that says to place the PYRUVATE sample in the instrument. Remove the **front cuvette**, discard the water, and replace it with about 3 ml of the 1 mM pyruvate solution. Place the cuvette back in the instrument and click OK to start the scan. Again, you should see the scan as it occur in a different color on the

screen. At the end of the scan, a printout of the scan should be made directly on the HP LaserJet P2035 printer.

10. Continue in this way until you have run all five samples and collected five separate scans. You can later cut out or copy these scans and put them together on a single piece of paper.

11. To see the actual absorbance values at the peaks in each scan, go back to the **Task** function and move down to **Results** and double click. You should see again a graph of the scan for the WATER sample. If you click on the PEAKS tab, you should see peaks listed in order and their absorbance values. Write down these values in your notebook. You may find that there are no identifiable peaks for this sample. Why not?

12. If you use the arrow near the middle of the page, you can move successively through all of the scans you have done and see the graph for each sample. Again, click on the PEAKS tab to get a listing of the peaks. Write down the results in your notebook. Continue until you have compiled a list of all of the peak values.

12. After doing all of your scans, carefully rinse out the cuvettes with water, clean the surfaces with a Kim-Wipe and put them in the box for the next group of students.

13. Compile a summary table of the results. At which wavelengths do the compounds absorb light? At which wavelength(s) does only one of the compounds absorb light?

C. Using Spectronic Genesys 20 spectrophotometers

For the next two parts of the experiment, you will use Spectronic Genesys 20 spectrophotometers. Unlike the PE Lambda 25, there are single-beam instruments that only detect light in the near UV and visible range. The basic components are shown below.

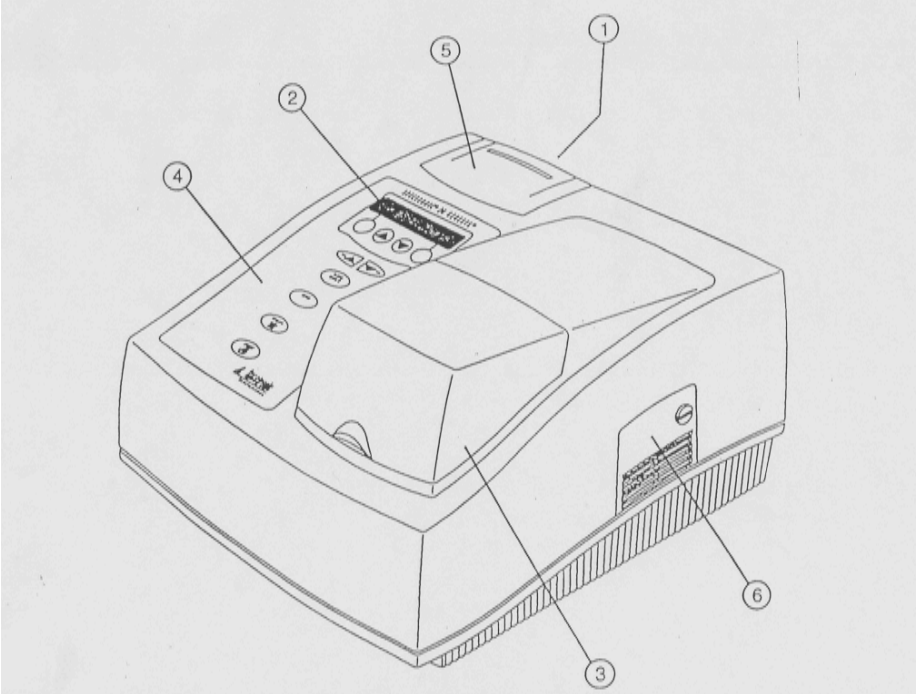


Figure 1 Components of SPECTRONIC® 20 GENESYS™ spectrophotometer

Components
1. On/Off switch
2. LCD display
3. Sample compartment door
4. Keyboard
5. Optional built-in printer
6. Lamp compartment door

The instrument should be used in the following way:

1. Be sure the power cord is plugged into a grounded 120 Volt outlet.

2. Turn on the power switch on the back of the instrument. The instrument will go through a short power-up sequence that takes about 2 minutes. Then allow the instrument to warm up for 15 minutes before taking any readings.

3. Press the A/T/C button on the key pad to select absorbance.

4. Press the nm(UP) or nm(DOWN) buttons on the key pad to select the wavelength to be used.

5. To set the instrument to zero absorbance, lift up the cover of the sample compartment and insert a plastic disposable cuvette containing the reference solution appropriate to your experiment. Be sure to wipe the outside of the cuvette first with a Kim-Wipe and insert the cuvette correctly so that light passes through the clear walls. Note the arrow in the sample compartment that indicates light passes from the back of the instrument towards the front. Close the cover of the sample compartment.

6. Press the 0 ABS/100% T button on the key pad to set the instrument to 0 absorbance. The zero reading will appear on the LCD display.

7. To make an absorbance reading, remove the cuvette with the reference solution and insert another cuvette containing the sample solution. Again, be sure the outside of the cuvette is clean and is oriented correctly.

8. The absorbance of the solution will appear on the LCD display. Record the number in your lab notebook.

9. Once the instrument has been standardized, it should remain stable and you can continue to take readings without resetting the instrument to 0 absorbance.

D. Using Beer's Law to Determine a Concentration

The purpose of this part of the experiment is to determine the actual concentration of a solution of NADH using its known absorption characteristics.

1. Follow the directions for **Part A** of **Experiment 3** beginning on page 76 of the lab manual. You will be provided with more of the 1.0 mM solution of NADH in water. Set Genesys 20 instrument to a wavelength of 340 nm. Then set the instrument to zero absorbance using a disposable semi-micro methacrylate plastic cuvette filled with about 2 ml of filtered deionized water. These cuvettes show less absorbance in the near UV region than those made of polystyrene.

2. Pour out the water and then measure the absorbance of the NADH solution using the same cuvette. If the absorbance is greater than **0.8**, make a 1/2 dilution of the stock 1 mM NADH solution in water and measure the absorbance again. If the absorbance is still too high, make additional serial 1/2 dilutions in water until you get a stable reading at 340 nm in the range of 0.1 and 0.8.

3. Use the molar extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm for NADH to calculate the actual concentration of the NADH solution. An example of this calculation is given in Practice Session 3.1 on page 66 of the lab manual. If you diluted the sample before making your final absorbance reading, correct for the dilution factor.

4. How does the actual value for the NADH concentration of your solution compare with the value on 1.0 mM on the tube? Why might the measured value differ from the concentration value as determined by weighing out some stock powder of NADH and dissolving it in water?

E. Preparation of a NADH Standard Curve

The purpose of this part of the experiment is to prepare a NADH standard curve. This can be used to determine NADH concentrations under the conditions that are actually used in the enzyme assays.

1. You will use the same 1.0 mM solution of NADH you used in Parts B and D. You will combine varying amounts of this solution with varying amounts 0.15 M CAPS buffer, pH 10, so that the total volume is always 1.0 ml (1000 µl). This is the same buffer that will be used in the LDH assays as part of next week's lab.

2. To improve the accuracy of your standard curve and to determine the limits of its sensitivity, you will a) do the assays in duplicate, b) test a large number of different amounts of NADH, c) use micropipetters to transfer the stock NADH solution, and d) wipe the outside of the pipet tips carefully with a Kim-Wipe

3. Set up 21 12 x 75 mm glass tubes as shown in the following table. Note that the total volume of each tube will be 1000 µl or 1.0 ml.

tube	0.15 M CAPS	1.0 mM NADH	A(340 nm)	ave	µmoles NADH
1	1000 µl	0	_____	_____	_____
2	990	10 µl	_____	_____	_____
3	990	10 µl	_____		
4	980	20 µl	_____	_____	_____
5	980	20 µl	_____		
6	950	50 µl	_____	_____	_____
7	950	50 µl	_____		
8	900	100 µl	_____	_____	_____
9	900	100 µl	_____		
10	850	150 µl	_____	_____	_____
11	850	150 µl	_____		
12	800	200 µl	_____	_____	_____
13	800	200 µl	_____		
14	700	300 µl	_____	_____	_____
15	700	300 µl	_____		
16	600	400 µl	_____	_____	_____
17	600	400 µl	_____		
18	500	500 µl	_____	_____	_____
19	500	500 µl	_____		
20	400	600 µl	_____	_____	_____
21	400	600 µl	_____		

4. Using a P-1000 micropipetter, add the CAPS buffer to the tubes first. Then add the stock 1.0 mM NADH solution to the tubes. Add the liquid to the buffer and draw the solution up and down several times to mix the solutions together. Use a P-100 for volumes of 100 µl or less; use the P-1000 micropipetter for volumes of 100 µl or more. It will help the accuracy if you use a new tip for each sample and wipe off the outside of the tip quickly with a Kim-Wipe.

5. When all of the samples have been prepared, cover each tube with part of a square of Parafilm and invert several times. This is better than vortexing the samples because it does not generate a lot of foam.

6. Measure the absorbance of each tube at 340 nm using a semi-micro methacrylate plastic cuvette. Again, this type of plastic absorbs less light in the UV region than polystyrene. Use tube # 1 to set the instrument to zero absorbance since this "blank" contains only CAPS buffer. Then read the remaining tubes in order using the same cuvette. If you measure the absorbance values of a series of samples in order of increasing amount or color, there is no significant carryover from one sample to the next. Record all of the values in your lab notebook using a chart like that shown in step # 3.

7. Calculate the average (mean) absorbance value for each of the duplicate samples.

8. Now calculate the amount NADH in the duplicate tubes. To do this, multiply the volume of NADH by the concentration in moles/liter. For example, for tubes # 10 and # 11 where you added 150 µl of 1.0 mM NADH, the amount of NADH is:

$$150\ \mu l \times \frac{1.0\ mmole}{liter} \times \frac{liter}{1000\ ml} \times \frac{1\ ml}{1000\ \mu l}$$

$$= 0.00015\ mmoles$$

Since this is a very small number, it will be easier to interpret the results if you express the amounts in µmoles instead.

$$0.00015\ mmoles \times \frac{1000\ \mu moles}{mmole} = 0.15\ \mu moles$$

Calculate the amounts for each of the volumes of NADH tested and add them to the table on page 6. What is the range of amounts represented by your standard samples?

9. Now make a graph in which you plot the average A_{340} value of the duplicate tubes as a function of the amount of NADH they contain. Use a sheet of precise linear graph paper, in which there are 1 mm divisions per cm.

10. Draw a "best fit" straight line through data points with a ruler. The line should go through the origin (0 NADH = 0 Absorbance) since you used tube # 1 to set the instrument to zero. The curve should be linear through at least some of the points. You might find, however, that the standard curve becomes nonlinear at high amounts of NADH.

11. Based on the linear portion of the curve, define a conversion factor relating absorbance at 340 nm to the amount of NADH. You can calculate this conversion factor by dividing the absorbance at any point on the line by the corresponding number of μmoles. This conversion factor is the same as the slope of the line, which has the units of A_{340}/μmole.

12. If you like, you can prepare the standard curve using Microsoft Excel or another software program. Section 1.8 in Chapter 1 of the lab manual tells you how to do this. Note that there are some significant differences in how Excel graphs are made if you are using the 2003 or 2007or 2010 versions of Microsoft Office.

    It is essential in making graphs with Excel to use a **X-Y scatterplot** and to add a **linear regression trendline** after you have made the graph. You can set the line so that it goes through the **origin** at (0,0). This is entirely appropriate, since you know that the first tube has no NADH in it. You can request that the **slope of the line** and its **regression coefficient (R^2 value)** be displayed on the graph. The slope is the same as the conversion factor mentioned in step # 11. The regression coefficient is a measure of how well the line fits the data points: a perfect fit gives an R^2 value of 1.0.

F. NADH Concentrations of Two Unknown Solutions

The purpose of this part of the experiment is to determine the NADH concentrations of several unknown solutions using both the Beer-Lambert law and the NADH standard curve. Four unknown solutions will be provided which will simply be labeled A, B, C, and D. Each group will do two (2) of these samples.

1. Since you do not know the concentration of these unknown solutions, you will need to make several dilutions so that some of your NADH samples will fall within the functional range of the spectrophotometer or the standard curve.

2. Make 2 serial 1/4 dilutions of one of your assigned samples in the following way. Add 6.0 ml of water to each of two large test tubes. Mix the unknown NADH suspension and add 2.0 ml (2 x 1000 μl) of the suspension to the first tube. Cover the tube with part of a square of Parafilm and invert several times to mix. Then add 2.0 ml (2 x 1000 μl) of the 1/4 dilution to the second tube to make a 1/16 dilution. Again, cover the tube with part of a square of Parafilm and invert to mix.

3. Measure the absorbance of the undiluted, the 1/4 dilution, and the 1/16 dilution of your first unknown solution at 340 nm in the Spectronic 20 Genesys spectrophotometer. Again, use a cuvette filled with water to set the instrument to zero absorbance. It will help if you read the 1/16 dilution first, then the 1/4 dilution, and finally the undiluted sample. To read each solution, you only need to add about 2 ml of it to the cuvette.

4. If you think about it, the concentration of a compound like NADH in a solution is really the same whether you put 1 ml, 2 ml, 3 ml, or 5 ml into a cuvette in order to measure it. **By convention in biochemistry, the absorbance of a solution is expressed per 1.0 ml.** For example, suppose you find that the absorbance at 340 nm of your undiluted NADH solution is 0.452. You would then say that this solution has 0.452 A_{340}/ml.

5. Now look to see which of these absorbance values falls within the linear portion of your NADH standard curve. It is possible that one or two of them will do so, but it is also possible that only one of them will do so.

6. Now convert this absorbance value to an actual amount of NADH using the conversion factor you found in step # 11 or 12 of Part C. For example, suppose that using the conversion factor from the standard curve, you find that an absorbance at 340 nm of 0.452 corresponds to 1.23 μmoles. You would then say that the concentration of the unknown solution is 1.23 μmoles/ml.

7. Note that if you have to use one of the dilutions of your stock suspension to get an absorbance value within the range of the standard curve, you need to correct for this dilution factor by multiplying by its inverse. For example, if the concentration of a 1/4 dilution of an unknown solution is 1.57 μmoles/ml, the concentration of the original solution would be:

$$\frac{1.57\ \mu\text{moles}}{\text{ml}} \times 4 = \frac{6.28\ \mu\text{moles}}{\text{ml}}$$

8. Finally, express the concentration of your unknown NADH solution in moles/liter (M) and mmoles/liter (mM). For example,

$$\frac{6.28\ \mu\text{moles}}{\text{ml}} \times \frac{1000\ \text{ml}}{1\ \text{liter}} \times \frac{1\ \text{moles}}{10^6\ \mu\text{mole}}$$

$$= \frac{6.28 \times 10^{-3}\ \text{moles}}{\text{liter}}$$

$$= \frac{0.00628\ \text{moles}}{\text{liter}}$$

$$= 0.00628\ \text{M}$$

$$= 6.28\ \text{mM}$$

9. If two of the samples of your unknown solution of NADH give absorbance values that fall within the range of the standard curve, calculate the molar concentration of the solution based on each reading and then take the average.

10. Now, using the absorbance value(s) that fall within the range of 0.1 and 0.8, determine the concentration of the unknown solution using the Beer-Lambert law and the molar extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm for NADH. This is the same type of calculation you did in Part D.

11. Again, correct for any dilution and then calculate the concentration of NADH in the original unknown solution. Compare the value for the concentration found in this way with the value determined from the standard curve. Why might the two values differ?

12. Repeat the entire process with your other unknown sample.