Biochemistry Lab
Spectrophotometry Lab

Note: You will work within your group throughout the laboratory experiments, but individually for all homework/assignment questions.

Purpose: Become familiar with the use of the UV/Vis spectrophotometer and plate reader, prepare NADH/NAD+ solutions, and determine concentrations using the extinction coefficient.

Materials: 10 mM K^+ phosphate buffer, pH 7.2, quartz and plastic cuvettes, 96 well plates, 100 mM stock solutions of NADH and NAD^+, 10 ml conical tubes, microfuge tubes

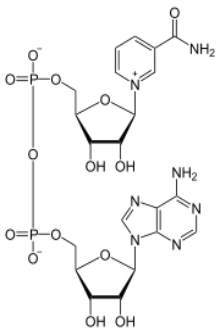

Fig 1 Structure of NAD^+

Structure of $NADH/NAD^+$: Nicotinimide adenine dinucleotide (NADH, Fig 1) has two oxidation states and is involved in electron and proton transfers in the cell. Enzymes that catalyze these reactions are dehydrogenases. NADH consists of two ribose carbohydrate bases and two nitrogen bases, adenine and nicotinamide (see Fig 2). There are two chromophores in the NADH molecule, the reduced nicotinamide ring (labeled) and the adenine ring. Each has a distinct absorption spectrum (plot of absorbance versus wavelength) as you will see in the second part of the experiment and the extinction coefficient of either absorption band can be used to quantitate the concentration of NADH in solution.

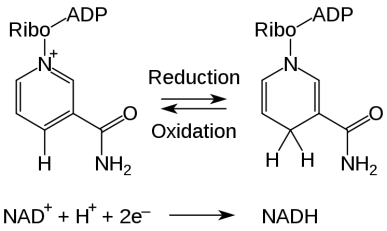

Fig 2. Reduced and oxidized form of the nicotinamide base of $NADH/NAD^+$

Introduction to Beer's Law: The extinction coefficient of an absorbing molecule is a fundamental parameter of that molecule and relates its concentration in solution to the absorbance of the solution at a particular wavelength and is related to Beer's Law, which states that absorbance is proportional to concentration and path length:

A is proportional to cl

Where c is the concentration and l is the path length of the cuvette, usually in cm.

For a particular compound at a given wavelength, under a standard set of conditions, the proportionality constant, ε, is the extinction coefficient, usually expressed in molar or millimolar terms (this constant is often referred to as the Molar Absorptivity).

Beer's law thus becomes:

$$A = \varepsilon cl$$

and is usually expressed as absorbance per cm.

The units of ε must be clearly defined and the conditions of the measurements of the absorbance stated.

TASK 1: Determine absorbance curve for NADH and NAD^+

Use the Vernier Spectrophotometer for this task.

1. Use the stock NADH and NAD^+ solutions to prepare 10.0 ml of 0.4 mM NADH and 2.0 ml of 0.4 mM NAD^+ solution in phosphate buffer. *You will use the unused NADH solution in Task 3, do not discard.*

2. Using a quartz cuvette, determine the absorbance of each solution by performing a wavelength scan from 240 - 400 nm. You only need 1.0 ml of solution in the cuvette.
    a. If the absorbance is significantly over 2.0 you may need to reduce the concentration by dilution and re-read the samples.

3. Determine the maximum absorbance peak(s) for each solution.

4. Record unique features of the absorbance spectrum to allow you to measure the absorbance of NADH in the presence of NAD^+.

TASK 2: Determine the extinction coefficient of NADH

Use the Vernier Spectrophotometer for this task.

1. Using the appropriate absorbance maximum from Task 1, you can now determine the extinction coefficient of NADH.

2. Prepare a series of 1.5 ml dilutions from 0.05 to 0.40 mM NADH in phosphate buffer. Use microfuge tubes for your dilutions. Including a blank or zero control, your curve should have 6-10 dilutions.

3. Using a Vernier spectrophotometer, record the absorbance of each solution at the appropriate wavelength.

4. Into a 96 well plate, transfer 200 µl of each sample into a well. Record the location of each addition in your notebook. Measure and record absorbance of each sample in your laboratory notebook.

5. For both sets of measurements, prepare a graph of the absorbance vs NADH concentration and determine the least squared line for each graph.

6. The slope of the line = $\varepsilon \times l$ (path length). **Note:** To determine the path length for the plate reader, you will need to determine the dimensions of the wells of a 96 well plate. There are a number of 96 well plates on the market; some have wells with straight sides, others are more conical. Assume the width of the cylindrical wells in our plates is 6.6 mm for your calculations and, using the volume, calculate the depth (length) of the solution.

7. Record the molar absorptivity for each method. Compare your results to published/established values for the extinction coefficient of NADH. Include units in cm^{-1}.

8. Consider the following for your results/discussion:
    a. Why not just use a single concentration of NADH to measure the molar absorptivity?
    b. Was there a difference in absolute value between using the plate reader vs the UV/Vis spectrophotometer? If so, why?
    c. What is the purpose of using a plate reader vs a UV/Vis spectrophotometer? Is there a key difference in how each instrument measures light transmittance / absorbance?

TASK 3: Impact of volume addition when using a 96 well plate

Use the Biotek Plate Reader for this task.

1. Dilute 2 ml of 0.4 mM NADH from Task 1 with an equal volume of phosphate buffer.

2. In triplicate, pipet into successive wells of a 96 well plate, 50, 100, 150, 200, 250 and 300 µl of the dilute 0.2 mM NADH solution.

3. Read the absorbance of the samples on the plate reader.

4. Consider the following for your results/discussion:
    a. Determine the linearity of NADH absorbance as a function of volume addition.
    b. Would you expect the trend you observed? How does this relate to the importance of pipetting when using a plate reader?
    c. Why is it less important to be precise and accurate when pipetting solution into a cuvette for measuring absorbance in a UV/Vis spectrophotometer?