

BIOC 462a 2007-2008

Experiment 2: Spectrophotometric determination of the pKa of para-Nitrophenol.

Reading: Chapter 2 in N&B.

Purpose: In this set of experiments we study, in detail, how the spectral properties of para-nitrophenol (PNP) change as a function of pH. First, we will look at the spectra of PNP under acidic and basic conditions. Second, we will determine the extinction coefficient of the deprotonated form of PNP (PNPO⁻) at a wavelength close to its λ_{max} (λ_{max}). Finally, using this spectral information, we will investigate the pH titration of PNP in aqueous buffer and in a buffer containing an alcohol (MeOH, EtOH, or isopropanol) in order to determine the effect of solvent polarity on the pKa of PNP.

Spectroscopy: Background Information:

Spectroscopy is a very important technique in biochemical studies and it is very important for an investigator to be well versed in both the theory and application of the technique. In these experiments, hopefully, you will develop a richer appreciation for spectroscopic techniques as applied to the study of biochemical systems.

Fundamentally, the absorbance spectrum of any chemical species is a measurement of how much light is absorbed at different wavelengths. As pointed out in your text, the absorbance (A) at any wavelength is a function of the chemical properties of the material (for UV-visible spectra this can correspond to molecular orbital electronic transitions) which is related to the (milli) molar absorptivity coefficient (see below), the distance the light travels through of a solution of the sample in a cuvette (path length), and the concentration of the material. This relationship is expressed mathematically in the well-known Beer-Lambert Relationship:

$$A_{\lambda} = (\epsilon_{\lambda})(c)(l)$$

Where ϵ_{λ} is the (milli-) molar absorptivity coefficient at a specific wavelength (λ), c is concentration, and l is the path length of the sample (typically 1 cm). The derivation of this relationship is sufficiently described in Ch. 2 of N&B.

Operationally, we typically use this equation for the determination of the concentration of material in a solution or for the calculation of the expected absorbance of a known concentration of the sample solution. However, in this experiment we will try to expand our understanding of the application of this equation.

It is very important to remember that every visible spectrum is actually the absorbance of the sample **minus** the absorbance of a reference (often referred

to as the baseline). In other words, at each wavelength the final or net absorbance is:

$$A(\text{net}) = A(\text{sample}) - A(\text{reference}) \\ = (\epsilon_{\lambda} \text{ sample})(c \text{ sample})(l) - (\epsilon_{\lambda} \text{ reference})(c \text{ reference})(l)$$

In most cases, the reference can be one of the following:

- Air.
- A solution of a non-absorbing species such as water or a buffer solution for which $\epsilon = 0$ at all wavelengths); a chemical species other than the sample that have absorbance, but are not altered by the reaction.
- A solution containing equimolar sample before any chemical transformation has occurred.

The method, by which the subtraction is carried out, depends upon the spectrophotometer being used. In single beam instruments like a Spec 20 you first calibrate the instrument at a given wavelength against a test tube containing the reference solution. In essence, you are adjusting a potentiometer that results in an absorbance of zero for the reference solution plus the test tube. In non-computer interfaced dual beam instruments, it is necessary to have the sample and reference present in the spectrophotometer at the same time. As the scan is made, a mirrored chopping motor sends alternating beams of light of the same wavelength through the sample and reference samples. Each beam of light impinges upon a photo-multiplier tube (PMT), which in turns generates a voltage or current. The electrical signal of the reference is electronically subtracted from that of the sample. The resulting signal is then sent to a strip-recording device that draws the spectrum on calibrated graph paper.

With the computerization of most modern spectrophotometers, it is seldom necessary to run both sample and reference simultaneously (even in dual beam instruments). Usually the spectrum of the reference solution (i.e., the baseline spectrum) is run first. The instrument (hardware and software) converts the electrical current coming from the detector to absorbance values that are stored in a numerical array. When the spectrum of the sample is obtained, the absorbance values for the reference are subtracted from the absorbance values for the sample. The final spectrum that you actually observe is this difference, ***assuming that you have chosen to do a baseline correction***. It should be pointed out that in modern research, there are some very specialized forms of spectroscopy in which both sample and reference must be present at the same time, but we will not be concerned with these applications here.

Based on the above explanation, **every** spectrum represents a difference between the sample and reference, even if the absorbance of the reference = 0 at every wavelength. However, spectroscopists define the type of spectrum based on the identity of the reference solution. An **absolute spectrum** is obtained when the reference solution does not contain the sample in any chemical form. That is, the solution in the reference cell or cuvette is buffer or water or solvent but the sample is not present. A **difference spectrum** is

obtained when the reference solution contains the sample, at the same concentration as in the solution in the sample cuvette. One then carries out a chemical reaction with the solution in the sample cell, while doing nothing to the reference solution. The difference spectrum obtained from this type of experiment represents the sample that has been modified minus the unmodified sample (the y-axis is therefore labeled **Delta Absorbance**). Both types of spectra can be very informative and are often complimentary, and therefore both absolute and difference spectroscopy have their special benefits to the researcher as we will see in this and future experiments.

It is worth pointing out that in addition to the absorption characteristics of the sample and reference solutions, the cuvette or spectral cell also contributes to the total absorbance. This is the result of the light absorbing properties of the material from which the cell is made and due to light scattering effects. The net result is that the intensity of light, which passes through the cell (even if it contains no solution) and hits the optical detector, is less than the incident light. The net effect is that there is an apparent increase in absorbance (or decrease in transmittance). Because of these problems, it is necessary to determine if your cuvette is suitable for specific spectral regions. The most common cuvette materials and the spectral regions over which they can be used are:

- Polystyrene (plastic): 340 – 800 nm
- Optical Glass: 334 – 2500 nm
- Spectrosil® Quartz: 170 – 2600 nm

For many applications where the observation wavelength is greater than 340 nm, disposable plastic cuvettes are well suited, especially when dealing with solutions such as Coomassie Blue, which tend to permanently stain glass. However, for determining the absorbance of proteins at 280 nm or DNA at 260 nm, it is necessary to use cuvettes made from Spectrosil® quartz.

Light scattering artifacts are typically not a serious consideration when using standard sized (3 mL) 10 mm cuvettes. Typically, this contribution can be cancelled by using carefully matched cells, or using the same cuvette for the reference and sample solutions (in a single beam instrument). Scattering artifacts can become significant when using small volume (< 1 mL) cuvettes, often referred to as micro or semi-micro cuvettes. This occurs in instruments in which the incident beam is rather wide or it does not precisely hit the center of the front face of the cuvette in an orthogonal manner. When these effects become significant, it is necessary to use cuvettes, which have been blacked out everywhere, except for the sample compartment.

Finally, high precision cuvettes are very expensive and should be handled with care. It is advisable to always clean the face of the cuvette with a soft tissue, handle the cuvette gently when placing it in the spectrophotometer, and ALWAYS rinse the cuvette out very well with tap water followed by deionized water. In order to avoid water drop spots on the glass, rinse the cuvette with EtOH.

Experiment 2-1: Measuring the Absorbance Spectra of the Acidic and Basic Forms of para-Nitrophenol.

Part A. Procedure for Absolute Spectra for Acidic (phenol) and Basic (phenolate) PNP:

1. Using two test tubes, dilute 1 mL of 0.1 mM PNP into 3 mL of monobasic phosphate (Tube 1) and dibasic phosphate (Tube 2) buffers. **(Note: the pH values for the two forms of phosphate buffers were determined in Expt. 1).**
2. Set up parameters for Cary 50:
SCAN: 500 nm to 300 nm
SCAN SPEED: MEDIUM or FAST
BASELINE CORRECTION: ON
3. Run a baseline spectrum of either buffer or H₂O.
4. Run a spectrum of the acidic PNP sample. Return sample to Tube 1.
5. Run a spectrum of the basic PNP sample. Return sample to Tube 2.
6. Record the λ_{\max} 's for both spectra and the absorbance value for each peak.
7. Determine the wavelength at which the spectra intersect (i.e., the isosbestic point or ip). **NOTE:** This data will be used in a subsequent experiment.
8. Using the absorbance values for the two peaks and the isosbestic point (ip), calculate $A_{\lambda_{\max}(\text{basic})}/A_{\lambda_{\max}(\text{acidic})}$ and $A_{\lambda_{\max}(\text{basic})}/A(\text{ip})$.
9. In Expt. 2-3, you will use the absorbance (Delta Absorbance) at a chosen wavelength, which may or may not correspond to the λ_{\max} , to calculate the extent of deprotonation of PNP. Why might you **NOT** want to use the λ_{\max} of the basic form of PNP (Hint: compare the acidic vs. basic spectra in this spectral region)?

Part B. Procedure for Basic – Acidic Difference Spectrum:

1. Use the same set-up parameters as above to run a **baseline** spectrum of the acidic PNP sample (Tube 1).
2. Run a spectrum of the basic PNP sample (Tube 2).
3. Determine the wavelength of the “well” or absorbance minimum and the absorbance value at this wavelength.
4. Determine the wavelength of the absorbance maximum, and the absorbance value at this wavelength.
5. How do these two wavelengths correspond to the peaks seen in the absolute spectra?
6. Remembering that the spectrum you have just obtained is a Difference Spectrum and the y-axis corresponds to Delta Absorbance, consider the shape and absorbance values over the chosen range of wavelengths. Now using the Beer-Lambert relationship, explain why some absorbance values are greater than 0, some are less than 0, and why at one wavelength $\Delta A = 0$. Hint: what parameter in the Beer-Lambert equation (besides A) is changing at each wavelength? How does this affect the resultant difference spectrum.

Experiment 2-2: Determination of the Extinction Coefficient for PNPO^- .

Purpose: In this experiment you will determine the extinction coefficient for PNP at 400 nm in a dibasic phosphate buffer. For each concentration, you will prepare 3 identical samples, then measure the absorbance of each sample. From this data, you should be able to determine the degree of error in your sample preparation techniques and, thus, the error (or certainty) in calculating this spectral parameter.

Procedure:

1. Prepare three replicate samples of PNPO^- at 10, 20 and 40 μM using the 0.1 mM PNP stock in dibasic phosphate buffer. Final volume for all samples is 4 ml.
2. Measure the absorbance at 400 nm for each sample.
3. For each concentration of PNPO^- , calculate the mean and standard deviation for the absorbance values for the three replicate samples. Plot the mean value for each concentration vs. $[\text{PNPO}^-]$. Draw error bars using the standard deviation.
4. Determine the extinction coefficient from the slope of line drawn through the plot of A_{400} vs. $[\text{PNPO}^-]$. Alternatively, one can use a linear regression analysis to fit the data. If possible, you should force the fit through the origin. Now that you have an accurate value for the extinction coefficient of PNPO^- at 400 nm, using the spectra obtained in Expt. 2-1 calculate the value for PNPOH at the same wavelength. For a $\text{PNPO}^- - \text{PNPOH}$ difference spectrum, what would the delta extinction coefficient be at 400 nm?
5. Questions: When doing linear regression analysis on this data, why does it make sense to “force” the fit through the origin? What would be a physical explanation for a non-zero intercept? For each concentration of PNPO^- , based on the standard deviation, what is the percent error? How is this related to the precision of the absorbance values (and calculations based on those values) you report? What are the most likely sources (i.e. spectrophotometer, pipettes, operator error, etc.) of error in this measurement?

Experiment 2-3: Determination of the pK_a for PNP in Phosphate Buffer (\pm 10% Alcohol).

Purpose: In this experiment we will use the spectroscopic information gained in the previous two experiments to determine the pK_a of PNP in phosphate buffers, at different pH values, alone and in the presence of 10% either EtOH or isopropanol.

Procedure: Using the phosphate buffers that you prepared in Expt. 1, prepare PNP solutions at each pH using the following guidelines:

- Choose an appropriate wavelength that you will use throughout the entire pH range. Consult the spectra you obtained in Expt. 2-1. (Consider the reaction we are studying, what the product of the reaction is, and how can we best observe product formation without complications arising from the spectral properties of the reactant).
- **Maximal** absorbance at this wavelength should be ≤ 1.0 .
- Final volume of each solution should be 3 mL.

1. Before measuring the absorbance for each sample, determine the pH of the solution after PNP has been added.
2. Obtain a spectrum of the appropriate baseline solution, prior to running a spectrum of any sample.
3. For the spectra at the different pH values, you can use either an absolute or difference spectral approach. If you choose the latter method, it is advisable to run your sample spectra from low to high pH. You also must consider which sample (at a specific pH) you will use for your baseline solution.
4. Repeat this process using the same phosphate buffers that will contain a final alcohol (EtOH or isopropanol) concentration of 10% (v/v) after PNP has been added. In order to prepare the alcohol-containing solutions, you will use 100% EtOH or isopropanol.

Lab report:

(Note: this lab report will combine the data from Expt. 1 and Expt. 2. The numbering system starts with the three figures you prepared for Expt. 1 and continues for the data you present for Expt. 2)

Compose the following figures:

Figure 1. The absolute spectra of PNP at pH 5 and 10.

Figure 2. The PNPO^- minus PNPOH difference spectrum.

Figure 3. Determination of the extinction coefficient for PNP at pH 10.

Figure 4. Determination of the pKa of PNP in phosphate buffer \pm 10% alcohol.

In the Results section of your report, thoroughly discuss each figure. For instance, for Figs. 1 and 2, you should mention the important wavelengths and the relative absorbance values. It is also worth noting the wavelength of the isosbestic point and its chemical or spectroscopic significance. In Figure 1, you should discuss the difference spectrum in terms of the Beer-Lambert equation (i.e., how positive and negative Delta Absorbance values are related to the magnitude of the extinction coefficients for the two forms of PNP at these wavelengths).

For Figure 2, there are two important considerations. First, the value for the extinction coefficient at the wavelength you chose. Second, how much error is there in your data for each concentration of PNP? How is this error related to the certainty of the value you determine for the extinction coefficient, which is related to the standard deviation? If the error is large, what are the likely sources of this error? Having determined an extinction coefficient for the basic form of PNP, you could now prepare a table that lists the λ_{max} values for the acidic and basic forms of PNP, and the extinction coefficients for each of these bands.

Figure 3 should show a plot of Absorbance vs. $[\text{PNP}]$ in either millimolar or micromolar concentration units where you choose the average value for absorbance for your three data points at each concentration. A linear regression analysis line should be drawn through your data with the y-intercept being "forced" to be zero. Why? The slope of this fit is the extinction coefficient determined at that wavelength in either units of $\text{mM}^{-1} \text{cm}^{-1}$ or $\mu\text{M}^{-1} \text{cm}^{-1}$. Obviously, an important consideration is how good do your data agree for each concentration of PNP? Hopefully, the error will be less than 2%, if not then to what do you attribute the variation? Secondly, how well do your data at the three concentrations fall on the linear least squares fit?

Figure 4 requires a more sophisticated plot. One of the problems with alcohol in water is that it changes the refractive index of the solution, so the absorptive properties of the solution in the absence vs. the solution in the presence of alcohol are different. Therefore, it is not possible to easily determine $[\text{PNPO}^-]$ using the same extinction coefficient for the sample containing 10% alcohol. In order to directly compare the two titration curves it is necessary to normalize the absorbance data. In this case, normalize means that the absorbance values will vary from 0 to 1. Normalization can be done by:

$$A_{\text{normalized}} = (A_i - A_{\text{initial}})/(A_{\text{final}} - A_{\text{initial}})$$

This needs to be done for both the PNP minus alcohol and the data for PNP plus the MeOH, EtOH, and isopropanol data.

In order to determine the most accurate value for the pKa for PNP **IN THE ABSENCE OF ALCOHOL ONLY** you will learn how to do simple curve fitting by the following procedure:

1. Draw the best smooth curve through the data by hand for the data set obtained in the absence of alcohol. An estimate of the pKa value can be determined directly from the plot.
2. Starting with the Henderson-Hasselbach equation and using the pKa value you have chosen, plus the pH at which the measurement was made, solve the equation for the F_{CB} , which in this reaction is F_{PNPO^-} :

$$pH = pKa + \log CB/WA$$

$$\log CB/WA = pH - pKa$$

$$CB/WA = (10^{pH-pKa})/1$$

Now solve for the F_{CB} using the mathematical values given in the above equation for CB and WA and taking into account the fact that $F_{CB} = CB/(CB + WA)$.

3. For each pH value (X) for the buffers you used, and the estimated pKa value from your initial plot, calculate a "theoretical" F_{CB} (a new Y value). Superimpose the plot of F_{CB} vs. pH on your normalized Absorbance plot as a **SOLID** line. What you have now done is generate a theoretical curve for your data using the estimated pKa value you initially obtained. In order to assess whether the fit is good, it is necessary to a simple statistical analysis.
4. The easiest statistical method for this non-linear data is least squares analysis, where you look at the variance between your experimental data (y) and the theoretical data:

$$S_y^2 = \frac{\sum (y_{obs} - y_{calc})^2}{N - 1}$$

Where N = the number of data points. In order for this variance to have any useful information, it is necessary to compare it with a second theoretical curve, generated as described above, in which you change the value for pKa.

For example, if you guessed that pKa = 6.9 from the original plot, then calculate a theoretical curve using this value for pKa. Calculate the least squares variance for this pKa. Recalculate a new theoretical curve using pKa = 7.0, and determine the variance for this data set. If the second variance is less than the first, you are moving in the right direction. If higher, then you are moving in the wrong direction, and you might want to decrease the initial pKa value to 6.8 and go through the calculations again.

5. For the purposes of your lab report, you are to show in the Figure **ONLY** the best-fit curve (i.e. the one with the smallest value for the variance) you obtained

superimposed on your data. In the Results section, you must list the pKa values for which you calculated a theoretical curve (a minimum of five) and the least squares variance for each one.

Typically you would also go through this same routine for the data sets obtained in the presence of the alcohol however for the purposes of this class we will forego such a pleasurable experience. For the (+) alcohol data, make your best guess at the value for pKa from a hand plot, then generate the theoretical curves for the alcohol using that pKa value and the equation you solved above, which will then be superimposed on your experimentally determined data.

You are at liberty to use whatever number crunching device you choose: calculator, Excel, Sigmaplot, Origin, etc.

Now that you have analyzed the effect of alcohol on the titration of PNP, discuss the results based on your knowledge of the behavior of a weak acid in the presence of a solvent less polar than water. Taking into account that you are working with a phosphoric acid buffer, what effect do you think the alcohol would have on the shift in pKa for an amino buffer like Tris, based on your understanding of the chemical (i.e. charged) nature of the WA and CB forms of Tris?