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

Reading: Chapter 3 (Sections 3.1 – 3.5) in NB&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 absolute 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 or EtOH) 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. 3 (pps. 67 – 68) of NB&B and in the lecture notes. Operationally, we can use this equation for the determination of the concentration of material in a solution, for the calculation of the expected absorbance of a known concentration of the sample solution, or for the calculation of the value of ϵ_{λ} for a chemical species.

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

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

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

- Air (very rarely used).
- A solution of a non-absorbing species such as water, a buffer solution for which $\epsilon \sim 0$ at all wavelengths, or a chemical species other than the sample that has absorbance, but is not altered by the reaction.
- A solution containing equimolar sample before any chemical transformation has occurred (this will yield a difference spectrum described below).

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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 turn 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 digitally 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. For difference spectra the y-axis is **ALWAYS** labeled **Delta Absorbance or ΔA** . Both absolute and difference spectra can be very informative and are often complimentary; both 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

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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 significantly stains 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 in order to scan down in the UV region of the spectrum.

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 a small window in 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 (Kimwipes), 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. **NEVER** dry the cuvette with Q-tip type cotton applicators or Kimwipes, which can leave macroscopic pieces of cotton or paper inside the cuvette, which will float around in the solution and can often have a deleterious effect on the spectra you obtain. **NEVER** use compressed air coming out of a lab bench outlet. This air often is often contaminated with pump oil from the compressor which will in turn contaminate your cuvette. Often one will use either N₂ or Ar gas from a tank to dry a cuvette that has been rinsed with EtOH.

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

Before getting down to the details, you might want to look at the Lab Report section (below to see what you will be showing in your figures).

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

1. Using two test tubes, dilute 1 mL of 0.1 mM PNP into 3 mL of either monobasic phosphate or TrisHCl (Tube 1) and a second solution prepared using either dibasic phosphate or Tris base (Tube 2). (**Note: the pH values for the two forms of the buffers should have been determined in Expt. 1. If not, do so now.**)

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, superimposing this spectrum on the acidic PNP spectrum. 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 two 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 $A_{\lambda_{\max}}(\text{basic})/A(\text{ip})$. These absorbance ratios can be a valuable diagnostic tool.

9. In Expt. 2-3, you will use the absorbance (or Δ 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 (in this case negative absorbance) 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. Remember that the spectrum you have just obtained is a **Difference Spectrum** and the y-axis corresponds to Δ **Absorbance**. Considering the shape and absorbance values over the spectral range, use the Beer-Lambert relationship to 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).

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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 either a dibasic phosphate or Tris base 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 either dibasic phosphate buffer. Final volume for all samples is 4 ml.
2. Run spectra over the same spectral region as used above, which the instructional staff will want to see once you have collected all of your data. **This provides you a visual means to determine how well you are pipetting!** 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. $[\text{PNPO}^-]$. If possible, 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. Logic dictates that your line should pass 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 extinction coefficient for PNPOH at the same wavelength. For a $\text{PNPO}^- - \text{PNPOH}$ difference spectrum, what would be the $\Delta\epsilon$ at 400 nm?

Questions: As stated above, 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?

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

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

Procedure: Using either the phosphate or Tris 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 titration experiment. 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.
- Determine what concentration of PNP you should have in each solution.

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 buffers that will contain a final alcohol (EtOH or MeOH) concentration of 10% (v/v) after PNP has been added. In order to prepare the alcohol-containing solutions, you will use 100% EtOH or MeOH.

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Lab report:

Beginning with this lab report, we will follow the format given in “Instructions to Authors” for the journal *Biochemistry* which will be handed out in class. Remember, your TA’s are the “journal editors”. If you fail to follow their format, they can choose to reject your manuscript! Before beginning to write, compose the following figures:

Figure 1. The **absolute spectra** of acidic and basic PNP **on the same plot**.

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

Figure 3. Plot of Absorbance vs. $[\text{PNPO}^-]$ for the determination of the extinction coefficient for PNPO^- .

Figure 4. Spectra of PNP obtained by titration using either phosphate or Tris buffers $\pm 10\%$ alcohol.

Figure 5. A plot of normalized (Δ)A vs. pH for the titration of PNP, with best fit curve drawn through your data (see below).

In the Introduction we expect to see a thorough understanding of the theoretical aspects of this experiment (i.e., the Henderson-Hasselbach equation) AND a sentence or two at the end of the Introduction describing the SCIENTIFIC reason for doing this experiment.

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 ΔA values are related to the magnitude of the extinction coefficients for the two forms of PNP at these wavelengths).

Figure 3 should show a plot of Absorbance vs. $[\text{PNP}]$ in either mM or μM 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? Remember, the error in your measurement is 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? Finally, 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 4 is straightforward and provides the basis for Figure 5.

Figure 5 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, “to normalize” means that the absorbance values will vary from 0 to 1. Normalization can be done using actual absorbance values by:

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

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Alternatively, one can use a metric ruler to measure the peak heights in millimeters and then calculate the normalized absorbance (varying from 0 to 1) from a simple ratio of these measurements. You will need to normalize your data for both the PNP minus alcohol and the PNP plus the MeOH or EtOH data. **This data is to be plotted as individual data points without connecting them.**

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, which bears the title of “**non-linear least squares regression analysis**”:

1. Hand draw the best smooth curve through the data (displayed by symbols that **ARE NOT** connected together) 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. You have now generated a theoretical curve for your data using the estimated pKa value you initially chose in order to calculate a theoretical F_{CB} as a function of pH. In order to assess whether the fit is good, it is necessary to do a simple statistical analysis.

4. The easiest statistical method for this non-linear data is least squares analysis, where you look at the standard deviation, S_y , between your experimental data (y) and the theoretical data (y_{calc}):

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

And

$$S_y = (S_y^2)^{1/2}$$

Where N = the number of data points and $y_{obs}-y_{calc}$ is the variance between the observed and theoretical F_{CB} at each pH. 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 standard deviation for this pKa. Recalculate a new theoretical curve using pKa = 7.0, and determine the standard deviation for this data set. If the second standard deviation is less than the first, you are moving in the right direction. If higher, then you are moving in the wrong direction,

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and you might want to decrease the initial pK_a value to 6.8 and go through the calculations again. For purposes of this report, you will perform five such calculations, preferably with two pK_a values less than and two values greater than that which gives you the **minimal standard deviation**. Obviously, you will also want to include the pK_a which gives you the minimum. *In this iterative process, ΔpK_a is to be no greater than 0.2 pH units!*

5. For the purposes of your lab report, you are to show in Figure 5 **ONLY** the best-fit curve (i.e. the curve you generated for the pK_a with the smallest value for the standard deviation, **S_y**) superimposed on your experimentally determined data (symbols only). In the Results section, include a table listing the pK_a values for which you calculated the theoretical curves (**a minimum of five**) and the standard deviation for each pK_a as a way to justify the value for pK_a that you are reporting. Alternatively, you can plot S_y vs. pK_a that can be included in lieu of the table.

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 pK_a from a hand plot, then generate the theoretical curve for the alcohol using that pK_a 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: slide rule, 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 (PNP) in the presence of a solvent less polar than water. It is worth noting that not only is the behavior of PNP altered by the alcohol, but so are the behaviors of the two buffers, Tris and phosphate. Based on your knowledge of the chemical nature of these two buffers, why do you see opposite shifts in the apparent pK_a values for these two buffers? How is this related to the shift in equilibrium between the WA and CB forms of these buffers in the presence of the less polar solvent?

In the Discussion section you can summarize your findings.

Additional Questions also to be turned in with your Lab Report:

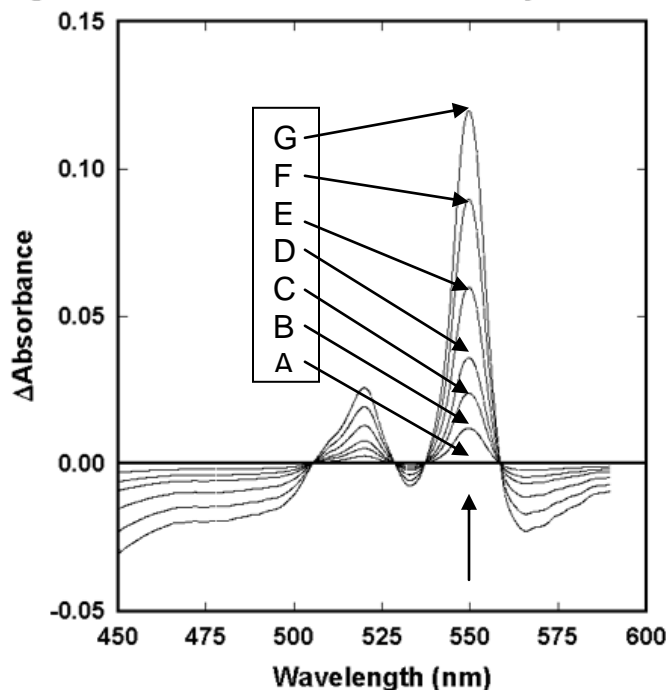
1. The operational definition of an extinction coefficient is that it is the absorbance that a 1 M, 1 mM, or 1 μ M solution would have. Thus, the mM extinction for reduced cytochrome c at 550 nm is $30 \text{ mM}^{-1} \text{ cm}^{-1}$; this solution would have an absorbance of 30 for a 1 mM concentration which is impossible to measure (why?). What would be the absorbance or extinction coefficient for a 1 M solution? Could you measure this absorbance? What would be μ M extinction coefficient (and yes, units are important in this class)? Could you measure the absorbance of this solution?

2. Researchers working in the photosynthesis field commonly make all their measurements in transmittance (or %T). As you titrate a sample with a strongly absorbing species, how would you expect transmittance to change? At the end of the experiment, $I/I_0 = 0.006$. What would be the absorbance of that solution and would you be able to measure that absorbance with most spectrophotometers?

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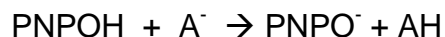
3. In the figure below for reductive titration of cytochrome c (see lecture notes) you see an increase in absorbance at 550 nm. The millimolar extinction coefficients for reduced and oxidized cytochrome are $30 \text{ mM}^{-1} \text{ cm}^{-1}$ and $8 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively. Calculate **precisely** (yes, you might want to use a ruler!) the cytochrome concentration from the final spectrum in the series (the one with the highest absorbance at 550 nm). Next, for each spectrum labeled A-G (corresponding to a specific voltage or potential applied to the system to reduce the cytochrome), calculate the percent reduced for that sample. Assume the solid line at $\Delta A = 0$ corresponds to the initial (or fully oxidized) solution and the final spectrum corresponds to the fully reduced protein.

Figure 5.1 Reductive Titration of cyts b and c



4. In the experiment done this week you have determined the pK_a of para-nitrophenol in an aqueous buffer. The amino acid, tyrosine, also has a phenolic group as the side chain (consult your Lehninger text book for the structure and pK_a for the dissociation of the proton from the side chain ROH group). Remembering your O-Chem, suggest why the the K_a of PNP is orders of magnitude greater (pK_a is lower) than that for the ROH of tyrosine. Using the Lehninger value for the pK_a for the ROH of tyrosine, what would be the ratio of RO^-/ROH for tyrosine at a pH equal to the pK_a you have determined for PNP?

5. Suppose PNP was involved in the following proton exchange reaction:



Assume AH has a very broad absorption spectrum, from 300 nm to 450, with a strong positive absorbance over all these wavelengths, while A^- has no absorbance over the same region. (It is a good idea to make your own drawing of the absolute spectra of all these species, based on the spectra for PNP you obtained and the description of A^-/HA). What would be the **best** single wavelength to observe the **formation of AH**, without contribution of the conversion of PNPOH to PNPO^- to the total absorbance change and why?