

Expt. 2: Spectroscopy and Determination of pK_a of PNP

Goals:

- 1. Learn how to use spectroscopy as a powerful tool in biochemical studies.**
- 2. Perform a pH titration of para-nitrophenol (PNP) using changes in the visible spectrum to determine the pK_a .**
- 3. Determine if the polarity of the solvent alters pK_a by adding alcohols of increasing number of carbons.**

Spectroscopy Over View

- Spectrophotometry: the process that determines the amount of light at a given wavelength absorbed by chromophores.**
- Chromophore: any material that absorbs light.**
 - PNPOH and $PNPO^-$.**
 - Protein amino acids (Trp, Tyr, cystines).**
 - Phe and nucleotide bases.**
 - Cofactors and prosthetic groups: Hemes, flavins, Fe-S, Cu^{2+} , NAD^+ /NADH.**

Uses of Spectroscopy in Biochemistry

- Product identification.
- Changes in structural/physical properties.
- Determining concentration.
- Measuring rate of product formation (steady-state kinetics).
- Enzyme activity assays.
- Ligand binding assays.

Absorption Process

Absorption of light (electro-magnetic radiation) occurs because the energy of some physical transition in the chromophore = the energy of the light.

Most common forms of light in biochemical studies:

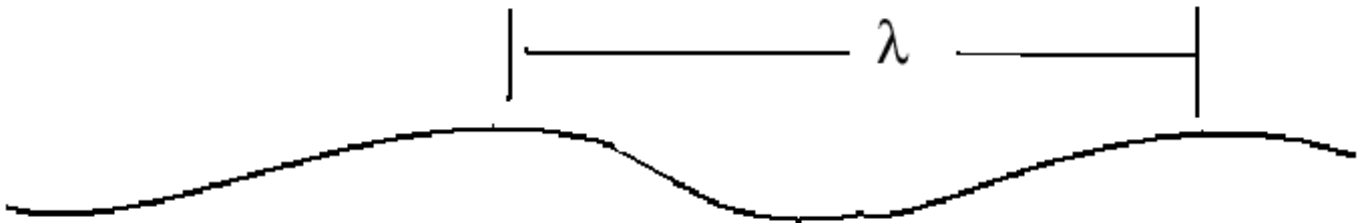
- UV-visible: 200 – 800 nm; electronic transitions ($\pi \rightarrow \pi^*$ and some $\sigma \rightarrow \sigma^*$).
- Infrared: $10^3 - 10^5$ nm; vibration, rotation about, bending of covalent bonds.
- Radio (NMR and EPR): 10^6 and 10^{10} nm; spin orientation of nuclei and unpaired electrons.

Review of Light and Energy

$$E = h\nu \quad \text{where } h = 6.63 \times 10^{-34} \text{ J s}$$
$$\text{and } \nu = \text{frequency in Hz (s}^{-1}\text{)}$$

Hz of light = the number of waves passing a fixed pt./sec.

Wavelength (λ): the peak to peak distance, in cm (10^{-2} m) or nm (10^{-9} m).



$$E = h\nu = h \frac{c}{n\lambda} \quad c = 2.99 \times 10^8 \text{ m s}^{-1}$$
$$n = \text{refractive index (= 1)}$$

NOTE: E is inversely proportional to λ .
As λ increases, the Energy of that light decreases.

For a mol of photons (= 1 Einstein) at 410 nm:

E =

$$\frac{(6.63 \times 10^{-34} \text{ Js})(2.99 \times 10^{10} \text{ cm/s})(6.02 \times 10^{23} / \text{mol})}{(410 \times 10^{-6} \text{ cm})}$$

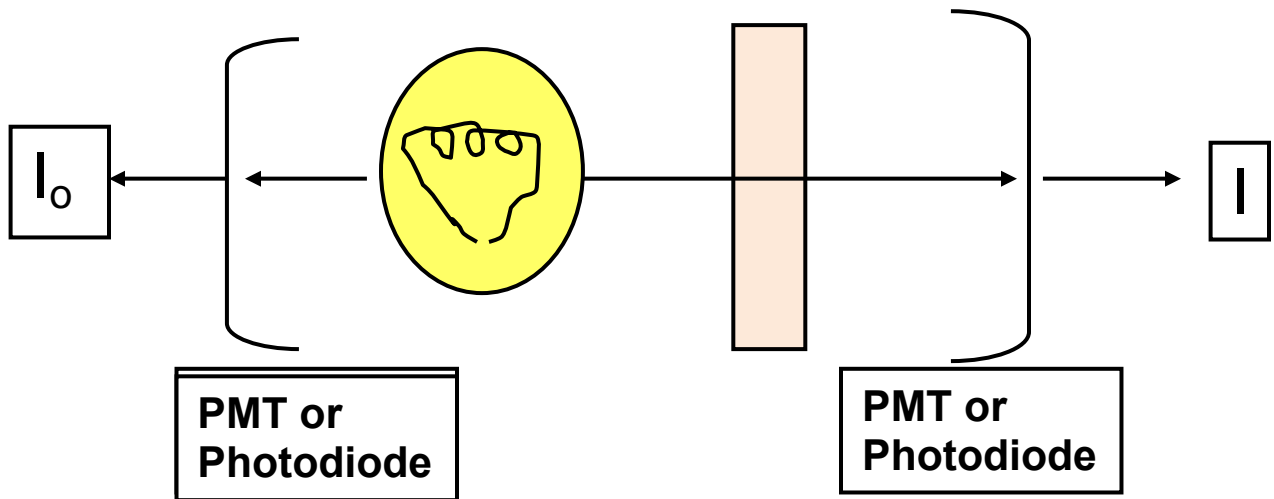
$$E = 2.9 \times 10^4 \text{ J/mol} = 29 \text{ kJ/mol.}$$

This represents a lot of free energy, if it can be captured!

For a species that absorbs light at 410 nm, such as PNPO⁻ (as we shall see), the energy of that light represents the difference the energy levels in the π -electronic configuration in that molecule, i.e. the energy of light absorbed = the energy difference between orbitals.

Absorption of UV-visible light energy results in electronic transitions in $\pi \rightarrow \pi^*$ and $\sigma \rightarrow \sigma^*$ MO's.

Light Intensity, Transmittance, and Absorbance



Transmittance = I/I_0 and varies from $1 \rightarrow 0$.
If nothing is in the beam, then $I = I_0$, but if a cuvette containing a solution is in the beam, then generally, $I/I_0 < 1$:

- Cuvette may absorb light.
- Cuvette scatters light.
- Buffer solution scatters light.

This DOES NOT take into consideration absorption of light by the solution!

(This is why you ALWAYS run a baseline spectrum!)

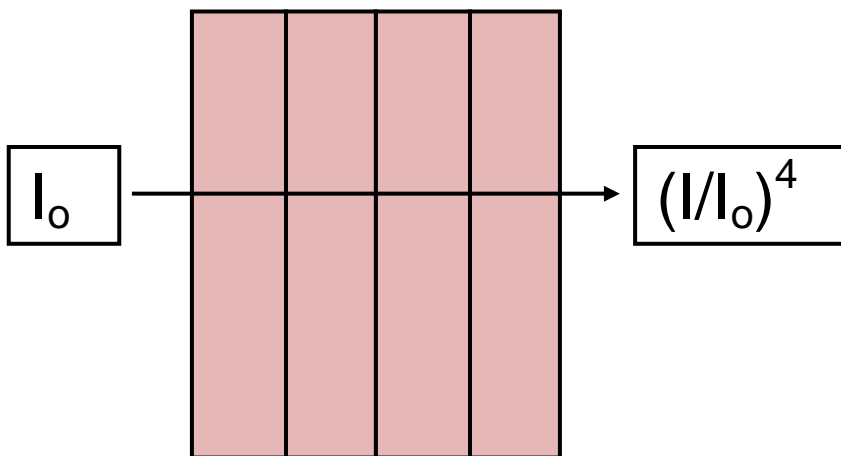
Path Length and Concentration Effects on I/I_0

If a solution is in cuvette that absorbs light of specific λ , then there is a path length and concentration dependency for I.

Lambert Relationship:

Consider the cell to be divided into layers, with the same concentration of solution in each layer. Then there is an I/I_0 value (<1) for each layer. Then: I/I_0 DECREASES EXPONENTIALLY with each layer:

$$\begin{matrix} 1^{\text{st}} & \rightarrow & 2^{\text{nd}} & \rightarrow & 3^{\text{rd}} & \rightarrow & 4^{\text{th}} & \rightarrow & \dots & \rightarrow & n^{\text{th}} \\ I/I_0 & \rightarrow & (I/I_0)^2 & \rightarrow & (I/I_0)^3 & \rightarrow & (I/I_0)^4 & \rightarrow & \dots & \rightarrow & (I/I_0)^n \end{matrix}$$



Beer's Relationship:

I/I_0 DECREASES EXPONENTIALLY with INCREASING CONCENTRATION in similar manner.

Combined Beer-Lambert Relationship:

$$\text{Transmittance (T)} = I/I_0 = 10^{-\epsilon c L}$$
$$-\log(T) = \epsilon c L = A \quad (= \text{Absorbance})$$

- ϵ : absorptivity or extinction coefficient
- c : concentration
- L : path length.

How Does T and A Relate?

I/I_0 (=T) A (= -logT)

1	0	
0.9	0.046	
0.5	0.3	Believable
0.1	1	
0.01	2	
0.001	3	Doubtful

Very low A values: limited by Signal/noise ratio.

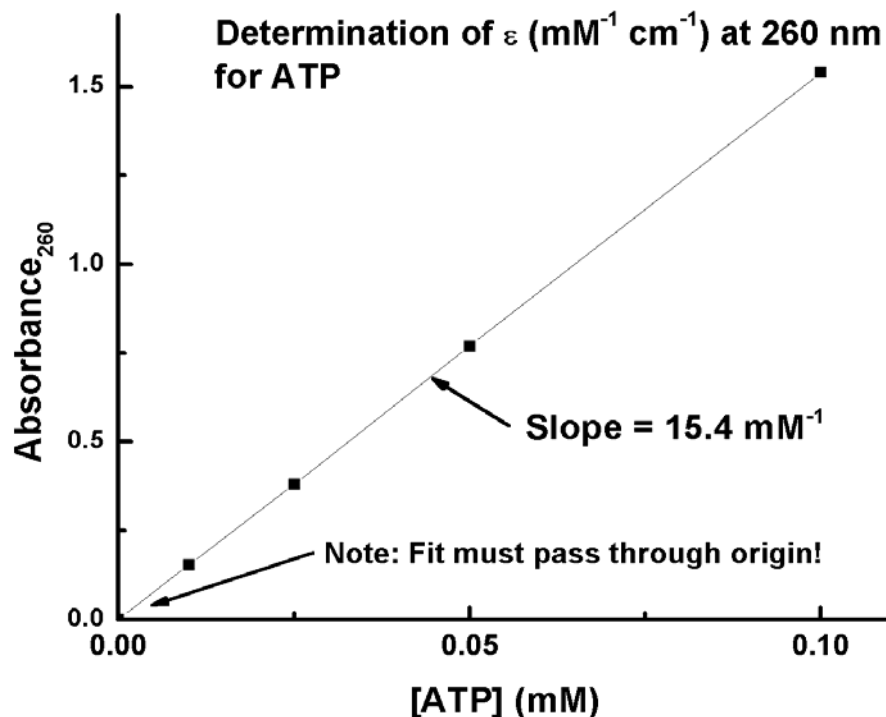
Very high A values: limited by sensitivity of detectors.

You pay a lot of money to optimize these two!

About ϵ :

- Commonly called an **Extinction** or **Absorptivity** Coefficient.
- Molar: $M^{-1}cm^{-1}$; milliMolar: $mM^{-1}cm^{-1}$; microMolar: $\mu M^{-1}cm^{-1}$.
- Is specific for chromophore, λ , and conditions (pH, oxidation state, etc.).
- It is the absorbance that a 1 M solution would have in a 1 cm path length cell.

Determination of Extinction Coefficient



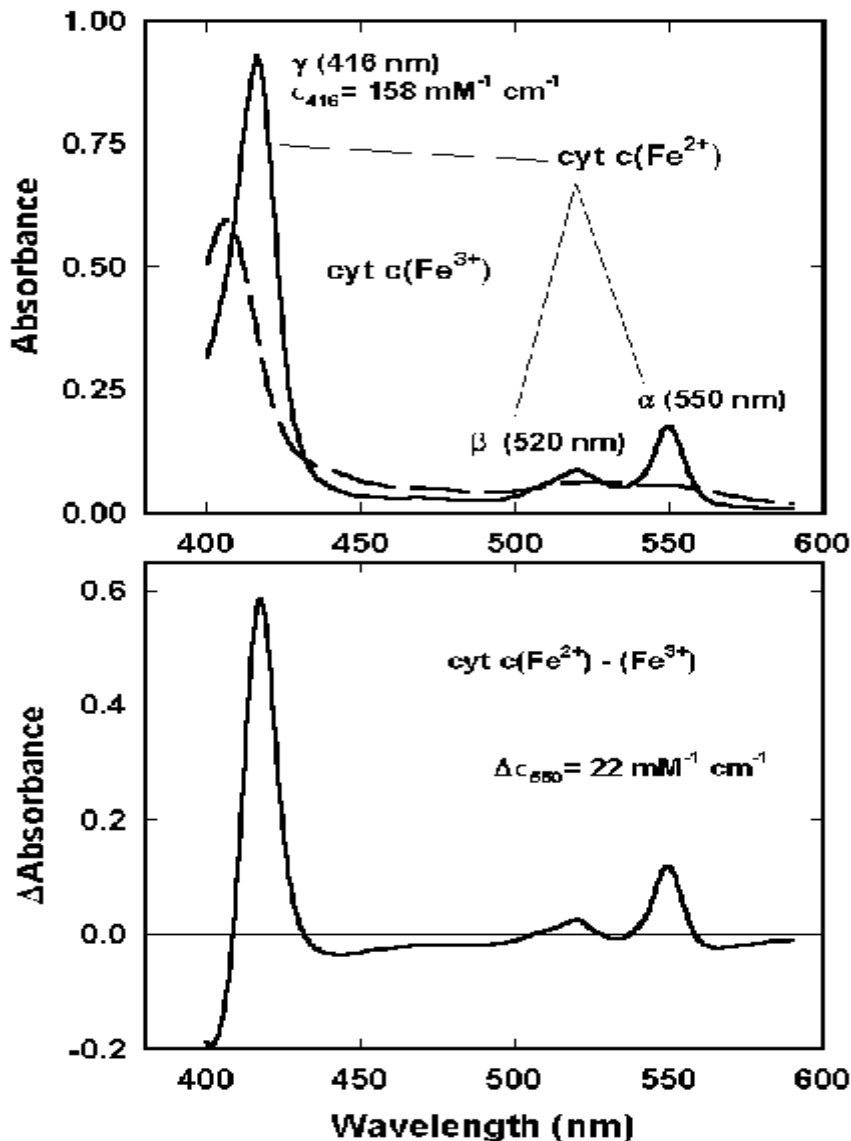
Measure absorbance at specific wavelength as a function of concentration. Determine ϵ from slope.

Values of ϵ for Biological Chromophores

Chromophore	λ (nm)	ϵ ($M^{-1} \text{ cm}^{-1}$)
RSSR	280	600
Tyr	280	1000
Trp	280	10,000
FeS(ox)	400-500	6000-8000
Flavins(ox)	440	14,000
Hemes	400-450	80,000-120,000

Examples of Visible Spectra:

Mitochondrial cytochrome c



Top panel: absolute spectra of reduced and oxidized cytochrome c.

Bottom panel: Reduced minus Oxidized difference spectrum of cytochrome c. Note that the intensity is expressed as Δ Absorbance.

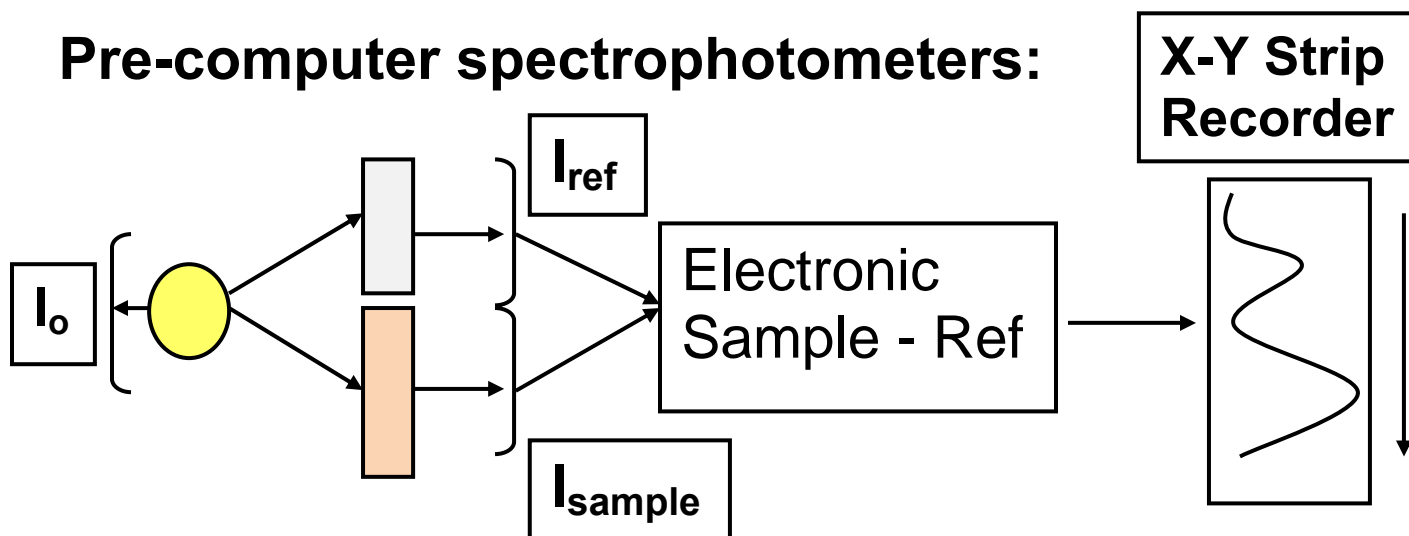
Obtaining Spectra of Samples

Every spectrum **ALWAYS** corresponds to a difference between the **A** of the sample and **A** of a reference or baseline solution:

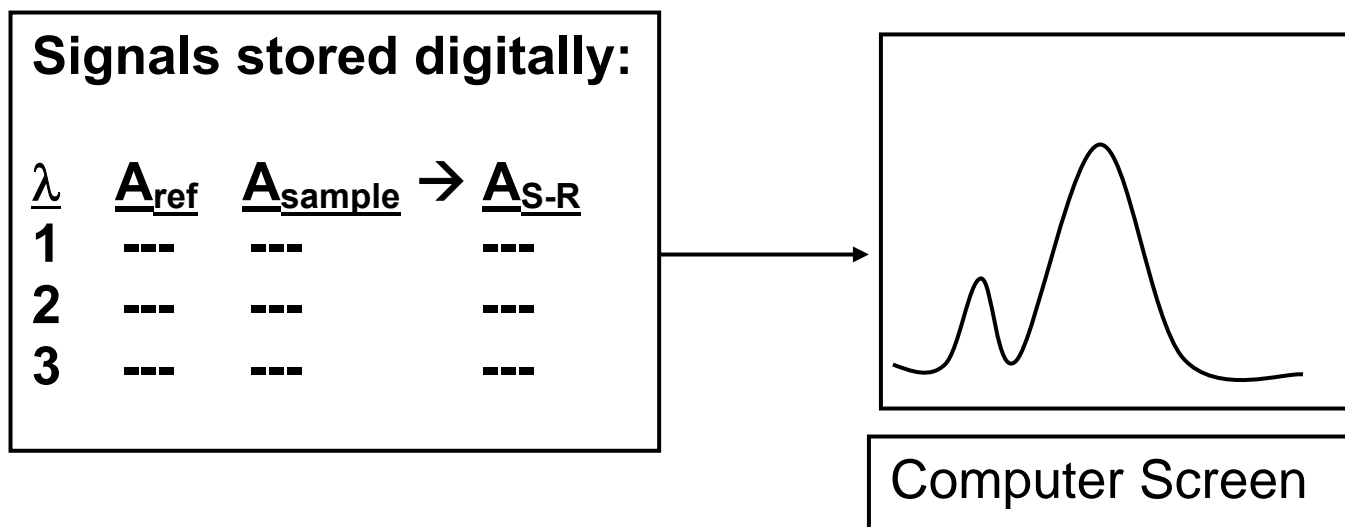
$$A_{\text{final}} = A_{\text{sample}} - A_{\text{reference}}$$

Done either electronically or in computer memory.

Pre-computer spectrophotometers:



Computer-driven spectrophotometers:



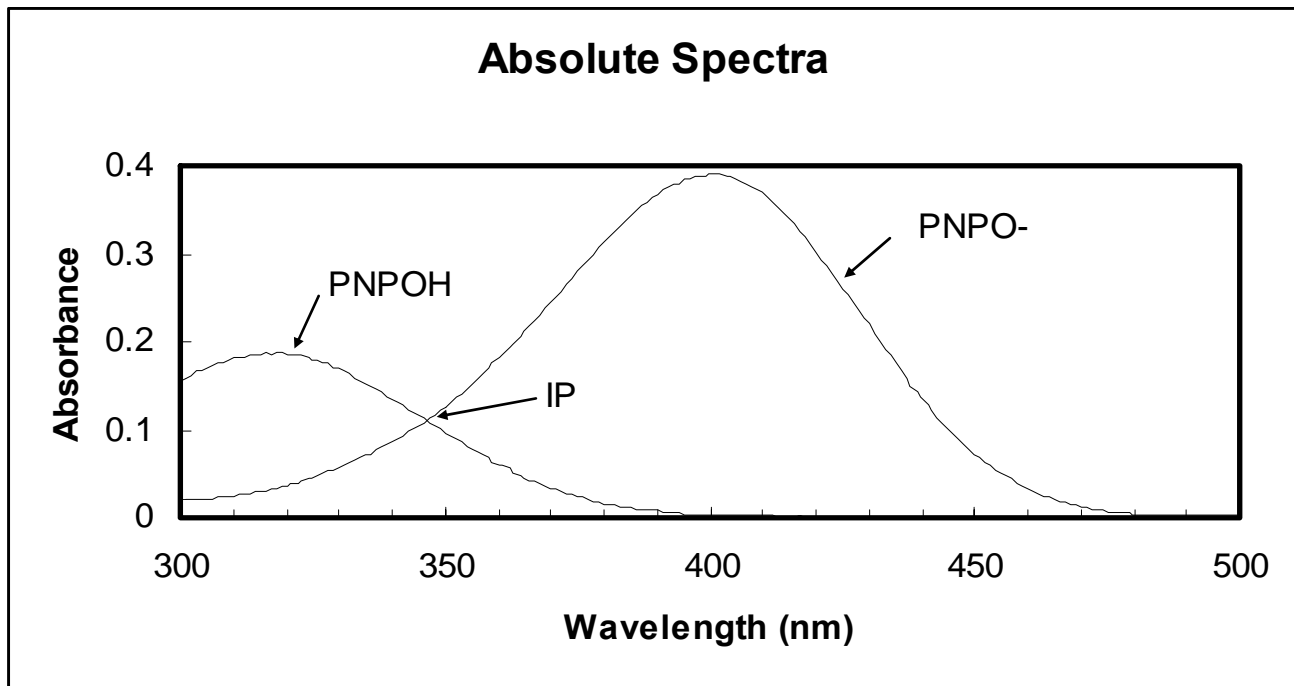
On Cary 50, scanning generates a numerical array of numbers (λ, A).

- **Baseline: numerical array for reference solution. Takes into account absorption (scattering) properties of cuvette and solution.**
- **As you scan sample a temporary array of signal coming from detector is generated.**
- **Spectrum seen on screen:**

Temporary array - Baseline array

Absolute vs. Difference Spectroscopy

Absolute: $A_{\text{Sample}} - A_{\text{Buffer}}$



The absolute spectra of PNPOH and PNPO⁻ are shown. IP corresponds to the isosbestic point (IP), the wavelength at which both spectra have equal absorbance values (see below).

At each wavelength (λ) for PNPOH:

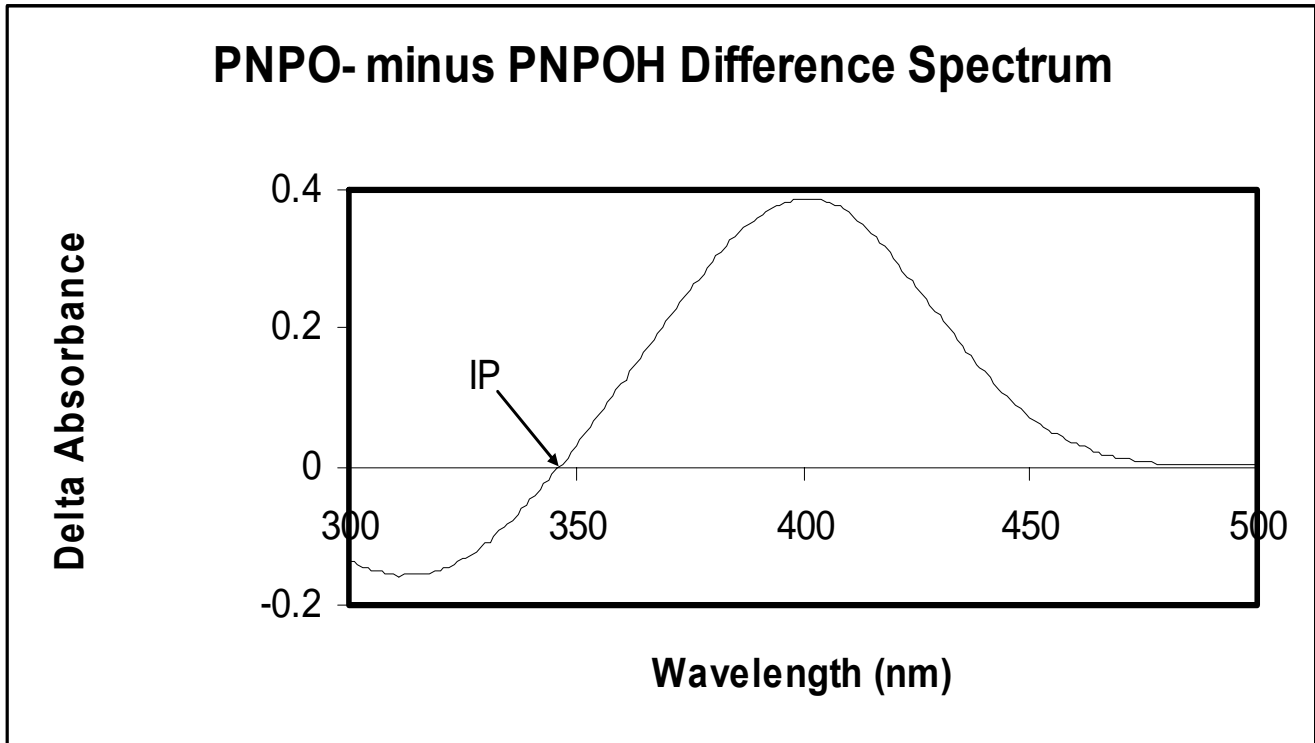
$$A_{\lambda} = (\epsilon_{\lambda, \text{PNPOH}})([\text{PNPOH}])(L)$$

For PNPO⁻:

$$A_{\lambda} = (\epsilon_{\lambda, \text{PNPO}^-})([\text{PNPO}^-])(L)$$

If $[\text{PNPOH}] = [\text{PNPO}^-]$, what do we know about ϵ_{PNPOH} and ϵ_{PNPO^-} at IP?

Difference: Sample in final state - Sample in initial state.



The difference spectrum from the pH titration of PNP was obtained by subtracting the spectrum of PNPOH (initial state) from that of PNPO⁻ (final state). Note that at some wavelengths $\Delta A < 0!$

At each λ :

$$\begin{aligned}\Delta A_{\lambda} &= A_{\lambda, \text{PNPO}^-} - A_{\lambda, \text{PNPOH}} \\ &= (\epsilon_{\lambda, \text{PNPO}^-})([\text{PNPO}^-])(L) - (\epsilon_{\lambda, \text{PNPOH}})([\text{PNPOH}](L))\end{aligned}$$

If $[\text{PNPO}^-] = [\text{PNPOH}]$ then

$$\begin{aligned}\Delta A_{\lambda} &= (\epsilon_{\lambda, \text{PNPO}^-} - \epsilon_{\lambda, \text{PNPOH}})([\text{PNPO}^-])(L) \\ &= (\Delta \epsilon_{\lambda})([\text{PNPO}^-])(L)\end{aligned}$$

Advantages and Uses of Two Types of Spectra:

Absolute:

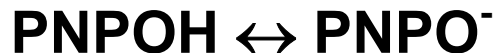
- **Determine λ_{\max} and λ_{\min} values.**
- **Determine concentration.**
- **Can easily find isosbestic points.**

Difference:

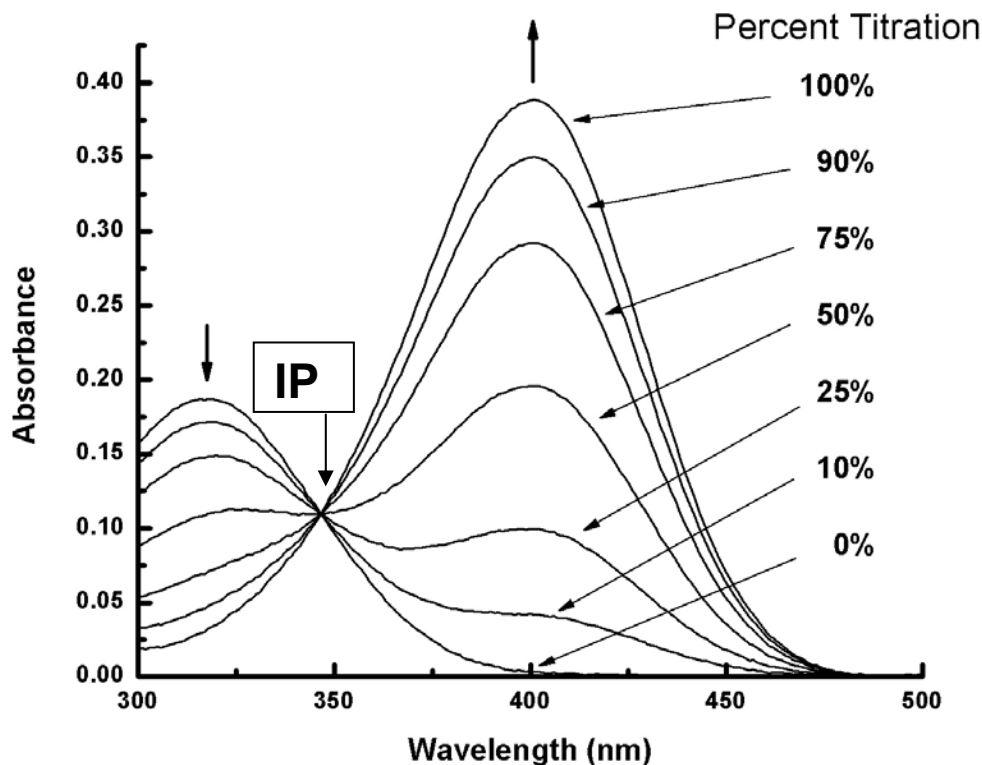
- **Allow you to monitor small changes in concentration of sample easily.**
- **Determine if you have simple chemical reaction.**
- **Almost all kinetic measurements are made using Difference Spectroscopy.**
 - Fixed wavelength monitoring.
 - Stopped flow and laser flash measurements.

Following Spectral Changes in Reactions

In this week's pH titration experiment we are looking at the conversion of:



Absolute spectra:



For each spectrum:

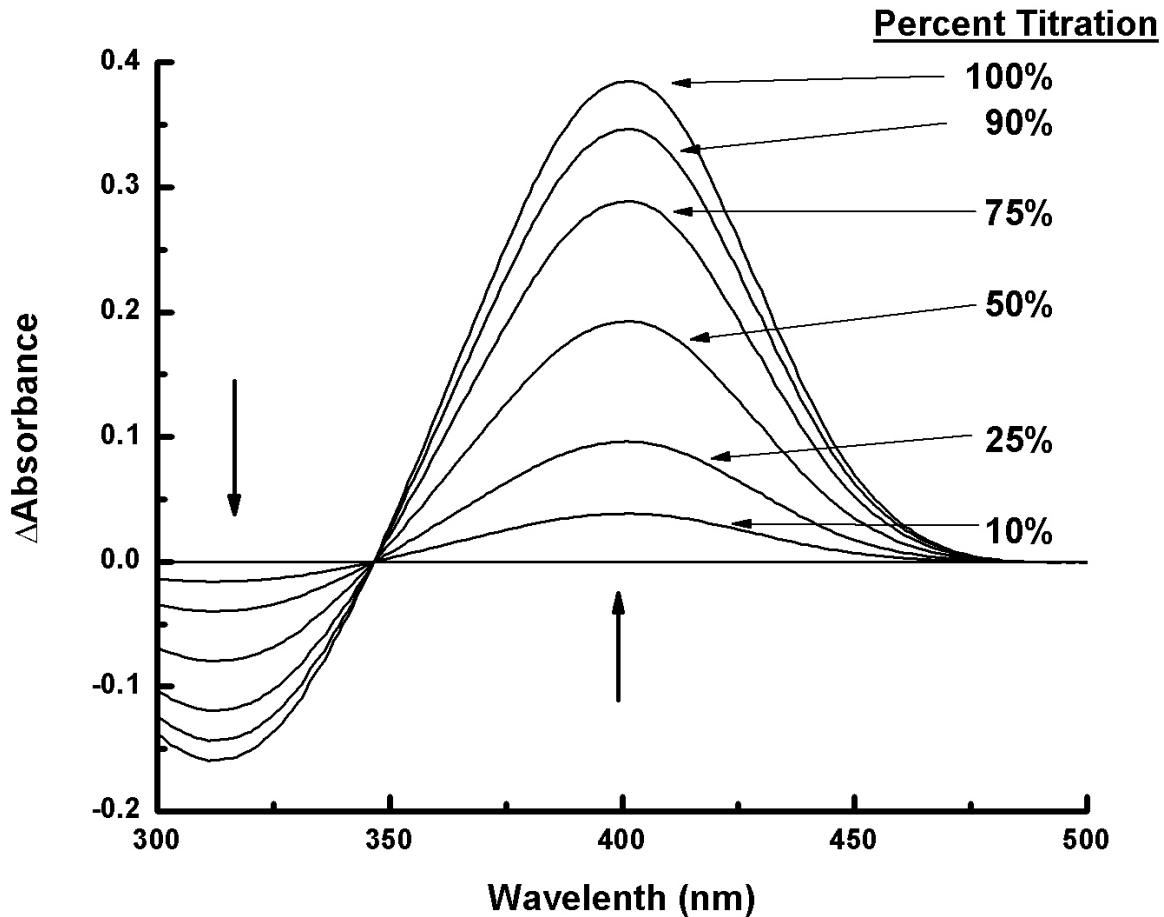
$$A_{\lambda} = A_{\lambda, \text{PNPOH}} + A_{\lambda, \text{PNPO}^-}$$

$$= (\epsilon_{\lambda, \text{PNPOH}})([\text{PNPOH}])(L) + (\epsilon_{\lambda, \text{PNPO}^-})([\text{PNPO}^-])(L)$$

How would you modify this eqn to take into account $[\text{PNP}]_{\text{total}}$, instead of individual concentrations?

At the IP, absorbance is constant throughout the titration, at every other λ it changes!

Difference Spectra: PNPO⁻ minus PNPOH



At every λ , you are looking at ΔA :

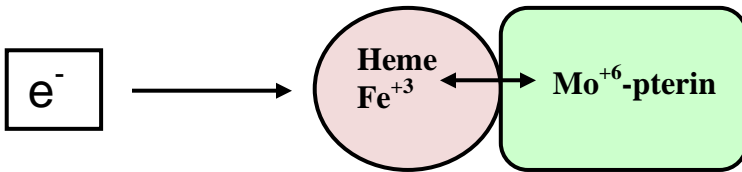
$$\begin{aligned} \Delta A_{\lambda} &= (\epsilon_{\lambda}^{\text{PNPO}^-} - \epsilon_{\lambda}^{\text{PNPOH}})([\text{PNPO}^-])(L) \\ &= (\Delta\epsilon_{\lambda})([\text{PNPO}^-])(L) \end{aligned}$$

When $\epsilon_{\lambda}^{\text{PNPO}^-} < \epsilon_{\lambda}^{\text{PNPOH}}$, then $\Delta A_{\lambda} < 0$.

When $\epsilon_{\lambda}^{\text{PNPO}^-} = \epsilon_{\lambda}^{\text{PNPOH}}$, then $\Delta A_{\lambda} = 0$.

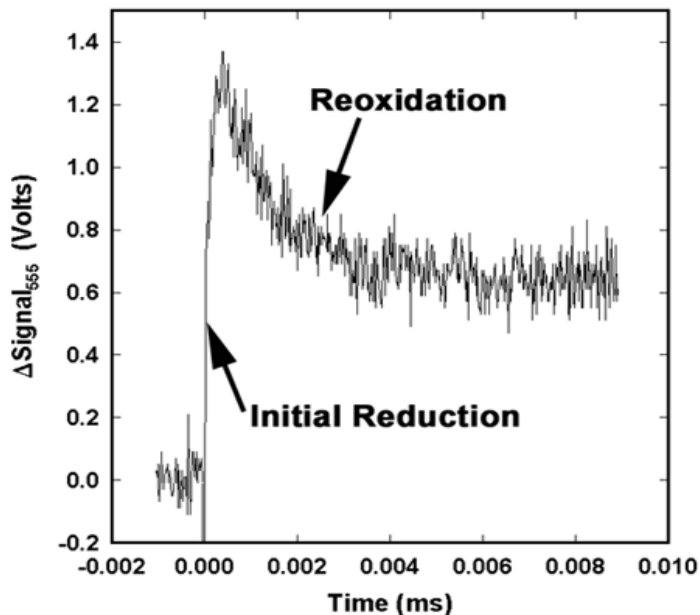
When $\epsilon_{\lambda}^{\text{PNPO}^-} > \epsilon_{\lambda}^{\text{PNPOH}}$, then $\Delta A_{\lambda} > 0$.

Making use of these spectroscopic concepts: Single wavelength difference spectroscopy following product formation in an electron transfer reaction:



- In this experiment, sulfite oxidase was rapidly reduced by in a laser flash photolysis experiment, resulting in rapid increase in absorbance at 555 nm.
- There is a second, slower oxidation reaction by another co-factor, resulting in a decrease in A_{555} .

Figure 5.2 Reduction of Sulfite Oxidase



The signal at $t = 0$ takes into account the sample before the laser flash, therefore is **difference spectroscopy**.

- Two useful pieces of information gained from trace:
 1. The **rate constants** for the reduction and oxidation of the heme AND the sequence of these reactions.
 2. The **equilibrium distribution** of the electron between the two co-factors, thus K_{eq} .