Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 5

More on UV-visible Spectrophotometry: The Beer-Lambert Law and Mixtures

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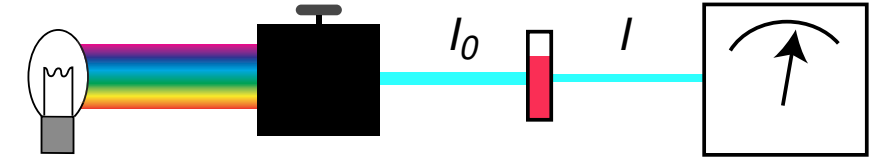
Lab Reports

- Due two weeks after the final lab session for the experiment.
- Link to schedule for experiments and lab reports is on Canvas.
- Due at 1:00 PM on the day of your lab session.
- Lab report for Experiment 1 is due next week (week of 20 January).
- To submit lab report:
  - Sign all of the pages for the experiment in LabArchives.
  - Export the folder as a pdf file.
  - Submit the pdf file on Canvas.

A UV-Visible Spectrophotometer



Direct Methods for Measuring Protein Concentration by Absorbance

1. Direct measurement of UV absorbance (usually at 280 nm)
   - Very useful for pure protein samples, but need to know the extinction coefficient.
   - Extinction coefficient is specific to the protein and depends primarily on the number of Tyr and Trp residues per molecule.

     Can be estimated reasonably well from the amino acid sequence or composition.
   - Not especially sensitive. Good for concentrations of $\approx$ 0.1 mg/mL or greater.
   - Absorbance from other compounds can interfere.
2. Direct measurement of visible absorbance.
   - Very useful for metalloproteins containing Fe or Cu.
   - Need to know extinction coefficient.

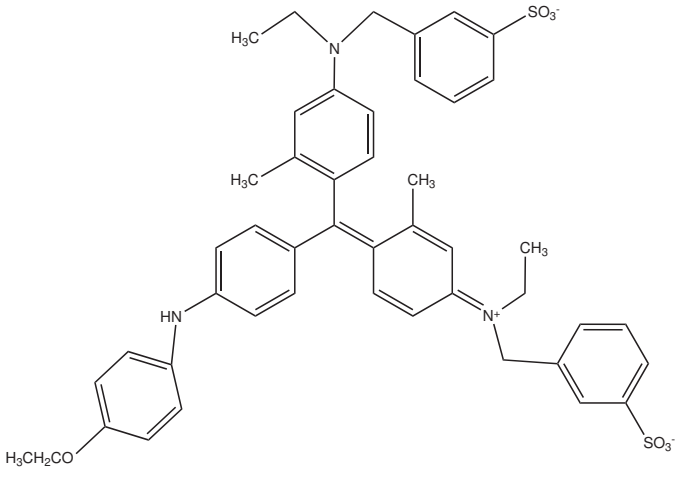
Indirect Methods for Measuring Protein Concentration

1. Formation of coordinated metal complexes, especially Cu.
2. Binding to dyes, leading to spectral shift of the dye.

Advantages

- Much more sensitive (10 $\times$ or more) than direct UV absorbance.
- Less sensitive to interference from other compounds.

The Bradford Dye-Binding Assay



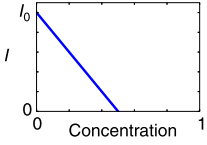
Coomassie blue G-250

- Extensive conjugation leads to absorption of visible light.
- For dye: $\lambda_{max} = 465\,\text{nm}$
- For dye bound to protein: $\lambda_{max} = 595\,\text{nm}$
- Absorption at 595 nm increases as protein is added to fixed amount of dye.
- Requires calibration for a particular batch of dye and solution conditions.

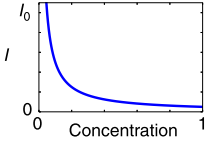
Clicker Question #1

How does transmitted light intensity change with concentration?

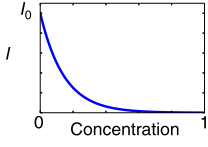
A) $I = I_0 - aC$

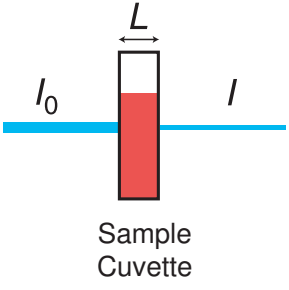


B) $I = \dfrac{I_0}{aC}$



C) $I = I_0 e^{-aC}$

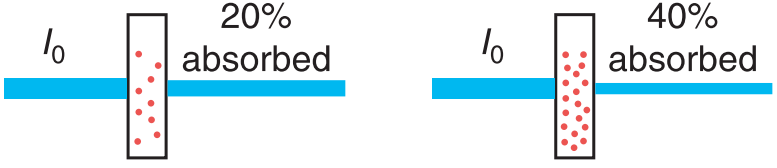




All answers count (for now)!

Clicker Question #2

If we double the concentration, does the number of photons absorbed double?

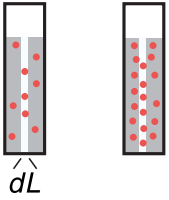


A) for Yes

B) for No

All answers count (for now)!

For a thin slice of the cuvette:



- The probability of a photon being absorbed is proportional to the concentration, C, and the thickness of the slice, dL:

$$p = kC\,dL$$

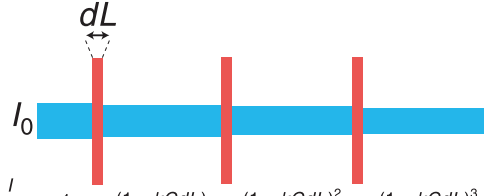
where k is a constant of proportionality.

- Fraction of light transmitted by a thin slice:

$$\frac{I}{I_0} = (1 - p) = (1 - kC\,dL)$$

- How thin does the slice have to be for this to be true?

Many Thin Slices



A Very Handy Approximation

- Consider the function $y = e^{-x}$
- For $|x| \ll 1$:

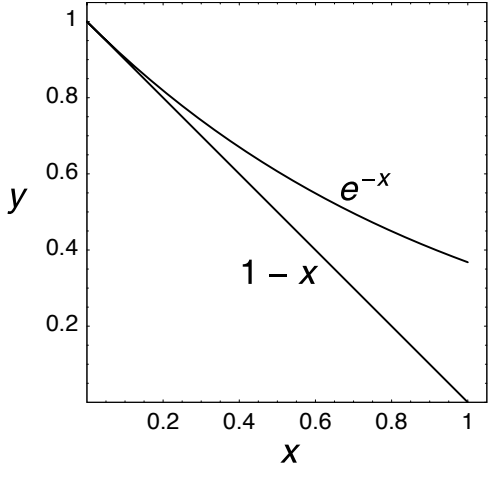
$$e^{-x} \approx (1 - x)$$
$$\ln(1 - x) \approx -x$$

- This only works for e (≈ 2.71828).

  For other numbers:

$$a^{-x} \approx (1 - x \ln a)$$

  for $|x| \ll 1$



Many Thin Slices (contd.)

$$\ln \frac{I}{I_0} = N \ln(1 - kCdL)$$

- Our approximation: For $|x| \ll 1$:

$$(1 - x) \approx e^{-x}$$
$$\ln(1 - x) \approx -x$$

- Substituting:

$$\ln \frac{I}{I_0} = -NkCdL$$

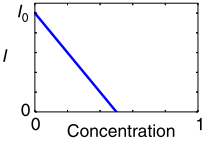
- $dL \cdot N = L$, total path length

$$\ln \frac{I}{I_0} = -C \cdot L \cdot k$$

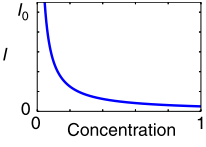
Clicker Question #3

How does transmitted light intensity change with concentration?

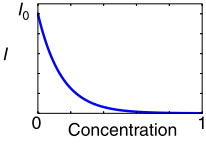
A) $I = I_0 - kC$

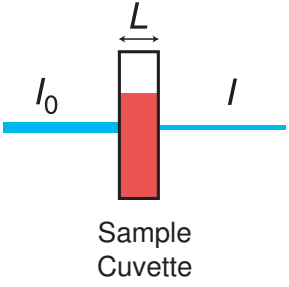


B) $I = \dfrac{I_0}{aC}$



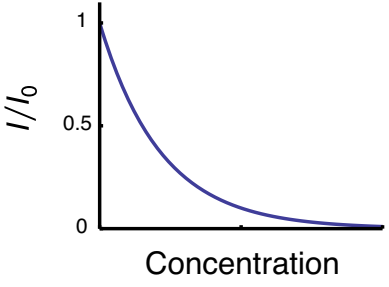
C) $I = I_0 e^{-aC}$

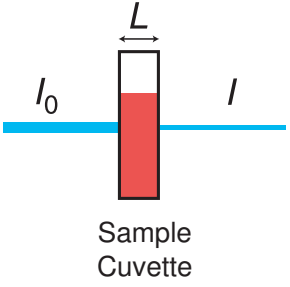




One correct answer!

The Beer-Lambert Law



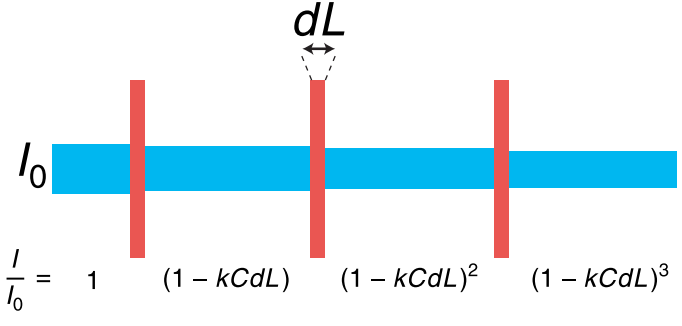


$$\ln \frac{I}{I_0} = -C \cdot L \cdot k \qquad \log_{10}(x) = \ln(x) \log_{10}(e)$$

$$\log_{10} \frac{I}{I_0} = \ln \frac{I}{I_0} \log_{10}(e) = -C \cdot L \cdot k \log_{10}(e)$$

$$\log_{10} \frac{I_0}{I} = C \cdot L \cdot \epsilon = A$$

A Quick Review of How We Got Here



- For N slices: $\frac{I}{I_0} = (1 - kCdL)^N$
- Take (natural) logarithms:

$$\ln \frac{I}{I_0} = \ln(1 - kCdL)^N = N \ln(1 - kCdL)$$

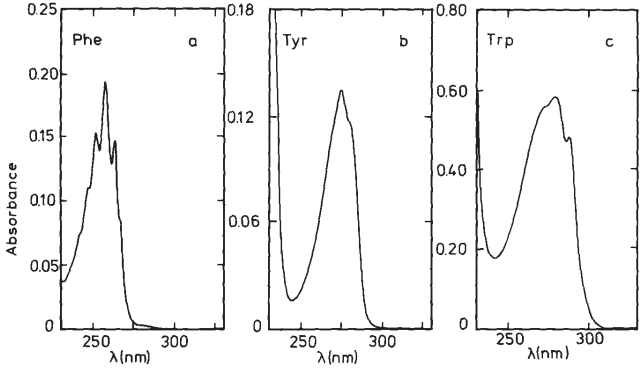
Units for the Extinction Coefficient

$$A = C \cdot L \cdot \epsilon$$

- A is dimensionless
- Most cuvettes have a path length of 1 cm, so it is convenient to use cm as the dimension of length.
- If concentration is expressed in molar units, then ϵ should have units of $\mathrm{M}^{-1}\mathrm{cm}^{-1}$, so that:
  $\mathrm{M} \times \mathrm{cm} \times \mathrm{M}^{-1}\mathrm{cm}^{-1}$ is dimensionless
- If concentration is expressed in units of mg/mL, then ϵ should have units of $\mathrm{cm}^{-1}(\mathrm{mg/mL})^{-1} = \mathrm{cm}^{-1}(\mathrm{mL/mg})$.
- If concentration is expressed as % (m/v) solute, then ϵ should have units of $\mathrm{cm}^{-1}\%^{-1} = \mathrm{cm}^{-1}(\mathrm{g}/100\mathrm{mL})^{-1} = \mathrm{cm}^{-1}(100\mathrm{mL/g})$.

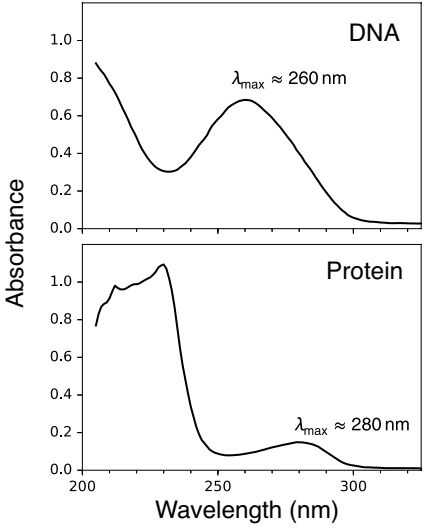
What if Solution Contains Multiple Compounds that Absorb Light?

- Peaks in UV-visible absorption spectra are quite broad:



- Peaks from different compounds often overlap.
- Absorption at a given wavelength may contain contributions from multiple compounds.

UV Absorption Spectra of Proteins and DNA



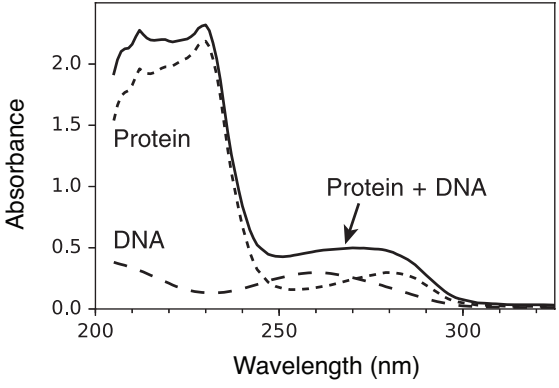
- DNA spectra do not depend much on sequence.
- Protein spectra do depend on amino acid composition, and a bit on three-dimensional structure.
- DNA and protein spectra, between 250 and 300 nm overlap extensively.
- Concentrations:

  $[\text{DNA}] \approx 0.03\,\text{mg/ml}$

  $[\text{Protein}] \approx 0.16\,\text{mg/ml}$

Spectra adapted from Spectrophotometry Handbook (2012) GE LifeScience

Spectra of DNA, Protein and a Mixture



- Absorbances of different components add.
- Assumes components don't interact.
- Can we interpret absorbance of mixtures?

Estimating Concentrations of Protein and DNA in a Mixture

- Between 250 and 300 nm

  For Protein: $\lambda_{max} \approx 280\,\text{nm}$

  For DNA: $\lambda_{max} \approx 260\,\text{nm}$

- At 260 nm (assuming 1-cm cuvette):

$$A_{260} = [\text{Protein}] \cdot \epsilon_{260}^{\text{Protein}} + [\text{NA}] \cdot \epsilon_{260}^{\text{NA}}$$

- At 280 nm:

$$A_{280} = [\text{Protein}] \cdot \epsilon_{280}^{\text{Protein}} + [\text{NA}] \cdot \epsilon_{280}^{\text{NA}}$$

- If all four extinction coefficients are known, and we measure A_{260} and A_{280}, we have two equations in two unknowns.

  Solve for [Protein] and [NA].

- What could go wrong?

Outline of Experiment

- Two samples:
  - A pure protein: Bovine serum albumin (BSA)
  - An *E. coli* extract, containing lots of proteins and nucleic acids
- Direct UV absorbance measurements at 260 and 280 nm
  - For BSA, estimate [Protein] from A_{280} and known extinction coefficient.
  - For both samples, estimate [Protein] and [NA] from extinction coefficients for "typical" proteins and nucleic acids.
- Bradford dye-binding assay
  - Use BSA to establish a standard curve, using [BSA] determined from A_{280}
  - Independent estimate [Protein] in *E. coli* extract, to be compared with estimate from $A_{280} : A_{260}$