Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2017

Lecture 5

More on UV-visible Spectrophotometry:

Beer's Law and Overlapping Spectra

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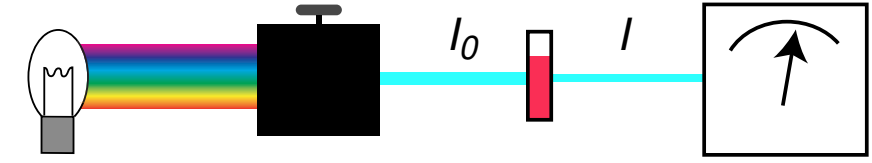
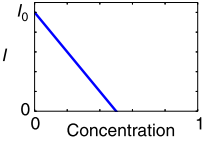
A UV-Visible Spectrophotometer



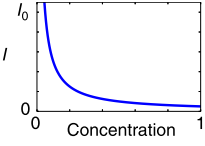
Clicker Question #1

How does transmitted light intensity change with concentration?

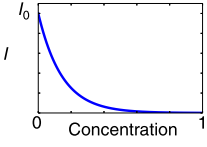
1. $I = I_0 - kC$

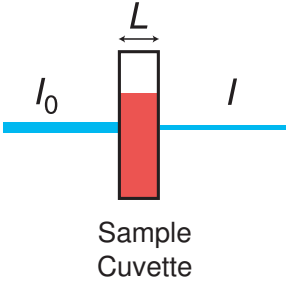


2. $I = \frac{I_0}{kC}$



3. $I = I_0 e^{-kC}$





No wrong answers (for points)!

Clicker Question #2

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

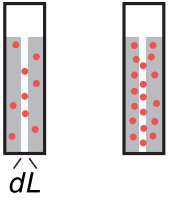
Click:

1 for Yes

2 for No

No wrong answers (for points)!

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:

$$p = kCdL$$

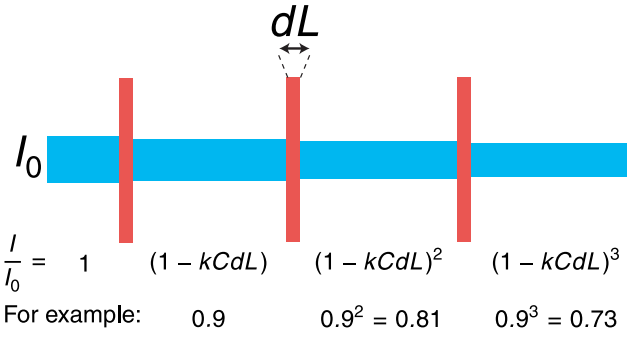
where k is a constant of proportionality.

- Fraction of light transmitted by a thin slice:

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

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

Many Thin Slices



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

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

A Very Handy Approximation

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

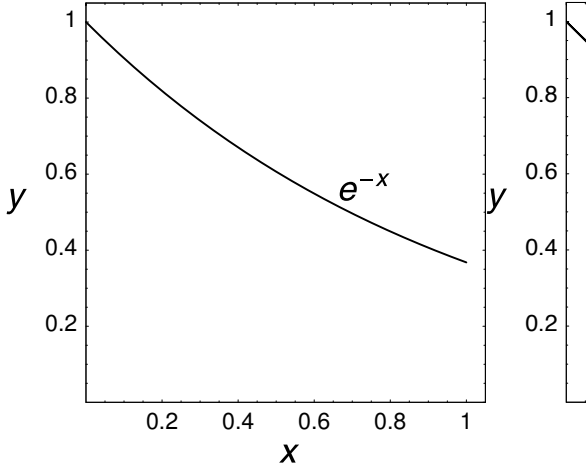
$$e^{-x} \approx (1 - x)$$
$$-x \approx \ln(1 - x)$$

- 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:

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

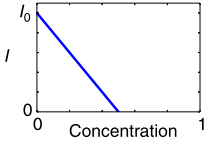
- $dL \cdot N = L$, total path length

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

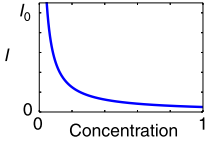
Clicker Question #3

How does transmitted light intensity change with concentration?

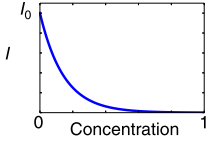
1. $I = I_0 - kC$

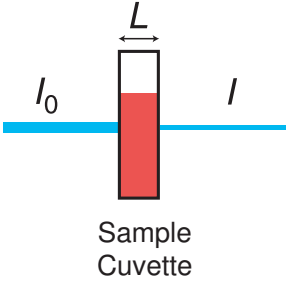


2. $I = \dfrac{I_0}{kC}$



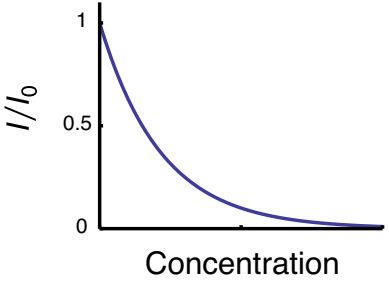
3. $I = I_0 e^{-kC}$

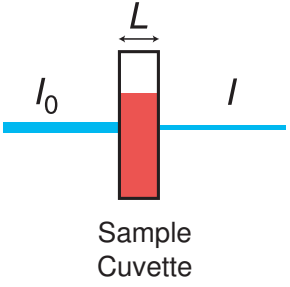




One correct answer!

The Beer-Lambert Law



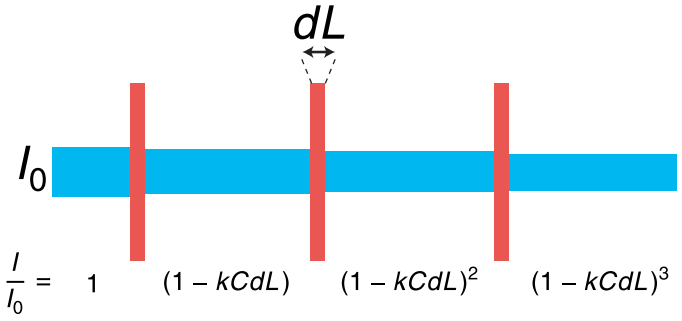


$$\ln \frac{I}{I_0} = -C \cdot L \cdot k \qquad \log_{10}(x) = \ln(x) \log_{10}(e)$$

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

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

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 $\mathrm{cm}^{-1}\%^{-1} = \mathrm{cm}^{-1}(\mathrm{g/100mL})^{-1} = \mathrm{cm}^{-1}(\mathrm{100mL/g})$.

Clicker Question #4

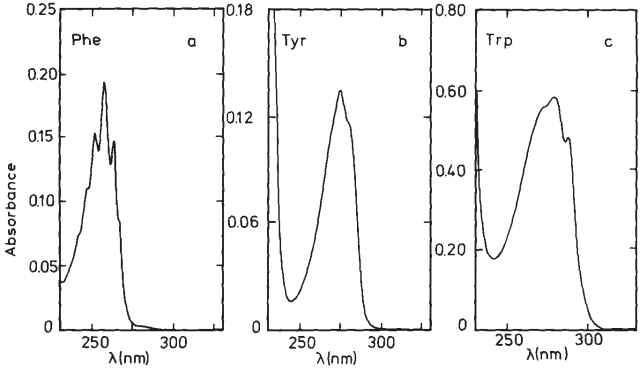
- Someone gives you a solution of a mystery compound and tells you that the extinction coefficient at 535 nm is $3\,\mathrm{cm}^{-1}(\mathrm{g/L})^{-1}$
- Using a 1 cm cuvette, the absorbance is 1.2.
- The concentration of the sample is:

1. 0.04 mg/mL
2. 0.4 mg/mL
3. 4 mg/mL
4. 0.04 g/mL
5. 0.4 g/mL
6. 4 g/mL

$$A = C \cdot L \cdot \epsilon$$
$$1.2 = C \times 1\,\mathrm{cm} \times 3\,\mathrm{cm}^{-1}(\mathrm{g/L})^{-1}$$
$$1.2 = C \times 3\,(\mathrm{g/L})^{-1}$$
$$C = 1.2 \div 3\,(\mathrm{g/L})^{-1}$$
$$C = 0.4\,\mathrm{g/L} = 0.4\,\mathrm{mg/mL}$$

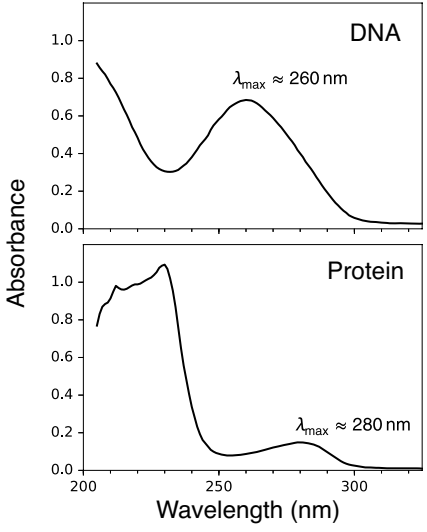
What if Solution Contains Multiple Compounds that Absorb Light?

- 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



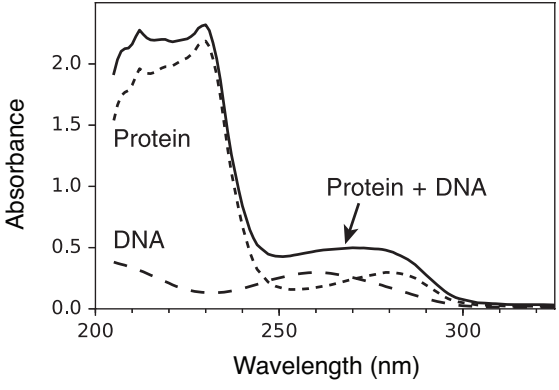
- DNA spectra do not depend much on sequence.
- Protein spectra do depend on amino acid composition, and a bit on three-dimensional structure.
- DNA and protein spectra, between 250 and 300 nm overlap extensively.
- Concentrations:

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

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

Spectra adapted from Spectrophotometry Handbook (2012) GE LifeScience
http://www.gelifesciences.com/file_source/GELS/Service%20and%20Support/Documents%20and%20Downloads/Handbooks/pdfs/Spectrophotometry.pdf

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 absorption 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 $A_{280} : A_{260}$