Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2018

Lecture 5

More on UV-visible Spectrophotometry:

Beer's Law and Measuring Protein Concentration

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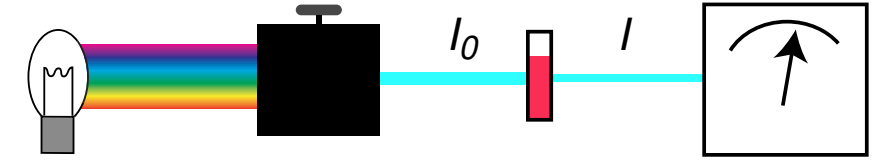
Why Do Errors Follow a Gaussian Distribution?

- Total error is made up of multiple small contributions, each of which can be positive or negative.
- There are more ways to have roughly equal numbers of positive and negative errors than there are to have a large excess of either positive or negative errors.

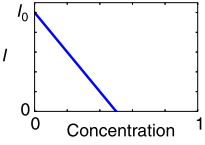
A UV-Visible Spectrophotometer



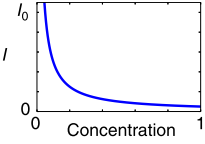
Clicker Question #1

How does transmitted light intensity change with concentration?

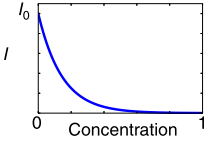
1. $I = I_0 - kC$

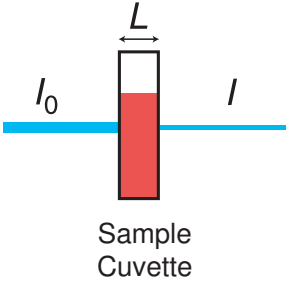


2. $I = \frac{I_0}{kC}$



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

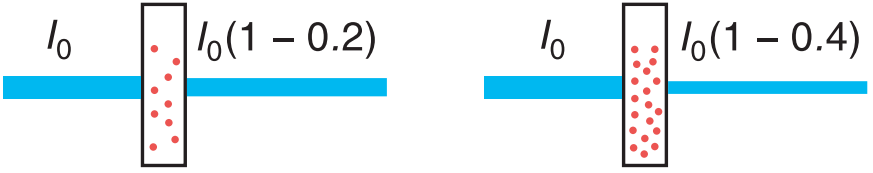




All answers count (for now)!

Clicker Question #2

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



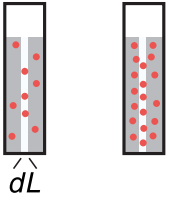
Click:

1 for Yes

2 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:

$$p = kCdL$$

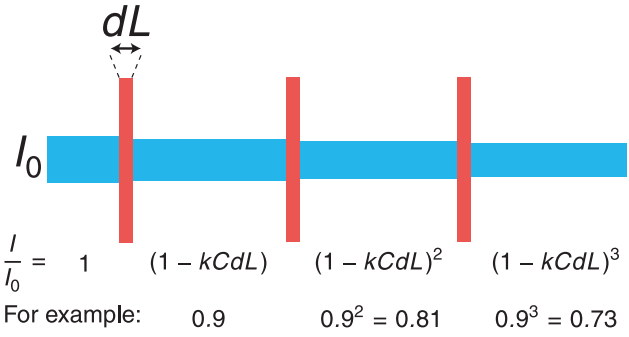
where k is a constant of proportionality.

- 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: $\frac{I}{I_0} = (1 - kCdL)^N$
- 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 $|x| \ll 1$:

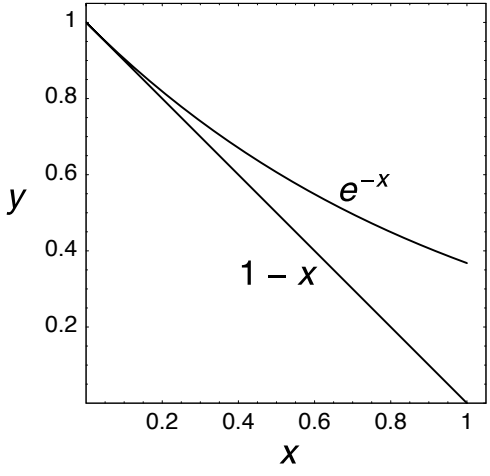
$$e^{-x} \approx (1 - x)$$
$$\ln(1 - x) \approx -x$$

- 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:

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

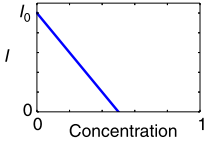
- $dL \cdot N = L$, total path length

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

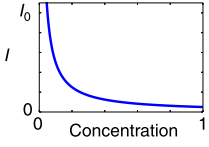
Clicker Question #3

How does transmitted light intensity change with concentration?

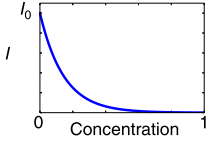
1. $I = I_0 - kC$

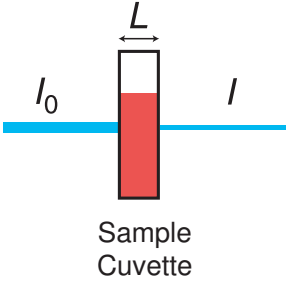


2. $I = \dfrac{I_0}{kC}$



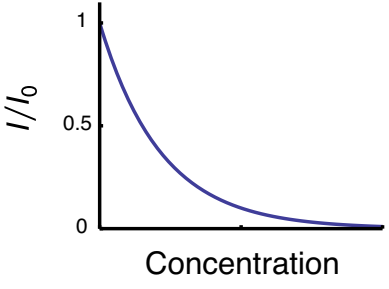
3. $I = I_0 e^{-kC}$

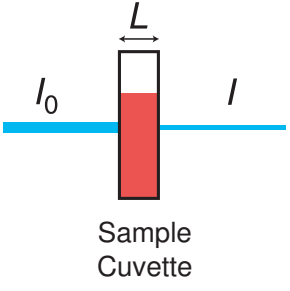




One correct answer!

The Beer-Lambert Law



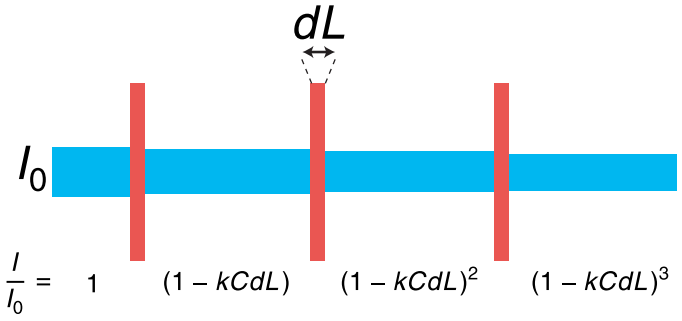


$$\ln \frac{I}{I_0} = -C \cdot L \cdot k \qquad \log_{10}(x) = \ln(x) \log_{10}(e)$$

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

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

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

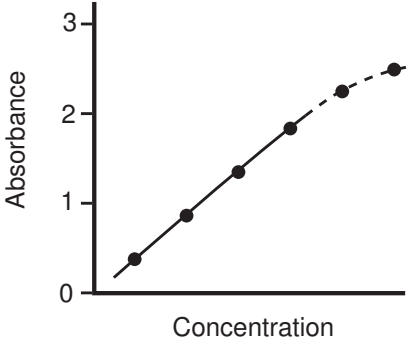
Clicker Question #4

- 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

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

Absorbance versus Concentration



The most reliable measurements are obtained when $0.1 < A < 1$

Some Practical Points

- The cuvettes must be transparent to light of the wavelength of interest.
  - Glass or plastic work well for visible light ($\lambda \gtrsim 350$ nm.)
  - Fused silica (quartz) ~~is~~ **was** necessary for UV light ($200 \text{ nm} < \lambda \lesssim 350$ nm). Quartz cuvettes are very expensive!
  - Very recent: There are UV-transparent plastic cuvettes! (down to about 220 nm)
- Absorbances are measured relative to that for a "blank" solution that contains everything *except* the compound of interest.

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.

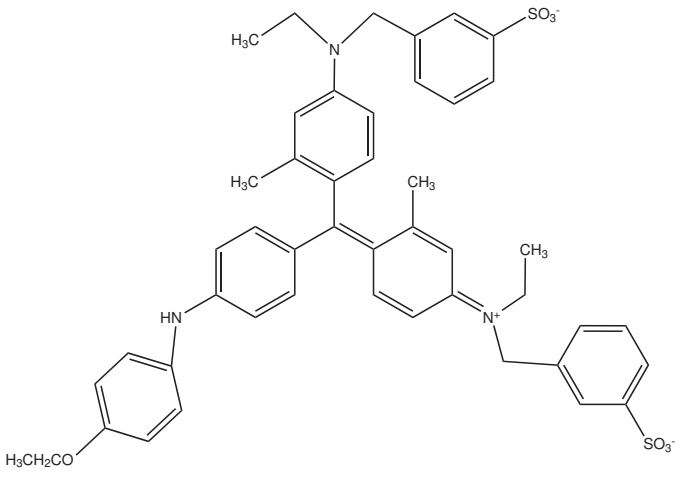
Indirect Methods for Measuring Protein Concentration

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

Advantages

- Much more sensitive (10 × 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.

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 $A_{280} : A_{260}$