Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 4:
A Bit More on Buffers

and

Introduction to UV-visible Spectrophotometry

Thursday, 19 January 2023

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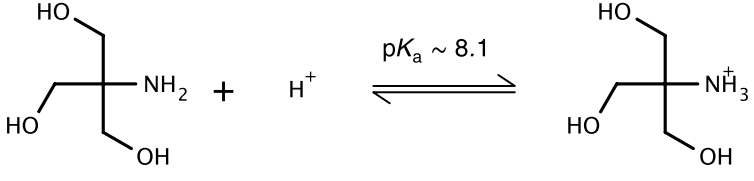
The Buffer We Will Use for Most of Our Experiments

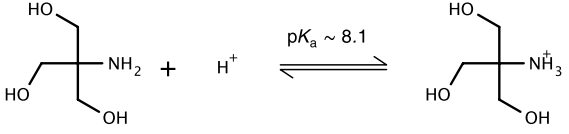
- Tris: tris(hydroxymethyl)aminomethane



- Works well at pH 8, where we will do most of our experiments.
- Largely unreactive with biological molecules.
- Relatively inexpensive.

Clicker Question #1

What will the pH be if we make a solution of 0.2 M tris base?



(A⁻) (AH)

A) pH 4

B) pH 6

C) pH 7

D) pH 8

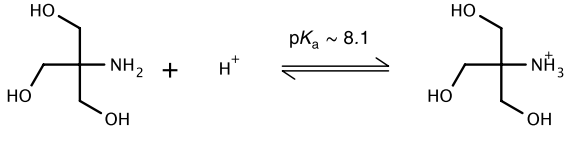
E) pH 10

Protocol for Preparing Tris Buffer

- Measure out tris base to make 50 mL of a 0.2 M solution.
- Dissolve tris in about 40 mL of water.
- Adjust pH to 8.0, at 25°C by adding HCl and monitoring with a pH meter.
- Adjust final volume to 50 mL, using a graduated cylinder.
- Filter solution and store in a carefully labeled vessel.

Clicker Question #2

How many mL of 1 M HCl should we add to a solution containing 0.01 moles of tris base to adjust the pH to 8.0?



A) $\sim$ 1 mL

B) $\sim$ 2 mL

C) $\sim$ 5 mL

D) $\sim$ 10 mL

E) $\sim$ 15 mL

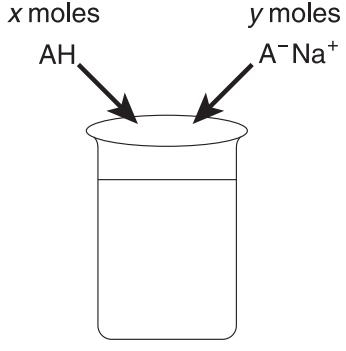
A General Approach to Making Buffers

$$AH \rightleftharpoons A^- + H^+$$

1. Choose buffer compound to match pK_a to working pH.
2. Start with a solution of weak acid (or base).
3. Adjust pH to desired value by adding strong base (or acid).
   Two ways:
   a. Follow pH with meter.
      - Advantage: No math required!
      - Disadvantage: Subject to pH-meter errors.
      - Variability in final salt concentration, because pH is relatively insensitive to amount of strong base (or acid) added. It's a buffer solution!
   b. Calculate the amount of strong base (or acid) to add.
      - Advantage: Independent of pH meter.
      - Advantage: More consistent with respect to final concentrations.
      - Disadvantage: Depends on accurately determined concentration of strong base (or acid).
      - Disadvantage: pK_a can depend on solution conditions.

Another Way to Make a Buffer Solution

- Directly mix weak acid and salt of weak base.
  (or weak base and salt of weak acid)



- Because they are weak, the acid and base do not release or take up a significant net number of H^+ ions when dissolved. So, their concentrations don't change significantly.
- Ratio of $[A^-]$ and $[AH]$

$$\frac{[A^-]}{[AH]} = \frac{y}{x}$$

- pH:

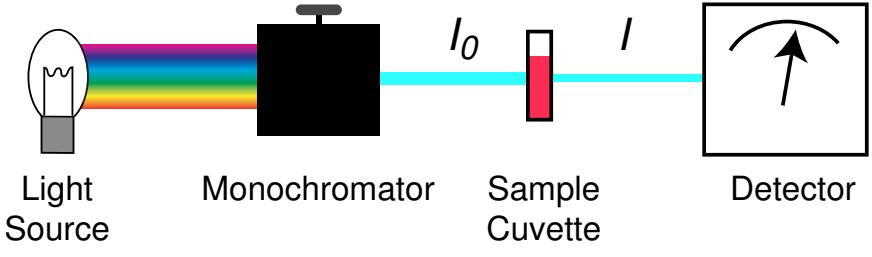
$$\text{pH} = \log \frac{y}{x} + \text{p}K_\text{a}$$

Warning!

Direction Change

UV-Visible Spectrophotometry

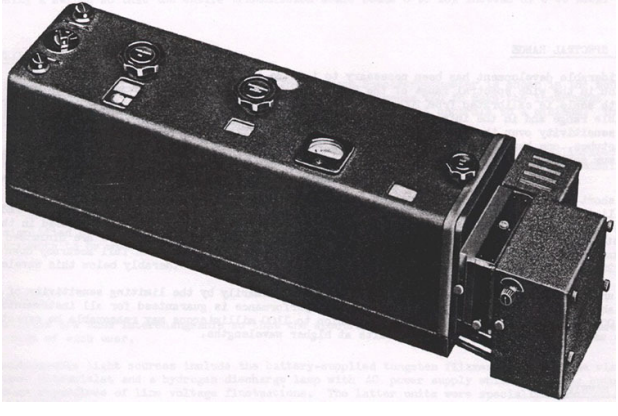
A UV-Visible Spectrophotometer



$$\% \, Transmittance = \frac{I}{I_0} \times 100$$

$$A = \log \frac{I_0}{I}$$

The *Classic* Spectrophotometer

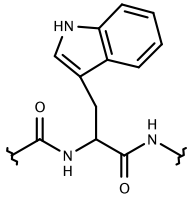


The Beckman DU: Produced from 1941 to 1975

Cary, H. H. & Beckman, A. O. J. (1941) *J. Opt. Soc. Am.* **31**, 682-689.

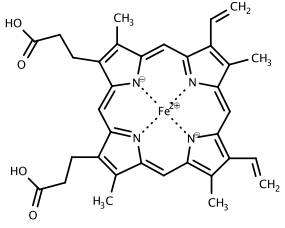
UV or Visible Absorbance Usually Arises from:

- Systems of conjugated double bonds
  - Peptide bonds in proteins.
  - Aromatic amino acid residues in proteins
  - Bases in nucleic acids



Tryptophan

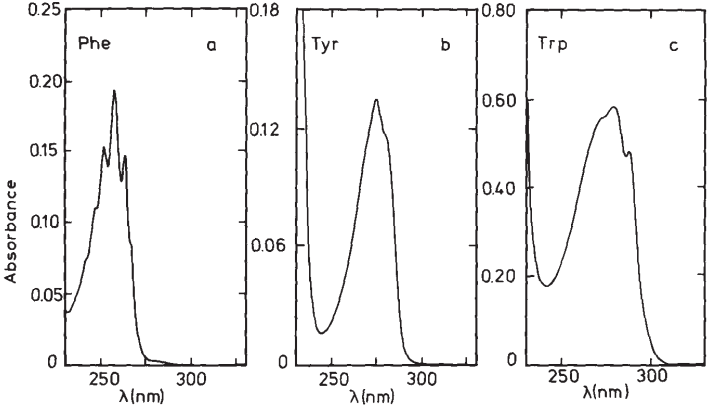
- Coordinated metal ions
  - Heme
  - Chlorophyl



Heme

Larger conjugated systems $\rightarrow$ longer wavelength of light absorbed

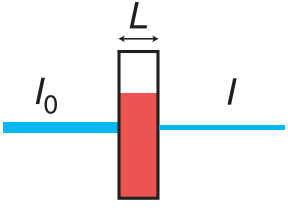
UV Absorbance of Aromatic Amino Acids



- λ_{max} Wavelength of maximum absorbance

Spectra from: Schmid, F. X. (1997). In *Protein Structure: A Practical Approach* (Creighton, T. E., ed.), pp. 261-297. IRL Press, Oxford. (A good introduction to optical spectroscopy of proteins)

The Beer-Lambert Law



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

$C =$ concentration (M)

$L =$ cuvette path length (cm)

$\epsilon =$ extinction coefficient ($M^{-1}cm^{-1}$)

$$C = \frac{A}{\epsilon \cdot L}$$

- Allows us to measure concentration by measuring absorbance.
- How do we know what the extinction coefficient is?

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

Clicker Question #3

- 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:

A) 0.04 mg/mL

B) 0.4 mg/mL

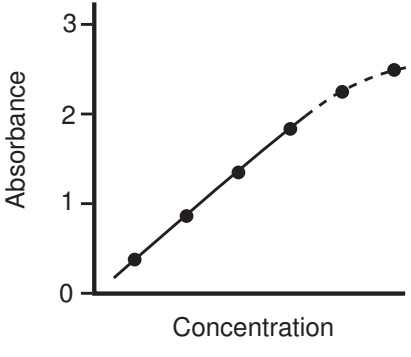
C) 4 mg/mL

D) 0.04 g/mL

E) 0.4 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 $0.1 \lesssim A \lesssim 1.5$

Some Practical Points

- The cuvettes must be transparent to light of the wavelength of interest.
  - Glass or plastic work well for visible light ($\lambda > \approx 350$ nm.)
  - Fused silica (quartz) ~~is~~ **was** necessary for UV light ($200 \text{ nm} < \lambda < \approx 350$ 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 $\times$ or more) than direct UV absorbance.
- Less sensitive to interference from other compounds.

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 $A_{280} : A_{260}$