5.3: Quantification of Protein Concentration

Review: the Beer-Lambert Law

\[ \log(1/T) = \epsilon c l = A \]
\[ T = 1/10^A \]

Values for \( \epsilon \) and \( l \)

- Path length \( l \) is usually in units of cm. (note: most spectrophotometers are designed to accept 1cm wide cuvettes)
- Molar extinction coefficient \( \epsilon \) has units of M\(^{-1}\) cm\(^{-1}\) and is a constant of proportionality that relates the absorption of molar solutions
- Mass extinction coefficient \( \epsilon \) \( \% \) refers to the absorbance of a 1\% by mass solution. Typically this refers to an aqueous solution that we can take to have a density of 1000g/L. A 1\% by mass aqueous solution would therefore refer to the dissolution of 10g/L, or a 10mg/ml solution of the molecule of interest.
- Since the absorbance of a molecule is a function of the wavelength (i.e. the absorption is not equal for every wavelength) the extinction coefficient must also reference a wavelength. This is typically done using a subscript:

\[ \epsilon \ 1\%_{280nm} = 14.5 \ g^{-1} \ \text{L cm}^{-1} \]

- In this case a 10mg/ml solution of the molecule will have an absorbance reading of 14.5 (dimensionless units) at \( l = 280\)nm (the absorption at other wavelengths may not be known). The units of concentration are g/L, thus \( \epsilon \) will have dimensions of g\(^{-1}\) L cm\(^{-1}\).
Why is it important to be able to quantitate protein concentration in a sample?

An important application of "Biotechnology" is the production of proteins as commercial products. Such products might have pharmaceutical applications (e.g. insulin, human growth hormone, tissue plasminogen activator, erythropoietin, blood clotting factor VIII), industrial applications (e.g. subtilisin (an enzyme in detergents), 2,5-diketo-D-gluconate reductase (an enzyme in vitamin C production), as materials (e.g. silk protein in textiles, barnacle adhesion protein as a glue). In these cases, there are various aspects of successful production that require quantititation:

- How much of the protein can be produced (i.e. what is the efficiency of production)?
- How pure is the protein that is produced (industrial applications may require 90% pure, pharmaceutical applications may require 99.999% pure)

Such proteins may be isolated from natural sources (e.g. blood clotting factor VIII may be extracted from human blood), or they may be produced recombinantly (e.g. E. coli bacterial cells can be genetically engineered to produce human growth hormone). In both cases, it may be necessary to purify the protein using a series of fractionation steps. We will go into more detail about such fractionation steps in a later lecture, but the general idea is that a heterogeneous mixture of molecules can be fractionated based upon some physical property of the molecules. The following are properties that can be used to fractionate a heterogeneous mixture of biomolecules:

- Molecular mass (i.e. "big" molecules can be separated from "small" molecules)
- pKa (i.e. "acidic" molecules can be separated from "basic" molecules)
- Hydrophobicity (i.e. non-polar molecules can be separated from polar molecules)

For such fractionation steps involving proteins, we need to keep track of how much of the contaminating proteins went into one fraction and how much of our desired protein went into the other fraction. Although the details are somewhat more complicated than this simple description, it is important to be able to quantitate protein concentration to be able to effectively purify a protein of interest.

Once a protein is pure, it may be of considerable economic interest to be able to quantify the yield (and, therefore, be able to determine how much it cost to produce a given mass of protein). For example, the only source for human growth hormone (to treat small stature) used to be to extract it from human pituitary glands harvested from the brains of cadavers. Suffice it to say, this made the protein extremely expensive. Furthermore, the isolation from human tissues meant that the sample could also be potentially contaminated with human pathogens (hepatitis, CJD, AIDS, etc.). With the advent of genetic engineering, the production of human growth hormone by bacterial cells (i.e. E. coli) meant that relative large quantities could be produced far cheaper (and with no threat of human pathogens).

Why not just weigh the protein?

- Most samples are typically quantities of milligrams or even micrograms, not grams, and thus, it is difficult to transfer and measure such small amounts
- Water is present in proteins, and it is extremely difficult to remove all the water (some water molecules hydrogen bond extremely tightly to proteins). Thus, the mass measurement would include some waters, and would increase the apparent mass of the protein
Absorbance spectra of biological molecules

Proteins

Proteins do not absorb in the visible wavelength unless they have a prosthetic group (e.g. Fe$^{2+}$) or an unnatural amino acid. However, the amino acids tryptophan, tyrosine and cysteine absorb light in the UV wavelength:

![The amino acid tryptophan](https://bio.libretexts.org/Bookshelves/Biochemistry/Supplemental_Modules_(Biochemistry)/5._Lab_Notes_Part_1/5.3%3A_Qu...

*Figure 5.3.1: Tryptophan absorption*

- Tryptophan has a peak of absorption at 280nm in the UV range
- This is a useful wavelength to quantitate the absorption of tryptophan
- Since the absorption is proportional to concentration, this is a useful way to quantitates protein concentration (for proteins containing Trp)

Nucleic acids

The aromatic rings in the bases of nucleic acids also absorb in the UV range:
Figure 5.3.2: dAMP Absorption

- Each DNA and RNA base has a slightly different absorption spectrum
- 260 or 280nm is a typically useful wavelength to monitor concentration of nucleic acids

Note that samples of nucleic acids and proteins can both absorb at 280nm, therefore, samples of biological molecules should be pure in order to quantitate using UV absorption spectroscopy (any contaminating nucleic acids in a protein sample will increase the apparent absorbance, likewise for contaminating proteins in a nucleic acid sample).

Important aspects of quantification of proteins using UV absorbance

- If a protein contains Trp, Tyr or Cys residues it will absorb in the UV. If it does not contain these amino acids, it will not absorb UV light, and we cannot quantify it using this method
- Multiple Trp, Tyr or Cys residues will contribute to the Extinction coefficient for the protein. Thus, we need to know how many of these residues are present in the protein to know the correct extinction coefficient
- Nucleic acids (DNA, RNA) contaminant will also absorb UV light, as will other proteins with Trp, Tyr and Cys residues. Thus, the sample must be pure to use UV absorption to quantify a protein

Molar extinction coefficients of Trp, Tyr and Cys amino acids:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>E_{280nm} (M^{-1} cm^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>5690</td>
</tr>
<tr>
<td>Tyr</td>
<td>1280</td>
</tr>
</tbody>
</table>
Example 5.3.1: Bovine insulin

Bovine insulin contains 4 Tyr residues, 6 Cys residues and 0 Trp residues. We can determine the expected molar extinction coefficient at 280nm, $E_{280nm}$, by the following calculation:

$$E_{280nm} = (0)(5690) + (4)(1280) + (6)(120)$$

$$E_{280nm} = 5840 \text{ M}^{-1} \text{ cm}^{-1}$$

Thus, a 1.0M solution of pure bovine insulin would give an absorbance of 5,840 at 280nm (obviously, it would have to be diluted considerably to be read accurately).

A useful expression relating the parameters of $E$, concentration ($C$) and $A$ are derived from the Beer-Lambert law (assuming 1cm path length):

$$A/E = C$$

For example, if a sample of bovine insulin was observed to give an absorbance at 280nm of 0.745 we could calculate the concentration to be:

$$0.745/5840 \text{ M}^{-1} \text{ cm}^{-1} = C$$

$$C = 1.28 \times 10^{-4} \text{ M}$$ (note: cm\(^{-1}\) drops out with 1cm pathlength)

It should be noted that a deuterium lamp is required for UV spectrophotometry, as well as quartz cuvettes (since glass absorbs UV light)

### Colorimetric (chromogenic) methods of protein concentration determination

Chromo means color and genesis mean creation, so chromogenic means the "creation of color". Color means color (duh…) and metric means to measure, so colorimetric is to "measure color". Both terms refer to the same sort of thing in the present case - we can modify the protein sample with appropriate reagents so as to produce a color reaction (in visible spectrum) and measure protein concentration using a VIS spectrophotometer. Advantages are:

- Cheap lamp! (tungsten light bulb versus deuterium for UV)
- Cheap cuvette! (cheap glass or plastic versus quartz)
- Not contaminating absorbance from proteins or nucleic acids! (no absorption in VIS spectrum)

We will consider three methods: The **Biuret, Lowry and Bradford** methods of colorimetric determination of proteins.
Under high pH (alkaline) conditions the copper II ion (Cu\(^{2+}\)) is believed to form a complex with peptide nitrogens of proteins:

![Cu\(^{2+}\) complex](image)

**Figure 5.3.3: Cu\(^{2+}\) complex**

This complex absorbs light at 550nm and has the following useful properties:

- It is dependent upon at least a dipeptide structure (see above), thus, contaminating amino acids will not contribute to the 550nm absorption
- The binding depends upon the peptide backbone nitrogen and not the side chain functional group. Thus, the binding is independent of sequence
- Nucleic acids do not interfere since they don't share the peptide backbone structure
- However, ammonia and certain amines can interfere. Thus, ammonium sulfate salts, and the common biological buffer TRIS (tris hydroxymethyl amino ethane) will provide a false positive and cannot be present in the sample
- Also, the absorption is relatively weak, thus, the method is somewhat insensitive and requires a relatively high concentration of protein

**Lowry**

The Lowry method is a modification of the Biuret method. After treatment with Copper II, the protein is treated with phosphomolybdotungstate mixed acids (acidic compounds of molybdenum and tungsten ions). These acids are known as the Folin-Ciocalteu (or just Folin) reagent. The Folin reagent is added under alkaline conditions, and the Folin reagent is subsequently reduced by the the Copper ions as well as Tyr, Trp and polar amino acid side chains. The product of this reaction is heteropolymolybdenum blue, which absorbs strongly at 750nm. This assay has the following properties:
• More sensitive than the Biuret assay (can detect lower concentrations of protein)
• Somewhat dependent upon amino acid composition (i.e. relative concentrations of Tyr, Trp and polar amino acids)
• Absorption reaction is linearly dependent upon protein concentration, but only at low concentrations of protein (i.e. the standard curve and assay must be performed at a low concentration regime).
• Sensitive to contaminants as with the Biuret method, as well as others related to the Folin reagent and redox reactions.
• More critical to timing and precision of person doing the assay

Bradford

A dye known as Coomassie Brilliant Blue was developed by the textile industry. It was noticed to stain skin as well as the textiles. Thus, this dye (which normally absorbs at 465nm) was known to bind to proteins and to absorb strongly at 595nm. The dye forms a wide variety of strong, but non-covalent, interactions including hydrogen bonding donor and acceptor interactions as well as hydrophobic (non-polar) interactions.

![Coomassie Brilliant Blue binding](https://bio.libretexts.org/Bookshelves/Biochemistry/Supplemental_Modules_(Biochemistry)/5._Lab_Notes_Part_1/5.3%3A_Qu...

The method is quite simple: a single step in which the dye is added to the protein solution under acidic conditions, and then the absorbance is read at 595nm. However, the method has the following properties:

• The response is generally independent of the amino acid composition
• The assay is sensitive, but somewhat non-linear. Thus, a standard curve must always be performed (using known concentrations of pure protein)
• The dye stains pretty much everything, including cuvettes, floors, countertops. It can be a real mess if spilled (I know this from personal experience). And since it is a textile dye, if you get it on your clothes, you will need to learn to like blue polka dots.