## Introduction to Absorbance Spectroscopy

A single beam spectrophotometer is comprised of a light source, a monochromator, a sample holder, and a detector. An ideal instrument has a light source that emits with equal intensity at all wavelengths, a monochromator that is equally efficient in splitting light into narrow groups of wavelength for all wavelengths, and a detector that is sensitive and responds equally to all wavelengths.
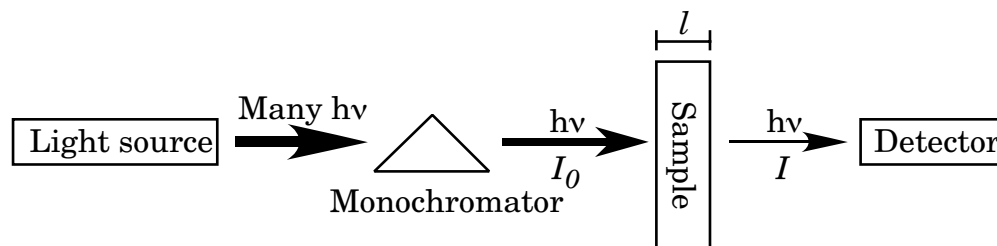
Light sources

Because no single light source with the appropriate characteristics exists, most spectrophotometers use two lamps, with one for the ultraviolet region and one for the visible region. The visible lamp is usually a tungsten lamp, while the ultraviolet lamp is a deuterium lamp. An alternative, relatively rarely used in spectrophotometers, although commonly used in other types of spectroscopic instruments, is a xenon arc lamp. In a xenon arc, the flow of electrons through an electrode gap in a pressurized xenon chamber ionizes the xenon atoms; the binding of electrons to the ionized xenon results in fairly consistent light emission over a large range of wavelengths. However, the light intensity from a xenon arc drops rapidly below 280 nm.

Monochromators

Although prisms can be used as monochromators, most instruments use diffraction gratings. Light shining on the closely spaced grooves of a diffraction grating at an angle is separated into different wavelengths in a consistent manner, assuming that the grooves are consistently produced.

Detectors

The most commonly used detector is a **photomultiplier tube** (PMT). An incoming photon hits a thin metal film inside a vacuum tube. The metal film is maintained at a large negative potential, and emits electrons. These collide with a series of dynodes maintained at progressively lower potentials; each dynode emits several electrons in response to each incoming electron, resulting in a large amplification of the signal. Because the initial photon is required to initiate the process, most PMTs have very little dark current ("dark current" is signal without light). Proper functioning of a PMT requires a constant voltage across the PMT; maintaining a constant voltage in the face of a high signal requires a well-designed instrument.

PMTs are wavelength dependent, with the degree of dependence being related to the metal used in the thin film; most PMTs exhibit the greatest sensitivity at ~400 nm.

An alternative type of detector uses **photodiodes**. Photodiodes are inexpensive but not very sensitive. Their low cost has allowed arrays of photodiodes to be set up to allow simultaneous detection of many wavelengths. In this type of spectrophotometer, the monochromator is located after the sample, so that it splits the multiwavelength light leaving the sample.

A **charge coupled device** (CCD) is a sensitive array detector. CCDs store charges released in response to photon impacts. Because the stored charges are stable for prolonged periods, a CCD can collect data for considerable time prior to readout of the signal. They are therefore potentially extremely sensitive. They will probably displace PMTs from some uses as their price decreases. CCDs are used in digital cameras and other consumer products and are rapidly becoming less expensive as a result of both economies of scale and the development of improved production techniques.

Recall that absorbance is a log function. An absorbance value of 1 is observed when 10% of the incident light reaches the detector. An absorbance value of 2 is observed when only 1% of the incident light reaches the detector. High quality PMT detectors may be able to yield accurate measurements at absorbance values of 5 or higher. Many CCD or photodiode instruments, and lower quality PMT instruments, however, tend to become unreliable at absorbance values above about 2 because too little light is reaching the detector to allow accurate measurements.

Although spectrophotometer readings are usually reported as absorbance, the instruments actually measure optical density. In the simplest terms, optical density (OD) has the same definition as absorbance:

$$OD = \log\left(\frac{I_0}{I}\right)$$

However, optical density result from any process that decreases the light intensity reaching the detector, and therefore has a variety of causes in addition to the quantum mechanical absorbance phenomenon discussed above. One major cause of optical density is light scattering, by either particles or gas bubbles that are present in the sample. Another major cause of optical density is the sample cuvette.

Cuvettes
Most samples studied using visible and ultraviolet spectroscopy are liquid. The sample must therefore be placed in a transparent container to allow measurement. These containers are called cuvettes.

Cuvettes are generally made from transparent plastic, glass, or quartz. Different cuvettes have different optical properties. Plastic cuvettes are increasingly popular because they do not shatter when dropped, and because their low price makes them disposable. However, plastic cuvettes tend to have considerable absorbance in the ultraviolet. Performing measurements in the far ultraviolet (below ~250 nm) requires relatively expensive (and relatively fragile) quartz cuvettes. The graph below shows absorbance spectra for cuvettes of different composition, where each

cuvette only contained water. It is apparent that the plastic cuvettes become opaque in the ultraviolet, and that only the quartz cuvette is useful below about 260 nm.

Absorbance Scans for Different Cuvettes
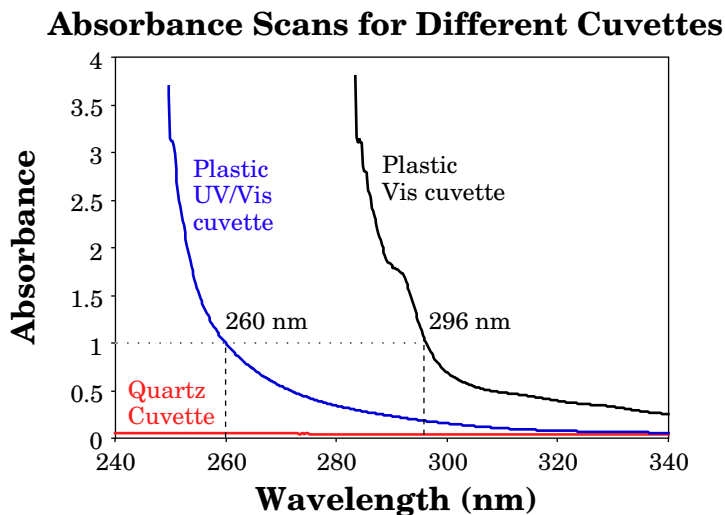
Glass cuvettes are also opaque in the ultraviolet; unlike plastic cuvettes, glass cuvettes are effectively identical in physical appearance to quartz cuvettes. An important control experiment therefore involves measuring the absorbance properties of the cuvette to ensure that these properties are appropriate for the experiment. One issue with most spectrophotometers is that the acquisition software allows "zeroing" of the instrument with a sample present. Note, however, that the optical density of the sample is still present: an optical density measurement of zero may result fomr a sample with a high optical density if the instrument is zeroed to the opaque sample. While generating an instrument baseline using air as a sample will alleviate this issue, it must be remembered that air absorbs light at wavelengths shorter than about 210 nm.

A second potential problem with cuvettes is that not all cuvettes have the same pathlength in all orientations. In the spectrophotometer layout below, note that the cuvette has a 1 cm pathlength as drawn. However, if the cuvette were inserted into the instrument in a different orientation, the pathlength would be considerably shorter (about 0.3 cm). Since absorbance depends on pathlength (the "l" in $A = \varepsilon c l$), the absorbance will also be smaller than expected for a sample of a given concentration.
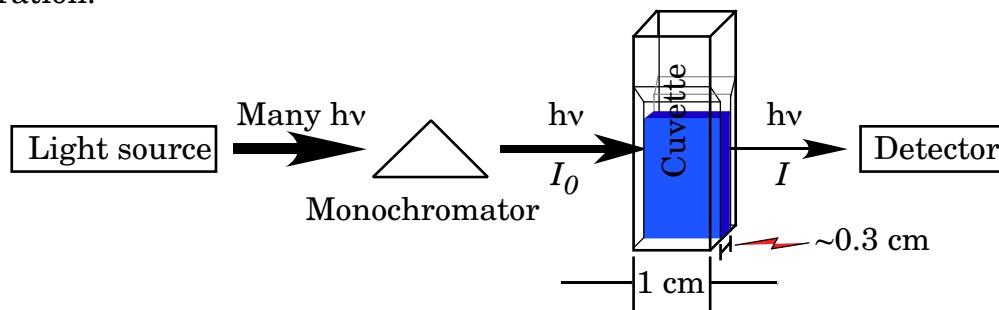
In addition to interference by the cuvette, solution components may absorb. Molecules containing disulfide bonds generally absorb at ~250 – 270 nm, which

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means that molecules such as oxidized β-mercaptoethanol or dithiothreitol will interfere with some measurements of proteins. In addition, nearly all molecules absorb light below 210 nm. Oxygen absorbance increases rapidly below 200 nm, and nitrogen absorbance is significant below 190 nm. Experiments requiring measurements below 200 nm are generally performed in an instrument purged with nitrogen; measurements below 185 nm generally require instruments in which the sample compartment and optical path are located in a vacuum chamber.

Advantages of absorbance spectroscopy

Optical density is measured as a ratio between the measured intensities of the incident light and light passing through the sample. Because this ratio can be measured at any desired wavelength, absorbance instruments automatically compensate for wavelength-dependent variations in lamp output, monochromator efficiency, or detector efficiency. Assuming that: 1) the lamp output and detector efficiency are constant during the measurement (a generally good assumption as long as the instrument had been running for more than about 15 to 30 minutes), 2) the monochromator wavelength output is calibrated properly and 3) the optical density value is within the dynamic range of the detector, any spectrophotometer will yield identical measurements for identical samples.

## Types of absorbance spectroscopic experiments:

Absorbance spectroscopy is frequently used to measure concentration. According to the Beer-Lambert law measurements of absorbance for molecules of known ε allow calculation of concentration. Alternatively, if the concentration of the absorbing species is known, the ε can be determined.

Absorbance is used to determine properties of the chromophore, such as the dipole strength. For proteins, because the chromophores are generally well characterized, absorbance spectroscopy is frequently used as a method for probing the environment around the chromophore. As an example, the absorbance spectra of tryptophan changes as a result of transfer from an organic to an aqueous environment.

**Spectra for 100 μM Trp and Tyr**
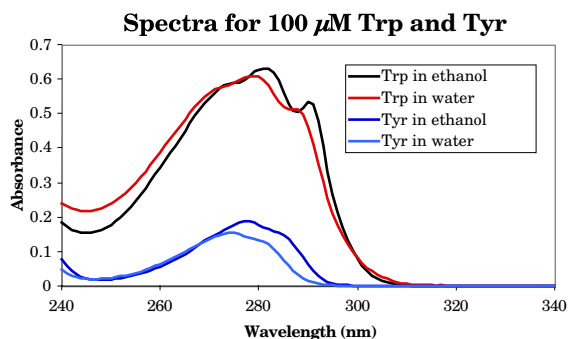
The change in absorbance spectra for a molecule in different environments or upon addition of a second molecule that may interact with the first is more apparent when **difference spectra** are measured. Although a difference spectrum can be obtained by sequentially measuring absorbance properties for two samples, and

mathematically subtracting the first data set from the second, difference spectra are much more readily measured using dual beam instruments. The layout of dual beam instruments varies. The two main methods of comparing the absorbance of the sample to the reference involve "dual beam in space" in which the instrument has two matched detectors, or "dual beam in time" in which the instrument uses a beam chopper to automatically monitor the sample and reference signals sequentially with short intervals between measurements.

Dual Beam in Space

Dual Beam in Time
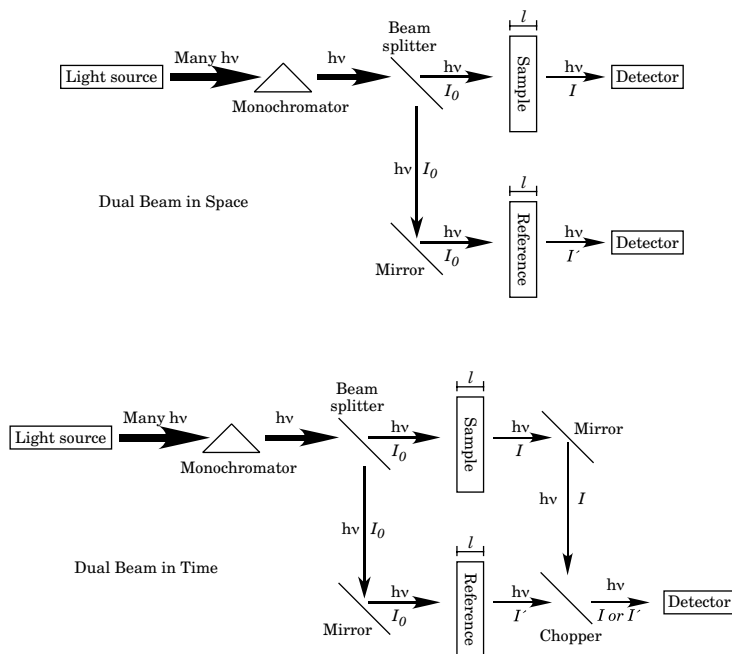
An example of an experiment measuring absorbance spectra and a difference spectrum for tryptophan in solvents (ethanol and water) is shown below. Notice how much more clearly the difference spectrum emphasizes the effects of the solvent environment.
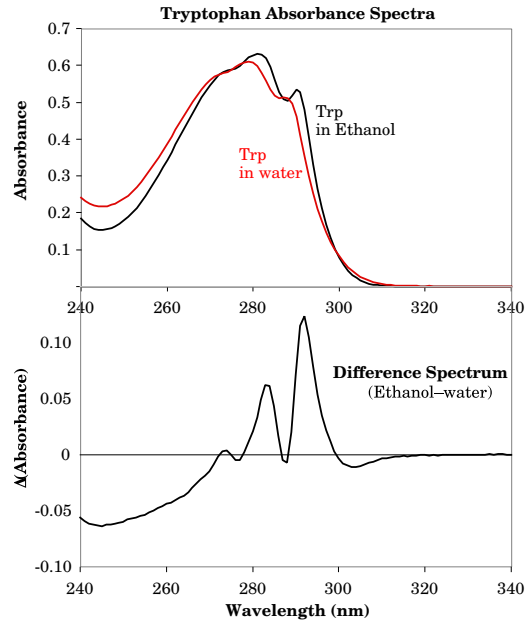
Absorbance Spectroscopy for Proteins

The peptide bond has a $\pi \rightarrow \pi^*$ transition with a maximum at 190 nm. It also has a forbidden $n \rightarrow \pi^*$ transition at 210-220 nm. Secondary structure can result in coupling of the dipoles, and somewhat altered spectra. However, many side-chains also absorb in this region, which makes interpretation difficult.

For proteins without prosthetic groups, the only absorbance at wavelengths above ~250 nm is due to aromatic amino acids (and disulfide bonds, but these are fairly rare and have ε of only ~300).

Tyrosine undergoes a large red-shift when deprotonated; because the pK_a of tyrosine is ~10.5 deprotonation is rare in native proteins. However, for proteins in high pH solutions, observation of spectral changes can yield information regarding the degree of tyrosine side-chain solvent exposure.

The extinction coefficient of tryptophan and tyrosine are fairly insensitive to environment. The $\Delta\varepsilon$ is usually ~100 for tyrosine and ~200 for tryptophan. This means that the extinction coefficient for a protein can be calculated based on the amino acid content:

$$\varepsilon_{280} = W \bullet 5615 \ (M \bullet cm)^{-1} + Y \bullet 1380 \ (M \bullet cm)^{-1}$$

In the equation above, W is the number of tryptophan residues, and Y is the number of tyrosine residues in the protein.[4]

Difference spectra can be used to assess binding of small molecules to the protein, or to assess the environment near aromatic residues. Some prosthetic groups have characteristic difference spectra. An example is the carbon monoxide difference spectrum for cytochrome P450 enzymes. The spectral shift (similar to the one shown

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[4] The values given are the average values from those published in Gill and von Hippel (1989) *Anal. Biochem*. **182**, 319-326, and Mach, Middaugh, and Lewis (1992) *Anal. Biochem*. **200**, 74-80.

below) is the source of the name for the enzyme: the difference spectrum (protein with carbon monoxide minus protein without carbon monoxide) has a large positive peak at 450 nm, although the absorbance peak is ~408 nm.
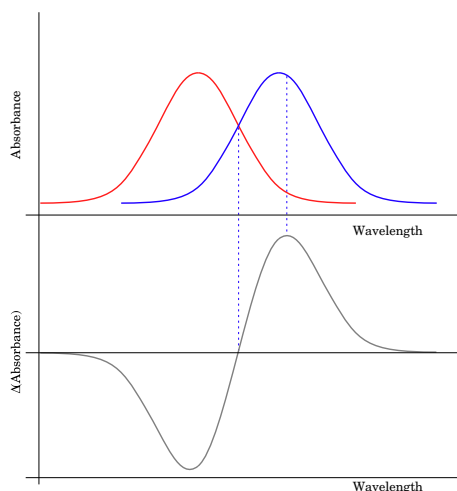
Many compounds exhibit spectral changes. If a single chromophore within the compound is changing its spectrum, the spectra will exhibit an **isosbestic point**, an unchanging point where the different spectra meet. The isosbestic point has a difference of zero on a difference spectrum.

In addition to measuring spectral properties intrinsic to a molecule, in many cases, spectroscopic probes can be attached to the molecule of interest. This is especially widely used for proteins. As an example, 5, 5´-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) changes its absorbance spectrum dramatically upon forming a disulfide bond to another molecule. It therefore can be used to measure the number of free cysteine sulfhydryl groups in a protein.

5, 5´-dithio-
bis-(2-nitrobenzoic acid)
(DTNB)
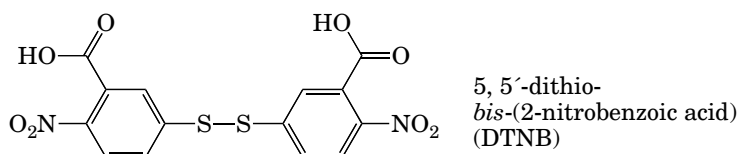
Similarly, fluorescent probes are widely used to monitor changes in environment in the close proximity to the probe binding site.

Photochemistry
The excited state of many molecules has altered reactivity. An intense light source may result in large numbers of molecules in excited states. If these molecules decompose or react with other molecules in solution, the absorbance spectrum will eventually change. This phenomenon is especially noticeable for very intense light sources such as xenon arcs. In some cases, the photochemistry is a useful phenomenon, which allows a better understanding of the parent molecule. In other cases, the photochemistry results in degradation of the sample, and may require alterations in experimental design, or a decrease in the incident light intensity to reduce the decomposition.

Photochemistry is occasionally a problem in absorbance spectroscopy. However, it is more frequently a problem in other types of spectroscopy, and can be a significant problem when performing fluorescence spectroscopy experiments for reasons that will be discussed in the sections on fluorescence spectroscopy.