

Introduction to Spectroscopy

Proteins are large molecules. Most contain well in excess of 100 amino acids, and therefore over 1000 non-hydrogen atoms. The large size of proteins means that their behavior in solution can be considerably more complex than simpler molecules. In addition, it means that a full understanding of most proteins cannot be accomplished by three-dimensional structure determination; while structure is important, the dynamic motions and functional changes are also important in understanding the protein.

Electromagnetic radiation

Many methods for studying proteins (and molecules in general) involve the use of electromagnetic radiation. Electromagnetic radiation can be generated with a wide range of wavelengths. Different wavelengths have differing energies, and interact with molecules in different ways.

The energy in a mole of 400 nm photons is calculated by:

$$E = h\nu = \frac{hc}{\lambda} = \frac{(6.626 \times 10^{-34} \text{ J} \cdot \text{sec}) \left(2.9979 \times 10^8 \frac{\text{m}}{\text{sec}} \right) (6.022 \times 10^{23} \frac{\text{photons}}{\text{mol}})}{400 \times 10^{-9} \text{ m}}$$

The table below gives the range of wavelengths and energies of photons in the electromagnetic spectrum. High-energy photons contain enough energy to break covalent bonds, although they only break bonds under certain conditions. Low-energy photons contain too little energy to disrupt covalent interactions, and are limited to contributing energy to molecules, usually without altering the molecular structure.

Wavelength Range (meters)	Photon energy (kJ/mol)	Spectroscopic region	Spectroscopic technique
10^{-13}	10^9	γ -radiation	Mössbauer
10^{-10}	10^6	X-radiation	X-ray diffraction and X-ray scattering
10^{-7}	1200	Far ultraviolet	Far UV spectroscopy
3×10^{-7} (300 nm)	430	Near ultraviolet	UV/Vis spectroscopy
4×10^{-7} to 7×10^{-7}	300 to 170	Visible	UV/Vis spectroscopy
10^{-6} to 10^{-4}	120 to 1.2	Infrared	IR spectroscopy
0.01	0.01	Microwave	Electron paramagnetic resonance
0.1	0.001	Radio	Nuclear magnetic resonance

The interaction of electromagnetic radiation with molecules is complex. An understanding of the process requires an understanding of some of the elements of quantum mechanics. The discussion below is based on quantum mechanical theory. If you have never had a course in quantum mechanics, you probably will have difficulty with the theoretical treatments, but will at least be able to see some of the theory that underlies the experimental techniques.

Review of Quantum Mechanical Concepts

A complex number is a number of the form: $a + bi$, where $i = \sqrt{-1}$. The complex conjugate of a complex number is: $a - bi$. Multiplying a complex number by its conjugate results in a real number: $a^2 + b^2$.

Molecular states are described by **wavefunctions**. Wavefunctions (ψ) are complex numbers that describe the position and spin of all of the particles in the system, as well as describing the external fields that may perturb the system. ψ cannot be measured directly. The **probability** that a system will have a particular property can be calculated by multiplying the ψ for that property by its complex conjugate ψ^* ; the result of this mathematical manipulation is a real number:

$$P = \psi^* \psi$$

A system must exist in some state. For convenience, probabilities are frequently considered to be numbers between zero and one. If the wavefunction is written properly, integration of the probability over all possible states yields an overall probability of 1. This is called **normalization**, because it acts as a constraint on the wavefunction.

$$\int P d\tau = \int \psi^* \psi d\tau = \langle \psi | \psi \rangle = 1$$

The expression within the angle brackets are the Dirac notation for integration over all states; in Dirac notation, the wavefunction on the left side of the expression is considered to be complex.

If a system can have two states (ψ_a and ψ_b), the overlap between the states is a measure of their similarity. If the **overlap integral** $\langle \psi_a | \psi_b \rangle = 1$, then the states are identical. If $\langle \psi_a | \psi_b \rangle = 0$ then the states do not overlap. It is worth considering ψ_a and ψ_b as vectors, with the overlap integral representing the dot product of the vectors. (Dot products are highest for parallel vectors, and are 0 for perpendicular vectors.)

If a system has two states, the overall wavefunction ψ will be:

$$\psi = C_a \psi_a + C_b \psi_b$$

The coefficients C_a and C_b are related to the probabilities that the system is in the corresponding state. For essentially all of the systems we will discuss (although not for all systems), $\langle \psi_a | \psi_a \rangle = 1$ and $\langle \psi_a | \psi_b \rangle = 0$. For these systems, the probability of state a is:

$$P_a = C_a^* C_a$$

It is often useful to consider portions of a molecule separate from the remainder. In these cases, the overall wavefunction can be factored into component wavefunctions. An example is the **Born-Oppenheimer** approximation, in which nuclei are assumed to be fixed while the electrons can move. For this approximation:

$$\Psi = \Psi_e(r,R)\Psi_N(R)$$

where r refers to the electron position, and R refers to the nuclear position. In this approximation, the electronic wavefunction Ψ_e is calculated for a fixed nuclear position, and the nuclear wavefunction Ψ_N is calculated for the time-averaged electronic state.

Operators

The wavefunction that describes a system can be studied by using mathematical operations on the wavefunction. Although many mathematical operations are purely mathematical constructs, most operators of interest in quantum mechanics are related to physically observable properties.

An eigenvalue equation is one in which the product of the specific wavefunction (eigenfunction) and a number (the eigenvalue) equals the effect of the operator on the wavefunction.

$$\mathbf{O}\Psi = \lambda\Psi$$

The expectation value for the operator = $\langle\Psi|\mathbf{O}|\Psi\rangle$. Substituting gives $\langle\Psi|\lambda\Psi\rangle$. Since $\langle\Psi|\Psi\rangle = 1$, $\langle\Psi|\lambda\Psi\rangle = \lambda$. Therefore, if Ψ is an eigenfunction, measurement of the effect of an operator will yield an observation of the eigenvalue.¹

The Schrödinger equation

The **time-dependent** Schrödinger equation has the form:

$$i\hbar d\Psi/dt = \mathcal{H}\Psi$$

In this equation, $\hbar = h/2\pi$, where h is Planck's constant. \mathcal{H} is the Hamiltonian operator. The Hamiltonian is defined as the operator that has the energy of the system as its corresponding eigenvalue.

$$\langle\Psi|\mathcal{H}|\Psi\rangle = E$$

In general, $\mathcal{H} = \mathbf{T} + \mathbf{V}$, where \mathbf{T} is the kinetic energy operator and \mathbf{V} is the potential energy operator.

¹ States that are eigenfunctions of one operator are not necessarily eigenfunctions of other operators. For example, $\mathbf{r}\mathbf{p}Y \neq \mathbf{p}\mathbf{r}Y$ (where \mathbf{r} is the position operator, and \mathbf{p} is the linear momentum operator). This is the basis of the Heisenberg uncertainty principle; in this case, position and momentum cannot simultaneously be measured accurately.

In some cases, \mathcal{H} is not time-dependent. In these cases, the **time-independent** Schrödinger equation has the form:

$$\mathcal{H}\psi = E\psi$$

If \mathcal{H} is constant, then E must also be constant. However, ψ may still vary.

Combining the two Schrödinger equations yields:

$$i\hbar d\psi/dt = E\psi$$

Integrating gives:

$$\psi(t) = \psi(0)e^{-itE/\hbar}$$

The probability for the wavefunction is then:

$$P = \psi(t)^* \psi(t) = (\psi(0)e^{+itE/\hbar})(\psi(0)e^{-itE/\hbar}) = |\psi(0)|^2$$

Because the exponential parts of the equation have opposite signs but equal magnitudes, these terms vanish. As a result, the probability is a function only of the initial condition and therefore is independent of time. **Stationary states** are states in which the properties do not change over time.

For stationary states, $\langle \psi_a | \psi_b \rangle = 0$ if $E_a \neq E_b$.

If a system can be in two states, $\psi = C_a\psi_a + C_b\psi_b$. The probability the system is in state ψ_a is:

$$P_a = |\langle \psi_a | \psi \rangle|^2 = |C_a|^2$$

Note that the other state does not appear explicitly in the equation.

For stationary states,

$$\psi = \sum_i C_i \psi_i$$

In other words, the specific two state system discussed here can be generalized to a multi-state system. In most cases, the C_i is very small. If a system in one state is perturbed, only a few C_i will have non-zero values, and probably all of these will be small except for one. (This is the basis for perturbation theory.)

If a stationary state is perturbed by a potential, the effect will be an alteration of the C_i values for some states (possibly by mixing some states). The expectation value for the new state will be:

$$\langle \psi | V | \psi_a \rangle = \sum_i C_i \langle \psi_i | V | \psi_a \rangle$$

Because stationary states are normally orthogonal, the terms would all be zero except for the identity term. However, the potential operator may make some terms non-zero; in other words, **the potential may induce a transition** between one state and another. The probability of the transition from state a to state i is given by the expectation value for that transition.

Interactions of electromagnetic radiation and molecules

Electromagnetic radiation consists of a time-varying electric field and a time varying magnetic field. For most electronic transitions, the effect of the magnetic field can be neglected. For most spectroscopy, the wavelength of the radiation is large compared with the size of the chromophore, and therefore it is usually unnecessary to consider the variation of field strengths across the molecule.

The electric field² has a maximum amplitude \mathbf{E}_0 ; the field experienced by a molecule is:

$$\mathbf{E}(t) = \mathbf{E}_0 e^{i\omega t}$$

The term ω is the circular frequency of the light, where:

$$\omega = 2\pi\nu = \frac{2\pi c}{\lambda}$$

Because the electric field is time-dependent, the time-dependent form of the Schrödinger equation must be used. However, the effect of the potential can be factored out, leaving a time-independent Hamiltonian and the potential operator.

The operator relevant to light interactions is the electric dipole operator $\hat{\mu}$.

$$\hat{\mu} = \sum_i e_i \mathbf{r}_i$$

where e_i is each charge, and \mathbf{r}_i is the position operator. (The Franck-Condon principle states that light interacts with molecules on such short time scales that the nuclear motions can be ignored; this is a modified restatement of the Born-Oppenheimer approximation. Thus, only the electrons must be considered.)

The effect of the light electric field is a distortion of the state a wavefunction into a state close to state b . Classically, $\mu_{\text{ind}} = \alpha \cdot \mathbf{E}$, where α is the polarizability of the molecule, and μ_{ind} is the induced dipole moment. For the interaction of light with a molecule, the integral $\langle \psi_b | \hat{\mu} | \psi_a \rangle$ is the dipole induced by light.

Extinction coefficient

The rate of absorption of light by a system is:

² Most textbooks use a capital “E” for both energy and electric field. To attempt to minimize confusion, I will use E to refer to energy, and \mathbf{E} to indicate electric field.

$$\frac{dP_b}{dt} = B_{ab}I(\nu)$$

where B_{ab} is the transition rate per unit energy density of the radiation and $I(\nu)$ is the energy density at the frequency ν . The energy density of light is $I(\nu) = |\mathbf{E}_0|^2/4\pi$; it is a function of the number of photons present at that frequency, because each photon contributes to the electric field strength. The transition rate B_{ab} (the Einstein coefficient for stimulated absorption) is:

$$B_{ab} = \frac{2}{3} \frac{\nu}{\hbar^2} \left| \langle \psi_b | \mu | \psi_a \rangle \right|^2$$

(The derivation of the expression for B_{ab} is complicated and I will not attempt to reproduce it here.)

The rate at which energy is removed from the light is dependent on the probability of absorption transitions, the rate of emission transitions of the same frequency, on the energy per transition ($h\nu = E_b - E_a$), and on N_a and N_b , which are the number of molecules per cm^3 in states a and b .³

$$\frac{-dI(\nu)}{dt} = h\nu(N_a B_{ab} - N_b B_{ba})I(\nu)$$

The absorption of light through a slice dl of the sample at concentration C (where dl is so thin that the light intensity does not change as it passes through the slice) is given by:

$$\frac{-dI}{I} = C\epsilon dl$$

Integrating from I_0 to I and 0 to l :

$$\int_{I_0}^I \frac{dI}{I} = \int_0^l C\epsilon dl \quad \text{gives:} \quad \ln \frac{I_0}{I} = C\epsilon l$$

Converting to base 10 gives:

$$\log \frac{I_0}{I} = C\epsilon l$$

where ϵ is the molar extinction coefficient, and l is the pathlength in centimeters. Abbreviating the log term as the absorbance, A , gives: $A = C\epsilon l$, which is the usual form of the **Beer-Lambert law**.

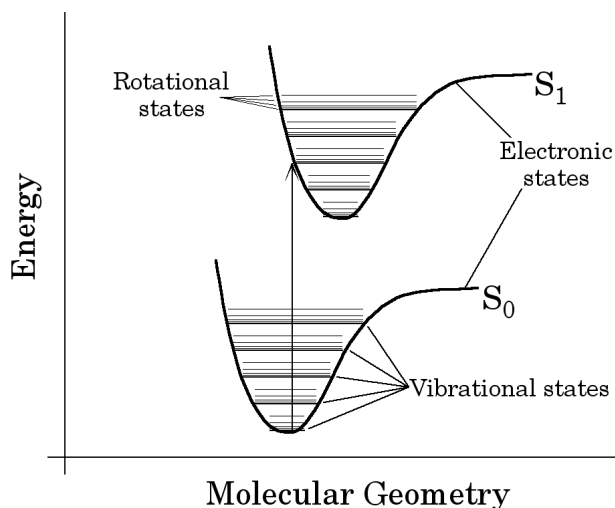
³ In most systems, $N_a \gg N_b$, and, for simple systems $B_{ab} = B_{ba}$. If, however, $N_b > N_a$, the change in light intensity will be positive. In other words, the light intensity exiting the system will be greater than the light intensity entering it. This observation is the basis of lasers.

Assuming that the molecules in solution do not interact, the extinction coefficient ϵ is a constant for a given molecule in solution at a given wavelength. (If the molecules interact with one another in a way that changes the energy levels of the transition, the extinction coefficient will change in a concentration-dependent manner.)

Absorption and energy states

Electromagnetic radiation only interacts with molecules at specific energies. In order for the electric field to induce a dipole, the energy difference between the two states must be equal to the energy contained in the photon, and the orientation of the electric field vector must match the orientation of the molecule. (The mathematical derivation of this is very complex; instead, we will consider the interaction with light using a more qualitative approach.)

The state of a molecule can be described by an energy diagram such as the one shown below. The energy diagram shows two electronic states, with each electronic state comprised of a variety of vibrational and rotational states. The difference in energy between one rotational state and the next is ~ 4 kJ/mol, which is in the range of thermal energy of the molecule, allowing molecules to adopt more than one rotational mode. However, the vibration energy levels are about 40 kJ/mol apart, and electronic states are 150 to 450 kJ/mol apart (depending on the molecule). Because these energies are well above the thermal energy range, in the absence of an external energy input only the lowest vibrational level of the lowest energy electronic level will be populated.



Each of the energy states the molecule can adopt is described by a wavefunction. The lowest energy state, the ground state, is ψ_0 . Other states within the ground electronic state are $\psi_{0,v,r}$, where the v and r subscripts refer to the specific vibrational and rotational states.

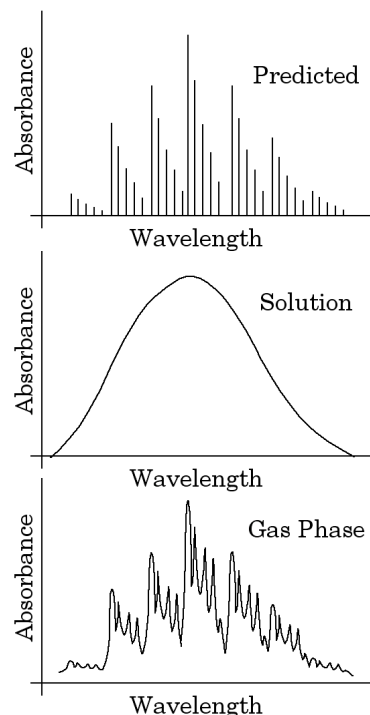
The interaction of a molecule with electromagnetic radiation in the ultraviolet or visible energy range induces a transition from one electronic state to another. The probability of any transition can be calculated using the overlap integral for the two

states. For an electronic transition, the overlap integral is $\langle \psi_{1,y,r} | \psi_0 \rangle$. The overlap integral can only be significantly different from zero as a result of the $\hat{\mu}$ operator; this will only occur if the energy of the photon is equal to the energy difference between the states ψ_0 and $\psi_{1,y,r}$, and even then only with a probability less than one if the electric field vector can induce a dipole in the molecule as it is oriented when the photon approaches (in other words, as indicated earlier, not all photons of the appropriate energy will induce transitions in all molecules).

In principle, the absorption spectrum of a molecule, the absorbance intensity as a function of the frequency (or more commonly, wavelength) of light should be a series of sharp bands, with each band corresponding to the transition from one energy level to another. The height of each absorbance band is related to the probability of that transition.

For many molecules, the effects of solvent environment, Doppler effects, and other influences tend to result in a relatively broad and featureless absorbance band that extends over ~ 50 nm. The overall absorbance band corresponds to a single electronic transition, which is broadened by the vibration and rotational transitions.

It is possible to see the absorbance fine structure for some molecules. This tends to be especially apparent in the gas phase, although for some molecules different vibrational bands are distinct enough to show spectral structure in solution.



Spectral analysis

An absorbance band represents a single electronic transition. (This assumes that the band is well enough separated from other electronic transitions; some molecules have more than one transition, and therefore may exhibit merged absorbance bands.) Analysis of the properties of the band allows assessment of some molecular properties.

In a 1 cm^3 volume containing a 1 molar solution, the rate of energy uptake is:

$$\frac{-dI(\lambda)}{dt} = \frac{h\nu N_0 B_{ab}}{1000 \frac{ml}{L}} I(\lambda)$$

Light travels at c , and therefore, the intensity change in distance dl is:

$$dI(\lambda) = \frac{1}{c} \frac{dI(\lambda)}{dt} dl = \frac{h\nu N_0 B_{ab}}{1000c} I(\lambda) dl$$

Because molecules do not absorb single wavelengths, the above equation cannot be solved using the Beer-Lambert law. Instead, it is necessary to calculate the intensity change over a range of wavelengths. Since intensity is a linear function of frequency, in most cases the integration is performed using frequencies rather than wavelength:

$$B_{ab} = \frac{1000c}{N_0 h} \int \epsilon_{ab} d\lambda$$

The parameter of interest is the dipole strength = $D = |\langle \psi_b | \mu | \psi_a \rangle|^2 = |\mu|^2$

Since, as was noted before, $B_{ab} = \frac{2}{3} \frac{\epsilon_0}{\hbar^2} |\langle \psi_b | \mu | \psi_a \rangle|^2 = \frac{2}{3} \frac{\epsilon_0}{\hbar^2} D$, it is possible to combine the equations to obtain an expression for D:

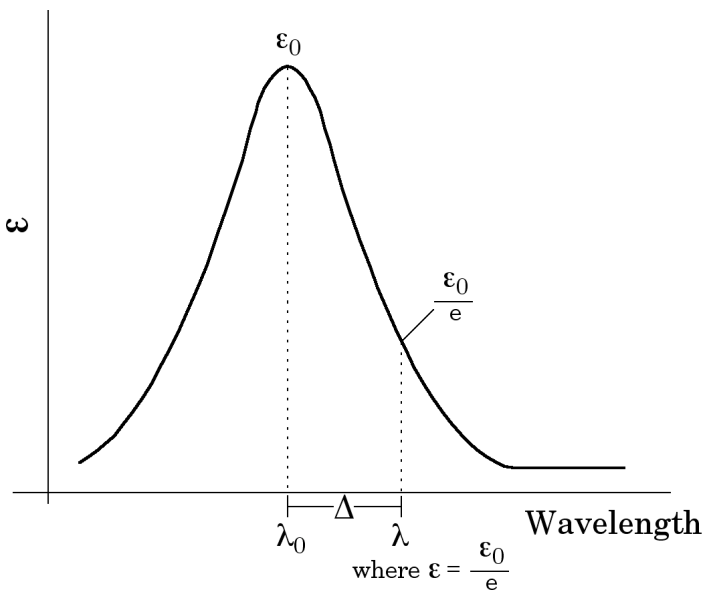
$$D = \frac{1000c}{\frac{2}{3} \frac{\epsilon_0}{\hbar^2}} \ln(10) \int \epsilon_{ab} d\lambda$$

where the "ln(10)" allows the conversion of ϵ to ϵ_0 . Evaluating the fundamental constants using cgs units results in:

$$9.18 \times 10^{39} \int \epsilon_{ab} d\lambda = 9.18 \times 10^{39} \int \epsilon_{ab} (debye)^2 d\lambda$$

where a debye = 10^{-18} electrostatic units • cm = 3.336×10^{-30} C • m. The dipole length is $|\mu| = \sqrt{D}$

An alternative method for performing the calculation uses the assumption of a gaussian absorbance band. Because a gaussian curve has a symmetrical shape, measurement of appropriate parameters in the curve avoid the necessity of converting to frequency or of integrating. In the graph at right, ϵ_0 is the maximal extinction coefficient, and e is the base for natural log (2.71828182846...)



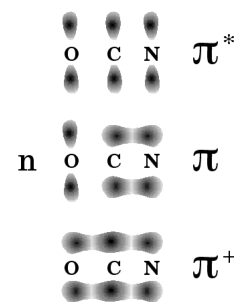
$$D = 9.180 \times 10^{-3} \frac{\epsilon_0}{\int \epsilon_0} e^{-((\lambda - \lambda_0)/\Delta)^2} d\lambda (debye)^2 = 1.63 \times 10^{-2} \frac{\epsilon_0}{\int \epsilon_0} (debye)^2$$

The oscillator strength of a transition, f_{ab} , is a unitless number between 0 and 1. When $f_{ab} = 0$, transitions do not occur. When $f_{ab} = 1$, transition always occurs when $\mu = \mu_0$.

$$f_{ab} = 7.5 \times 10^{-2} \frac{\mu^2}{\mu_0^2}$$

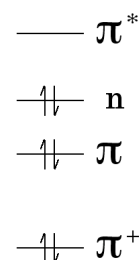
Electronic transitions in molecules

As an example of how light affects an actual molecule, we will consider the peptide backbone. The peptide backbone contains σ bonds, π bonds, and non-bonding electrons. For a given peptide bond, a total of six electrons are distributed among the three highest energy molecular and atomic orbitals. In addition, we need to consider the lowest energy unoccupied molecule orbital.

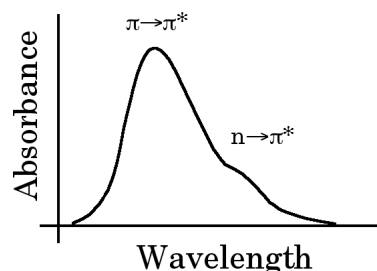
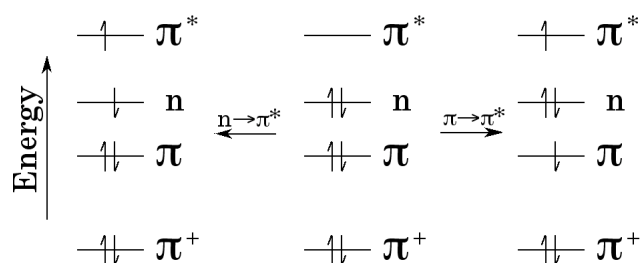


The strongly bonding π^+ orbital has the lowest energy. The π orbital is a more weakly bonding orbital; the non-bonding n orbital contains the lone pair of electrons associated with the carbonyl oxygen. The highest energy orbital of interest is the anti-bonding π^* orbital.

Each orbital can contain two electrons of opposite spin. Distributing the six electrons to the lowest energy states results in the diagram at right. As mentioned above, the π^* orbital is unoccupied; each of the other orbitals contains two electrons.



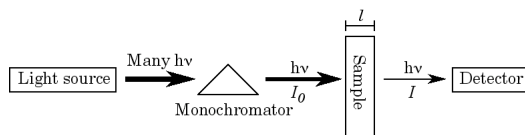
If a photon contains an energy equal to the difference in energy ΔE between one occupied orbital and the first unoccupied orbital, a transition may occur. For this system, two transitions are possible at reasonable energies: $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$.



The absorbance spectrum shown at right reflects the results of exposing the peptide bond to light. The energy difference between the π and π^* orbitals is greater than that between the n and π^* orbitals, and therefore requires shorter wavelength photons. However, the overlap between the π and π^* wavefunctions is much greater than that for the n and π^* orbitals. The transition probability $f_{n\pi^*}$ is very small (about 10^{-2} to 10^{-3} , compared to ~ 0.25 for $f_{\pi\pi^*}$). The limited overlap between the n and π^* wavefunctions make the transition “forbidden” (in the diagram, the $n \rightarrow \pi^*$ peak is exaggerated to make it visible). In quantum mechanics, forbidden processes are possible, although the probability is low.

Performing spectroscopic measurements

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 spectrum 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

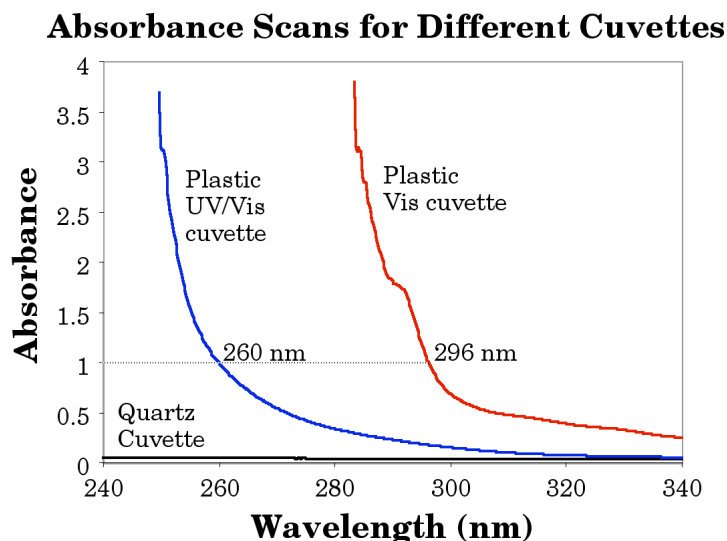
spectrophotometer, the monochromator is located after the sample, so that it splits the multiwavelength light leaving the sample.

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.

Cuvettes

Most samples studied using spectrophotometry 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 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.



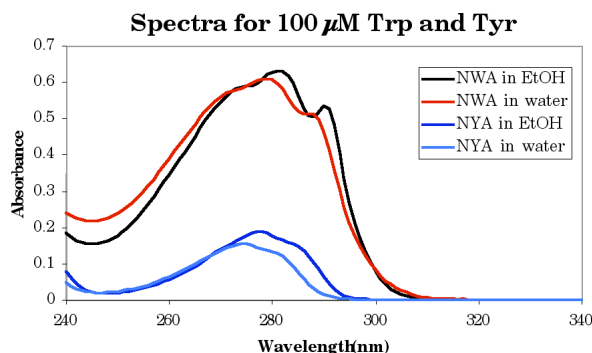
In addition to interference by the cuvette, buffer components may absorb. Disulfide bonds absorb at ~250 – 270 nm, which means that oxidized β -mercaptoethanol or dithiothreitol will interfere with some measurements of proteins. In addition, many buffer components absorb light below 210 nm. Oxygen absorbance 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 containing a vacuum chamber.

Types of absorbance spectroscopic experiments:

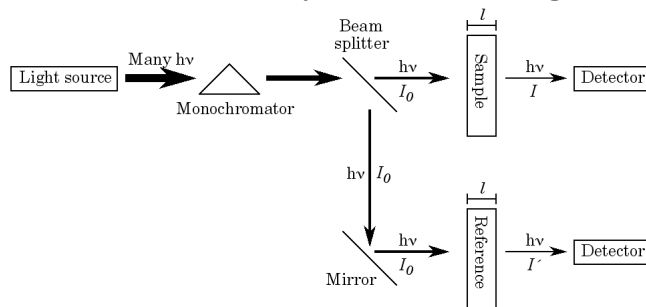
Absorbance spectroscopy is frequently used to measure concentration. According to the Beer-Lambert law measurements of absorbance for molecules of known ϵ allow calculation of concentration. Alternatively, if the concentration of the absorbing species is known, the ϵ can be determined.

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 both tryptophan and tyrosine change as a result of transfer from an organic to an aqueous environment.



This change is more apparent when **difference spectra** are measured.

Difference spectra are much more readily measured using dual beam instruments.



Protein spectroscopy

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.

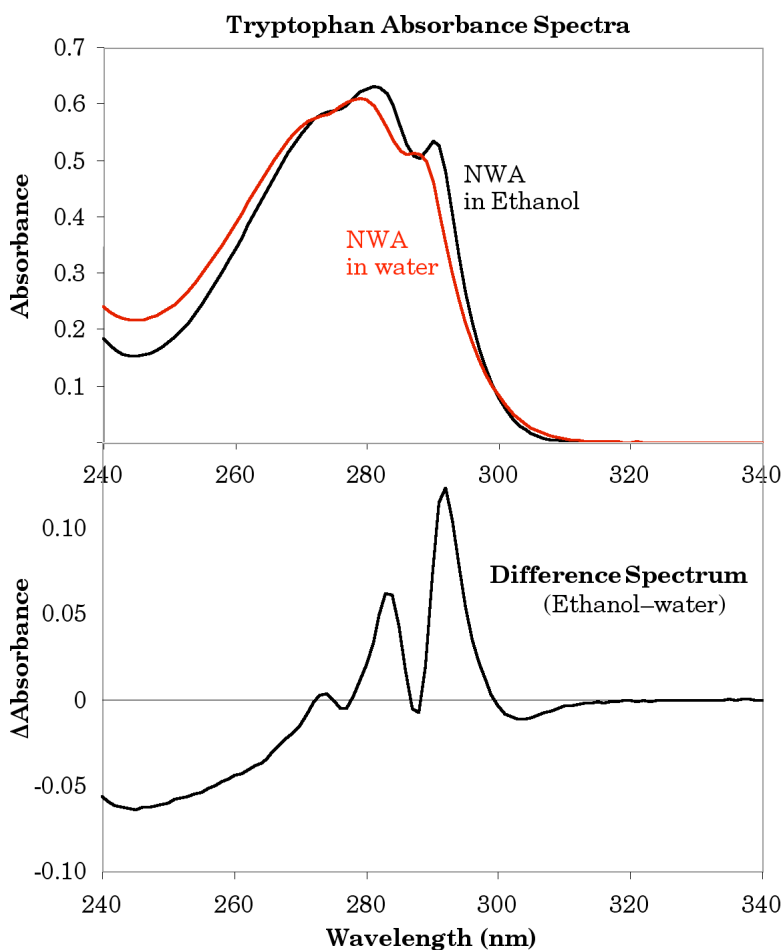
For proteins without prosthetic groups, the only absorbance beyond ~250 nm is due to aromatic amino acids (and -S-S-, but these are fairly rare and have ϵ of only ~300).

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 media, observation of spectral changes can yield information regarding the degree of tyrosine side-chain solvent exposure.

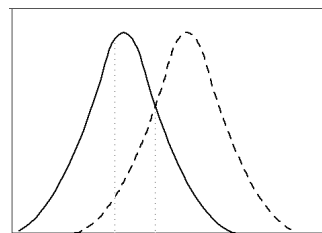
The extinction coefficient of tryptophan and tyrosine are fairly insensitive to environment. The $\Delta\epsilon$ is usually ~100 for tyrosine and ~200 for tryptophan. This means that the extinction coefficient for a protein can be calculated based on the amino acid content:

$$\text{For proteins: } \epsilon_{280} = \# \text{ Trp} \cdot 5615 + \# \text{ Tyr} \cdot 1380$$

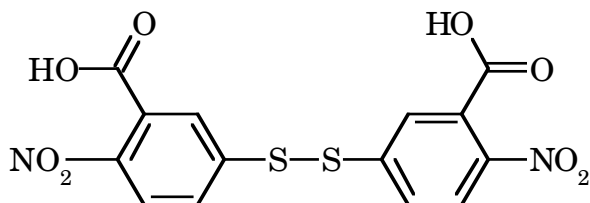
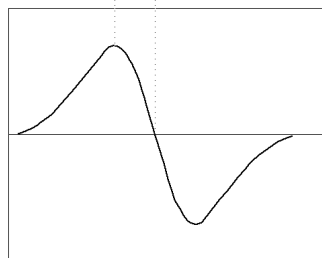
Difference spectra can be used to assess binding of small molecules to the protein, or to assess the environment near aromatic residues.



Some prosthetic groups have characteristic difference spectra. An example is the carbon monoxide difference spectrum for cytochrome P450 enzymes. The spectral shift (shown at right) 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.



Spectroscopic probes can be attached to 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)

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.

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.