F. FLUORESCENCE & CIRCULAR DICHROISM SPECTROSCOPY – AN INTRODUCTION

Introduction to Spectroscopy

Electronic transitions

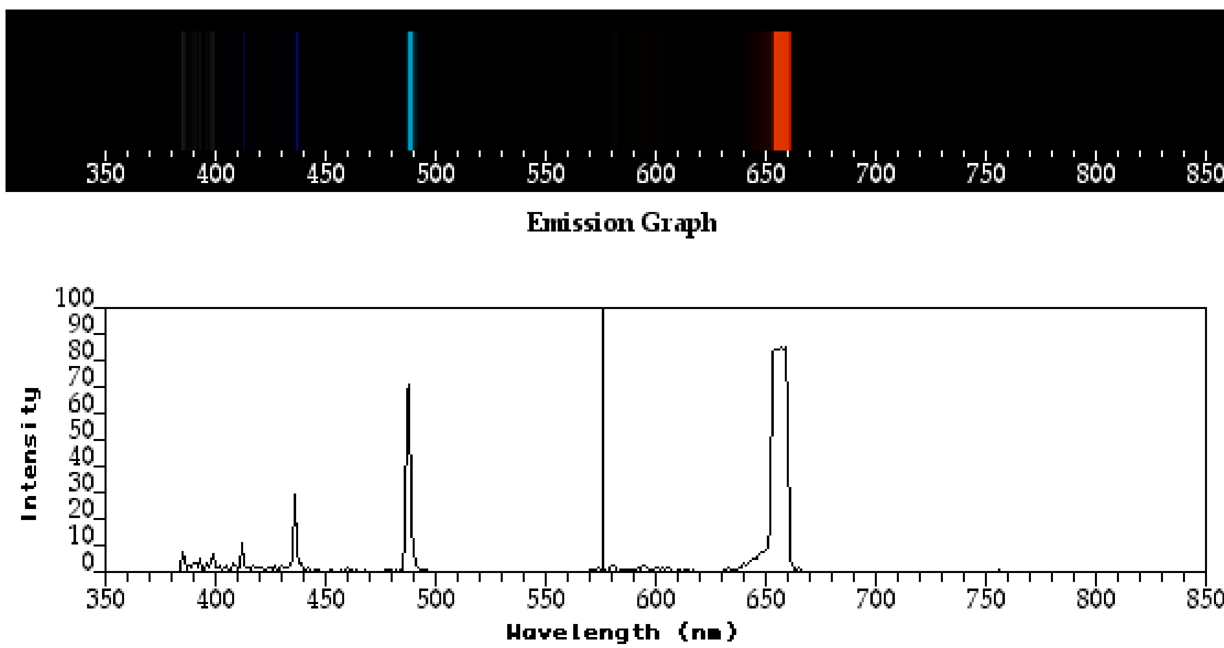

Figure F.1. Atomic emission spectrum of hydrogen. Note that the absorption spectrum possesses sharp lines equivalent to those seen in the emission spectrum.

UV/Vis spectroscopy monitors the excitation of a molecule from a ground electronic state to an excited electronic state. A photon of an appropriate energy is absorbed and an electron is advanced from a low lying orbital to one higher in energy. The simplest example is in atomic spectroscopy, where an electron in the hydrogen 2s orbital may be advanced to the 3p orbital by absorption of a photon with a wavelength of 654 nm.[1]

In molecules, one can think of a similar transition, but in this instance from one molecular orbital (MO) to another – usually from the highest occupied MO (HOMO) to the

[1] Note that the electron must change from an s to a p orbital, reflecting a change of +1 in the angular momentum quantum number, *l*, during the transition. There is a quantum mechanical rule that says the angular momentum of an electron must change by ±1 during an electronic transition. More on that later.

lowest unoccupied MO (LUMO). Although only a single electron is changing orbitals, the redistribution of electron density of the molecule with the electron in the ground state to a molecule with an electron in a higher energy orbital changes the energy of the molecule as a whole. Thus, instead of talking about the energy of an individual electron, we talk about the energy of the molecule. It is the *molecule* that advances from the ground state to the excited state upon absorption of a photon. The absorption event is a quantum phenomenon, but unlike narrow atomic absorption bands, one obtains a broad absorption band. That is because the vibrational energy levels for a molecule are superimposed on the electronic energy levels. In OChem, you used low-energy infrared light to excite a molecule *vibrationally* but not *electronically*. In electronic spectroscopy, such as UV/Vis, you can't help but excite a molecule both electronically and vibrationally. Although a pair of electronic energy levels may be separated by a fixed gap, one has the possibility of exciting a molecule to a variety of different vibrational levels as well. The breadth of distribution in vibrational energy levels is seen in the breadth of the absorption band (Figure F.2).

Application of UV/Vis Spectroscopy to Proteins

There are several naturally occurring chromophores in proteins: the peptide bond, the aromatic rings of Phe, Tyr and Trp (and His to a lesser degree), and the disulfide bond in cystine linkages. The most common use of these chromophores is in determining the presence and/or quantity of protein present. In that respect, Tyr and Trp are the most important contributors to absorbance in the ultraviolet spectrum (Table 2.4). With absorption maxima at 275 nm and 280 nm respectively, they lead to the common practice of using absorption of light at 280 nm to determine the quantity of protein present. UV/Vis spectroscopy plays additional roles when natural or artificial chromophores are present, but we won't get to that here. UV/Vis is not typically used for structural information, though occasionally there is information in the position of λ_{max} that can be valuable (see fluorescence spectroscopy below).

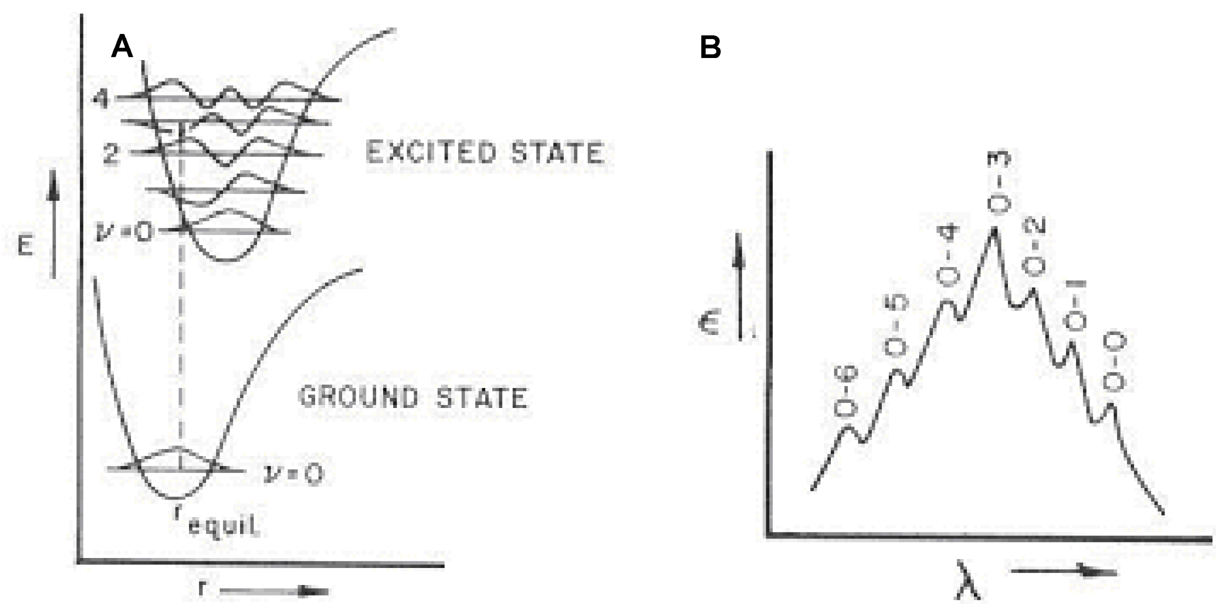

Figure F.2 A. Excitation from the ground state to the excited state of a molecule. Note that the energy "levels" are not level. The x-axis relates to molecular geometry, and the energy of the ground state varies as you apply more vibrational energy and distort geometry. Likewise, in the excited state, multiple vibrational energy levels (labeled ν=0, 1, 2…) mean that the excited state has no fixed energy either. (B) The absorption spectrum reflects the uncertainty of the transition. Starting the ν=0 vibrational state of the ground state, you can make the transition to any number of vibrational states in the excited state. That leads to a number of different wavelengths of light that can promote the transition. Hence the broad absorption band.

Circular dichroism spectroscopy

Spinning Photons

In organic chemistry, you learned that solutions of chiral compounds rotate the plane of polarization, so that a beam in one orientation passes through the sample and exits in a different orientation. The degree of rotation is measured and is reported as the **optical rotation** of the sample. Typically one uses light at 580 nm for this purpose. Organic molecules do not absorb photons at this wavelength, at least not typically. So, why is the plane of the incident radiation rotated?

To answer than, you need a quick lesson in the nature of the photon. It is a boson because it has spin 1. Electrons are fermions with half-integer spins. Just as electrons can be assigned a spin of ±1/2, photons can carry spin of ±1. We interpret the spin of a photon as its angular momentum. Remember from above that, in the hydrogen atom, electrons must be excited from the 2s to 3p orbital, reflecting a change in angular momentum of +1? The reason is that conservation of angular moment requires that the angular momentum of the absorbed photon be conserved in the electron. How does this relate to plane polarized light? It turns out that there is no such thing as a single photon with an electric field oscillating in a single plane. Instead, the electric field vector rotates about an axis defined by the direction of travel, essentially tracing out a spiral. Depending on the sign of the spin, +1 or –1, we can interpret the rotation as clockwise or counterclockwise.

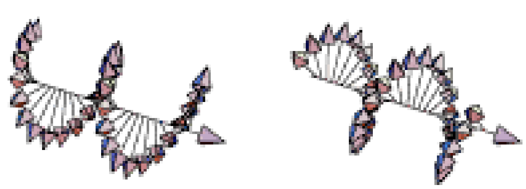

Figure F.3 Two photons of opposing spin. Each line perpendicular vector to the line of travel represents the position of the electric field vector at one given time. Note that the side on view would appear to be an oscillating wave.

Plane polarized light is a beam of photons that contain an equal population of spin +1 and spin –1 photons synchronized so that that their rotating fields reach alignment in the vertical orientation twice per cycle. In Figure F.4, the rotating vectors can be viewed as though you are looking into the path of two on-coming photons. At t=1 and t=5 the electric fields are perfectly aligned, and sum up positively in the plane of polarization. At t=3, the electric field vectors cancel each other out, and appear not to exist. Thus, as the electric field rotates about the axis of travel, the net electric field of the beam of photons remains in one plane of polarization.

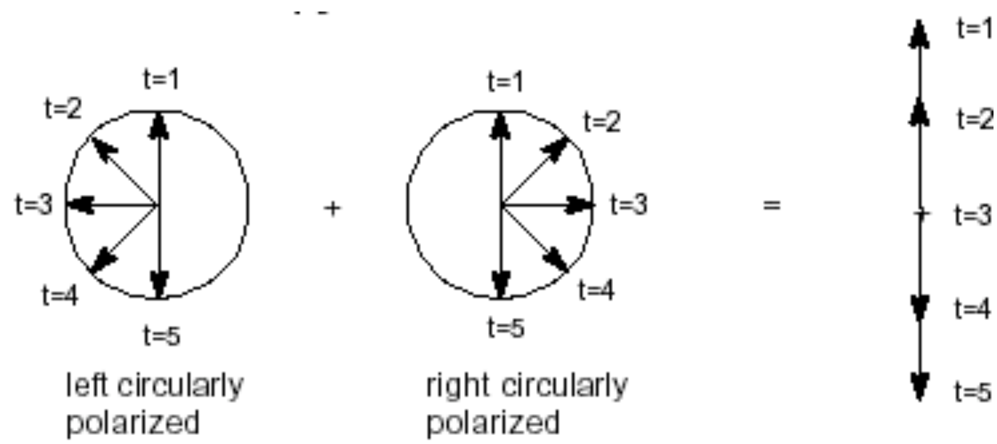

Figure F.4. Two photons of opposite spin traveling towards the observer will sum up to give the appearance of a wave oscillating in the vertical plane over time.

In **optical rotatory dispersion**, no absorption of light takes place, but when plane polarized light passes through a solution of a chiral compound, photons with one spin pass more rapidly through the sample than the other (that is, the two types of photons have different indices of refraction). The result is the appearance that the plane of polarization has been rotated by some angle α. In Figure F.5, it appears the right circularly polarized photon is has been retarded relative to the left circularly polarized photon to yield a counterclockwise rotation of the plane of polarization.

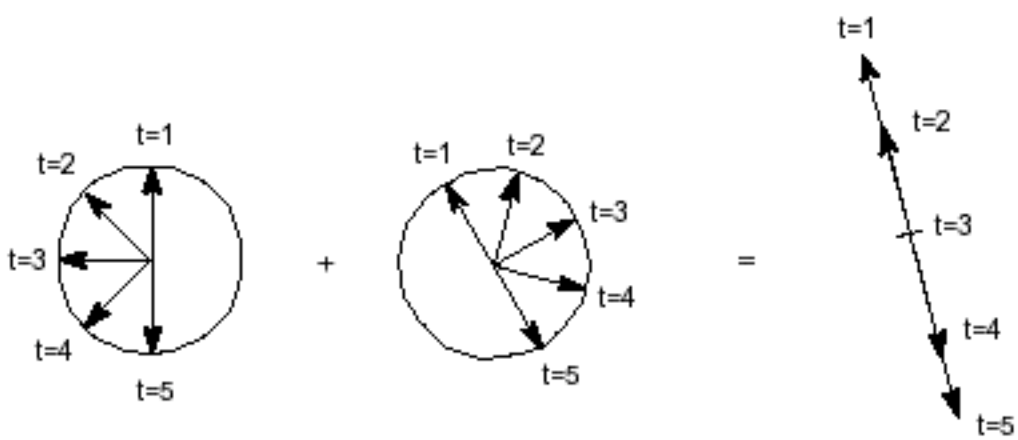

Figure F.5. Rotation of the plane of polarization takes place when photons with one spin travel through a sample at a different speed than photons of the opposite spin.

In **circular dichroism spectroscopy**, there is absorption of light passing through a chiral sample, as well as a difference in the index of refraction for the two circularly polarized forms of light. The result is both a tilting of the axis of polarization and a loss of "planarity". Instead one obtains "ellipticity" (Figure F.6). Chromophores – objects that absorb light – in chiral environments will display a circular dichroism (CD) signal. While it is possible to calculate the CD spectrum of a sample from first principles, for our purposes we can take this as a phenomenological attribute of chiral samples and use it to determine something of the structure of biochemical molecules.

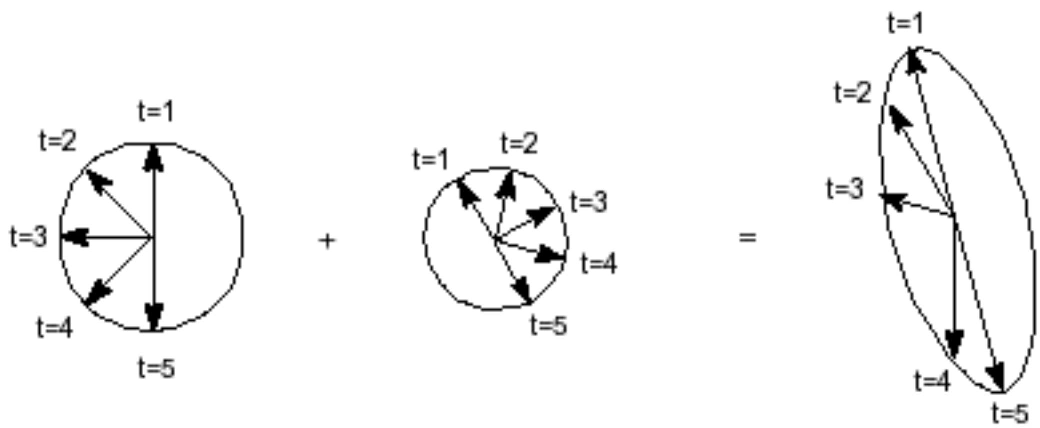

Figure F.6. In CD spectroscopy one form of circularly polarized light travels less rapidly than the other through a chiral sample and is absorbed preferentially. The resulting sample of light traces out an ellipse with its bulk electric field vector.

Circular Dichroism and Protein Secondary Structure

All polypeptides contain an important chromophore – the amide bond. Amides absorb light strongly at about 220 nm due to the excitation of a non-bonding electron in the HOMO of the amide to a π anti-bonding orbital (the LUMO). Fortuitously, virtually every amide group in the protein backbone experiences a chiral environment. Part of the chiral environment springs from the neighboring side chain groups on the alpha carbons of each residue, but more subtly, secondary structure creates a chiral environment for the chiral residues. Alpha helices possess a right hand twist, like a cork screw, and beta strands have a slight left hand twist. The result is that amide bonds in these conformations display readily identifiable CD spectra that are indicative of the secondary structure they inhabit (Figure F.7). Notably, alpha helices display a distinct negative ellipticity between 210 and 230 nm (described as the far UV), with a double minimum. The minimum at 222 nm is a particular signature of an alpha helix. Likewise, β strands and random coil possess their own typical spectra. The CD spectrum of a protein sample can often be used to estimate quantities of secondary structure present in a sample and also to observe gain or loss of secondary structure during protein folding or unfolding.

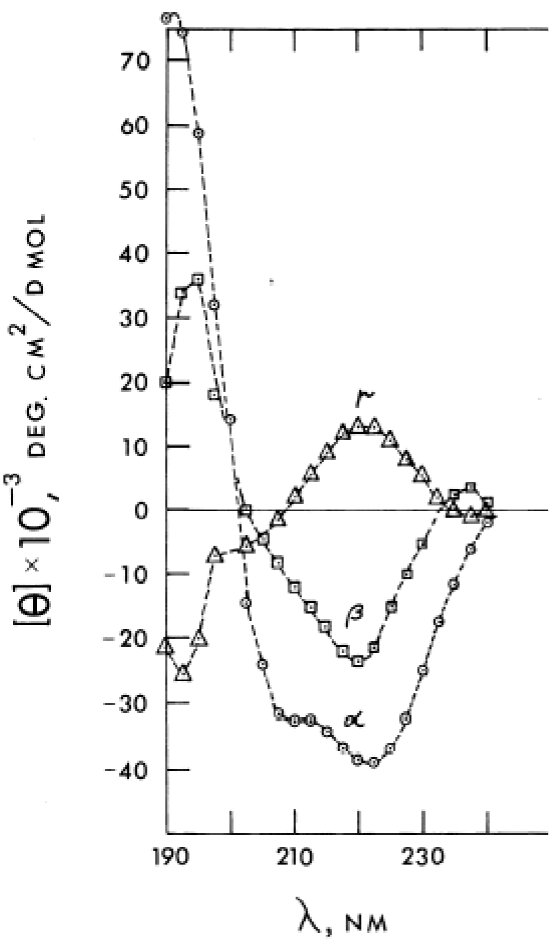

Figure F.7. Far UV CD spectra of β-strand (β), α-helix (α) and random coil (r). Note the strong double minimum in the α-helical spectrum.

Fluorescence spectroscopy

Fluorescence

In UV/Vis spectroscopy, one monitors absorption of a photon. In most instances, the absorption of the energy provided by the photon heats the sample an no light escapes. For some select molecules, however, re-emission of a photon follows the absorption event. Fluorescence and phosphorescence are both examples of this phenomenon and together they are classified as luminescent phenomena. We will restrict our conversation to fluorescence, which is the most commonly used technique in biophysical chemistry. In fluorescence, electronic excitation is accompanied by vibrational excitation as we saw in Figure F.2. If the lifetime of the excited state is long enough, the molecule may "relax" to the *ground* vibrational state of the *excited* electronic state. This is a small change in energy relative to the difference in electronic energy states and can be achieved simply by transferring vibrational energy through collision with another molecule. Some of the energy of the incident photon has been lost as heat, but not all of it. If, now, the remaining energy is lost as a photon in what is called *radiative emission*, a photon of longer wavelength that the incident photon is emitted and can be observed (Figure F.8). That is fluorescence. A common example is in Day-Glo pigments. They excited in the near UV (light just below 400 nm in wavelength) and emit in the visible (at wavelengths greater than 400 nm). This effect has been particularly entrancing to 8th graders for as long as I can remember.

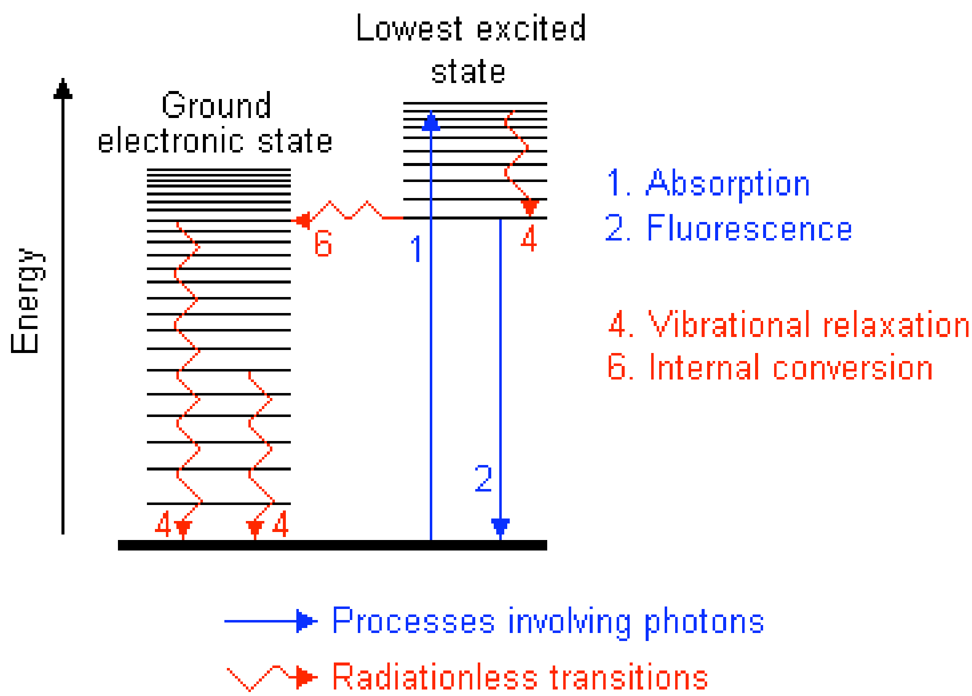

Figure F.8. The ground electronic state is depicted here as a dark black line. When the a photon is absorbed (1) the molecule advances to an excited vibrational state in the excited electronic state. Vibrational relaxation (4) emits some of that energy as heat, and then fluorescence (2) emits the rest of it as a long wavelength photon.

Fluorescence Spectroscopy in Proteins

Fluorescence can be an exceptionally useful tool in studying protein structure, because the local chemical environment can lead to significant changes in the intensity of fluorescence, as well as smaller changes in the spectrum of the emitted light. As luck would have it, both Tyr and Trp are fluorescent species, and it is possible to do fluorescent spectroscopy on virtually any protein, though the enhanced fluorescence of Trp makes it a particularly valuable tag.

While the emission spectrum of the indole ring of tryptophan may be altered by chemical environment (the peak is typically shifted to shorter wavelengths in a polar environment), the most useful observation is that Trp residues exposed to solution fluoresce less efficiently than those buried from solution. This is due to "collisional quenching". While the mechanism is not entirely clear, there are a number of dissolved species in water (most notably O_2) that can collide with Trp residues in the excited state and relax the Trp to the ground state without fluorescent emission. When a Trp residue becomes buried in the interior of a protein (say during protein folding), the fluorescence of the protein decreases. Fluorescence is a very useful probe of the folded structure of proteins.