Chem 728 Lecture Notes – Part 1a – Optical Spectroscopy

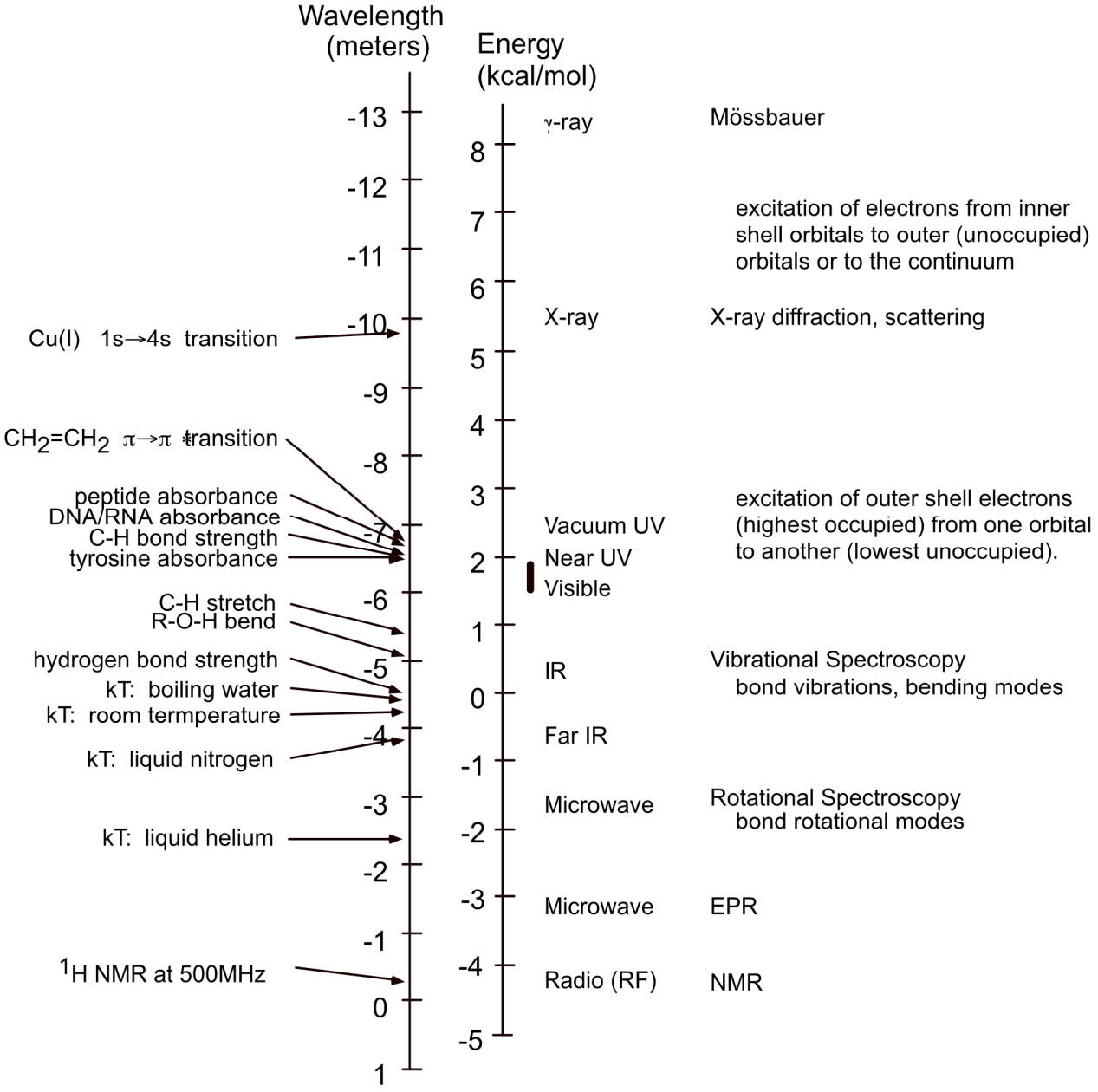
The following are lecture notes for Chem 728 (by C. Martin, with minor modifications by L. Thompson). Much of the material is taken directly from the indicated references (old vH, some refs updated to new). This is not intended to replace the original references, but is made available solely for the convenience of students in the class.

VH = "Principles of Physical Biochemistry," Kensal E. van Holde, W. Curtis Johnson, & P. Shing Ho, Prentice Hall, NJ, 1998 (ISBN 0-13-720459-0)

CS = "Biophysical Chemistry, Volumes I-III" Charles R. Cantor & Paul R. Schimmel, W. H. Freeman, NY, 1980 (ISBN 0-7167-1188-5, 0-7167-1190-7, 0-7167-1192-3)

General Properties of Light and Matter (VH 8; CS 7)

Range of energy: spectroscopies and molecular processes



very high energies - electrons from inner to extreme outer shells or to continuum
x-rays: scattering from atomic centers
UV: excitation of electrons from one orbital to another
visible: excitation of electrons from one "big" orbital to another
IR: bond vibrations, bends
microwave: bond rotational modes, EPR
RF: nuclear magnetic resonance

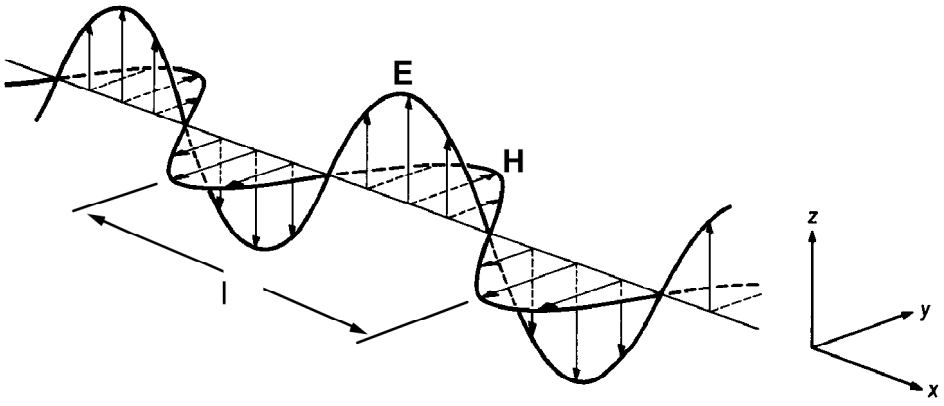
"Light" and energy

$$E = h\nu = hc/\lambda$$

$h = 6.63 \times 10^{-27}$ erg s $c = 3.0 \times 10^{10}$ cm s^{-1} $N_o = 6.02 \times 10^{23}$ $mole^{-1}$

$m_e = 9.11 \times 10^{-28}$ g; $k = 1.38 \times 10^{-16}$ erg K^{-1} $R = N_o k = 1.987$ cal mol^{-1} K^{-1}

erg = g cm^2 s^{-2} cal = 4.18×10^7 erg $\hbar = 2\pi h$ $\omega = 2\pi\nu$



Plane-polarized radiation

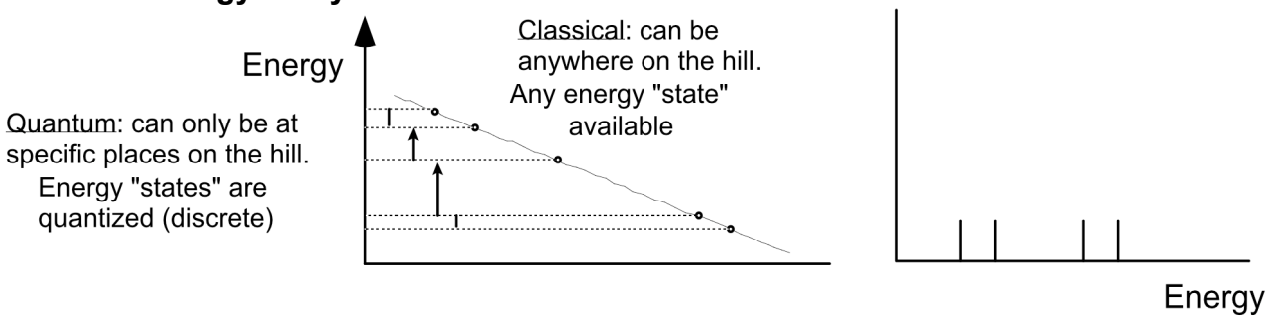
Electromagnetic radiation ("light") composed of electrical and magnetic components.

E and B components are perpendicular and vary in space with time. Light can be represented as a spatial "wave." Frequency of oscillation is ν, and wavelength λ.

picture here....

This is the classical representation and we will mostly stick with this kind of explanation, however, there are times when classical analogies fail, and only quantum mechanics will correctly predict nature.

Quantization of energy - only discrete states available



Quantum mechanics tells us that systems can only exist in discrete states.
There is no classical analogy here.
In classical mechanics, a **spring** of a specified length, width, and physical makeup can vibrate at any of an infinite number of frequencies. In quantum mechanics only discrete states are available, which are defined by the physical properties of the spring. Since states are discrete, energy gaps are discrete, and spectroscopic band widths *would* be very narrow, but heterogeneity in sample and environment broadens most bands significantly.

Each component in an ensemble has a discrete transition energy, but heterogeneity within the ensemble of microsystems results in a broad band associated with the macrosystem.
Later: Dynamics will also be seen to effect linewidths.
We will also see later that spin states of nuclei and electrons are also quantized ("up" and "down").

Brief summary of quantum mechanics

The state of a system (atom, molecule) is described by a <u>wave function</u>

$\psi(x,y,z,spin,t)$ (ψ is, in general, a complex function - imaginary numbers...)

The probability of finding the system at a particular set of conditions of a state is given by the product of ψ and its complex conjugate ψ^*. $P = \psi\,\psi^*$

The probability of the system being "anywhere" in state ψ is determined by averaging over all conditions of that state: $P(t) = \int P\,dx\,dy\,dz\,d(spin) = \int\int \psi\,\psi^*\,d\tau$. Because QM tells us that systems exist discretely in a given state, then $\int\int \psi\,\psi^*\,d\tau = \langle\psi|\psi\rangle = 1$. ($d\tau$ means "over all space - dx dy dz)

Operators - The Result of a Measurement

An observable quantity (e.g. energy, dipole moment, location in space) is governed by a mathematical device known as an operator.

The Hamiltonian H is used to describe the energy of a state.

The result of a measurement on a state (e.g. measure the energy) can be calculated by taking the average value of the operator operating on that state: the expectation value.

$$\int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\int_{-\infty}^{\infty} \psi\,H\,\psi\,dx\,dy\,dz = \int\int \psi\,H\,\psi\,d\tau = E \qquad \text{in other words,} \qquad \langle\psi|H|\psi\rangle = E$$

A transition between two states can be *induced* by a perturbation.

The effectiveness of this induction is governed by the extent to which the perturbation can deform the initial state to make it resemble the final state (*i.e.*, mix the states)

Assume a perturbation which can *mix* states, described as a potential **V**. The expectation that it will induce a system in state ψ_1 to effect a transition to state ψ_2 is given by

$P(\text{mixing}) = \langle\psi_2|\mathbf{V}|\psi_1\rangle$ remember that as $\mathbf{V}|\psi_1 \rightarrow \psi_2$, then this goes to $\langle\psi_2|\psi_2\rangle = 1$

Light can be a perturbation

The ability of light to induce transitions can be calculated by its ability to induce dipole moments that oscillate with the light.

The electric component of light can induce dipoles in electronic states.

In this case, the probability of an electric vector $\boldsymbol{\mu}$ inducing a transition is $\langle\psi_2|\boldsymbol{\mu}|\psi_1\rangle$

Perturbations have a directionality

The preferred directions for inducing dipole moments are determined by and fixed with respect to the geometry of the molecule.

ψ is a function of space (x,y,z), as is $\boldsymbol{\mu}$, therefore the ability of the electric dipole (vector) to induce transitions is dependent on their relative geometries.

Light can *induce* transitions between states (CS 7.1)

Energy match is required

Probability of light-induced transition is related to the matching of the light energy to the energy difference between levels

From quantum mechanics, the probability of a system initially in state *a* being found in state *b* (ie. a$\rightarrow$ b) is given by the following:

$$P_b = |C_b(t)|^2 = \text{Error!}$$

$$P_b = |C_b(t)|^2 = \frac{|\langle\psi_b|\underline{\mu}|\psi_a\rangle\cdot\mathbf{E}_0|^2}{\hbar^2}\;\frac{t^2\sin^2[(E_b-E_a)/\hbar - \omega)\,t/2]}{2[(E_b-E_a)/\hbar - \omega)\,t/2]^2}$$

$\underline{\mu}$ induces a dipole in state a, perturbing it to "resemble" state b. This term reflects this ability.

as this term $\rightarrow 0$, $P_b \rightarrow 1$

where $\boldsymbol{\mu}$ is the electric dipole operator, $\mathbf{E_o}$ is the electric field (a vector), and E_b and E_a are the energies of systems in states ψ_b and ψ_a, respectively.

Note that since $\hbar\omega$ is the energy of the light, transitions from a to b will be most likely when the denominator $((E_b-E_a)/\hbar - \omega)$ is small, that is when

$\hbar\omega = \hbar\omega = E_b - E_a$ energy of light matches energy difference between states

The amount of *net* light absorption is dependent on the number of molecules in each state

It can be shown that for radiation centered at frequency ω, the rate at which molecules are transformed from state a to state b is given by

$$\frac{dP_b}{dt} = \frac{d}{dt}\left[\int d\omega\, |C_b(t)|^2\right] = \frac{1}{2\hbar^2}\, |\langle \psi_b|\boldsymbol{\mu}|\psi_a\rangle \bullet \mathbf{E_o}|^2$$

for polarized light; a specific orientation of the molecule, and single frequency ω.

More generally, integrating over all orientations, it can be shown that

$$|\langle \psi_b|\boldsymbol{\mu}|\psi_a\rangle \bullet \mathbf{E_o}|^2 = (1/3)\, |\langle \psi_b|\boldsymbol{\mu}|\psi_a\rangle|^2\, |\mathbf{E_o}|^2$$

then $$\frac{dP_b}{dt} = \frac{1}{6\hbar^2}\, |\langle \psi_b|\boldsymbol{\mu}|\psi_a\rangle|^2\, |\mathbf{E_o}|^2$$

From classical E & M:

$$I(\omega) = \frac{|\mathbf{E_o}|^2}{4\pi}$$ Intensity: energy density incident on the sample at frequency ω

We can then define

$B_{ab} = \frac{2}{3}\frac{\pi}{\hbar^2}\, |\langle \psi_b|\boldsymbol{\mu}|\psi_a\rangle|^2$ transition rate ($a \rightarrow b$) per unit energy density

So that $\frac{dP_b}{dt} = B_{ab}\, I(\omega)$ the two components are separable

Note: $V = k\,[C]$ analogy to a first order kinetic "reaction"

This result is for any system initially in state a. It defines the probability of finding that system in state b at some time t (as a result of interaction of the system with the electric field component of the light. It contains two components: 1) B_{ab}, the transition rate (analogous to a chemical rate constant) and 2) $I(\omega)$, the energy density, or intensity, of the light - roughly the density of photons hitting the sample (analogous to "concentration of photons"). Note that at this point we have said nothing about which state is higher in energy.

A similar expression can be written for the transition from state b to state a. So that light is both emitted and absorbed. The *net* change then depends on the population of each level, such that the *net* absorption of light can be written as:

$$-\frac{dI(\nu)}{dt} = h\nu (N_a B_{ab} - N_b B_{ba})\ I(\nu)$$ The rate at which energy is removed from the light

where $h\nu = E_b - E_a$ (the energy of the transition between states)
For simple cases of interest to us, the Einstein coefficients $B_{ab} = B_{ba}$, so that

$$-\frac{dI(\nu)}{dt} = h\nu (N_a - N_b)\ B_{ab}\ I(\nu)$$ **N.B.**

Thus, through the factors N_a and N_b, light absorption depends not only on the concentration of the species, but also on the <u>difference in population</u> between the levels. A *very* important result of this is that no matter how much light you shine on the system, the most you can ever do is equally populate each of the levels. You can not (without getting fancy) "pump" all of the systems from state *a* to state *b*. We will see this later.

Light induces dipoles in molecules

B_{ab} $\langle \psi_b | \boldsymbol{\mu} | \psi_a \rangle$ = the dipole moment induced by the light = <u>transition dipole moment</u>

Classically, electromagnetic radiation (light) possesses an electric field component, $\mathbf{E}_o$ above. Since light is an oscillating wave, the electric field also oscillates. The oscillating field can then interact with an existing dipole or induce an oscillating dipole in the molecule, $\boldsymbol{\mu}$ above.

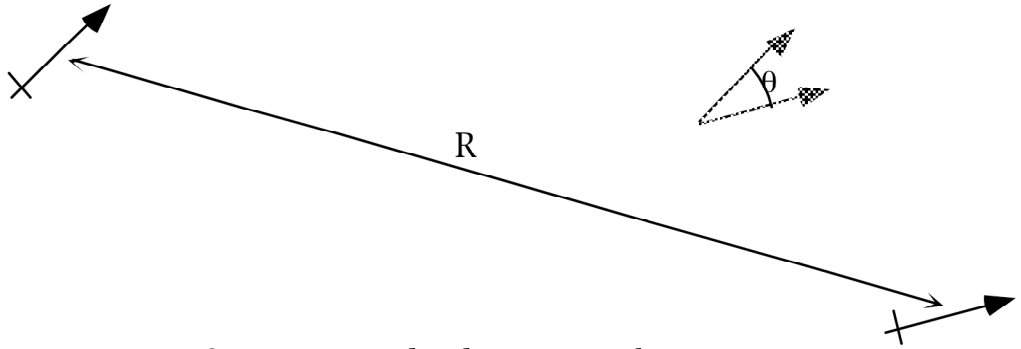
The integral $\langle \psi_b | \boldsymbol{\mu} | \psi_a \rangle$ (<u>transition dipole moment</u>, it is a vector) describes the ability of light to distort a molecule in state *a* so as to produce a system which resembles state *b*. Oscillations have a time component, and so have *phase*. This will be important when two dipoles are simultaneously excited. Relative phases important.

Transitions induced in *both directions* equally.

Purely a result of quantum mechanics

Net absorption *usually* observed, due to population of states (see below).

Dipole-dipole interaction (CS p. 263-265)



The interaction energy for two point dipoles is given by:

$$E_d = \varepsilon^{-1} \left[\frac{\boldsymbol{\mu}_A \cdot \boldsymbol{\mu}_B}{r^3} - \frac{3(\boldsymbol{\mu}_A \cdot \mathbf{r})(\boldsymbol{\mu}_A \cdot \mathbf{r})}{r^5} \right]$$

In this *point dipole* approximation, the charge separation (distance) within each dipole is assumed to be much smaller than the distance separating the two dipoles. This is not necessarily true in chemical systems, but a more rigorous calculation is generally prohibitive. A further approximation is generally made in dropping the second term in the equation. This assumption is generally not too bad. **< End of lecture 1 '90 >**

Note that the <u>dielectric</u> constant, ε, is involved. Pictorially, this means that if the intervening medium has its own dipoles between the two point dipoles, their effect will lessen the interaction between the two dipoles.

Note also that there is an angular dependence, such that dipoles oriented 90° with respect to one another have no interaction energy.

Finally, the large distance dependence ($\frac{1}{r^3}$) in this equation shows that only near neighbor atoms in a molecule interact substantially.

Boltzmann distribution (VH 2; CS 8.2)

Boltzmann equation specifies thermal distribution:

$$n_b = n_a\, e^{\frac{-(E_b - E_a)}{kT}} \qquad \frac{n_b}{n_a} = e^{\frac{-\Delta E}{kT}}$$

Thermal energy allows for transitions between states, but this is, of course, limited by the energy separation of the states relative to the thermal energy (*ie*. the temperature). The temperature equivalent of photons are called phonons. Thermal energy (the phonon bath) generally spans a wide range of energies, and so heat can usually bring a system to thermal equilibration.

Implications:

For states separated by substantial energy gap, lower energy state will be exclusively populated, *i.e.* as $\Delta E \gg$, $n_b/n_a \to 0$.

For closely spaced states, populations can be *almost* evenly distributed, *i.e.* as $\Delta E \to 0$, $n_b/n_a \to 1$.

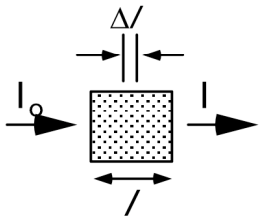
Temperature dependence: as $T \to 0$ ($\to$), high energy states depopulated, $n_b/n_a \to 0$.

Optical Spectroscopies

Absorption Spectroscopy

Beer-Lambert Law (CS 7.2)

The fraction of light absorbed:



$$\frac{-\Delta I}{I} = C \cdot \Delta l$$

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

$$\int_{I_o}^{I} \frac{-dI}{I} = \int_{0}^{l} C \cdot dl$$

$$\ln \frac{I_o}{I} = C \cdot l$$

$$\log \frac{I_o}{I} = C \cdot l$$

$$A(\lambda) = \log \frac{I_o}{I} = C \cdot \varepsilon(\lambda)\, l$$

ε, extinction coeff units ($M^{-1}\,cm^{-1}$, $(mg/ml)^{-1}\,cm^{-1}$)

Note that ε is a function of wavelength. $\varepsilon(\lambda)$.

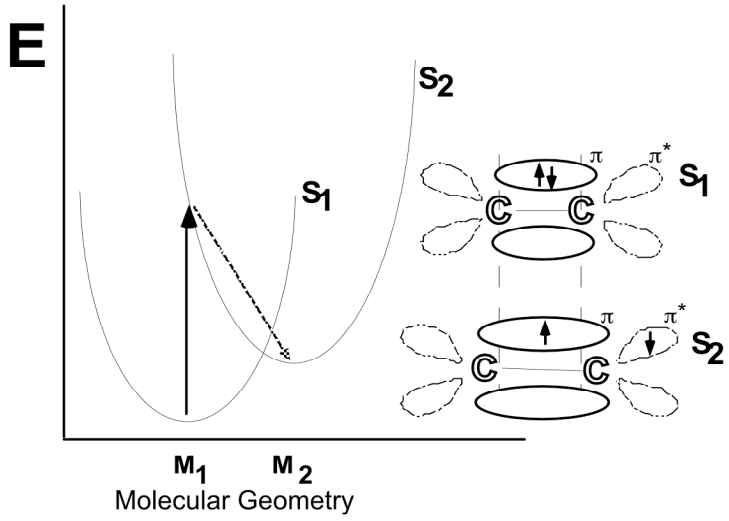
Always pay attention to units!! Always specify units, but when others don't, assume $M^{-1}\,cm^{-1}$.

Electronic transitions - geometry of states and the time scale of the transition

Idea of two states - "molecular geometry" axis

molecular geometry generally is the positions of all atoms and the spin states of the nuclei and electrons.

More typically, one aspect dominates a given transition. For example, in ethylene, the molecular geometry might be simplified to the carbon-carbon internuclear distance. For the case in which the π bond is fully occupied, there is a preferred internuclear separation (short - double bond). For the case in which one electron has been promoted to the π^* orbital, the preferred internuclear separation will be larger (longer - one and one half bond).



Frank-Condon/Born-Oppenheimer: electrons move much faster than nuclei. Nuclei do not move during an electronic transition. They *subsequently* relax to preferred position. The arrows above indicate that the molecule *first* absorbs a photon of light to promote the electron, *after* which the nuclei move to the preferred configuration.

Vibration and rotation are included in the precise definition of the molecular "state"

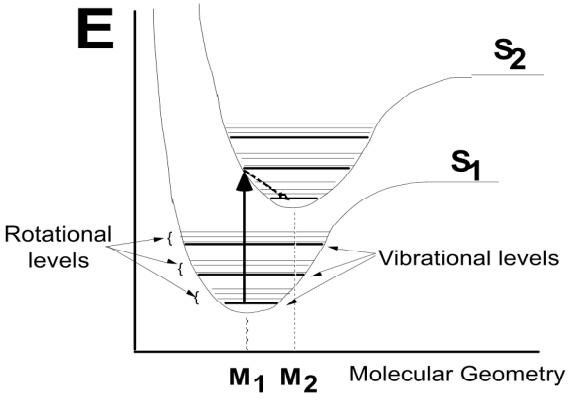
Vibrational sublevels (quantized) exist for each electronic state.

Rotational sublevels (quantized) exist for each vibrational state.

Excited vibrational states are usually not well-populated in the ground state, but transitions to excited vibrational levels within the excited electron states are likely.

Excited ground state rotational levels *are* often significantly populated, but...

Normally the linewidth of the electronic transition due to the inherent heterogeneity of the states makes resolution of vibronic and rotational levels impossible. In these cases, the sublevels are ignored and we speak of the system in electronic terms only.



Particle in a box: energy levels in delocalized systems

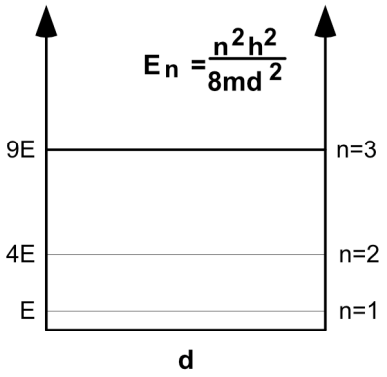
(Not in book...)

$$\mathbf{E_n = \frac{n^2h^2}{8m_ed^2}} \quad \mathbf{n=1,2,3,4...} \quad (m_e = \text{mass of the electron})$$

Note that the size of energy gap between the levels is inversely proportional to the square of the size of the box, d.

Also note to always count the total number of electrons and fill the lowest levels first. The lowest energy transition will then be from the highest occupied orbital (HOMO) to the lowest unoccupied orbital (LUMO).

Back to ethylene. Expansion of conjugated system lowers energy, as predicted.

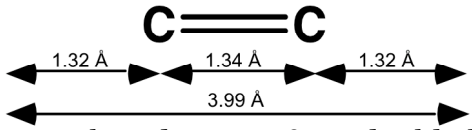


175 nm
ε =15,000 M^{-1} cm^{-1}

217 nm
ε =21,000 M^{-1} cm^{-1}

258 nm
ε =35,000 M^{-1} cm^{-1}

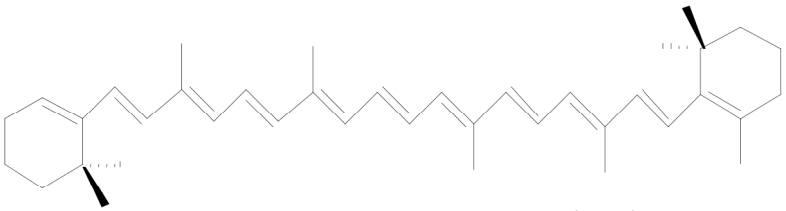
Using the equation above, we can calculate the size of the "box" for the case of ethylene. Noting that each level can contain two electrons (Pauli exclusion principle), we find that the n=1 state is fully populated and that the lowest energy transition will be from n=1 to n=2. The difference in energy, $E_2 - E_1$, can be obtained from the observed wavelength of the transition, finally yielding d = 3.99 Å.



Since we know that the carbon-carbon distance for a double bond is 1.34 Å, the box must extend 1.32 Å on either side.

distances for other boxes are 4.44 Å and 4.84 Å.

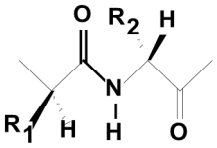
There are many examples in biology of large π systems: nucleic acid bases, porphyrins, visual and pigment chromophores, ... Building a "bigger box" is nature's way of bringing absorptions into the "visual" region of the spectrum, or rather, of bringing "vision" out from under the opaqueness of the UV region of the biological spectrum. size of box is 6.41 Å



452 nm $\varepsilon = 15.2 \times 10^4$ $M^{-1}cm^{-1}$

β-carotene (found in carrots)

The peptide bond can also be thought of as a "box." Note that there is a π system extending from the carbonyl oxygen, through its carbon, and across to nitrogen. Delocalization onto the electronegative oxygen and into its and nitrogen's lone pairs, makes the box bigger than you might first think.



Peptide Bond

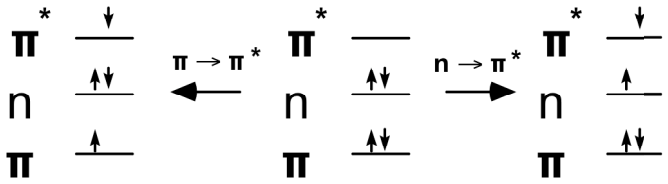
$\pi \rightarrow \pi^*$ 190 nm $\varepsilon \approx 7{,}000$ M^{-1} cm^{-1}
$n \rightarrow \pi^*$ 220 nm $\varepsilon \approx 200$ M^{-1} cm^{-1}

Note that there are two transitions in the UV region, but that one is much more allowed than the other. The absorption at 190 nm is more commonly monitored, although it is getting very close to the vacuum region of the UV where everything absorbs.

We now have a qualitative feel for transition <u>energies</u> (the wavelength of the transition). What about the <u>probability</u> of a transition?

<u>Symmetry dictates transition probabilities</u>: Formaldehyde as a simple model. (CS p. 370)

We've learned about geometrical constraints on transition <u>energies</u> (absorption wavelength). What about transition <u>probabilities</u> (absorption intensities)?

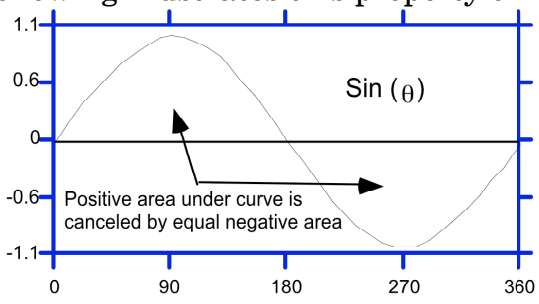


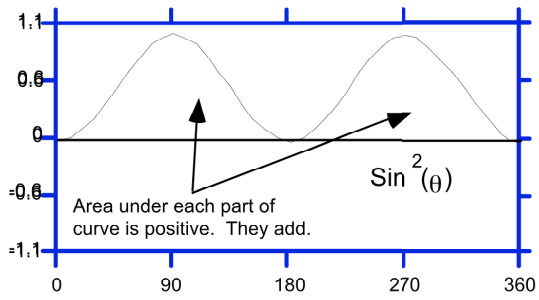
An sp^2 hybridized carbon can combine with a simple unhybridized oxygen to form π and π^* orbitals.

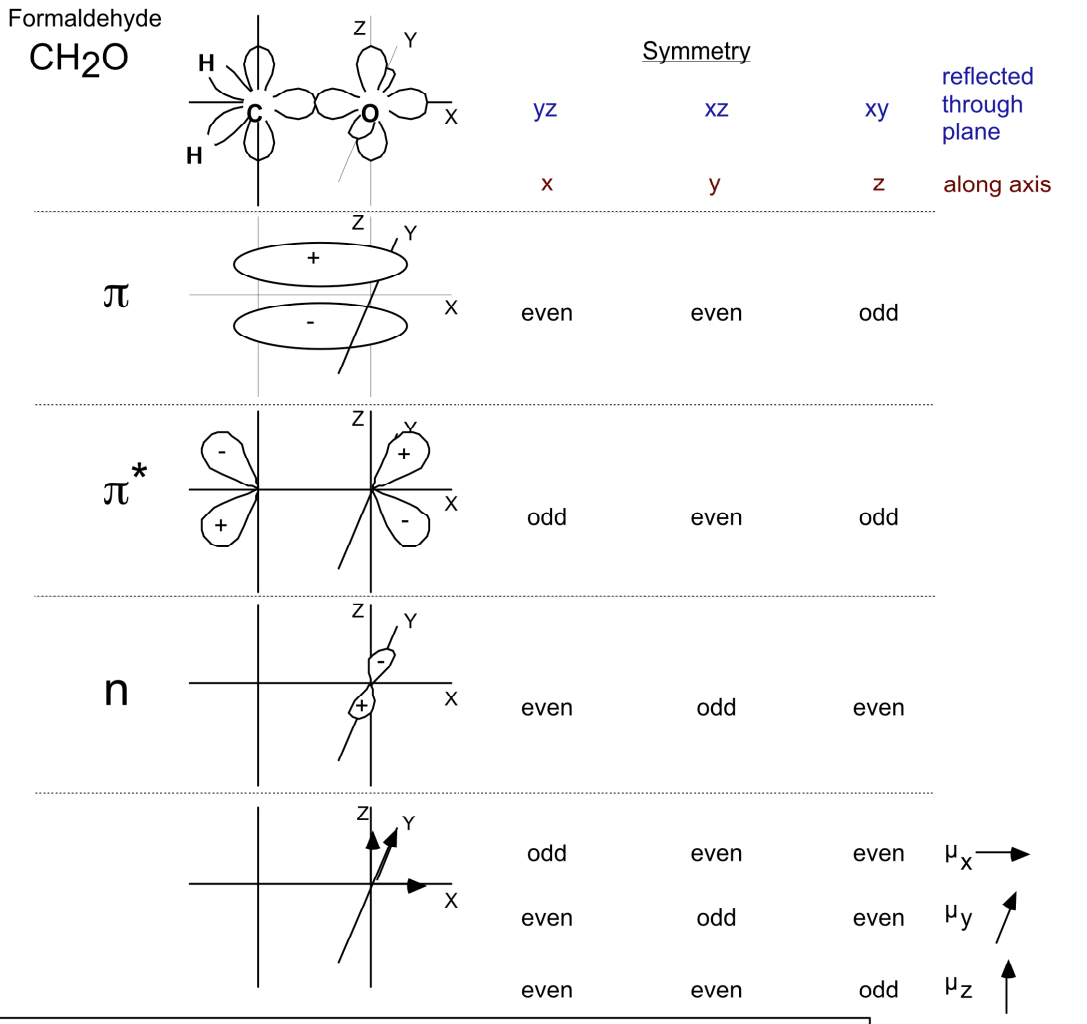
The ground state of formaldehyde can be represented as $n^2\pi^2$ (highest occupied orbitals only).

Remember that the formula for the transition probability contains the term $|\langle \psi_b | \boldsymbol{\mu} | \psi_a \rangle$, and note that this term is an <u>integral over all space</u>. If the integrand has odd symmetry, the integral will go to zero, whereas if it has even symmetry, the integral will be non-zero. We can examine the symmetry of each orbital and of each of the electric dipole operators associated with the light.

The following illustrates this property of integration:







$$\langle\psi_i|\boldsymbol{\mu}|\psi_f\rangle = \langle\psi_i|\mu_x|\psi_f\rangle + \langle\psi_i|\mu_y|\psi_f\rangle + \langle\psi_i|\mu_z|\psi_f\rangle$$

$$\iiint\psi_i\,\mu_x\,\psi_f\,\partial x\partial y\partial z + \iiint\psi_i\,\mu_y\,\psi_f\,\partial x\partial y\partial z + \iiint\psi_i\,\mu_z\,\psi_f\,\partial x\partial y\partial z$$

$\langle\pi|\boldsymbol{\mu}|\pi^*\rangle = \langle\pi|\mu_x|\pi^*\rangle + \langle\pi|\mu_y|\pi^*\rangle + \langle\pi|\mu_z|\pi^*\rangle$

$[(oeo)(eee)(eoo)]$ + 0 + 0 **non-zero**

$\neq 0$ **(allowed)**

Example:

$[(oeo)(eee)(eoo)]$

$\int(oeo)\partial x$

$\int(eee)\partial y$

$\int(eoo)\partial z$

$\langle n|\boldsymbol{\mu}|\pi^*\rangle = \langle n|\mu_x|\pi^*\rangle + \langle n|\mu_y|\pi^*\rangle + \langle n|\mu_z|\pi^*\rangle$

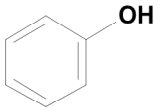
$[(eoo)(oee)(eeo)]$ + 0 + 0

$= 0$ **(forbidden)**

Aromatic groups can be more complicated, but nevertheless, symmetry is often very important. Extinction coefficient depends on symmetry. Benzene is "too symmetrical." A dipole does not exist and is not easily induced. Consequently the transition probability (and therefore the extinction coefficient) is very small.

Benzene

256 nm ε = 400 M^{-1} cm^{-1}

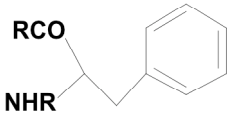


Phenol

271 nm ε = 2,000 M^{-1} cm^{-1}

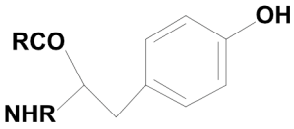
Introduction of the hydroxyl group in phenol, breaks the symmetry in one plane, producing a dipole in the molecule. The extinction goes up dramatically.

The amino acids phenylalanine and tyrosine are analogous to benzene and phenol. The effects of symmetry are similarly illustrated.



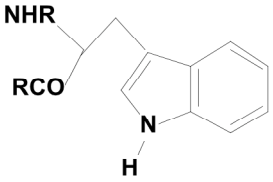
Phenylalanine

250 nm ε = 200 M^{-1} cm^{-1}



Tyrosine

274 nm ε = 1,400 M^{-1} cm^{-1}



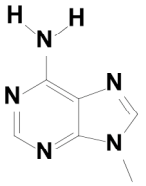
Tryptophan

3 transitions 240-290 nm

ε_{280} = 5,700 M^{-1} cm^{-1}

Tryptophan is more complex. In particular, there are three transitions in the region of the UV spectrum from 240 to 290, each with its own characteristic extinction. The net absorption "band" represents the sum of these transitions. The quoted extinction coefficient does not reflect one single transition.

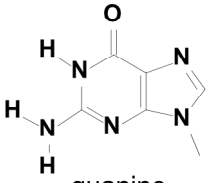
Nucleic acids are similarly "big boxes." The spectra of several nucleotides actually show the composite nature of the absorption band, as shoulders on the main envelope can be seen. The sugar and phosphate components have negligible effect, but base-base interactions can have significant effects (we'll see later).



adenine

259 nm

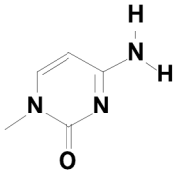
ε = 15,400 M^{-1} cm^{-1}



guanine

253 nm

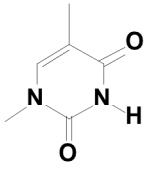
ε = 13,700 M^{-1} cm^{-1}



cytidine

271 nm

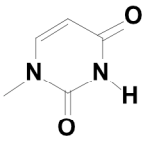
ε = 9,000 M^{-1} cm^{-1}



thymidine

267 nm

ε = 9,600 M^{-1} cm^{-1}



uracil

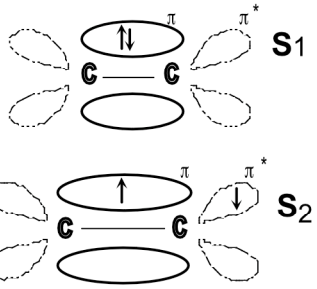
262 nm

ε = 10,000 M^{-1} cm^{-1}

Structure from spectroscopy - whole not the sum of the parts.

Solvent effects

If you know something about the nature of a transition, you can often predict the effect of solvent on the absorption properties of a chromophore. In general, solvent interactions stabilize both the excited and the ground states. If this effect is equal for the two, the energy of the transition will not change.

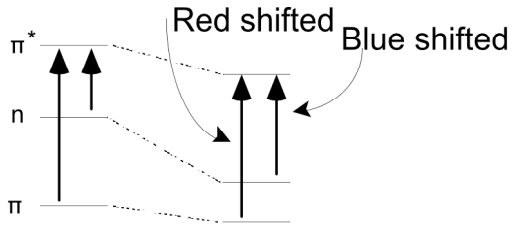


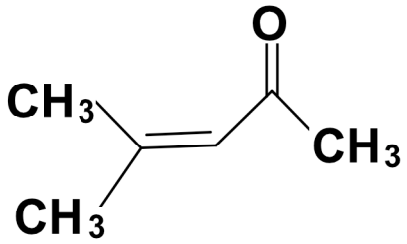
Solvent polarizability is important. If a particular electronic state is characterized by a large dipole moment, then a highly polarizable solvent will stabilize that state more than a less polarizable solvent. **N.B.**

Note in the structures at right for ethylene: the π^* orbital is more diffuse than the π orbital. Consequently it will have a larger inducible dipole moment. The π^* orbital will be stabilized more than the π orbital by interaction with a polarizable solvent.

Example: mesityl oxide (CS p. 387).

Non-polar **Polar**





Solvent	$\pi \rightarrow \pi^*$ λ_{max}	$n \rightarrow \pi^*$ λ_{max} (nm)
Hexane	230	327
Ethanol	237	315
Water	245	305

Note: the textbook is wrong!!!

As for formaldehyde, 3 energy levels dominate the low energy transitions, yielding $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. Look at solvent polarizability.

The electron in the π orbital is localized primarily between the involved nuclei.

In contrast, the π^* orbital is more diffuse and extends well beyond the central nuclei, consequently one expects the π^* orbital to be influenced much more by solvent than the π orbital. In particular, a more polar solvent should stabilize the dipole in the π^* orbital much more than it stabilizes the π orbital - this leads to a reduction in their energy separation so that the $\pi \rightarrow \pi^*$ absorption will shift to lower energy (longer wavelengths - to the red) in a more polar solvent.

An electron in a non-bonding orbital (for the simplest case, a lone pair) is readily available for interactions with solvent (*e.g.* as a H-bond acceptor). Therefore we expect the shift to be even larger for the $n \rightarrow \pi^*$, however in this case the ground state is stabilized relative to the excited state, so that the difference in energy between the two states will *increase* (the transition will move to lower wavelength - a blue shift).

Solvent perturbation spectroscopy.

In limited cases, one can monitor exposed surface amino acids by their response to changing solvent. Amino acids such as tyrosine and tryptophan which are exposed to solvent will show well characterized shifts as the polarity of the solvent is reduced (by addition of alcohol for example). In contrast, buried residues may be relatively insulated from changes in the solvent. In ideal cases, this can be used to determine the number of each type of aromatic residue exposed to solvent.

More interestingly, if a protein undergoes a major conformational reorganization, previously buried groups may become exposed to solvent and vice versa. This will be observed as an abrupt change in the spectrum accompanying the conformational change.

An extreme example of this is the change in the exposure of buried aromatic residues accompanying the unfolding of a native protein structure. In fact, this approach is used in many studies of thermal denaturation (although calorimetry provides more information).

The environment in a protein can vary dramatically.

The local environment that a given electron sees is very dependent on the environment and is usually quite *anisotropic* (not homogeneous). By this we mean that polarizability and the distribution of dipoles is not uniformly distributed around a point in space, as in solution. One end of an electronic orbital may be situated near a carbonyl oxygen from the peptide bond, while the other end is in close proximity to a hydrophobic methyl group from alanine. Thus it is very difficult to predict quantitatively the energy levels within a protein from studies of solvent shifts.

Protonation state can significantly effect absorption maxima, extinction coefficients.

Tyrosine has a pK_a of 10.9. Deprotonation leads to a significant shift in the absorption maximum towards the red. This can be exploited to titrate Tyr residues in a protein.

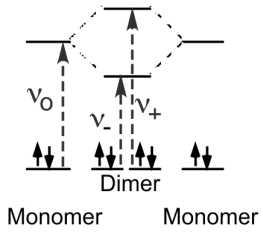
For nucleic acid bases, protonation of C or G leads to large red shifts. Deprotonation of U or T also results in a large red shift. Protonation of A leads to a small change.

Interactions *between* chromophores - the *exciton* effect.

For a theoretical description, see CS 390-395.

What happens when we have two identical chromophores? For two non-interacting monomeric chromophores, the result is intuitively obvious - the resulting spectrum is that of the monomer, but with twice the intensity.

For two *interacting* monomers, we again note that in general the ground states of a molecule are less polar than the excited states. Consequently excited states of molecules can interact with each other through dipolar interactions, in the same way that the excited state of a monomer interacts with polar solvent. The result is that the energies of the ground states for the monomers are effected much less by their interaction than are the energies of their excited states. Combining the excited states in much the same way as one combines atomic orbitals to form molecular orbitals, we see that the two resulting excited states are equally higher and lower in energy than the excited state of the non-interacting monomer. In equation form:



$\nu_+ = \nu_o + V_{12}$ $\nu_- = \nu_o - V_{12}$ $2V_{12}$ is called the exciton splitting.

The interaction energy V_{12} depends on various factors, but to a first approximation falls of as R_{12}^3 (that is, it falls off rapidly with distance).

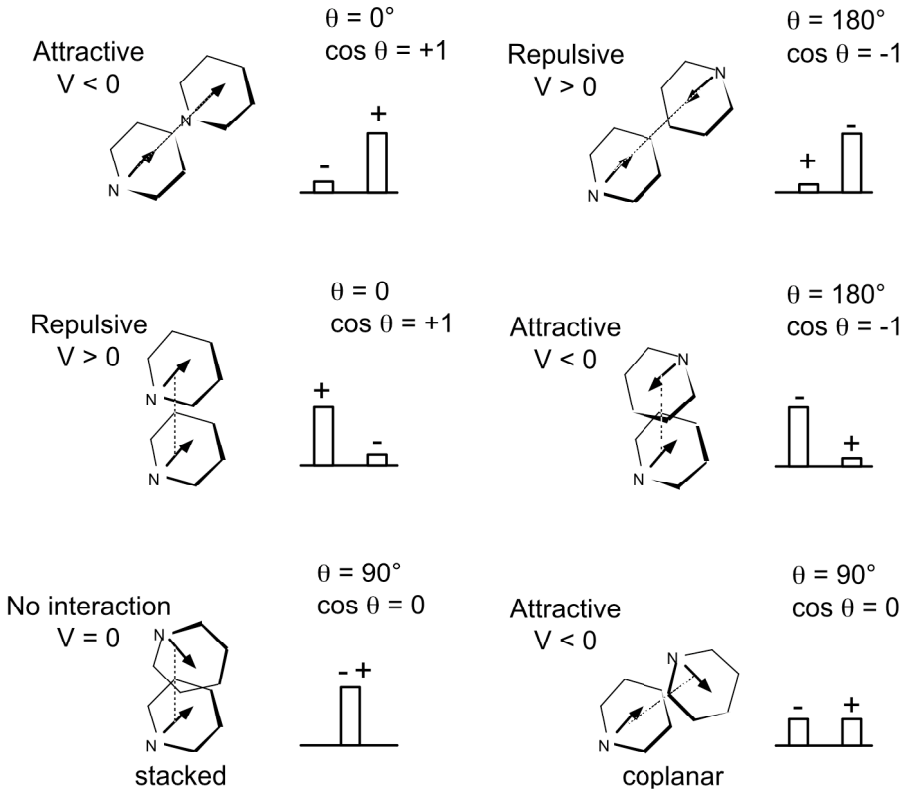
Similarly, it can be shown that the relative intensity of each transition is split about the original intensity (*i.e.* $I_+ + I_- = 2I_o$), but their relative intensities depend on the angle between the two transition dipoles, θ

$D_\pm = D_o \pm D_o \cos\theta$ See page 396 in CS for a description of this effect.

Interactions between identical dimers.

The spectrum of two *identical* and *non-interacting* chromophores is simply the spectrum of one, but with twice the intensity.

We saw above that the ground and excited states of different molecules can interact with each other. What happens when two identical chromophores interact? One important feature of such an interaction can be seen when we consider the transition dipole vector of each chromophore. The interaction has a strong angular dependence (the dipole-dipole interaction).



The intensities and the splittings depend on the angle ; the intensity of the resulting band is proportional to the magnitude of the vector resulting from the addition (or subtraction) of the contributing transition dipole vectors.
So we see that chromophores can interact positively or negatively, resulting in split transitions, not necessarily of the same intensity.

Extension to systems of more than two chromophores.

By analogy, we see that 3 interacting monomers will give rise to 3 absorption bands, and so on for larger groups of interactions. What happens for a very long polymer? For a regular structure such as an -helix, symmetry considerations require that almost all of the bands will have zero intensity. Only two allowed π π^* transitions remain: 1 parallel to the helix axis and 1 perpendicular.

Example: (CS p. 369) oriented poly-L-glutamic acid film. The molecule forms long -helices which align parallel to one another. Measurement of absorption spectra using light polarized parallel or perpendicular to the helix axis shows distinctly different absorption maxima and extinction coefficients.

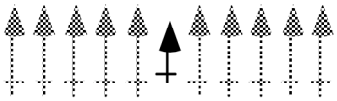
Hypochromism (*less color*): interaction between *different* transitions in different molecules.

Simplistically, for a long polymer of interacting monomers, the overall integrated absorption intensity should be the sum of that of the individual monomers. This is often not true, as displayed in DNA. The intact double helix absorbs 30% less than a mixture of the same monomers. The best explanation of the effect, by Tinoco and Rhodes, is (according to CS) "not simple."

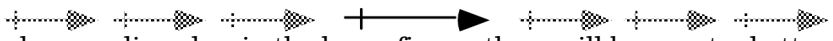
So far, when looking at monomer-monomer interactions, we have considered interactions between two identical transitions within each monomer. But a transition in one monomer can be influenced by ***other*** transitions in its neighbor.

Very simplistically, we can see the effect as the effects of induced dipoles. Remember that an electric field will induce a dipole in each orbital's electronic clouds. If such dipoles are all aligned parallel to each other as shown in the first case below, there will be a mutual repulsion. This makes the induction of each dipole more difficult.

Hypochromism



Hyperchromism



Similarly, if the dipoles are aligned as in the lower figure, there will be a mutual attraction, making the induction of dipoles easier. We can now see that in the first case, the transition probability will be reduced (extinction coefficient smaller - hypo (under)), while in the second case it will be increased (extinction coefficient larger - hyper (over)),.

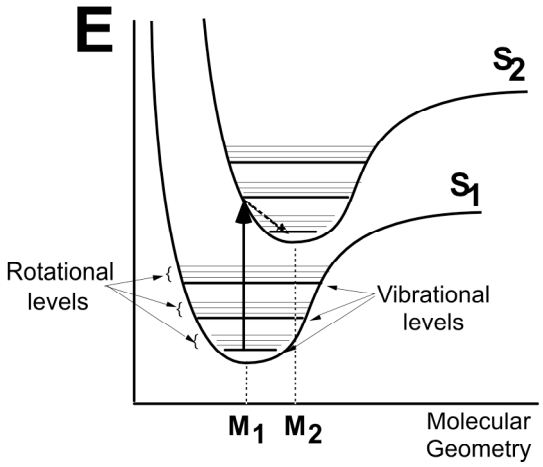
Since this is a dipole-dipole interaction, the strength of the interaction goes as $1/r^3$. The chromophores must be relatively close in space. As we saw above, the angle of the chromophores' transition dipoles is also critical. It also requires a polarizability in both molecules.

The former case (hypochromism) occurs for duplexes of DNA, hence we observe a hypochromic decrease in the extinction coefficient for duplex DNA vs. melted DNA (or the component free nucleotides).

Fluorescence and Phosphorescence Spectroscopies

Introduction - emissive transitions

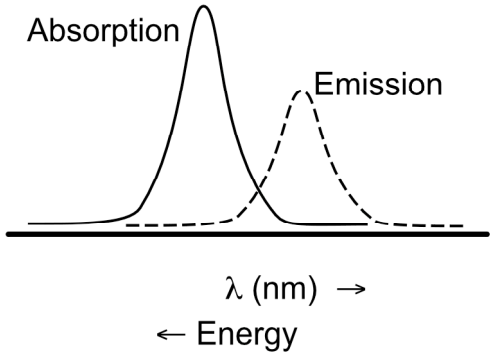
Review: We saw before that when a molecule in the ground vibronic level of the ground state is influenced by light of the appropriate energy, transitions are induced between that state and higher lying excited electronic states. We also saw that during the time course of the electronic excitation, nuclei don't move appreciably. Consequently excitation is often to excited vibronic and rotational levels within the molecule.



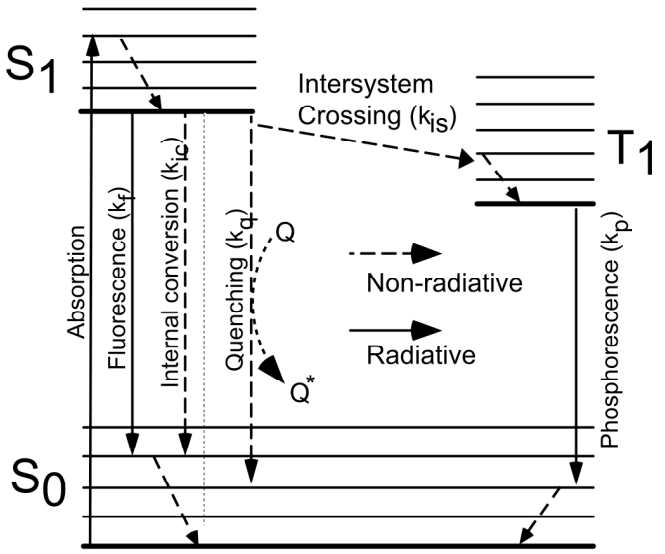
Non-radiative and radiative processes

The excitation from S_o to S_1 is a radiative absorption. Radiative: involving light. Subsequent to this excitation, the molecule relaxes to the ground vibronic (and rotational) sublevels within the excited electronic state. This process is *non-radiative*, that is, it is not associated with light energy, but rather represents a transfer of thermal energy to the surroundings. For example, a diatomic molecule in an excited vibrational state may collide with a solvent molecule and transfer that vibrational energy to the solvent molecule.

The resulting molecule, in its ground vibronic sublevel within the excited electronic level, may now emit a photon of light and *spontaneously* revert to the ground electronic state S_o (**fluorescence** - a radiative emission process). But again, the electronic transition is faster than the movement of nuclei, so that it relaxes to an excited vibronic sublevel of the ground electronic state. Consequently, light emitted in fluorescence is always of lower energy than that originally absorbed.



Note that there exist pathways for *non-radiative* transitions between electronic energy levels. It is due to these processes that we can rarely excite enough molecules between states (with illumination at the transition energy) that we equalize the populations in the two electronic states. In fact, with most spectroscopies, the populations of the individual levels remain very close to their Boltzmann distributions, despite our induction of transitions between states. Non-radiative pathways predominate over radiative ones.



Another non-radiative process which can occur is called inter-system crossing. In this process, the singlet excited state is converted into a triplet. Remember that during the initial absorption transition, we took an electron from a fully occupied and therefore spin-paired level (a singlet level). During that transition the spin does not change, so that the resulting excited state is still a singlet. However, in this excited state, the two electrons are in singly occupied orbitals. We know that the triplet state will always be lower in energy (with both spins aligned, we say they are more correlated and stay away from each other better - see an introductory quantum mechanics text). Such a transition, however, is formally forbidden (remember that there must be a mechanism to induce the transition), so that the rate of intersystem crossing is generally very slow. For systems that do make this transition, the relaxation back to the original singlet ground state is also formally forbidden, so that the lifetime of the triplet state can be much longer than that of the corresponding excited singlet state. The radiative transition back to the singlet ground state is called **phosphorescence**. In the case that non-radiative pathways between electronic states (internal conversion and intersystem crossing) are slow relative to the rate of spontaneous emission, fluorescence and/or phosphorescence can be observed.

Radiative processes summary:

absorption - as discussed previously

fluorescence - transition back to the ground electronic state, with emission of a photon of the corresponding energy.

phosphorescence - transition from an excited **triplet** back down to the ground state **singlet**, with emission of a photon of the corresponding energy. This transition is formally forbidden, so that for most systems the rate of this transition is very low - the triplet state once generated has a much longer life time in general than does the excited singlet state.

Non-radiative processes summary:

vibrational relaxation (v.r.): transitions with the vibronic sublevels of an electronic state. The transition energies are usually near kT for room temperature. The rate of such transitions is generally very high ($t_{1/2} = 10^{-12}$ sec).

internal conversion: occurring at a rate k_{ic}. The associated energy released is transmitted to the environment via non-radiative energy transfer, such as collision with solvent or with other molecules. In general k_{ic} will increase with temperature (at the expense of other transitions, such as fluorescence and phosphorescence).

intersystem crossing: in this case a formally *forbidden* "spin flip" converts the system from a singlet state (all electrons paired) to a triplet (a system with a net electron spin of 1). Note that direct excitation from the ground state singlet to the excited state triplet is strongly forbidden.

quenching: like internal conversion, this processes often derives from processes such as molecular collisions (or chemical reaction), but in this case the energy transfer is large enough to lead to transitions between *electronic* states of the molecule. See below for more.

Excited Electronic State Lifetimes

Singlet excited state (S_1): Generally the rate of spontaneous emission from this state is fast. Consequently, the lifetime of this state is very short (typically $t_{1/2} = 10^{-12}$ sec).

Triplet excited state (T_1): Since the transitions back down to the singlet ground state are formally forbidden, the lifetime of this state is significantly longer than rates for transitions from singlet to singlet).

Quantum yield

Each process of "deexcitation" has associated with it a rate constant. The relative yield of each pathway depends critically on the rates of each of these processes. The quantum yield for a particular pathway is defined as the ratio of the rate of that pathway to the sum of the rates for all pathways from that state (or, alternatively, the denominator can be expressed as the rate of excitation to that state).

$$\Phi_i = \frac{k_i}{n} = \frac{k_i}{k_{all\ deexcitation}} = \frac{k_i}{k_{excitation}}$$

$$\sum_{j=1} k_j$$

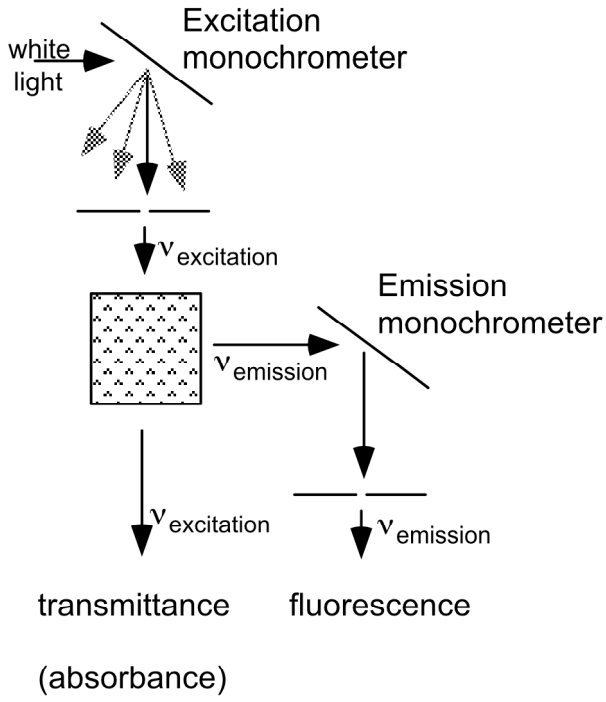
In other words, the quantum yield for fluorescence is the ratio of the photons emitted in fluorescence to the photons absorbed by the original transition.

$$\Phi_F = \frac{k_F}{k_F + k_{ic} + k_{is} + k_q[Q]} = \frac{\text{deexcitation via fluorescence}}{\text{all deexcitation}} = \frac{\text{photons emitted}}{\text{photons absorbed}}$$

It is the latter description of quantum yield that is typically measured experimentally.

The fluorescence experiment

Experimentally, fluorescence is a **very sensitive** technique. In absorption, we detect the light which passes through the sample. In a very dilute solution, we may have 0.01% of the light absorbed, such that the light that passes through may represent 99.99% of the incident light. Consequently, the absorption measurement is a very small difference of two much larger numbers. In fluorescence, we directly detect the emitted photons in the absence of any other light. This is a much more sensitive measurement. In the fluorescence experiment we can record both the absorption spectrum and the emission spectrum. In both cases, we measure the emitted light.



1) **Fluorescence excitation**. In the first case, we set the emission monochrometer to the wavelength of the fluorescence emission maximum and scan the excitation monochrometer.

2) **Fluorescence emission**. In the second case, we excite with only light corresponding to the maximum excitation, and then we scan the emission monochrometer.

In practice, for an unknown sample, one must iterate back and forth between these approaches to determine the maxima for both excitation and emission.

Within an electronic transition, the largest signal will generally be lowest in energy and will correspond to transitions from the lowest vibronic level of the ground state (excitation) to the lowest vibronic level of the excited electronic state.

Internal Conversion

In internal conversion, a molecule in an excited vibrational state can collide with a solvent molecule, transferring its energy to the solvent molecule and reverting to a lower energy vibronic level. The solvent molecule gains kinetic energy (it is "heated").

Quenching via Chemistry

The energy difference between the ground and excited states (the excitation energy) can be used to perform chemistry. A second molecule can interact with the excited state of the absorber, so as to allow relaxation of the absorber coupled to the input energy into a chemical reaction involving the quencher. This type of reaction will generally show a

dependence on the concentration of the quenching molecule (as well as the absorber). Studying the concentration dependence of the quenching can provide support for and further information on the quenching mechanism.

A variation of quenching is direct chemistry involving the absorber. The excited state of a molecule is chemically different, often being characterized by weakened bond character. As an example, in ethylene the $\pi \rightarrow \pi^*$ transition takes a system with a double bond between carbon, and converts it to a system with a net single bond between carbons. In more complex systems, if other stresses pre-exist which strain that single bond, the reduction in bonding associated with the $\pi \rightarrow \pi^*$ transition might lead to bond cleavage.

It is this mechanism which is usually exploited in "photo-bleaching." The product of the chemical reaction no longer has the absorption properties of the original "reactant." We will mention this later.

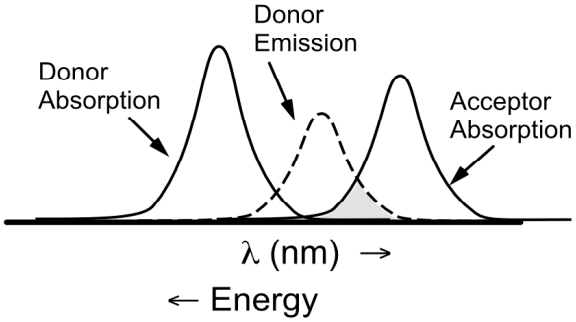
Transfer of excitation energy

Finally, a molecule in an excited electronic state can transfer its excitation energy to another molecule. In the process, the donor molecule relaxes from the its excited to its ground electronic state, while the acceptor molecule is excited from the ground to its excited electronic state. We can write the efficiency of transfer as

$$E = \frac{k_T}{k_T + k_F + k_{ic} + k_{is}}$$ where k_F, k_{ic}, and k_{is} are properties of the donor.

For appreciable transfer of energy, two requirements hold:

1) A mechanism for transfer must exist.
   Typically, a dipole-dipole interaction can lead to a coupled transition between electronic states. The dipole of the donor's excited state can interact with the ground state of the absorber in a manner similar to the interaction of light's electric field with the ground state of the absorber. In the quenching pathway of energy transfer, the donor is de-excited to the lower electronic state, while the acceptor is excited to its higher excited electronic state.
2) The involved energies must match (as for light (**E**) induced transitions).
   Re-stated: there must be an appreciable overlap of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor.



A quantitative theory for such energy transfer has been developed by T. Förster (see CS 451-453). In this theory, the rate of energy transfer is given by

$$k_T = \frac{1}{\tau_D}\left(\frac{R_o}{R}\right)^6 \qquad \tau_D = \frac{1}{k_F + k_{ic} + k_{is}}$$

NOTE: Cantor & Schimmel is ***wrong*** for k_T!!

where τ_D is the lifetime of the donor in the absence of the acceptor, R is the distance between donor and acceptor.

R_o is a factor called the "characteristic transfer distance." It contains contributions from

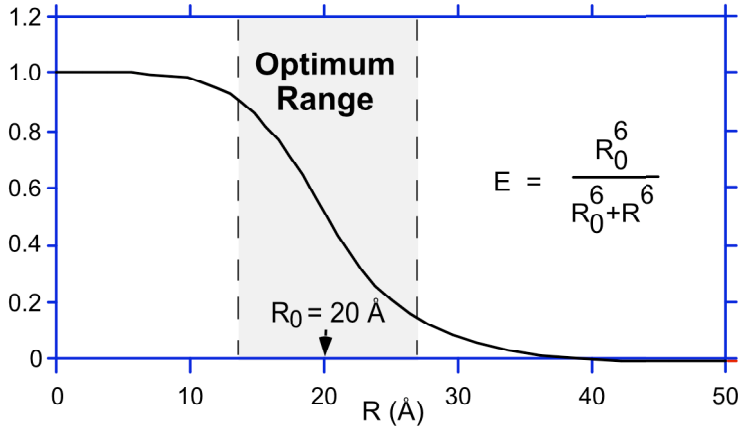
1) the spectral overlap between donor and acceptor (energies match)
2) the refractive index of the medium between the two
3) the orientation of the two with respect to each other - dipoles must be able to interact with each other (this component can average out for rapidly tumbling molecules).

Note that the distance, R, comes into this equation as the inverse of the 6th power. This is due to the fact that coupling mechanism is a dipole-dipole interaction. The leading term in the dipole-dipole interaction is dependent on R^3, and this term is squared in computing the transition probability.

If we rewrite the efficiency of energy transfer as $E = \frac{k_T}{k_T + 1/\tau_D}$

then substituting for k_T from before:

and substitute in for k_T, then we have $E = \frac{R_0^6}{R_0^6+R^6}$



It can be seen that this is most sensitive for distances R near R_o. Values of R_o typically range from 10 Å to >50 Å. Distances between chromophores of as much as 80 Å between chromophores have been measured.

More thorough explanation:

The transition we are looking for is $\psi_{D0}\psi_{A1}$ $\psi_{D1}\psi_{A0}$ (where 0 refers to ground state and 1 to the excited state) and arises through a dipolar interaction between donor and acceptor. The probability of this transition is proportional to

$$Rate \propto \left| \left\langle \psi_{D_0}\psi_{A_1} \left| \frac{\vec{\mu}_D \cdot \vec{\mu}_A}{R^3} - \frac{3\left(\vec{\mu}_D \cdot \tilde{R}\right)\left(\tilde{R} \cdot \vec{\mu}_A\right)}{R^5} \right| \psi_{D_1}\psi_{A_0} \right\rangle \right|^2$$

Assume that one can separate out the angular dependence of the dipolar term as below:

$$\frac{\vec{\mu}_D \cdot \vec{\mu}_A}{R^3} - \frac{3\left(\vec{\mu}_D \cdot \tilde{R}\right)\left(\tilde{R} \cdot \vec{\mu}_A\right)}{R^5} = \frac{(\cos\theta_{DA})\mu_D\mu_A}{R^3} - \frac{3(\cos\theta_D)(\mu_D R)(\cos\theta_A)(R\mu_A)}{R^5}$$

$$= (\cos\theta_{DA})\frac{\mu_D\mu_A}{R^3} - 3(\cos\theta_D)(\cos\theta_A)\frac{(\mu_D R)(R\mu_A)}{R^5}$$

$$= \left[\cos\theta_{DA} - 3\cos\theta_D\cos\theta_A\right]\frac{\mu_D\mu_A}{R^3} = \alpha\frac{\mu_D\mu_A}{R^3}$$

$$Rate \propto \left| \left\langle \psi_{D_0}\psi_{A_1} \left| \alpha\frac{\mu_D\mu_A}{R^3} \right| \psi_{D_1}\psi_{A_0} \right\rangle \right|^2 = \frac{\alpha}{R^6}\left| \left\langle \psi_{D_0}\psi_{A_1} \left| \mu_D\mu_A \right| \psi_{D_1}\psi_{A_0} \right\rangle \right|^2$$

$$= \frac{\alpha}{R^6}\left| \left\langle \psi_{D_0} \left| \mu_D \right| \psi_{D_1} \right\rangle \right|^2 \left| \left\langle \psi_{A_1} \left| \mu_A \right| \psi_{A_0} \right\rangle \right|^2$$

Caveats... Note that in a complicated system such as biology often throws at us, there may be multiple chromophores present. If the absorption maximum of one is close to the emission maximum of the other, the former can absorb the light emitted by the latter. This is normally a problem only when the absorber is present at high concentrations and/or has a very large extinction coefficient. This is a <u>fundamentally different</u> process than that seen above, since it involves no direct interaction between the two molecules. In this case the fluorophore emits photons independent of the acceptor. The absorbing molecule may, of course, not fluoresce. This latter situation is rare.

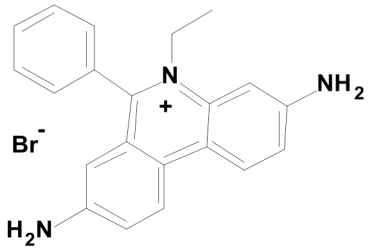
If we examine the experimental design for fluorescence spectroscopy presented above, we can see that if the fluorescent molecule absorbs light VERY strongly, then a large percentage of the excitation light may be absorbed in that part of the cuvette very near

the source. If your fluorescence detection is focused towards the center of the cuvette, you may be looking at molecules which are in the "shadow" of the molecules near the source. This is called the "inner filter effect." One should always check the absorption spectrum of the sample to see that absorption is not too large.

Fluorescence and the Environment

Just as we saw in absorbance, the environment of a fluorophore can have dramatic effects on the electronic states of the molecule, and hence on its fluorescence properties. Stabilizing and destabilizing interactions with excited and ground state wavefunctions can alter not only the energies of the transitions, but also the rates of each of the radiative and non-radiative processes. We have seen, for example, how neighboring groups can quench or transfer fluorescence through a dipole-dipole mechanism. Although the absorption process for any given moleculre is complete within 10^{-15} sec (remember to distinguish this from the rate at which photons are absorbed in a *bulk* solution, probability), there is often an appreciable delay between absorption and fluorescence - generally 10^{-9} to 10^{-8} sec. During this time, other process can occur: collisional quenching as we've just seen, protonation/deprotonation, local conformational changes, etc.

Many fluorescent molecules, such as ethidium bromide (EtBr), undergo rapid solvent quenching of their excited singlet states. Consequently, EtBr in water does not have a large quantum yield for fluorescence in solution. However, when it intercalates into DNA, it not only interacts with the DNA base π systems, but also is relatively protected from solvent. The result is that the fluorescence quantum yield goes up significantly. This is the basis of EtBr staining of duplex DNA in gels.



Fluorescent probes: many of the common "Bio-Absorbers" are only weakly fluorescent if at all (see p. 443 CS). This can work to our advantage. In many cases, one can introduce a fluorescent probe into one's system, for example bound near the active site of an enzyme.

Time-dependent measurement of fluorescence.

For the processes discussed above, we can write:

$$\tau_R = \frac{1}{k_F} \qquad \tau_F = \frac{1}{k_F + k_{ic} + k_{is} + k_q(Q)} \qquad \phi_F = \frac{\tau_F}{\tau_R}$$

Where τ_R is termed the "radiative lifetime" and τ_F the "fluorescence lifetime." Note that $\tau_F \leq \tau_R$. The radiative lifetime is related simply to the probability of a molecule in the excited state emitting a photon. The fluorescence lifetime represents the lifetime of the excited state as a result of *all* de-excitation processes (radiative and nonradiative).

So far we have discussed only "steady-state" measurements of fluorescence. Modern spectroscopic techniques now allow us to look at fluorescence in real time. We can, for example, excite a chromophore with a very short (1 nsec), intense pulse of light and then "watch" the fluorescence decay slowly away as the excited state becomes depopulated. If we call $S_b(t)$ the concentration of excited state singlets, we can write:

$$\frac{-d(S_b)}{dt} = [k_F + k_{ic} + k_{is} + k_q(Q))]\,(S_b) = \frac{1}{\tau_F}(S_b) \quad \text{(compare with V = k [C])}$$

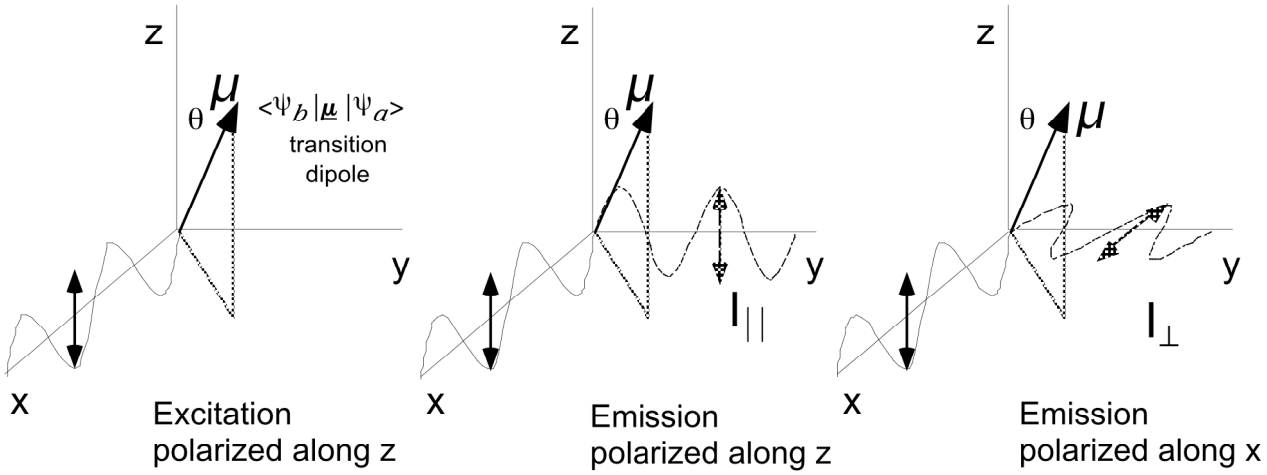
$$S_b(t) = S_b(0)\, e^{-t/\tau_F} \quad \text{(integrated as for 1st order kinetics)}$$

$$I(t) \quad k_F\, S_b(t) = k_F\, S_b(0) e^{-t/\tau_F}$$

So that, as expected, the decay follows a simple exponential. One distinct advantage of time-resolved fluorescence spectroscopy is that multiple chromophores can often be distinguished. Two fluorophores with different values of τ_F will produce a decay curve

consisting of two exponentials. Curve fitting can resolve these components. This would not be possible in a steady-state measurement.

Fluorescence Anisotropy



We saw before that molecules can have very different transition probabilities in different directions. The electric field component of the light must be able to induce dipole changes in the electronic orbital being excited.

If we illuminate a molecule with light polarized along the z-axis (the **E** vector oscillating along the z-axis), the probability of excitation is a function of $|\boldsymbol{\mu} \bullet \mathbf{E}|^2$ and so is proportional to $\cos^2\theta$, as shown above. Consequently, only a fraction of the molecules in a randomly oriented solution or powder will be excited - selective excitation. If the sample is rigid, then the emitted fluorescence will come from this sub-population and if the fluorescence is associated with the same transition as the original excitation, then the emitted light will retain this same polarization distribution. Emitted light which retains the original polarization is denoted by $\mathbf{I}_{||}$ (fluorescence intensity parallel to the excitation polarization). No fluorescence will be observed perpendicular, $\mathbf{I}_{\perp}$, to the excitation polarization.

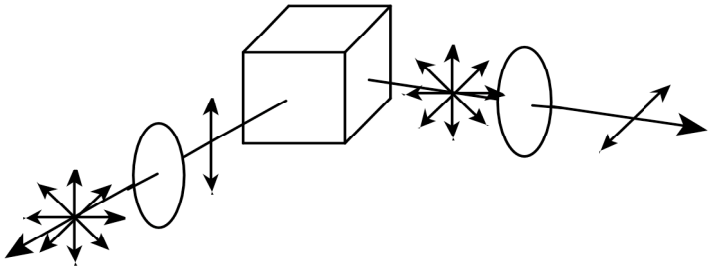
If the lifetime of the excited state is long enough, the molecule will have randomly re-oriented before subsequent fluorescence. The emitted light will have all directions of polarization. The fluorescence intensity will be equal in the parallel ($I_{||}$) and in the perpendicular ($I_{\perp}$) directions.

If re-orientation is comparable to the excited state lifetime, we can define the fluorescence anisotropy as

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}}$$

where again, $I_{||}$ is the intensity of the emitted light observed parallel to the polarization of the excitation light and $I_{\perp}$ is the intensity observed perpendicular. Examining our two extremes, if reorientation is complete before significant fluorescence, then $I_{||} = I_{\perp}$ and A = 0. If reorientation of the molecule is infinitely slow, then $I_{\perp} = 0$ and A = 1.

Experimentally, we typically start with non-polarized light and pass it through a polarizing filter. To detect light with a particular polarization component, we use a second filter in front of the detector. By rotating these filters with respect to each other, we can determine $I_{||}$ and $I_{\perp}$.



We have assumed that the orientations of the transition moments in the ground and excited electronic states coincide. This is not necessarily the case, such that even in the complete absence of reorientation, a component of the emitted light will be perpendicular to the excitation polarization. In this case, the anisotropy will have a maximum value (A) less than 1. This depolarization is called the *intrinsic* polarization. In proteins, there are two types of motions to consider.

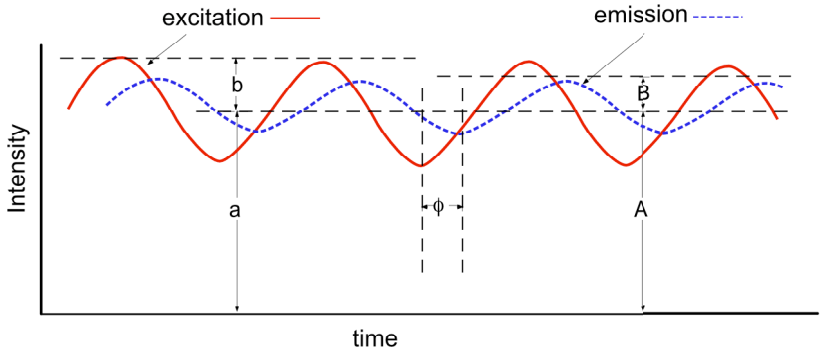
For a fluorophore completely immobilized within a protein, the rotation measured will be the overall rotation of the protein itself.

Rarely is a fluorophore *completely* immobilized within a protein. A tryptophan residue near the surface of the protein will be relatively free to rotate about its axis, even if the rotation of the protein to which it is attached is quite slow. Fluorescence will reflect this local mobility.

Phase Modulated Fluorescence - An alternative to time domain fluorescence decay

The obvious way to measure fluorescence lifetimes is to excited with a brief flash and then watch (in time) the decay of the fluorescence. An alternative, and very useful, approach is a steady state approach which exploits the delay in fluorescence following excitation. If the excitation light is modulated in a sinusoidal fashion, the fluorescence response will reflect this modulation, but as a result of the delay, will be phase shifted (ϕ) relative to the modulation of the excitation.

The plot below shows an overlay of the excitation and emission profiles:



The phase lifetime is related simply to the phase shift by: $\tan\phi = \omega\tau_p$

where ω is the frequency of the modulation of the excitation.

Remembering that not all excited states deexcite via fluorescence (quantum yield < 1), the intensity of the fluorescence emission will be less than the intensity of the light absorbed. Noting that the modulation of the excitation is not complete, we can define the demodulation factor: $m = \frac{\text{fractional modulation of emission}}{\text{fractional modulation of excitation}} = \frac{(B/A)}{(b/a)} = \frac{Ba}{bA}$

with terms as described in the figure above.

The modulation lifetime is related to the demodulation factor by: $m = \frac{1}{(1+\omega^2\tau_m^2)^{1/2}}$

For a simple single exponential decay, $\tau = \tau_p = \tau_m$

As an aside, let's derive the above relationships for a single exponential decay:

We can write the time dependent intensity of the excitation light as: $f(t) = a + b\sin(\omega t)$

And note that the fluorescence response will be similar: $N(t) = A + B\sin(\omega t - \phi)$

The time derivative of the response is then: $\frac{\partial N(t)}{\partial t} = B\omega\cos(\omega t - \phi)$

We know that for an instantaneous excitation of a fluorophore, the rate of decay of the excited state population (and therefore of the fluorescence) is a simple exponential with rate constant $k = k_f + k_{ic}$.

$$\frac{\partial N(t)}{\partial t} = -kN(t) = -\frac{1}{\tau}N(t)$$

Adding continuous excitation, we add the intensity of the light

$$\frac{\partial N(t)}{\partial t} = -\frac{1}{\tau}N(t) + f(t) = -\frac{1}{\tau}\left[A + B\sin(\omega t - \phi)\right] + a + b\sin(\omega t)$$

Equating this to the expression earlier, we have:

$$B\omega\cos(\omega t - \phi) = -\frac{1}{\tau}\left[A + B\sin(\omega t - \phi)\right] + a + b\sin(\omega t)$$

Expanding:

$$B\omega\left[\cos(\omega t)\cos(\phi) + \sin(\omega t)\sin(\phi)\right] = -\frac{1}{\tau}\left[A + B\left[\sin(\omega t)\cos(\phi) - \cos(\omega t)\sin(\phi)\right]\right] + a + b\sin(\omega t)$$

$$B\omega\cos(\phi)\cos(\omega t) + B\omega\sin(\phi)\sin(\omega t) = -\frac{1}{\tau}A - \frac{1}{\tau}B\cos(\phi)\sin(\omega t) + \frac{1}{\tau}B\sin(\phi)\cos(\omega t) + a + b\sin(\omega t)$$

$$\left[B\omega\cos(\phi) - \frac{1}{\tau}B\sin(\phi)\right]\cos(\omega t) + \left[B\omega\sin(\phi) + \frac{1}{\tau}B\cos(\phi) - b\right]\sin(\omega t) + \left[\frac{1}{\tau}A - a\right] = 0$$

For this to be true at all times t requires:

$$B\omega\cos(\phi) - \frac{1}{\tau}B\sin(\phi) = 0 \qquad B\omega\sin(\phi) + \frac{1}{\tau}B\cos(\phi) - b = 0 \qquad \frac{1}{\tau}A - a = 0$$

From the first equation we have:

$$\frac{\sin(\phi)}{\cos(\phi)} = \tan(\phi) = \omega\tau$$

Squaring the first two equations and adding the resulting equations yields:

$$B^2\omega^2\cos^2(\phi) + \frac{1}{\tau^2}B^2\sin^2(\phi) - \frac{2}{\tau}B^2\omega\sin(\phi)\cos(\phi) = 0$$

$$B^2\omega^2\sin^2(\phi) + \frac{1}{\tau^2}B^2\cos^2(\phi) + \frac{2}{\tau}B^2\omega\cos(\phi)\sin(\phi) = b^2$$

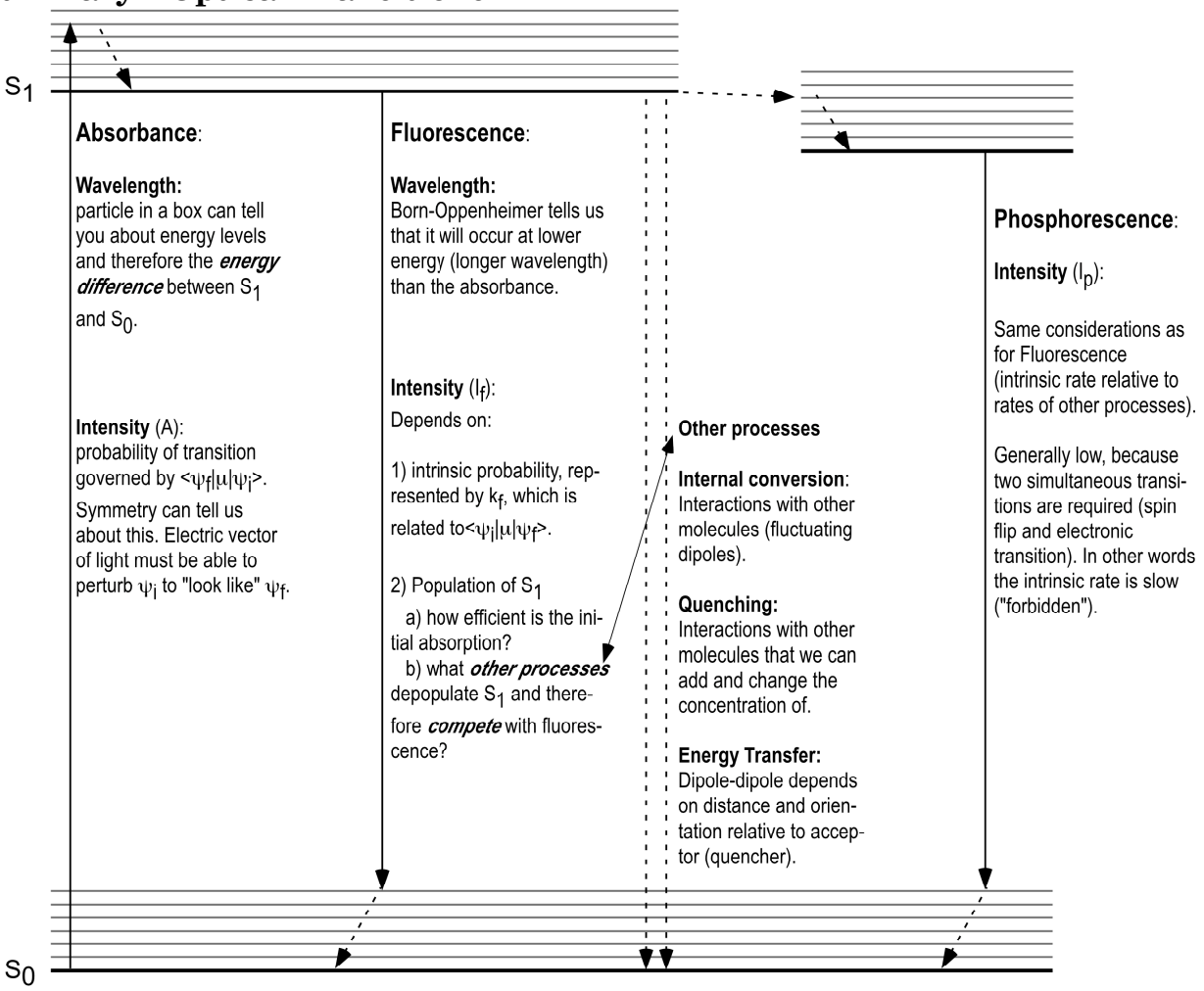
add: $$B^2\omega^2\cos^2(\phi) - \frac{1}{\tau^2}B^2\sin^2(\phi) + B^2\omega^2\sin^2(\phi) + \frac{1}{\tau^2}B^2\cos^2(\phi) = b^2$$

$$\left(B^2\omega^2 + \frac{1}{\tau^2}B^2\right)\cos^2(\phi) + \left(B^2\omega^2 + \frac{1}{\tau^2}B^2\right)\sin^2(\phi) = b^2$$

$$\omega^2 + \frac{1}{\tau^2} = \left(\frac{b}{B}\right)^2 \qquad \text{but } \omega = \frac{A}{a}$$

$$m = \frac{Ba}{bA} = \frac{1}{\omega}\left(\omega^2 + \frac{1}{\tau^2}\right)^{-1/2} = \left(\omega^2\tau^2 + 1\right)^{-1/2}$$

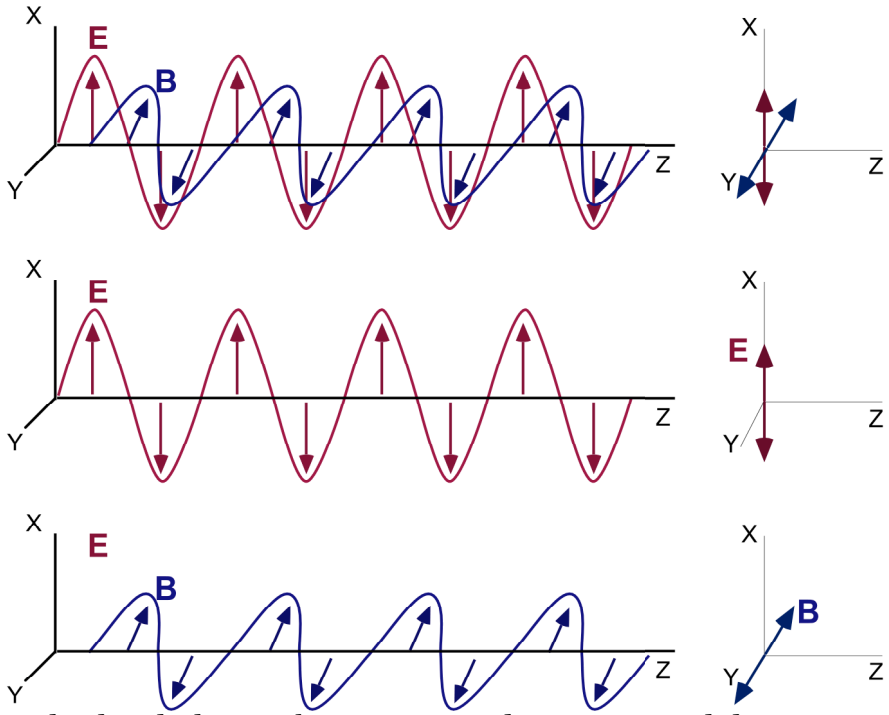
Summary – Optical Transitions



Circular Dichroism and Optical Rotation (CS 8.1, vH 10)

Return to a look at *absorption* processes.

Polarization of light



We saw previously that light can be represented as a sinusoidal wave representing its electric field component. In linearly polarized light, all of the electric field component is oriented in a single direction (z above), with the electric field vector oriented uniquely in one direction (x above).

Linear dichroism

We also saw that transitions can be "allowed" along one direction of the molecule and "disallowed" (forbidden) along another. For example, only one orientation of the electric field can induce $\pi \rightarrow \pi^*$ transitions in formaldehyde.

We also saw that for such a molecule in solution, excitation with polarized light will selectively excite only those molecules oriented which are properly oriented. If fluorescence occurs before rotational reorientation, then the emitted light will also be polarized.

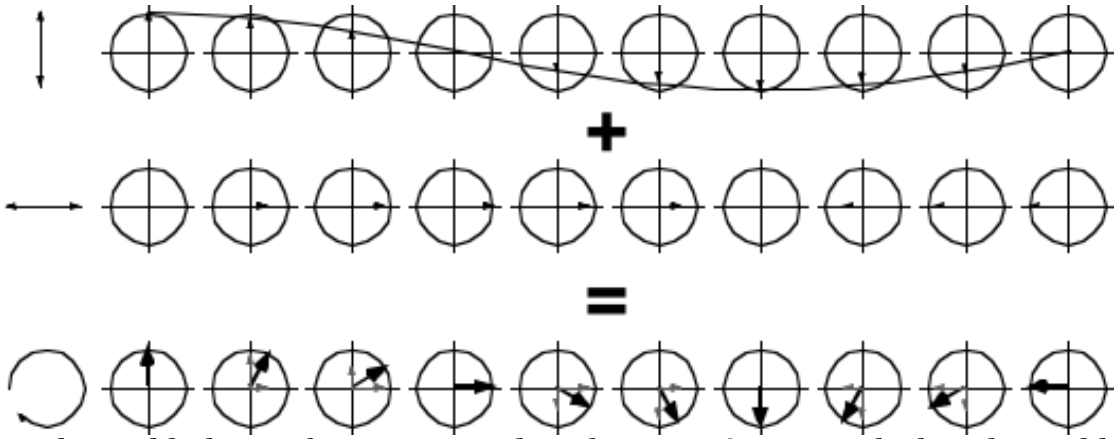
More simply, if we look at absorption (*not* fluorescence) in an oriented sample, in particular a sample oriented along one axis, but not along the other two, we can define the dichroic ratio

$$d = \frac{A_{\parallel} - A_{\perp}}{A_{\parallel} + A_{\perp}}$$

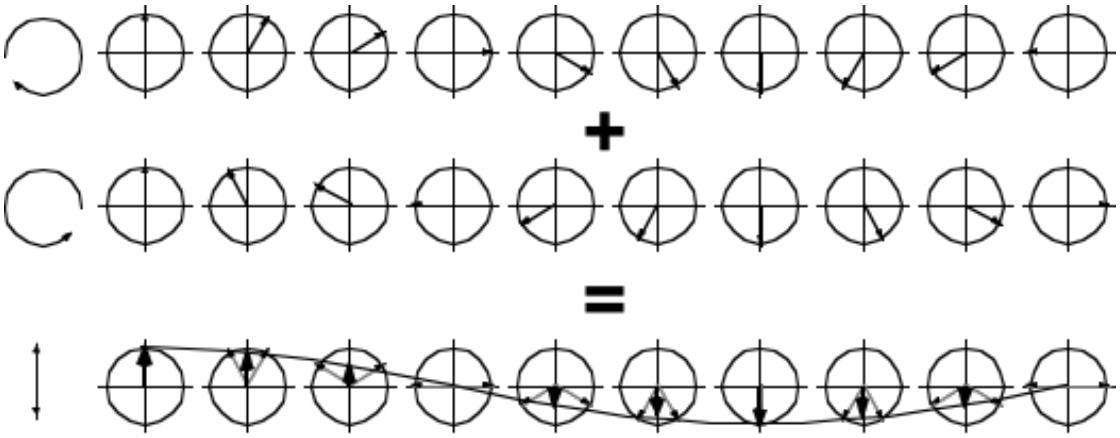
Example: In DNA, the bases are all oriented with their planes parallel to one another, but rotated to varying degrees within the plane. Since the transition dipole lies in the plane of the bases, only light polarized with its electric field component in that plane will result in an allowed transition, so that $A_{\parallel} = 0$ and $A_{\perp} > 0$. Therefore $d < 0$ and we say that DNA has negative dichroism.

Circular Polarization of light

Circularly polarized light can be represented as the sum of two linearly polarized light waves.



Plane polarized light can be represented as the sum of two circularly polarized light waves.



Absorption phenomenon

Circular Dichroism $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$

An optically active molecule can preferentially absorb left or right circularly polarized light. For the moment, accept the fact. We define the **circular dichroism** of a molecule as the difference in the extinction coefficients for left and right circularly polarized light.

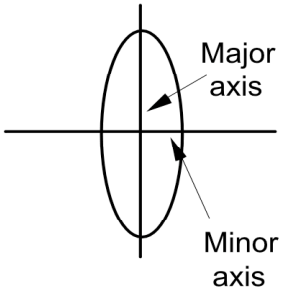
For a given absorption band, this property can very across the band. Such that we can define the overall rotational strength of the molecule as the integrated extinction coefficient difference across the entire band.

$$R = \int \frac{\Delta\varepsilon}{\nu} d\nu$$

Note that CD requires *absorption of light*. But the CD can be positive or negative.

Readily measured. Alternately measure absorption of pure left versus pure right circularly polarized light. Compare numerically.

Ellipticity: $\theta = \arctan \frac{\text{minor axis}}{\text{major axis}}$



Starting with linearly polarized lamp incident on a sample.

Remembering that linear light can be viewed as the sum of equal parts right and left circularly polarized light, we can predict what will happen when the right circularly polarized component is absorbed more than the left circularly polarized light. The result is elliptically polarized light. The degree of ellipticity can be presented as the ration of the minor axis to the major axis (0, for no difference in absorption for left and right circularly polarized; 1, for complete absorption of one of the components).

Equivalent to circular dichroism: $\theta = 2.303(A_L - A_R)\frac{180}{4\pi}$

Refraction phenomenon

Refractive index: $n_r = \frac{c}{v} = \frac{\text{speed of light in a vacuum}}{\text{speed of light in a medium}}$

This depends on the strength of the interaction between the electromagnetic field of light and the molecules in the medium.

Such interactions depends on the *polarizability* of the molecule.

The refractive index varies strongly with frequency near the frequency of an absorption transition, because the EM field and the light have similar energies.

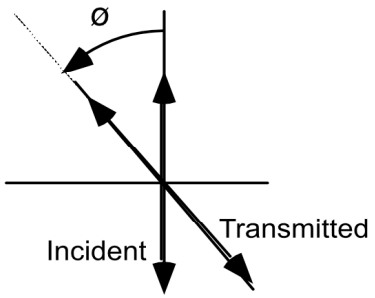
Circular Birefringence $n_L - n_R$

Due to symmetry (or lack thereof) in a molecule, the scattering interaction with it may be different for light polarized circularly either right or left (see below for some insight as to why). As we saw before, one can measure this difference in scattering by separately measuring the light scattered for either case or by measuring the effect of the sample on plane polarized light.

Circular birefringence is the difference in index of refraction for left and right circularly polarized light. The refractive index difference $n_L - n_R$ results in a difference in phase between the left and right circularly polarized light.

Optical Rotation: ø

Optical rotation is defined as the angle that the major axis makes with the y-axis. That is, it is a rotation of the plane of polarization. Optical rotation as a function of wavelength is called optical rotatory dispersion (**ORD**).



Equivalent to circular birefringence. $\text{ø} = \frac{180\,l}{(n_L - n_R)\,\lambda}$

Readily measured. Proteins and DNA at ≈0.1 mM typically rotate the plane of polarization by 0.01-0.1° Current instruments can measure Δø = 0.0001°

Summary

Absorption	Refraction
CD: $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$	Circular birefringence: $n_L - n_R$
Intensity (E_o) altered differentially	Phase (ϕ) altered differentially
Ellipticity: $\theta = \arctan\frac{\text{minor}}{\text{major}}$	Optical Rotation: ø

Molecular basis for optical activity

Introduction:

In absorption spectroscopy, the oscillating electric dipole component of light induced a dipole in the absorbing molecule. Moreover, we required that the electron involved be moved into a distribution resembling the excited state. This lead to the quantum mechanical result that the electron went from one *quantized* state to another. For light energies away from the exact required energy, the electric dipole influenced the electron distribution, but not in the exact way to make the probability of the transition very large.

Since the electric dipole of the light is oscillating, so then is this induced oscillating dipole in the molecule.

Note that for an oscillating electric field propagating through space, there is a magnetic component perpendicular to it. We write these:

$\mathbf{E} = \mathbf{E}_o \cos 2\pi\nu(t - \frac{x}{c})$ $\mathbf{H} = \mathbf{H}_o \cos 2\pi\nu(t - \frac{x}{c})$ where x is the position of the molecule.

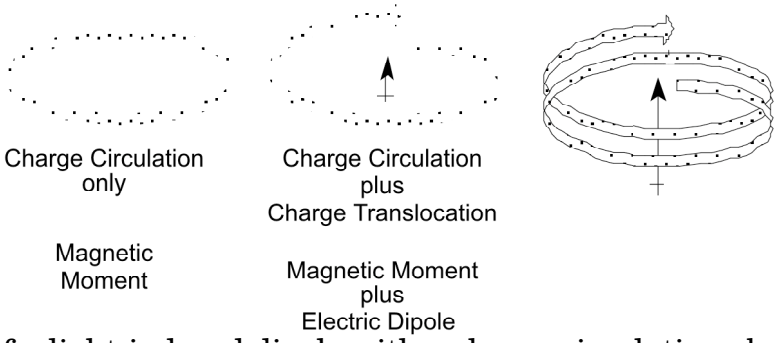
We saw before that the rotational strength in a CD transition is related to the integrated area of the CD spectrum. Quantum mechanically we can describe the rotational strength in a manner similar to the way we described the absorption strength (or probability) in the more simple absorption spectroscopy.

R_{oa} $\langle\psi_b|\boldsymbol{\mu}|\psi_a\rangle \bullet \langle\psi_a|\mathbf{m}|\psi_b\rangle$ (dipole strength $\langle\psi_b|\boldsymbol{\mu}|\psi_a\rangle \bullet \langle\psi_a|\boldsymbol{\mu}|\psi_b\rangle$)

where $\boldsymbol{\mu}$ = electric dipole operator (of the exciting light)

$\mathbf{m}$ = magnetic dipole operator (of the exciting light). This is proportional to the orbital angular momentum of the electron and corresponds to a charge circulation, ie. a "light-induced current loop" analogous to the light-induced electric dipole.

The result of the above dot product is that for a non-zero rotational strength, there must be a light-induced dipole **and** light-induced charge circulation. **Moreover**, the two vectors must have a parallel component (that is, they must not be perpendicular).



The combination of a light-induced dipole with a charge circulation about that dipole is a helical movement of the charge about the common axis. In order to get helical charge movement in a net direction, the molecule must be appropriately **asymmetric**.
Note that the light-induced magnetic dipole is generally much smaller than the light-induced electric dipole, therefore optical activity is typically a **small** effect.

Multi-chromophore interactions

As an example, look at dimer interaction. We have seen this before in the **exciton effect** in absorption spectroscopy. Two induced dipoles in neighboring chromophores can interact via a dipole-dipole interaction.
The rotation strength resulting from the dimer interaction can be expressed as

$$R_{oa} \quad 0.5\ \mathrm{imag}[\langle \psi_{1b}|\boldsymbol{\mu_1}|\psi_{1a}\rangle \bullet \langle \psi_{1a}|\underline{\mathbf{m}}_{\mathbf{1}}|\psi_{1b}\rangle + \langle \psi_{2b}|\boldsymbol{\mu_2}|\psi_{2a}\rangle \bullet \langle \psi_{2a}|\underline{\mathbf{m}}_{\mathbf{2}}|\psi_{2b}\rangle]$$

$$\pm\ 0.5\ \mathrm{imag}[\langle \psi_{1b}|\boldsymbol{\mu_1}|\psi_{1a}\rangle \bullet \langle \psi_{2a}|\underline{\mathbf{m}}_{\mathbf{2}}|\psi_{2b}\rangle + \langle \psi_{2b}|\boldsymbol{\mu_2}|\psi_{2a}\rangle \bullet \langle \psi_{1a}|\underline{\mathbf{m}}_{\mathbf{1}}|\psi_{1b}\rangle]$$

$$\pm \frac{\pi}{2\lambda}\ \underline{\mathbf{R}}_{12} \bullet \langle \psi_{2b}|\boldsymbol{\mu_2}|\psi_{2a}\rangle \times \langle \psi_{1b}|\boldsymbol{\mu_1}|\psi_{1a}\rangle$$

The important thing to note from the above is that there is a strong distance and orientational dependence on the chromophore-chromophore interaction. Hence CD is very sensitive to the precise geometry of the interacting chromophores.
In fact, this is the basis for most applications of CD. For example, we have seen that the peptide bond is a π system with absorption bands at 220 and 190 nm. For an isolated peptide bond, the extinction coefficient (simple absorption) is very small at 220 nm, but larger at 190□m.

Secondary structural information

In ordered protein structures such as an □-helix, we have a large number of interacting peptide bonds at characteristic fixed distances and angles. In a □-sheet, the angles and distances are quite different, yet characteristic for that structure. Consequently, the CD spectrum can often be used to extract information regarding protein secondary structure (percent □-helix, □-sheet, random coil, etc.)
For a protein which shows optical activity, the unique combination of circular dichroic contributions is also characteristic of a particular structure for that protein (think of it as a structural "fingerprint"). Even relatively small changes in structure (a conformational change) can have large effects on the CD spectrum. Consequently, CD can be used as a probe of conformational change (in ideal cases, we can sometimes reach conclusions about the disruption of □-helical structure, etc).