II. Introduction to Spectroscopic Terms- with examples from Uv-Vis

I. Orbitals used in Uv-Vis (An overview; formaldehyde; Fig II-1, see also Cantor and Schimmel Vol. 2, p. 370, Dwek & Cambell p. 65). Electronic transitions that occur in uv-vis use the most loosely bound electrons. In most molecules these are

![Diagram of orbitals](image)

\[ \frac{1}{\sqrt{2}} (\psi_1 + \psi_2) \]

bonding

\[ \frac{1}{\sqrt{2}} (\psi_1 - \psi_2) \]

antibonding

Fig. II-1

a) \( \pi \) and \( \pi^* \) (the **Highest Occupied Molecular Orbitals** and the **Lowest Unoccupied Molecular Orbitals** respectively).

b) n (non-bonding, i.e. lone-pairs)

Transitions from \( \pi \rightarrow \pi^* \) involve double bonds exclusively, simple double bonds as in ethylene-or conjugated double bonds as in benzene; \( n \rightarrow \pi^* \) originate on heteroatoms. Only \( \pi \rightarrow \pi^* \) transitions have high intensity. The peptide bond and the heterocyclic rings of the purine and pyrimidine bases have both \( \pi \rightarrow \pi^* \) and \( n \rightarrow \pi^* \) transitions between 180-300 nm. The disulfide bond has a weak band at 250 nm (\( A_m = 300, \ n \rightarrow \pi^* \)).

c) d-orbitals of transition metal ions; d \( \rightarrow \) d (weak).

d) Charge Transfer (CT) involving metal d orbitals and ligand (L) orbitals (strong):

\( d \rightarrow L(\pi^*), L(\pi) \rightarrow d \).

*We speak of the chromophore:* The fragment of the molecule responsible for the absorption process. A molecule may have more than one chromophore e.g. the several chromophores of hemoglobin
1. Peptide Bond at ca 200 nm ($\pi \rightarrow \pi^*$, $n \rightarrow \pi^*$).
2. Aromatic sidechains at 280 nm ($\pi \rightarrow \pi^*$). Additional bands present at lower wavelength but obscured by the many peptide bonds.
3. Heme at 400-700 nm (porphyrin is highly conjugated (18 atoms), $\pi \rightarrow \pi^*$ moves to visible, spectrum modified by Fe)
4. Fe at ca 1000 nm ($d \rightarrow d$).
5. Heme CT, porphyrin $\pi \rightarrow$ iron d (1000-2000) nm.

II. The Basics.

A. The Spectroscopic Event: A physical process unique to each spectroscopy called either a transition or a promotion/demotion depending on the spectroscopy under consideration. Transitions originate in the ground state and terminate in the excited states. For high energy spectroscopy ($\Delta E$ (the energy gap between the ground and excited states) $\gg$ RT e.g. Uv-Vis) the excited states are only occupied in the presence of the light source. By contrast, in magnetic resonance both ground and excited states are occupied at room temperature, though with a very small excess in the ground state.

B. Model: In conventional absorption spectroscopy the basic event is the translation of electron density as the electron is promoted from the ground state to the excited state. For example, in the $\pi \rightarrow \pi^*$ transition the process can be thought of as the movement of electron density from a region of space between two bonded atoms (thus creating a deficiency of electron charge) to a second region of space which acquires an excess of electron charge. The simplest example is an atomic $1s \rightarrow 2p$ transition driven by the electric dipole component of the electromagnetic wave.

C. Selection Rules for allowed transitions
   (1) Uv-Vis $\Delta L = 1$ ($s \rightarrow p$ or $p \rightarrow d$ but not $s \rightarrow s$ or $s \rightarrow d$).
   (2) IR: Dipole moment must change.
   (3) Raman: electric polarizability must change.
   (4) Epr: $\Delta m_s = 1$, $\Delta m_I = 0$
   (5) Nmr: $\Delta m_I = 1$, $\Delta m_s = 0$

D. Dichroism. The possibility of inducing a transition depends on the orientation of the chromophore with respect to the axis of polarization of the incident light beam and this can be examined using polarized light: e.g. $xz$ polarized light causes $s \rightarrow p_x$ but not e.g. $s \rightarrow p_y$.

III. Parameters of spectra.

A. **Position** of absorption/emission event with respect to some scale; the scale is usually in units of energy.
   1.UV-Vis: Position is $\nu$ (\(\alpha\) energy) or $\lambda$ (\(\alpha\) reciprocal energy)); scale is cm$^{-1}$ or nm.
   2. epr: g-values; magnetic field (\(\alpha\) reciprocal energy).
   3. nmr: chemical shifts; frequency (\(\alpha\) energy) or sometimes magnetic field.
   4. Others: wavenumbers (energy), electron volts (energy).
B. Intensity

1. At maximum. UV-Vis: In the following we define absorbance (A), the Beer-Lambert Law and hence the absorbance of a 1M solution in an optical path of 1 cm ($A_m$ or $\epsilon_m$ (extinction coefficient.)). It is common practice but not essential that $A_m$ be measured at the wavelength corresponding to a peak (this wavelength is usually specified).

\[ I(l) = I_o \exp(-\beta l) \]

\[ \ln \frac{I(l)}{I_o} = -\beta l \]

\[ \log \frac{I_o}{I(l)} = 2.303 \beta l \rightarrow A = A_m c l \]

Two practical points:

(a) Spectral Bandwidth (SBW). The purity of the incident "monochromatic" radiation; this is assumed to have a Gaussian profile centered on the wavelength used for the measurement. The SBW is the FWHH (Fig. II-2). It should be no more than 10% the natural bandwidth (NBW) of the absorption line of the sample being measured. Remember that SBW refers to the light and NBW to the sample.

(b) Straylight. Due to limitations in optical equipment there is always small amounts of unwanted light present in $I_o$. This "stray light" (SL) will contain many wavelengths (far removed from the nominal wavelength of the measurement) not absorbed by the sample. This leads to an underestimate of the absorbance. The full definition of absorbance including the possible contribution of stray light is

\[ A = \log \left[ \frac{(I_o + SL)}{(I + SL)} \right]. \]
2. **Integrated**: The fundamental quantity in spectroscopy is not the amplitude but the area under the curve. In most spectroscopies the absorption curve is Gaussian with respect to energy (i.e. frequency). However Uv-Vis data are usually recorded with respect to wavelength (reciprocal energy) and are thus asymmetric Gaussians, though this asymmetry is usually only obvious with broad bands.

In Uv-Vis a Gaussian that is symmetric with respect to wavelength is usually assumed: using data normalized to a molar solution in a 1cm. pathlength

\[
A_\lambda = A_m \exp\left\{-(\lambda - \lambda_0)/\Delta^2\right\}
\]  

(II-1)

where \(A_\lambda\) and \(A_m\) are the molar absorbances at \(\lambda\) and \(\lambda_0\) respectively. (For most other spectroscopies the abscissa is in energy units e.g. \(\nu\)). As \(\Gamma\) (eqn II-2) is the half-width at 0.5 \(A_m\) (HWHH) and \(\Delta\) is the half-width when \(A\) has decreased to 0.37 \(A_m\) (i.e. \(A_m/e\)) we find that \(\Delta = 1.2*\Gamma\). (Note that some people take \(\Gamma\) as the FWHH; then the numeric factors should be divided by 2).

The integrated area of this curve can be obtained quickly via the rectangular approximation

\[
\text{Area} = 2.12 A_m \Gamma
\]  

(II-2)

or somewhat more accurately via the Gaussian approximation
Area = $\pi^{1/2} A_m \Delta$  \hspace{1cm} (II-3)

**Significance of the area under the curve.** (a) Uv-Vis. The area under the curve has a fundamental significance. The rate constant for the transition from $G \rightarrow S$ (see Jablonski diagram, Fig I-3) is denoted symbolically by $B_{gs}$. For this simple two-level case the rate constant ($B_{sg}$) for the reverse transition ($S \rightarrow G$) is numerically equal to $B_{gs}$ (These are the transition rates per unit energy density i.e. normalized to a standard amount of energy (not photons)). These $B$'s are called the Einstein coefficients for stimulated absorption and emission respectively. In optical spectroscopy the $B$'s are directly proportional to the dipole strength, $D$, of the transition. $D$ can be calculated from the area under the absorption curve; it is approximately proportional to $A_m$.

Using the Gaussian approx. and with spectral parameters in nm:
$$D = 0.0163 A_m \Delta / \lambda_0$$  \hspace{1cm} (II-4)

In the Rectangular Approximation replace $\Delta$ by $1.2 \Gamma$.

The dipole strength is a direct measure of the amount of *transient* dipole ($\mu_e$) created during the transition. A "perfect" transition is approximated by a single electron ($q = 4.8 \times 10^{-10}$ esu) being translated through an Angstrom ($10^{-8}$ cm); the equivalent *transition dipole moment* ($\mu_e$) is $4.8 \times 10^{-18}$ esu cm or 4.8 Debye (The Debye has had various units over the years; most commonly it is given as $10^{-18}$ esu cm.). $D$ and $\mu_e$ are simply related:
$$D = (\mu_e)^2.$$  \hspace{1cm} (II-5)

The units of $D$ are esu$^2$cm$^2$ or Debye$^2$. Note that the Debye is always written out to avoid confusion with $D$.

How does one interpret the transition dipole moment?

Wavefunction $\phi$ )  \hspace{1cm} Electron Density $(\phi^2)$

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<tr>
<td>1s</td>
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<td>2p</td>
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<td>1s + 2p</td>
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Fig. II-3

A simple example is given by the atomic 1s $\Rightarrow$ 2p transition (Fig. II-3). Neither the 1s nor the 2p orbital has a dipole moment but as the electron leaves the 1s function and distorts to the 2p function the transiently formed (1s + 2p) hybrid is asymmetric and thus has a dipole moment.

{A quantity related to $D$ more common in the older literature is the oscillator strength ($f$); this is the area under the curve divided by the area expected for an electron behaving as an ideal three
This is calculated from the equation for the forced harmonic oscillator (see appendix) using the parameters for the electron \( \omega_0 = \text{natural frequency} \), \( D = \text{friction} \) and scaling to 1 mole/liter. For allowed transitions \( f \) approaches 1; summed over all absorption bands in a molecule \( f \) should equal the number of valence electrons. Using the Gaussian approx. and with spectral parameters in nm:

\[
f = 0.077 \frac{A_m \Delta}{(\lambda_o)^2}
\]

Example: Adenine has a \( \lambda_o \) at 260 nm, with \( A_m \) of 15000 \( M^{-1} cm^{-1} \) and wavelengths of half-maximal absorbance at 246 and 280 nm. So \( f = 0.34 \), \( D = 19 \text{ Debye}^2 \) and \( \mu_e = 4.3 \text{ Debye} \). Thus the \( \pi \to \pi^* \) transition responsible for the principal UV-absorption of adenine is associated with the change in the center of gravity of the electron cloud as an electron goes from the ground to the excited state, essentially equivalent to the displacement of 1 electron through 1Å.

b). Epr and nmr: Area under curve directly quantifies number of unpaired electrons or number of protons in one's sample.

C. The magnitude of the spectral linewidth (FWHH) \( \rightarrow \) Lifetime (LT) of excited state. This is especially useful in nmr, epr and in fluorescence spectroscopy (related to topic E below). For Lorentzian absorption curves \( LT = \frac{2}{2\pi \text{FWHH}} \) where the Full Width at Half Height is expressed in Hertz; the \( 2\pi \) exists to convert Hz to radians. For non-Lorentzian lines the calculation yields the lower limit on the LT.

D. Fine-Structure. These can be due to
1. Overlapping Transitions
2. Vibrational Contributions (UV-Vis)
3. Parent and satellite nuclear moments or unpaired electrons (epr)
4. Adjacent nuclear moments (nmr)

E. Relaxation Times. Some spectroscopic processes occur sufficiently slowly that the kinetics of the process can be monitored. This is of particular value in fluorescence, nmr and epr.

F. Solvent effects (most common in uv-vis)

\( n \to \pi^* \): Polar solvents stabilize the lone-pair on the heteroatom and make the promotion more difficult. The transition moves to higher-energy (shorter wavelength, a blue-shift a.k.a. hypsochromism). The antonym is bathochromism

\( \pi \to \pi^* \): Immediately after promotion the excited state has a solvent cage appropriate to the ground-state but a new charge distribution. The consequences are complicated depending upon whether the solute or solvent is polar/non-polar (detailed in J. Phys. Chem. 58 1002 and 1006 (1954)).

The utility of solvent effects in general is
(i) comparison of the spectrum of chromophore present in macromolecule with spectra of some small molecule containing the same chromophore in a variety of solvents and hence establishing the polarity of the binding site e.g. flavins.
(ii) By studying the response of the chromophore to a change in the solvent (e.g. \( D_2O \), dielectric, pH) the accessibility of the chromophore to solvent and/or its state of protonation can be assessed. This is called the solvent perturbation technique.
G. Dipole-Dipole Interactions.

For two point dipoles (electric or magnetic) oriented arbitrarily in space the expression for the energy of interaction, $V_{12}$, is:

$$V_{12} = \frac{1}{R^3} \{ \mu_1 \cdot \mu_2 - 3(\mu_1 \cdot r)(\mu_2 \cdot r) \} \tag{II-6}$$

where $\mu_1$ and $\mu_2$ are the two dipoles (usually either 2 electrons or two protons.), $r$ is a unit vector aligned in the direction connecting the midpoints of $\mu_1$ and $\mu_2$ and $R$ is the distance between these two midpoints. The first dot product reflects the extent to which the two dipoles are co-linear; the second and third dot products measure the extent to which $\mu_1$ and $\mu_2$ are parallel to the line connecting their midpoints.

A dipole-dipole interaction between identical chromophores is called Exciton Coupling. For optical transitions it can lead to:

a) Band Splitting (usually too small to see directly but can increase the linewidth).
b) Induced optical activity (to be covered in CD lecture).
c) Band intensification (*hyperchromism*).
d) Band weakening (*hypochromism*, DNA = 30%).

This dipole-dipole coupling is also the origin of energy transfer (fluorescence) and spin-spin interactions in epr and nmr. Much of 2D and 3D nmr depends on these couplings (the NOESY technique).
H. Applications of Uv-Vis:

1. Concentrations measurements:
   a. Proteins.
   b. Nucleic Acids.
   c. NADH.

2. Assays:
   a. NADH.
   b. cytochrome c.

3. Oxidation and/or spin-state:
   a. Heme enzymes.
   b. Flavin, copper, iron-sulfur centers.
   c. Geometry (Co(II), Tetrahedral, ($T_d$) 650 nm, $A_m = 500$; Octahedral ($O_h$), 550 nm, $A_m = 10$).

4. Ligand binding
   a. Heme proteins e.g. hemoglobin.

5. Conformational changes e.g. DNA melting.

Instruments: Single Beam **versus** Double Beam.

**Single Beam:** A measurement is first made on the reference material—if there is one. Then the reference is replaced by the sample and a separate measurement made on it. If the sample data is to be corrected for contributions by the reference these corrections are done manually (with the help of a computer).

**Double Beam:** The reference and sample are placed in separate locations within the instrument. The measurement is made simultaneously on both and the difference between them computed automatically.

I. Difference Spectroscopy (tandem cells). A technique to enhance sensitivity!
Three kinds of difference scan be obtained.

1. A change in size with no change in shape.

2. The spectrum shifts to shorter or longer wavelength with relatively little change in shape; there may also be a change in size. These shifts are usually small, less than the FWHH. Only rarely are the shifts large enough to separate the bands.

3. One or more new bands may appear -to shorter or longer wavelength.
Some Guidelines For Using a UV-Vis Spectrophotometer.

1. Does this instrument have 1 or 2 monochromators (or gratings). If 1 do not make absorbance readings above 2.0; if 2 you can go to 3.0. If you cannot find out either (i) assume 1 or (ii) run a Beer's Law test on the sample and restrict your measurements to the linear range.

2. Am I making a reading at a single wavelength (e.g. for concentration determinations). If so use the maximum in the spectrum or a well pronounced shoulder. Do not make a reading in a region where the absorbance changes rapidly with wavelength.

3. If recording a spectrum what is the instrument time constant (also called response time, filter or RC). The time to scan through the FWHH of the narrowest band should be at least 10x the value of the time constant.

4. What is the spectral bandwidth (SBW). It should be less than 1/10'th the natural bandwidth (NBW).

5. Am I making a reading at wavelengths less then 340 nm; if yes you must use a quartz cuvette, otherwise try to use the much cheaper glass cuvettes.

6. When the blank is colorless and all readings are at wavelengths greater then 400 nm it is OK to use water in the reference cuvette.

7. Never touch the transparent sides of the cuvette with your fingers-always handle cuvettes using the frosted surfaces.

8. Unless you are using a "greasy" sample clean cuvettes with by rinsing thoroughly with distilled water. If they are not to be used soon store inverted in a cuvette rack so they can drain dry and dust cannot enter. If to be used immediately follow the water with methanol (not acetone) and dry using a stream of nitrogen (not compressed air which contains droplets of oil). Greasy samples should be soaked in warm detergent-a few drops of conc. detergent in hot tap water. Really dirty cuvettes should be treated for several hours with hot acid (see a lab manual and be careful); labs that do these measurements routinely often have a covered container in the hood with the appropriate acid cleaning fluid.

9. Before taking a measurement hold the cuvette up to the light to be sure it is free of bubbles and debris. Bubbles can usually be removed by tapping the bottom of the cuvette GENTLY on the bench. If any debris is in the light path (see next item) recover the sample and filter it using a minifilter attached to a disposable syringe. If you observe finger marks on the optical surface breath on the surface and wipe with a clean Kimwipe.

10. It is sometimes useful to know precisely where the light beam impacts the cuvette surface; an example is given below. This can be done as follows:

    Set the monochromator to 550 nm, turn out the room lights and place a white card where you expect the beam to strike the cuvette. If you cannot see a green vertical line open the optical slit (sometimes called bandwidth) until the green line is easy to see. Replace the card with a white plastic ruler located so that the bottom rests on the floor of the cuvette holder. Read the height of the bottom and top of the green line off the ruler.

    If you wish to conserve solution you can place a small object in the cuvette holder so that the cuvette is raised by an amount just enough that the upper surface of the base of
the cuvette is just below the bottom of the slit. Then you can pipette different volumes into
the cuvette until you find the volume such that the meniscus of the liquid is just above the
top of the slit. In this way you can reduce the amount of solution required from the 3.0 mL
normally used to about 1.0 mL; this can be very useful when your reagents are precious.