Electronic Spectra

(i) Absorption Spectra
(ii) Circular Dichroism
(iii) Fluorescence Spectra

(i) UV/visible spectroscopy
13.2 not 13.2(a), 13.7, 13.8
(1st half)
The absorption of light is due to the interaction of the oscillating electromagnetic field of the radiation with the charged particles in the molecules. If the electromagnetic force is high enough, then the electrons within the molecule will get shifted to a higher energy level ($10^{-15}$ s). The excited state is returned to its ground state either by emission of heat or emission of radiation which is equal or lesser to the absorbed radiation ($10^{-9}$ s).

Emission spectroscopy, a molecule undergoes a transition from a state of high energy $E_2$, to a state of lower energy $E_1$ and emits the excess energy as a photon.

Absorption spectroscopy, measures the absorption of radiation as a function of frequency or wavelength, due to it’s interaction with a sample. Absorption occurs when the energy of the photons match the $\Delta E$ between two states of the material.
Beer-Lambert law

\[
\log\left(\frac{I_0}{I}\right) = \text{Abs} = \varepsilon[J]l
\]

$\varepsilon$ units are L mol$^{-1}$ cm$^{-1}$ or M$^{-1}$ cm$^{-1}$

where $T = \frac{I}{I_0}$

$I_0$ intensity of light entering (incident)
$I$ intensity of light leaving (transmitted)
\(\varepsilon\) molar extinction coefficient (M$^{-1}$cm$^{-1}$)
J concentration (M or mol L$^{-1}$)
l pathway length (cm)
T = transmission

Typically values of $\varepsilon$ for strong transitions are of the order of $10^4$-$10^5$ L mol$^{-1}$ cm$^{-1}$
Problem  The molar absorption coefficient of tryptophan

Radiation of wavelength 280nm passed through 1.0mm pathlength of tryptophan 0.5 mmol L\(^{-1}\). The light intensity is reduced by 54% of its initial value. Calculate the molar extinction coefficient of tryptophan at 280nm. If the pathlength was 2.00 mm, what would the absorbance be?

\[
A = \log(I_0/I) = \varepsilon[J]l \quad \text{and} \quad \frac{T}{I_0} = \frac{I}{I_0} \quad \text{so} \quad A = -\log T = \varepsilon[J]l
\]

\[
\varepsilon = -\frac{\log T}{[J]l} = \frac{\log 0.54}{(5.0 \times 10^{-4} \text{ mol L}^{-1}) \times (1.00 \text{ mm})} = 5.4 \times 10^{-2} \text{ L mol}^{-1} \text{ mm}^{-1}
\]

The absorbance of a sample of length 2.0 mm is

\[
A = (5.4 \times 10^{-2} \text{ L mol}^{-1} \text{ mm}^{-1}) \times (5.0 \times 10^{-4} \text{ mol L}^{-1}) \times (2.00 \text{ mm}) = 0.54
\]
The ‘width’ of an electronic transition ($\lambda$, $\tilde{v}$ and $v$) results from contributions from the simultaneous excitation of molecular vibrations – “spectral line”.

Broadening of the individual lines is due to the lifetime of the states in the transition.

When the Schrödinger equation is solved for system changing over time, we no longer have precisely defined energies.

State of decay (lifetime of the state) is $e^{-t/\tau}$, $\tau$ is a time constant
Then its energy levels are blurred by $\delta E$

$$\delta E = \frac{\hbar}{\tau}$$ results in “line broadening”

$$\delta \tilde{v} = 5.3 \text{ cm}^{-1}$$ where $\tau$ is expressed in ps the wavenumber $\tilde{v} = 1/\lambda = v/c$ (cm$^{-1}$)

Two reasons for $\tau$ (a) collisional deactivation
(b) spontaneous emission dependent on the transition $v$

Natural lifetime ($\tau$) of electronic excited state $\sim 10^{-8}$ s, vibrational $\sim 10^{-13}$ s
Franck Condon principle

Electronic transitions occur, accompanied by excitation to different vibrational energy levels.

In the electronic ground state: nuclei in a molecule take up location in response to the electrons and other nuclei -> after an electronic transition, then nuclei are subject to different forces and may respond by vibrating.

Broad electronic absorption band consists of many superimposed bands from unresolved vibrational structure.
Franck Condon principle: electronic transitions occur so rapidly that during the transition the nuclei cannot respond.

Eg electron density is lost rapidly from some regions of the molecule and built up in others.

Thus, all electronic transitions are vertical transitions (internuclear distance doesn’t change).

Although, one should note that electronically excited molecule maybe formed in one of several vibrational states, so the absorption occurs at several different frequencies.
Electronic Spectroscopy

The absorption of a photon can often be traced to the excitation of an electron that localizes on a small group of atoms.

Groups with characteristic optical absorptions are called **chromophores**.

C=O bond absorbs at 290nm ie in a peptide bond.

d-d transitions (charge-transfer transitions) in d-metal complexes are usually characterized by relatively high $\lambda$, 500–700 nm.

The transition of the lone pair of e-’s in O of C=O, from a $n \rightarrow \pi^*$ orbital of the carbonyl group is usually responsible for its absorption.

C=C can absorb a photon to excite a $\pi$ electron into a $\pi^*$ orbital, if unconjugated corresponds to an absorbance of ~180nm. When part of a conjugated chain, energies of the molecular orbitals lie closer together and transition shifts to the visible range.
Absorption Spectroscopy

Biological chromophores
1. The peptide bonds and amino acids in proteins
   • The electrons of the peptide group are delocalized over the carbon, nitrogen, and oxygen atoms. The n-π* transition is typically observed at 210-220 nm, while the main π-π* transition occurs at ~190 nm.
   • Aromatic side chains contribute to absorption at λ > 230 nm

2. Purine and pyrimidine bases in nucleic acids and their derivatives

3. Highly conjugated double bond systems ie β-carotene, heme from hemoglobin.
### Biological Chromophores

1. Amino acids

<table>
<thead>
<tr>
<th>Molecule</th>
<th>λ (nm)</th>
<th>ε ( \times 10^{-3} ) (cm(^2).mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>280, 219</td>
<td>5.6, 47</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>274, 222, 193</td>
<td>1.4, 8.48</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>257, 206, 188</td>
<td>0.2, 9.3, 60</td>
</tr>
<tr>
<td>Histidine</td>
<td>211</td>
<td>5.9</td>
</tr>
<tr>
<td>Cystine</td>
<td>250</td>
<td>0.3</td>
</tr>
</tbody>
</table>
Biological Chromophores

Tryptophan absorption is used as the basis for protein concentration measurements.

Figure 11.1. Absorption spectra of the aromatic amino acids. (From Ref. 6.)
## Biological Chromophores

### 2. Bases and their derivatives

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$\lambda$ (nm)</th>
<th>$\epsilon$ (x10^{-3}) (cm^{2}.mol^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>260.5</td>
<td>13.4</td>
</tr>
<tr>
<td>Adenosine</td>
<td>259.5</td>
<td>14.9</td>
</tr>
<tr>
<td>NADH</td>
<td>340, 259</td>
<td>6.23, 14.4</td>
</tr>
<tr>
<td>NAD+</td>
<td>260</td>
<td>5.9, 18</td>
</tr>
<tr>
<td>FAD+</td>
<td>450</td>
<td>11.3</td>
</tr>
</tbody>
</table>
So when ligands bind to protein, the $\varepsilon$ is sometimes changed….this is very true for heme bound to protein.

The absorbance in the beam containing the mixture of enzyme (E) and ligand (L) and EL is the enzyme complex.

$$A_1 = \varepsilon_{EL}[EL] + \varepsilon_{E}[E] + \varepsilon_{L}[L]$$

$$= \varepsilon_{EL}[EL] + \varepsilon_{E}([E_0] - [EL]) + \varepsilon_{L}([L_0] - [EL])$$
If you have a complex of proteins which have different wavelengths of maximum extinction coefficients, then you can calculate the concentration of both components by measuring the absorbance at 2 \( \lambda \)'s, \( \lambda_1 \) and \( \lambda_2 \)

Total absorbance at a given \( \lambda \):
\[
A = A_A + A_B = \varepsilon_A l[A] + \varepsilon_B l[B] = (\varepsilon_A [A] + \varepsilon_B [B]) l
\]

\[
A_1 = (\varepsilon_{A1} [A] + \varepsilon_{B1} [B]) l, \quad A_2 = (\varepsilon_{A2} [A] + \varepsilon_{B2} [B]) l
\]

\[
[A] = \frac{\varepsilon_{A2} A_1 - \varepsilon_{B1} A_2}{(\varepsilon_{A1} \varepsilon_{B2} - \varepsilon_{A2} \varepsilon_{B1}) l} \quad [B] = \frac{\varepsilon_{A1} A_2 - \varepsilon_{B2} A_1}{(\varepsilon_{A1} \varepsilon_{B2} - \varepsilon_{A2} \varepsilon_{B1}) l}
\]

There maybe an isosbestic wavelength, \( \lambda^o \), where the molar extinction coefficient of the two species is equal then:

\[
A^o = \varepsilon^o ([A] + [B]) l
\]
A sample of RNA is hydrolyzed and separated into three fractions by column chromatography. Two of the three fractions are pure, the third contains both adenylic and guanylic acids. At pH 7.0, the absorbance of the mixture is 0.305 at 280 nm and 0.655 at 250 nm in 1 cm cells.

<table>
<thead>
<tr>
<th></th>
<th>$\varepsilon_{280}(M^{-1}cm^{-1})$</th>
<th>$\varepsilon_{250}(M^{-1}cm^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylic acid</td>
<td>2300</td>
<td>12,300</td>
</tr>
<tr>
<td>Guanylic acid</td>
<td>9300</td>
<td>15,700</td>
</tr>
</tbody>
</table>

\[
A_{280} = 0.305 = 2300x + 9300y \quad (1)
\]
\[
A_{250} = 0.655 = 12300x + 15700y \quad (2)
\]

From (1) \((0.305 - 2300x)/9300 = y\), place in Eqn (2)
\[
0.655 = 12300x + 15700((0.305 - 2300x)/9300)
\]

\[
x = 1.66 \times 10^{-5} \text{ M and } y = 2.86 \times 10^{-5} \text{ M}
\]
Spectroscopy Techniques Using Plane-Polarized Light
13.8 2nd half
Light is plane polarized when the electric and magnetic components each oscillate in a single plane.

Electromagnetic radiation consists of mutually-perpendicular electric and magnetic waves.

In a light source, the electric vectors are randomly orientated around the beam axis.
Chirality in Molecules

Pairs of isomers (D-alanine and L-alanine) are non-superimposable mirror images of each other and are called enantiomers or optical isomers.

\( \alpha \)-carbon center is called an optical center.
Chirality in biochemistry

Many biomolecules are chiral

- All amino acids in proteins are the L-enantiomer.
- L-amino acids result in right-handed helices.
- Enzymes can distinguish between L- and D- amino acids, through specific active sites.
- Monosaccharides are mostly D-enantiomers.
- D-amino acids sometimes are present in antibiotic peptides.

Most chemical reactions are carried out in the presence of 50% D- and L-enantiomers.
Fertility drug - Thalidomide

The drug was administered as a mixture of the S- and R-enantiomer forms,

The (\textit{R}) enantiomer is effective against morning sickness. The (\textit{S}) is teratogenic and causes birth defects. Unfortunately, if you administer one isoform, the enantiomers can interconvert \textit{in vivo}.

There is considerable interest in the pharma industry for enantioselective synthesis of drugs.
Two types of measurements are used to determine the effect of molecules on polarized light.

- Optical rotation is a measure of the rotation of linearly polarized light by a molecule, and wavelength dependence of the rotation is called optical rotary dispersion (ORD).

Which amino acid would give the optical rotation of zero?

- Circular dichroism (CD) is the difference in absorption of left-hand or right-hand circularly polarized light.
Circularized polarized light

We can select either the right or left vector of the circular polarized light. If we trace the path of the electric vector along the axis of a beam of circularized polarized light, it retains each magnitude but describes a helical pathway.

The $E$ vector of linearly polarized light (also called plane-polarized light) has a constant direction and a modulated amplitude. By contrast, the $E$ vector of circularly polarized light has a constant amplitude but a modulated direction.

The tip of the electric vector traces out a helical path (which can be either left- or right-handed) through space.
The differential absorption of left- and right-circularly polarized light is called CD.

In terms of absorbance for the two components, $A_L$ and $A_R$

$$\Delta \varepsilon_\lambda = \varepsilon_L - \varepsilon_R = (A_L - A_R)/[J]l$$

For reasons of historical CD has been expressed ellipticity

$$\theta_\lambda = 3330 \Delta \varepsilon_\lambda$$ and has units of deg cm$^2$ dmol$^{-1}$
Secondary Structure Elements of Proteins
Actual fit of a sample data against the Fasman poly-lys standards (left) using the program CDFIT. The R-value of the fit is 6%, with a total helix content of 80% and 20% random coil. The actual value is 77% total helix content.
Measuring protein denaturation by CD

Measuring the difference at 205 nm, and then calculating the fraction of protein folded = \((\theta_L - \theta_D)/(\theta_N - \theta_D)\)

\(K_{eq}\) is when 50% of the protein is folded, therefore the fraction of folded protein is equivalent to 0.5, and will give you the concentration of GdnHCl required to unfolded 50% of protein.
Electronic Spectra

Fluorescence Spectroscopy
Pgs 567, 568,
& Sections 13.13, 13.14
The excitation energy of a molecule that has absorbed a photon is returned to its ground state either by emission of heat or emission of radiation which is equal or lesser to the absorbed radiation.

Emission of heat is termed non-radiative decay.

Emission of radiative decay has two principle modes, phosphorescence and fluorescence.

*Lifetime of electrons in excited state is short: $10^{-13}$ (absorption) and $10^{-9}$ s (fluorescence).*
Jablonski diagram

Vibrational relaxation (nonradiative)

Energy

Absorption

Fluorescence
The measurement of fluorescence is more complex than absorption.

Sample must be excited at specific $\lambda_1$ and emission measured at a specific $\lambda_2$.

Emitted light must be at a longer wavelength than the absorbed light as

$$\Delta E_{\text{emission}} < \Delta E_{\text{absorption}}$$

Note: downward electronic transition is vertical in accordance with Franck-Condon principle.
**Fluorophore** = a molecule that absorbs light but then returns to the ground state by emitting some of the light as a photon rather than losing all the energy as heat. Wavelength and intensity of emitted light both very sensitive to the environment of the fluorophore (e.g., hydrophobic vs. aqueous environment can shift emission spectrum) measurements very sensitive so can detect small amounts of protein or other fluorophore.

**Fluorophores in proteins**

Trp (maximum wavelength of fluorescence emission ($\lambda_{\text{max}} \sim 340$ nm) is the strongest source of intrinsic fluorescence in proteins without fluorescent prosthetic groups, but tyrosine also contributes to intrinsic fluorescence. Some ligands and prosthetic groups are fluorescent, e.g. the chromophore in green fluorescent protein.

**USES of fluorescence spectroscopy**

- detect conformational changes
  - e.g. during protein folding (environment of chromophore affects $\lambda_{\text{max}}$ and intensity of Trp fluorescence; the more hydrophobic the environment, e.g. as Trp residues get buried in the interior of the protein during folding, the shorter the wavelength of maximum fluorescence emission)
- * detect and quantitate ligand binding