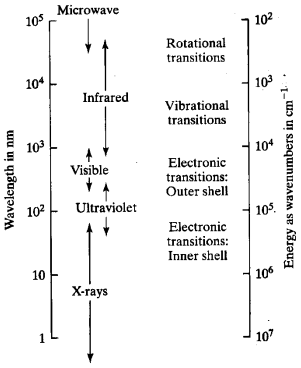
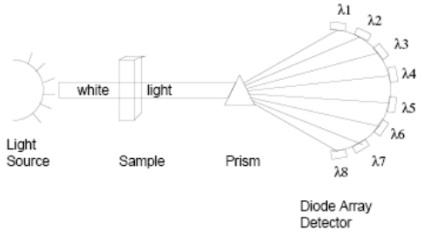
## Spectroscopy

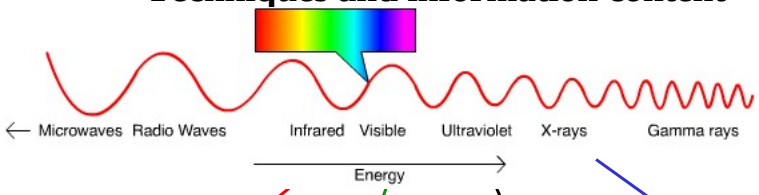
| Spectroscopy | Light Source | Dispersion Element | Detector |
|---|---|---|---|
| X-Ray | Electron Bombardment of metals | Diffraction Grating | Film/Ionization Chamber |
| UV-Vis | Tungsten(Visible) Deuterium, Hg (UV) | Prism or Diffraction Grating | Photomultiplier<br>Diode array detection |
| IR | Heat Source | Interference effects | Photomultiplier |
| ESR | Microwave cavity | Change in Magnet Field | microwave receiver |
| NMR | Radio Transmitter | Change in Magnet Field or Fourier Transform | radio receiver |




## Spectroscopy

### Techniques and information content




Molecular Libration (hindered rotations) → **Microwave**

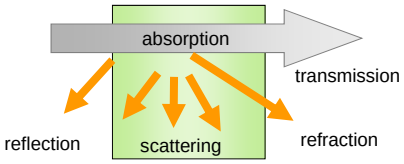
Molecular vibrations → **Infrared, Raman**

Electronic Absorption → **Visible Fluorescence Luminescence**

Valence band and shallow electronic levels (atoms) → **UV absorption UV photoemission Electron loss**

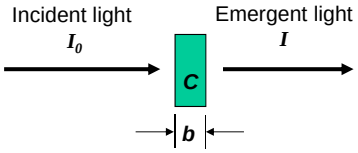
Deep electronic core levels (atoms) → **X-ray photoemission (XPS, ESCA) Auger Electron (AES)**

- **Atomic spectra**
  - The level and quantities of energy supplied to excite electrons can be measured & studied in terms of the frequency and the intensity of an electromagnetic radiation - ***absorption spectroscopy***
  - The level and quantities of energy emitted by excited electrons, as they return to their ground state, can be measured & studied by means of the ***emission spectroscopy***
  - The level & quantities of energy absorbed or emitted ($\nu$ & intensity of electromagnetic radiation) are specific for a substance
  - Atomic spectra are mostly in UV (sometime in visible) regions




- **Theory of light absorption**

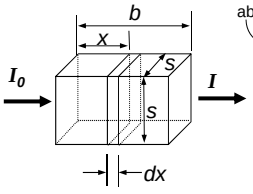
  Quantitative observation
  - The thicker the cuvette
    - more diminishing of light in intensity
  - Higher concentration the liquid
    - the less the emergent light intensity




These observations are summarised by **Beer's Law:**

***Successive increments in the number of identical absorbing molecules in the path of a beam of monochromatic radiation absorb equal fraction of the radiation power travel through them***

Thus




light absorbed; fraction of light

$$\frac{dI}{Ncs^2dx} = -k'I \quad \Rightarrow \quad \frac{dI}{I} = -k'Ncs^2dx = -acdx$$

$$\Rightarrow \int_{I_0}^{I_b}\frac{dI}{I} = -ac\int_0^b dx \quad \Rightarrow \quad \ln\frac{I_b}{I_0} = -acb$$

$$\Rightarrow \quad \log\frac{I_0}{I} = abc \equiv A$$

number of molecules *N*-Avogadro number

Absorbance

In UV absorption Spectroscopy

EM radiation in the UV-visible region of the spectrum is employed:

$$\lambda_{UV} = 420\text{nm} - 3\text{nm}$$
$$\nu_{UV} = 4.3 \cdot 10^{14} - 9.9 \cdot 10^{16}\,\text{Hz}$$

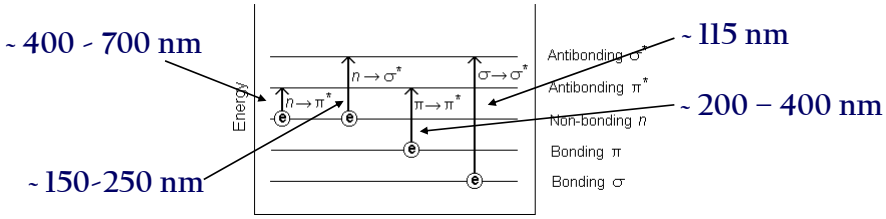
$$\lambda_{vis} = 700\text{nm} - 420\text{nm}$$
$$\nu_{vis} = 4.3 \cdot 10^{14} - 7.1 \cdot 10^{14}\,\text{Hz}$$

## Absorption: Physical Basis

Absorption occurs when the energy contained in a photon is absorbed by an electron resulting in a transition to an excited state

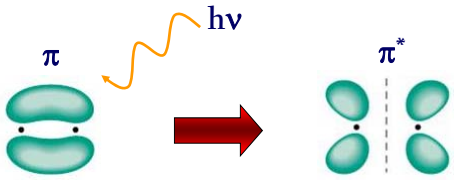
Since photon and electron energy levels are quantized, we can only get specific allowed transitions

$E = h\nu$ ($h = 6.626 * 10^{-34}$ Js)

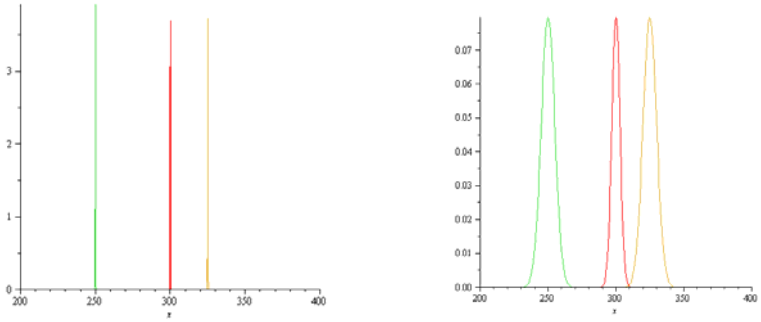



http://teaching.shu.ac.uk/hwb/chemistry/tutorials/molspec/uvvisab1.htm

## Absorption: Lineshape

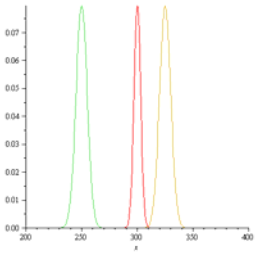



Absorption spectrum

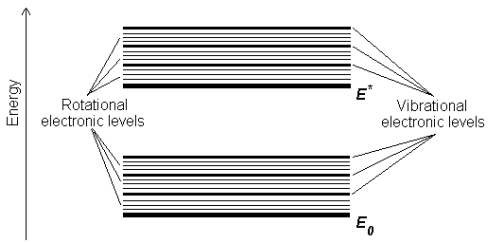


## Absorption: Lineshape

The lineshape of absorption spectra is normally distributed



Molecules are always rotating and vibrating. Each rotational or vibrational state slightly changes the energy of the transition.



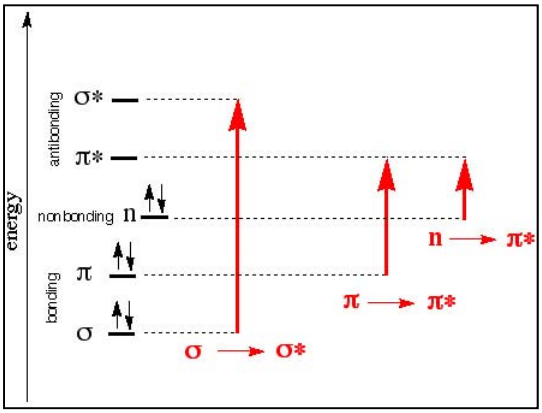
# *Electronic Transitions*

The following electronic transitions are possible:

$\pi \rightarrow \pi^*$ (pi to pi* transition)

$n \rightarrow \pi^*$ (n to pi star transition)

$\sigma \rightarrow \sigma^*$ (sigma to sigma star transition)

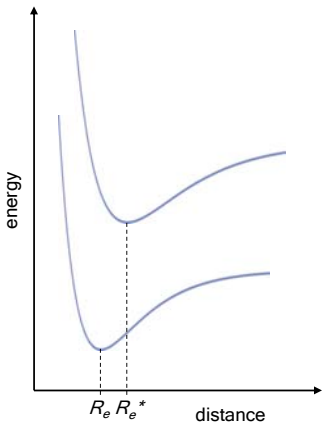



The **sigma to sigma*** transition requires an absorption of a photon with a wavelength which does not fall in the UV-vis range. Thus, only **pi to pi*** and **n to pi*** transitions occur in the UV-vis region are observed.

The absorption of EM radiation in the UV range has the effect of altering the distribution of the electrons (the external ones particularly) in the molecule.

As a consequence, the bond energy in the excited state of the molecule is in general different than in the ground state.

For the same reason, we can expect internuclear distances and vibrational force constants to be different in the ground state and excited states.



*B* - **Releasing E as *heat***

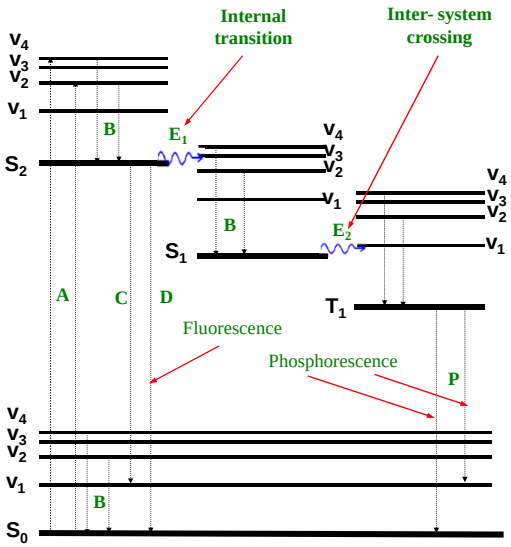
C - **Transfer remaining E to other chemical species by collision**

D – **Emit photons when falling back to the ground state - *Fluorescence***

$E_1$ - **Undergoing *internal transition* within the same mode of the excited state**

Process $E_2$ - **Undergoing *intersystem crossing* to a triplet sublevel of the excited state**

–Process P - **Radiating E from triplet to ground state (triplet quenching) - *Phosphorescence***




**Jablonski diagram**

•**Two types of *molecular emission spectra***

–***Fluorescence***

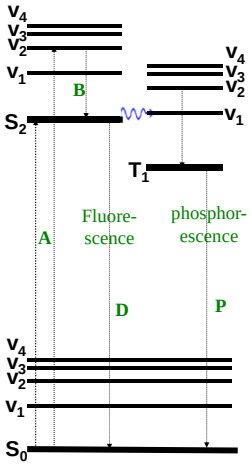
•In the case fluorescence the energy emitted can be the same or smaller (if heat is released before radiation) than the corresponding molecular absorption spectra.

e.g. absorption in UV region - emission in UV or visible region (the wavelength of visible region is longer than that of UV thus less energy)

•Fluorescence emission is generally short-lived (e.g. μs)

–***Phosphorescence***

•Phosphorescence generally takes much longer to complete (called *metastable*) than fluorescence because of the transition from triplet state to ground state involves altering the electron spin. If the emission is in visible light region, the light of excited material fades away gradually



# Definitions etc

- Chromophores
  - Part of molecule responsible for absorption
- Auxochromes
  - Groups that modify absorption of neighboring chromophores
  - Often have lone pairs, e.g. –OH, -OR, -$NR_2$, -halogen
  - Bathochromic shift: towards longer wavelength
  - Hypsochromic shift: towards shorter wavelength
  - Hyperchromic effect: increase in peak absorbance
  - Hypochromic effect: decrease in peak absorbance
- Typical for unconjugated organic molecules

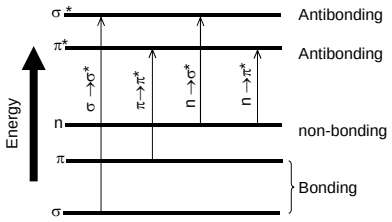
| | |
|---|---|
| $\sigma \rightarrow \sigma^*$ | below 150 nm |
| $n \rightarrow \sigma^*$, $\pi \rightarrow \pi^*$ | below 200 nm |
| $n \rightarrow \pi^*$ | 200-400nm |

**•Bonding appear in $\sigma$ & $\pi$ molecular orbitals; non-bonding in *n***

**•Antibonding orbitals correspond to the bonding ones**

**•Electronic transition can occur between various states; in general, the energy of transition increases in the following order:**

**$(n \rightarrow \pi^*) < (n \rightarrow \sigma^*) < (\pi \rightarrow \pi^*) < (\sigma \rightarrow \sigma^*)$**



The UV spectrum of a macromolecule never coincide with the sum of the contributions of the isolated chromophores. This observation suggests that the absorption properties of a chromophore are sensitive to the environment and, particularly, to:

-the interaction with the solvent,

-the interaction with other chromophores.

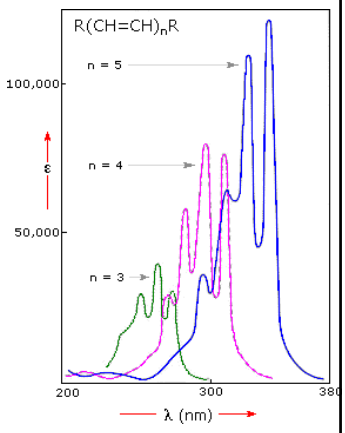
Both type of interactions are difficult to analyze and therefore their effects can not be easily predicted. Since they generally modify position, intensity and shape of absorption bands, they can be used to monitor the structural changes of a macromolecule during a physical-chemical process.

## Electronic Spectra of Polyatomic Molecules

UV absorption spectrum of a complex molecule can be related to the absorption properties of some chemical groups (*chromophores*) present in the molecule.

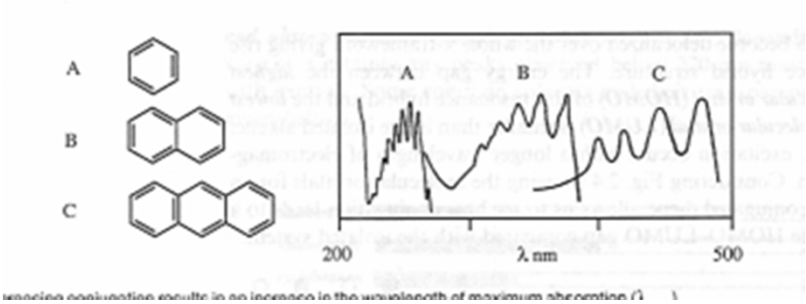
A chromophore is a chemical group which absorbs UV-visible radiation at a specific wavelength, with little influence from the other groups in the molecule.

Typical chromophores in organic molecules are C=C double bonds, C=O carboxylic groups and aromatic rings. Only when two or more of these groups are conjugated, a relevant change in their absorption properties is observed.



# Polycyclic aromatic hydrocarbons

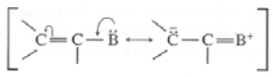
- Extensive conjugation leads to large bathochromic shift

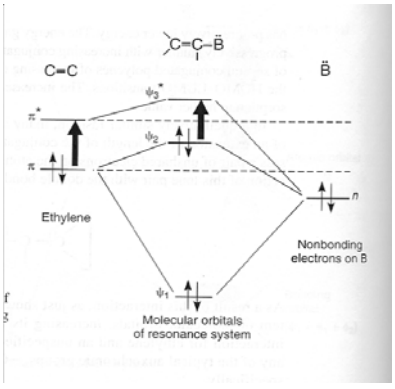



increasing conjugation results in an increase in the wavelength of maximum absorption ($\lambda_{max}$)

## Auxochromes

- Auxochromes with lone pairs often lead to increased delocalization (and conjugation) and therefore a bathochromic shift





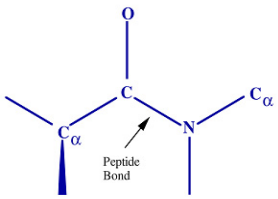
## UV Absorption of Biomolecules

For the majority of proteins and nucleic acids, the most biologically significant solvent is water, buffered at pH close to 7.0, in the presence of salt (~100 mM) to mimic conditions in vivo.

UV measurement are performed at wavelength $\lambda > 170$ nm.

UV spectra in water are usually broad, with practically no vibrational structure.

This is due to the strong polarity of the water molecule: because of the strong interactions with water, the energies of individual solute molecules differ from one another.




The Amide Chromophore

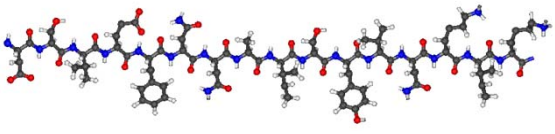
## UV Absorption of Biomolecules
## Proteins

Protein chromophores can be divided into two classes:

the peptide bond itself, and the amino acid sidechain.

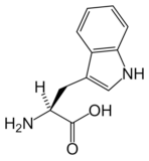
The UV spectrum of the peptide bond consists essentially of two bands:

- a strong band at ~ 190 nm ($\varepsilon_{max}$ ~ 7000)
- a weak (forbidden) band at ~ 210-220 nm ($\varepsilon_{max}$ ~ 100)

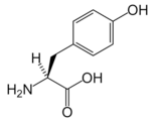


## UV spectroscopy of polypeptides and nucleic acids

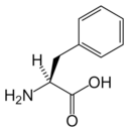
- Peptide bond absorption at ~ 190nm (not really usable)
- Tryptophan absorption at 280nm (ε=5600)
- Tyrosine, phenylalanine and cystine also have appreciable absorbances over 250 nm
- RNA/DNA at 250-275nm
- ProtParam (http://ca.expasy.org/tools/protparam-doc.html) allows one to calculate ε based on sequence – useful for concentration determination



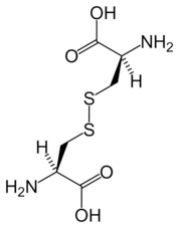
Tryptophan



Tyrosine



Phenylalanine

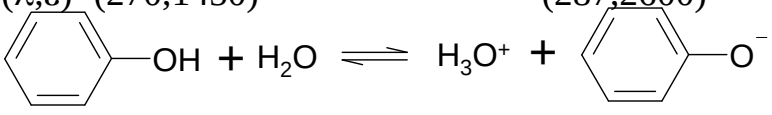


Cystine

## Solvent pH: effect on phenol spectrum

- Increasing pH shifts equilibrium to right
- More non-bonding electrons in phenoxide ion
  → higher extinction coefficient
  → greater delocalization → bathochromic shift

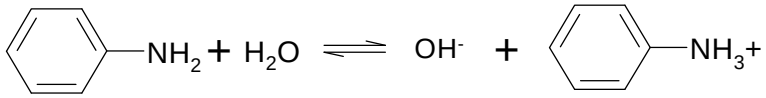
(λ,ε)=(270,1450) (287,2600)




Phenol Phenoxide ion

## Solvent pH: effect on aniline spectrum

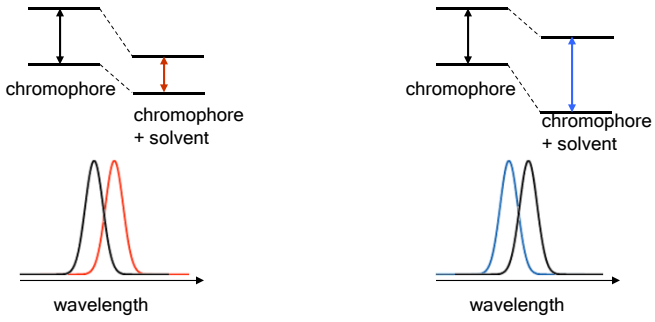
- Decreasing pH shifts equilibrium to right
- No non-bonding electrons in anilinium ion
  → lower extinction coefficient
  → less delocalization → hypsochromic shift
  (λ,ε)=(280,1430) (254,169)



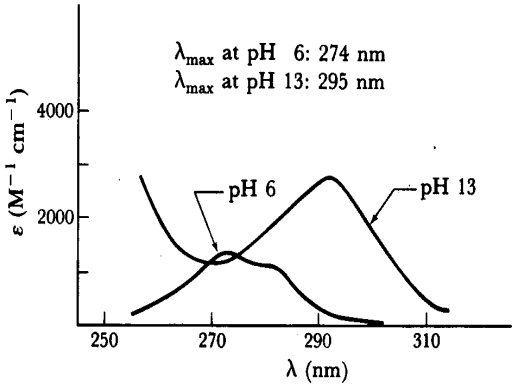

Aniline Anilinium ion

## UV Absorption of Biomolecules
## Effects of local environment

The interaction with the solvent has the effect of stabilizing (destabilizing) the electronic excited states relative to the ground state of the chromophore. Whether the interaction results in a stabilization rather than destabilization depends on the chromophore, on the solvent, and on the electronic transition in exam.



Dependence of tyrosine spectrum on pH




Exposure of tyrosine can also be detected by examining the spectrum of a protein at higher pH. If a tyrosyl residue is solvent-accessible, the side-chain will be deprotonated with a $pK_a$ of approximately 10, resulting in a large "red shift" (shift in $\lambda_{max}$ to higher wavelength) and substantial increase in intensity. As in other solvent-perturbation experiments, it is assumed that the overall conformation is unchanged by the increase in pH.

# Polarity effects of the solvent

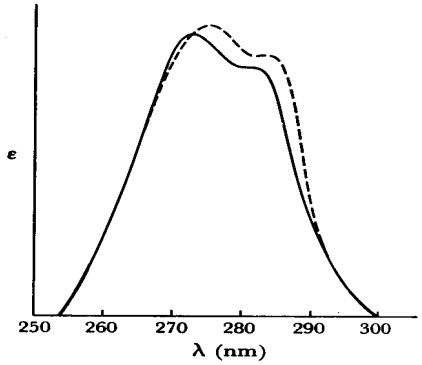
- $\pi^*$ more polar than $\pi$ in polar molecules
  $\rightarrow \pi^*$ better stabilized than $\pi$ in polar solvent
  $\rightarrow \pi \rightarrow \pi^*$ transition undergoes bathochromic shift with increasing polarity of solvent
- Hydrogen bonding stabilizes n more than $\pi^*$ in polar solvents
  $\rightarrow$ n $\rightarrow \pi^*$ undegoes hypsochromic shift
  $\rightarrow$peak absorbance is reduced due to stabilization of non-bonding electrons

Determine whether chromophores are solvent-accessible.

Solvent-perturbation studies. Nonpolar solvents are added to the macromolecular solution (typically at concentrations between 5-20%), and the resulting alterations in absorbance are recorded.

Assumptions of technique:

i. Only exposed chromophores are affected.
ii. No preferential solvation of macromolecule occurs
iii. Perturbing solvent does not alter native conformation.




The impact of a less polar solvent on the spectrum of tyrosine. Notice the increase in $\lambda_{max}$ in the presence of 20% ethylene glycol (dashed line).

## Difference spectrum

- Perturbing solvent: 80% water + 20% substance of reduced polarity (e.g. ethylene glycol)
- $\Delta A = A_{ps} - A_{water}$
- $\Delta A = 0 \rightarrow$ sample unaffected by perturbant
- Ex: $\Delta A = 1$ for free tryptophan but in a protein with 5 Trp it is 0.6 when measured using eth. gly. (4.3Å) and 0.4 when measured using glycerol(5.2 Å):
  for eth gly: (0.6/1) x 5 = 3 Trp accessible
  for glycerol: (0.4/1) x 5 = 2 Trp accessible
  $\rightarrow$ 3 – 2 = 1 Trp lies in a crevice which is between 4.3 Å and 5.2 Å in diameter

# Main points

- Main use is the determination of concentration
- Relatively broad spectra – not as useful as many other techniques for the identification of molecules
- Conjugation of double bonds leads to a bathochromic shift of the absorption spectra
- Environmental factors such as solvent pH and polarity may influence the absorption, and can therefore be used to extract information indirectly about the absorbing species

A molecule absorbs EM radiation with $\lambda_{max}$=280 nm in cyclohexane. Assume that, in water, the only solvent effect is due to the formation of a hydrogen bond between water and the excited state (but not the ground state) of the molecule. If the hydrogen bond has 5 kcal/mol of energy, predict $\lambda_{max}$ of the molecule in water.

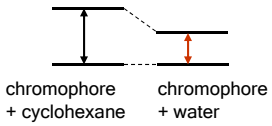
$$E_{water} = E_{cyclohexane} - E_{Hbond}$$

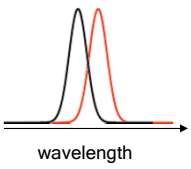
$$E_{Hbond} = 5 \cdot 10^3 \cdot \frac{4.184}{6.023 \cdot 10^{23}} = 3.474 \cdot 10^{-20}\ \text{J}$$

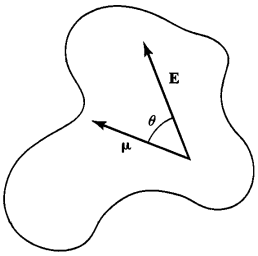
$$E_{cyclohexane} = h\frac{c}{\lambda} = 7.094 \cdot 10^{-19}\ \text{J}$$

$$E_{water} = E_{cyclohexane} - E_{Hbond} = 6.747 \cdot 10^{-19}\ \text{J}$$

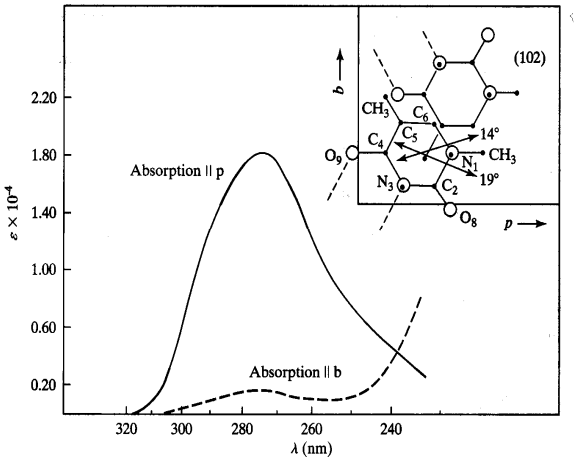
$$\lambda_{\text{max}}^{\text{water}} = h\frac{c}{E_{water}} = 294\,\text{nm}$$





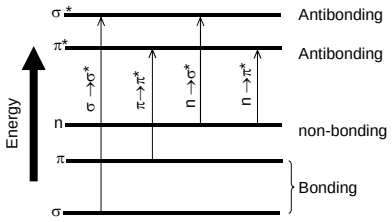

When the electric vector of the light is aligned with the transition dipole of the molecule the probability of absorption is a maximum




Polarized absorption spectra for crystals of 1-methylthymine.

- **Bonding appear in $\sigma$ & $\pi$ molecular orbitals; non-bonding in *n***
- **Antibonding orbitals correspond to the bonding ones**
- **Electronic transition can occur between various states; in general, the energy of transition increases in the following order:**

$$(n \rightarrow \pi^*) < (n \rightarrow \sigma^*) < (\pi \rightarrow \pi^*) < (\sigma \rightarrow \sigma^*)$$



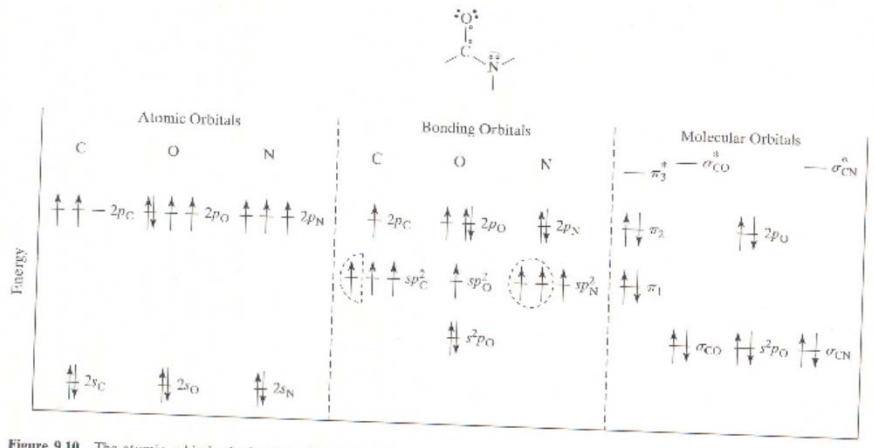
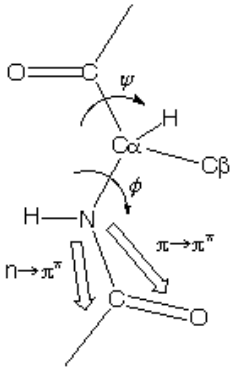



**Figure 9.10** The atomic orbitals, the hybrid orbitals formed for bonding, and resulting molecular orbitals for the three atoms in the amide chromophore. [Reprinted from W. C. Johnson, "Circular Dichroism and its Empirical Application to Biopolymers." *Methods of Biochemical Analysis*, vol. 31, ed. D. Glick, copyright 1985 by John Wiley & Sons.]




**n -> π* centered around 220 nm**

**π -> π* centered around 190 nm**

**n -> π* involves non-bonding electrons of O of the carbonyl**
**π -> π* involves the π-electrons of the carbonyl**

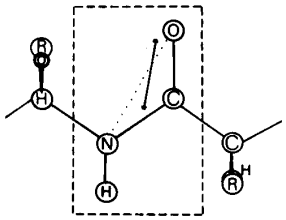
**The intensity and energy of these transitions depends on ϕ and ψ**

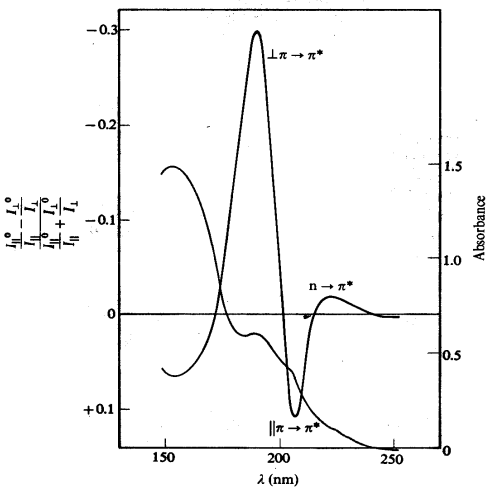
Biological Applications of Electronic Spectroscopy

a. Biologically relevant chromophores.

Peptide bond can undergo n→π*, π→π* transitions

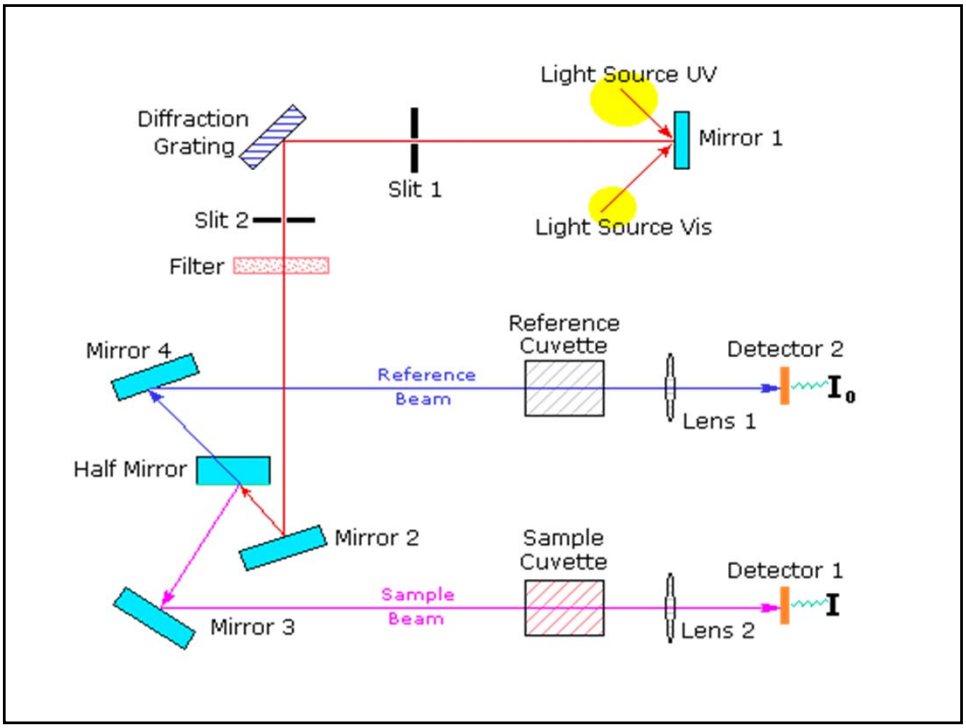
Existence of multiple transitions revealed by *linear dichroism*.





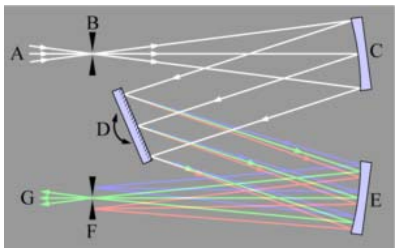

## UV/Visible Spectroscopy: Instrumentation

- In absorption spectroscopy, we measure ε as a function of wavelength λ
- The instrument we use to do this is called a UV/visible Spectrophotometer
- The Major Components Are:
  - A light source
  - A monochromator
  - A Sample Compartment
  - A detector




## Monochromators

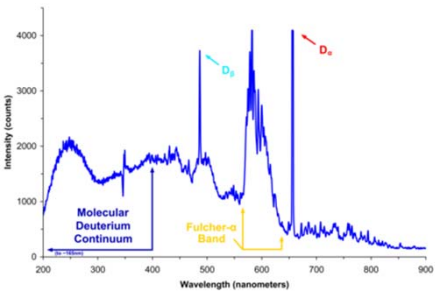
- The light sources we use produce continuous emission spectra. To get single wavelengths...
  - Czerny-Turner setup
  - B and C set up a beam that is at infinite focus
  - D is either a prism or a diffraction grating




  - E refocuses one wavelength on slit F
  - Different wavelengths can be focused on F by rotating D or E (usually D).

## UV/Visible Spectroscopy: Light Sources

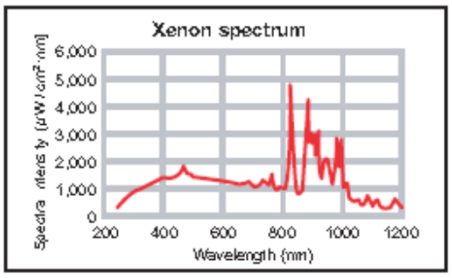
- Deuterium




- $D_2$ gas is discharged by contact with a high voltage tungsten cathode
- Continuous spectrum from ~150 nm - ~370 nm
- Usually used in conjunction with a tungsten/halogen source, which handles the visible spectrum

## UV/Visible Spectroscopy: Light Sources

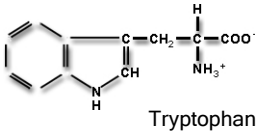
- Xenon, Mercury/Xenon



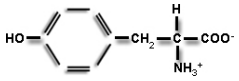

- Flash Arc-Lamps – light generated from Xe plasma
- Pure Xenon has very wide emission spectrum ~200 – 1200 nm
- Xenon/Mercury is blue shifted for more power in the UV region

## UV Absorption of Biomolecules
## Proteins

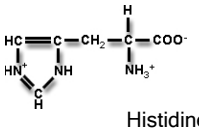
Some of the sidechains absorb UV radiation in the same region as the peptide bond (Asp, Glu, Asn, Gln, Arg and His). More interesting are the aromatic sidechains (Trp, Phe, Tyr, His) which absorb in the region 230nm –300nm (near UV) and therefore do not overlap with the strong peptide absorption band.



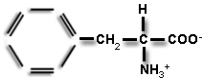
Tryptophan



Tyrosine



Histidine

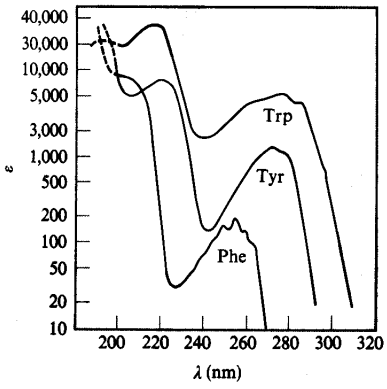


Phenylalanine

Aromatic amino acid side-chains. The side-chains of Phe, Tyr and Trp absorb in the near UV range between 250-300 nm.

Forbidden transitions can become "allowed" by two mechanisms which effectively lower the symmetry of the system:

- "vibronic coupling": coupling of an electronic transition with a change in vibrational energy level.
- mixing of orbitals



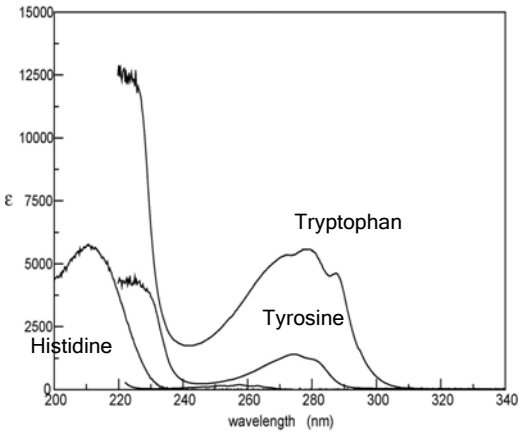

| residue | wavelength | ε ($M^{-1}cm^{-1}$) |
|---|---|---|
| phenylalanine | 258 | 200 |
| tyrosine | 274 | 1400 |
| tryptophan | 280 | 5600 |
| cystine (-S-S-) | 280 | 300 |

# UV Absorption of Biomolecules
## Proteins

In the absence of prosthetic groups, an average protein has maximum absorption in the near UV at approx. 280 nm. If the extinction coefficient $\varepsilon^{280}$ of the protein is known, it is possible to derive the protein concentration.

If $\varepsilon^{280}$ is not known, it is possible to estimate the concentration.

At this wavelength only Tyr and Trp contribute to the absorption spectrum, with absorption coefficients of ~ 1300 and 5700 $M^{-1}$ $cm^{-1}$, respectively. It is possible to roughly estimate the resulting extinction coefficient as:




$$\varepsilon_{prot}^{280} = n_{Trp}\varepsilon_{Trp}^{280} + n_{Tyr}\varepsilon_{Tyr}^{280}$$

# Nucleic Acids

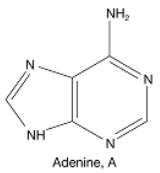
Absorbance between 250 - 300 nm due to the aromatic purine and pyrimidine bases.

- Undergo a series of overlapping $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions.

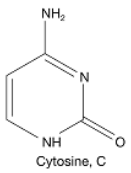
- Absorption is relatively strong, due to inherently lower symmetry:

| | |
|---|---|
| Adenosine | 14,900 $M^{-1}$ $cm^{-1}$ |
| Cytidine | 9,100 $M^{-1}$ $cm^{-1}$ |
| Guanosine | 9,000 $M^{-1}$ $cm^{-1}$ |
| Thymidine | 9,700 $M^{-1}$ $cm^{-1}$ |

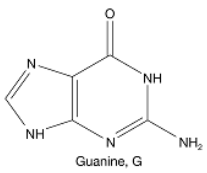
- Absorption properties strongly influenced by interactions with neighboring chromophores.



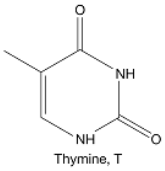
Adenine, A



Cytosine, C



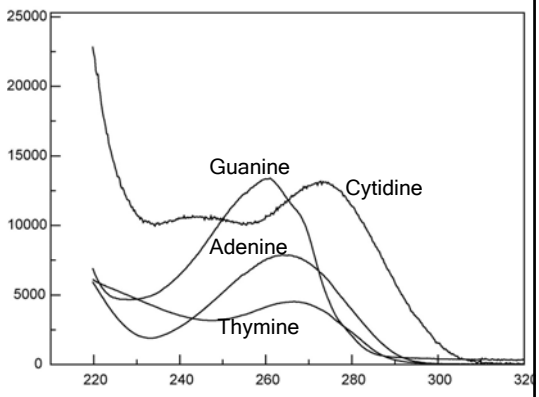
Guanine, G



Thymine, T

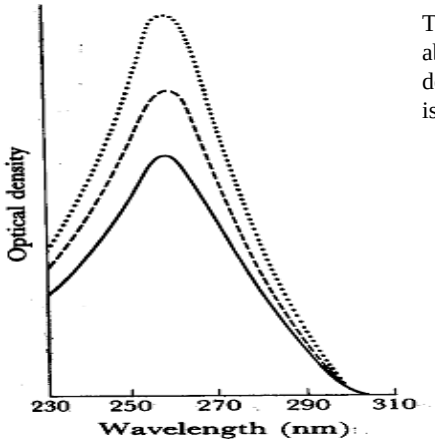
UV Absorption of Biomolecules
Nucleic Acids

Despite their rather simple appearance, these four bands result from a number of different electronic transitions taking place in the four aromatic rings. Usually in a DNA or RNA fragments their spectra merge into a single band with $\lambda_{max}$ at ~260 nm so that it is not easy to separate the different contributions. Since $\varepsilon^{280}$~10000 on average, accurate measurement of the UV spectra at concentrations as low as few $\mu g\ ml^{-1}$ are possible.



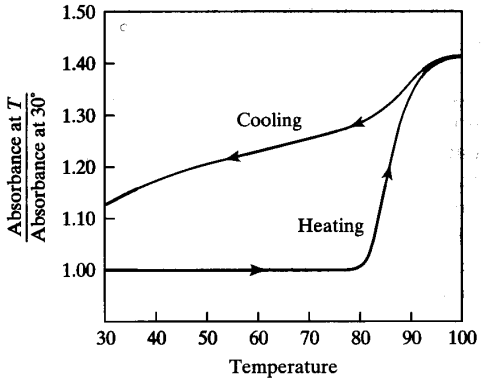

**Examine macromolecular stability.**

Denaturation of nucleic acids and proteins can be monitored by UV spectroscopy.




The figure shows the relative absorbance of double-stranded, single-stranded and isolated nucleotides

When double-stranded nucleic acids denature, their absorbance at 260 nm increases. The figure shows the relative absorbance of double-stranded (solid), single-stranded (dashed), and isolated nucleotides (dotted).



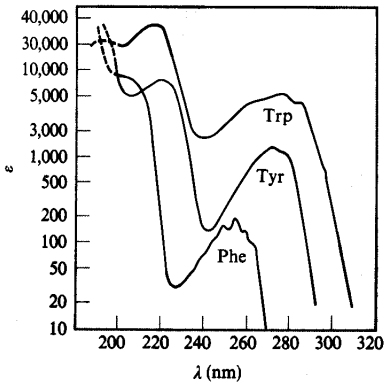

As a result of the pronounced hypochromism of double-stranded DNA, denaturation (strand-separation) is accompanied by a substantial increase in absorbance at 260 nm.

The melting temperature ($T_m$) is defined as the temperature at which the denaturation is half-complete. The steepness of the melting curve is an indication of the *cooperativity* ("all-or-none" character) of the denaturation.

Aromatic amino acid side-chains. The side-chains of Phe, Tyr and Trp absorb in the near UV range between 250-300 nm.

Forbidden transitions can become "allowed" by two mechanisms which effectively lower the symmetry of the system:

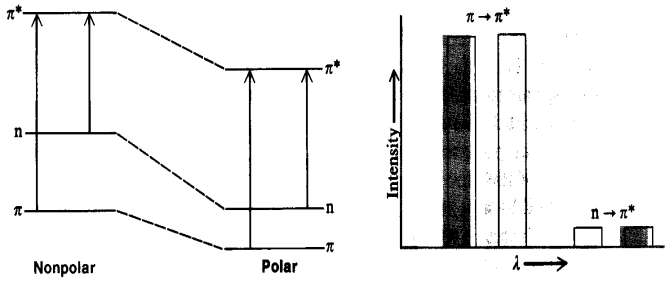
- "vibronic coupling": coupling of an electronic transition with a change in vibrational energy level.
- mixing of orbitals




| residue | wavelength | ε ($M^{-1}cm^{-1}$) |
|---|---|---|
| phenylalanine | 258 | 200 |
| tyrosine | 274 | 1400 |
| tryptophan | 280 | 5600 |
| cystine (-S-S-) | 280 | 300 |

The influence of nonpolar solvent addition is generally attributed to the differential polarity of the ground- and excited state orbitals.
Relative polarities of n, π, and π* orbitals: **π < π* < n**.
**n orbitals interact the most strongly with polar solvents, followed by π *, then by π.**

Based on these considerations, π→π* transitions should exhibit a blue shift with addition of nonpolar solvent; n→π* transitions should exhibit a red shift.



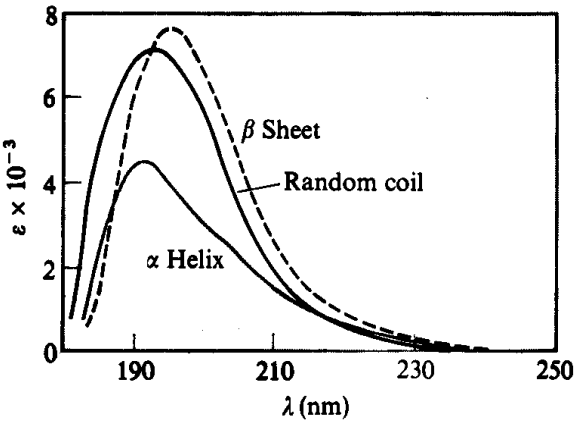

Sometime the π→π* transitions of tyrosine and tryptophan in proteins are red shifted in less polar solvents.
Influence of hydrogen bonding, which may preferentially stabilize the ground state in the purely aqueous solution, not considered

**poly-L-lysine {$(Lys)_n$} can adopt 3 different conformations merely by varying the pH and temperature**

**random coil at pH 7.0**

**α-helix at pH 10.8**

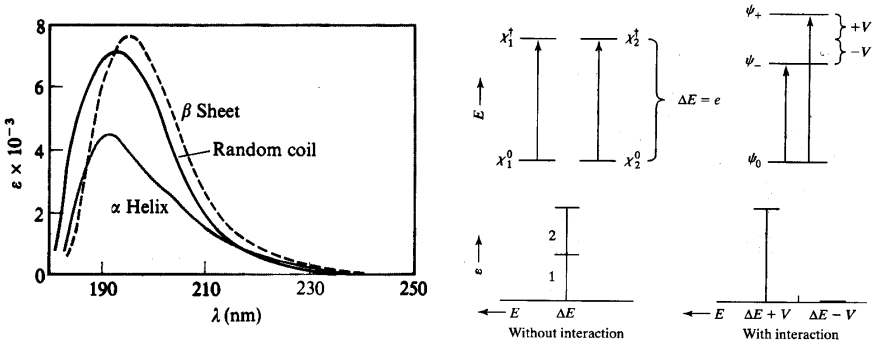
**β-form at pH 11.1 after heating to 52°C and recooling**




The unfolding of proteins can also be monitored by absorption spectroscopy. The exposure of buried aromatic chromophores that accompanies denaturation can produce detectable changes.

If the protein has substantial helical content, denaturation will be accompanied by a significant increase in absorption in the far UV because the random coil peptide bond absorbs more strongly than the α-helical peptide bond.

The peptide bond absorbance spectrum is strongly influenced by secondary structure, a consequence of *exciton splitting*. This means that an interaction between the transition dipoles of neighboring chromophores can cause an absorbance band to split into two components that will differ in intensity.



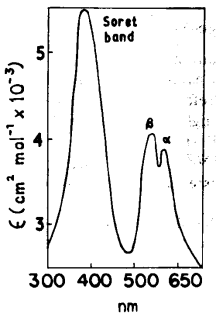

Spectroscopic properties of proteins containing prosthetic groups

| Protein | Prosthetic group | Longest-wavelength absorption band | | Second-longest absorption band | |
|---|---|---|---|---|---|
| | | $\lambda_{max}$ (nm) | $\varepsilon_{max}$ ($\times 10^{-4}$) | $\lambda_{max}$ (nm) | $\varepsilon_{max}$ ($\times 10^{-4}$) |
| Amino acid oxidase, rat kidney | FMN | 455 | 1.27 | 358 | 1.07 |
| Azurin, *P. fluorescens* | $Cu^{II}$ | 781 | 0.32 | 625 | 0.35 |
| Ceruloplasmin, human | 8 Coppers (3 distinct classes) | 794 | 2.2 | 610 | 1.13 |
| Cytochrome *c*, reduced, human | $Fe^{II}$-heme | 550 | 2.77 | — | — |
| Ferredoxin, *Scenedesmus* | (2 $Fe^{III}$, 2 sulfide) cluster | 421 | 0.98 | 330 | 1.33 |
| Flavodoxin, *C. pasteurianium* | FMN | 443 | 0.91 | 372 | 0.79 |
| Monoamine oxidase, bovine kidney | Flavins plus Cu | 455 | 4.7 | — | — |
| Pyruvic dehydrogenase, *E. coli* | FAD | 460 | 1.27 | 438 | 1.46 |
| Rhodopsin, bovine | Retinal-Lys | 498 | 4.2 | 350 | 1.1 |
| Reubredoxin, *M. aerogenes* | ($Fe^{III}$, 4 Cys) tetrahedron | 570 | 0.35 | 490 | 0.76 |
| Threonine deaminase, *E. coli* | 4 Pyridoxal phosphates | 415 | 2.6 | — | — |
| Xanthine oxidase | Fe, Mo | 550 | 2.2 | — | — |

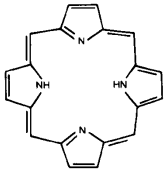
Conjugation of double bonds shifts the wavelength of maximum absorption to longer wavelengths.

Using the particle-in-a-box analogy, each additional conjugated bond significantly increases the size of the box. As a rule of thumb, each additional double bond shifts the wavelength maximum 25-30 nm to the red.

Thus,
ethylene absorbs at 195 nm,
1,3-butadiene at 220 nm,
1,3,5-hexatriene at 250 nm and
the porphyrin ring system at 550-600 nm.



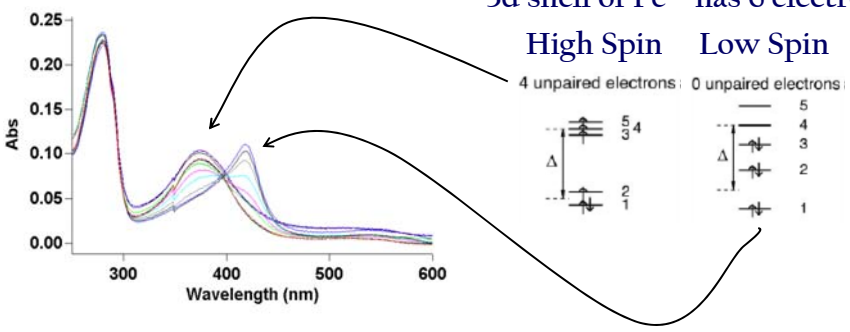

Spectrum of Zn-porphyrin




## UV/visible: Applications

- UV/visible - use in current research for heme-containing proteins, which have absorbance in the Soret region that is sensitive to the state of the protein

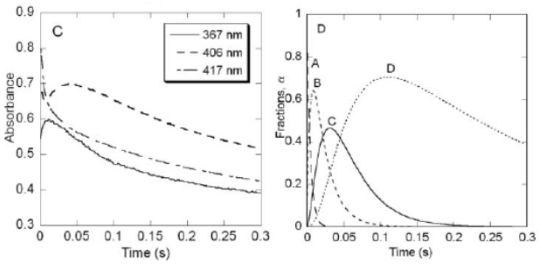
3d shell of $Fe^{2+}$ has 6 electrons

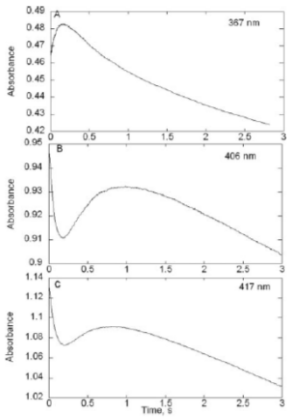


# Time-resolved UV/visible

The main protein signal (at 280 nm) doesn't change much with protein folding/activity

But Soret region absorption does (cytochrome $P450_{CAM}$)

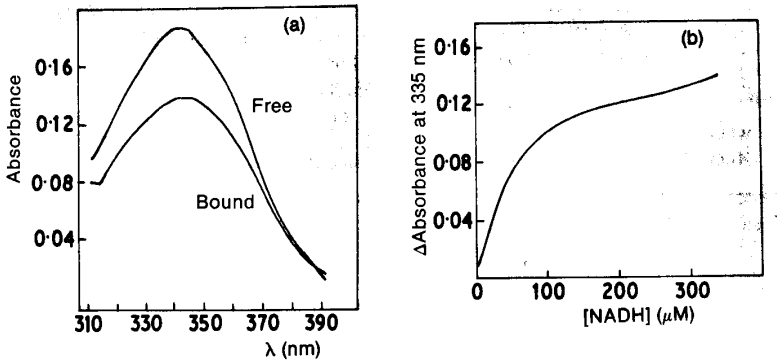



*J. Biol. Chem.* 280 (21): 20300-20309 (2005)

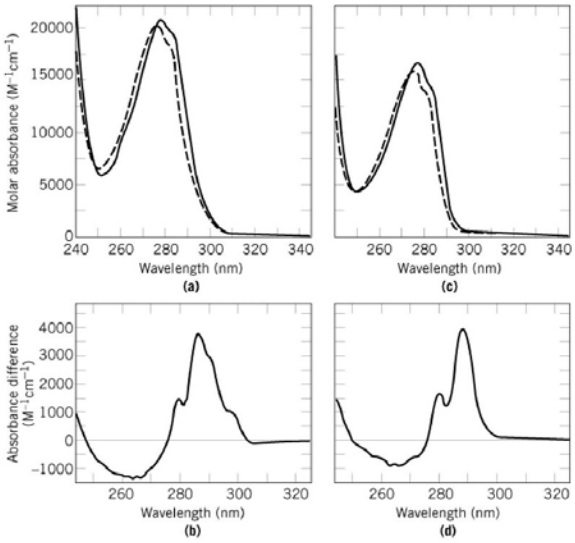
Identification. The absorption spectrum of a substance can offer insight into its identity.
E.g., Cytochromes are distinguished on the basis of their absorbance spectra.
The absorption spectrum can also be used to monitor purification progress.

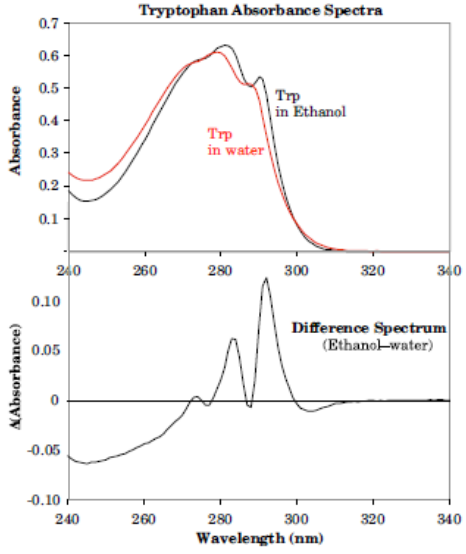
Monitor ligand-binding. Requires only that the spectrum of either the macromolecule or ligand undergo a measurable alteration (change in λ or ε) upon binding.




(a) Spectra of NADH (30 μM), both free and when bound to an excess of the tetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase. (b) Progress of titration monitored at 335 nm.

**Ultraviolet absorption spectra of (a) the wild-type form and (c) the Trp59Tyr variant of RNase Tr**



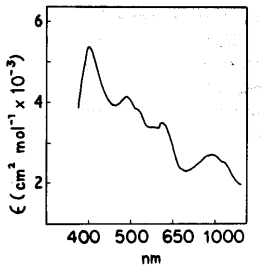
Metal Ions. Absorbance by transition metal ions can be due to either of two types of transition

i. **d-d transitions** -- i.e., transitions between the occupied and unoccupied d orbitals
   - Symmetry forbidden, so generally weak.
   - Spectral properties depend on the ligands and their arrangement around the metal ion.
   - Ligands may be placed in a spectrochemical series:

$I^- < Br^- < Cl^- < SCN^- < F^- < OH^- <$ oxalate $< H_2O < NCS^- < NH_3 \approx$ pyridine < ethylenediamine < dipyridyl < o-phenanthroline $< NO_2^- < CN^- \approx CO$.

From left to right , the effect of the ligands is to shift the absorption bands to higher energy (shorter $\lambda$)

ii. **Charge-transfer transitions** -- occur when an electron on the metal ion is promoted into an unoccupied orbital on the ligand.
   - Very intense ($\varepsilon_{max} = 10^3 - 10^4\ M^{-1}\ cm^{-1}$), since symmetries of metal ion and ligand orbitals are quite different.
   - Most commonly appear in near UV and tail into the visible region. However, charge transfer bands of iron (III) porphyrin extend into the near IR (1000 nm)




Spectrum of Fe(III) heme protein

# UV/Visible Applications

- iron-sulfur clusters in a native and a mutant protein

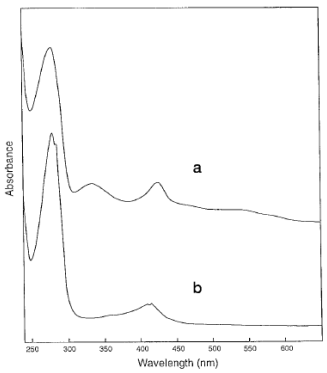
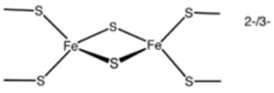



FIG. 1. UV-visible absorption spectra of purified recombinant *Drosophila* ferrochelatase (a) and C196S mutant human ferrochelatase (b).




Absorption band between 400-600 nm which is diagnostic of an 2Fe-2S cluster

C196S Mutant?

*JBC* (1998)Vol. 273, pp. 22311–22316

**Applications**

a) Requires only a knowledge of the extinction coefficient

b) In principle, solutions containing more than a single component can also be quantitated. Requires only that the spectra of the components of interest differ and that their $\varepsilon$ values are known at a suitable number of wavelengths.

c) As a rule of thumb, $A_{280}$ = 1 for a 1 mg/mL protein solution.
$A_{260}$ = 20 for a 1 mg/mL solution of DNA.

d) Enzyme assays (quantitation of product formation or substrate utilization).
e.g., measure production/utilization of NADH at 340 nm ($\varepsilon$ = 6.2 $mM^{-1}$ $cm^{-1}$)

e) Monitoring growth of microorganisms by monitoring $OD_{600}$. (light scattering.)
$OD_{600} = \tau c l$ where $\tau$ is analogous to $\varepsilon$

**Disadvantages:**

i. Must work in absorbance range in which concentration is proportional to absorbance (i.e., Beer's Law is obeyed.)
   - Threshold for deviations is strongly system- and instrument-dependent.
   - Causes of deviations include: "stray light", scattering, interactions.

ii. Absorbing impurities can cause error (detergents; other proteins, DNA).

iii. Single-wavelength measurements (at low absorbance values) can have errors.

The D and A usually form 1:1 complexes according to:

$$A + D \leftrightarrow DA$$

where the donor and acceptor are in equilibrium according to:

$$K_a = \frac{[DA]}{[A][D]}$$

The objective of the experiment is to obtain the equilibrium constant for a CT complex and the molar absorptivity of its CT transition.

## OPTICAL SPECTROSCOPY OF CT COMPLEXES

The total amount of donor and acceptor molecules, regardless of association, are $[D]_o$ and $[A]_o$, respectively. Then the equilibrium constant becomes:

$$K_a = \frac{[DA]}{([A]_o - [DA])([D]_o - [DA])}$$

At the CT transition frequency, the Lambert-Beer Law gives the concentration of the complex:

$$[DA] = \frac{\mathrm{A}_{DA}^{\lambda_{CT}}}{\varepsilon_{DA}^{\lambda_{CT}}\,\ell}$$

Inserting the spectroscopic values for $[DA]$ into the equilibrium constant expression gives:

$$\frac{[A]_o\,\ell}{\mathrm{A}} = \frac{1}{K_a\,\varepsilon_{DA}^{\lambda_{CT}}\,[D]_o} + \frac{[A]_o}{[D]_o\,\varepsilon_{DA}^{\lambda_{CT}}} + \frac{1}{\varepsilon_{DA}^{\lambda_{CT}}} + \frac{[DA]}{\varepsilon_{DA}^{\lambda_{CT}}\,[D]_o}$$

This may be simplified by taking $[D]_o >> [A]_o$ but keeping both of these concentrations relatively small.

These assumptions produce the Benesi-Hildebrand equation:

$$\frac{[A]_o \ell}{\mathrm{A}} = \frac{1}{K_a \varepsilon_{DA}^{\lambda_{CT}} [D]_o} + \frac{1}{\varepsilon_{DA}^{\lambda_{CT}}}$$

Linear plot with a slope of $1/K_a\varepsilon$ and an intercept of $1/\varepsilon$

Measurement of the absorbance of a series of solutions of differing concentrations of donor and acceptor will provide both the equilibrium constant for the complex as well as the molar absorptivity of the CT transition.

**Table 2**
Diffusion coefficients of APA-1, APA-2 and APA-3, before and after the addition of DNA at pH 4.7 and 7.4 and at body temperature (37 °C).

| Compound | $D_o$ ($cm^2\ s^{-1}$) at pH 4.7 | | $D_o$ ($cm^2\ s^{-1}$) at pH 4.7 | |
|---|---|---|---|---|
| | Before the addition of DNA | After the addition of DNA | Before the addition of DNA | After the addition of DNA |
| APA-1 | $2.46 \times 10^{-10}$ | $1.25 \times 10^{-11}$ | $1.09 \times 10^{-09}$ | $6.82 \times 10^{-12}$ |
| APA-2 | $2.70 \times 10^{-10}$ | $2.66 \times 10^{-11}$ | $6.53 \times 10^{-10}$ | $1.74 \times 10^{-11}$ |
| APA-3 | $1.02 \times 10^{-10}$ | $5.85 \times 10^{-11}$ | $2.11 \times 10^{-10}$ | $4.41 \times 10^{-11}$ |

*3.4. Evaluation of binding constants of compounds to DNA by UV-spectroscopy*

Binding constant "$K_b$" of compound–DNA complex can be determined from the variation in absorbance in UV–visible spectra before and after the addition of DNA. Binding constants for the three compounds were evaluated spectrophotometrically under physiological conditions of pH (4.7 and 7.4) and temperature (37 °C) by applying Benesi–Hildebrand equation [4,5,39].

$$\frac{A_0}{A - A_0} = \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} + \frac{\epsilon_G}{\epsilon_{H-G} - \epsilon_G} \frac{1}{K_b[\mathrm{DNA}]} \quad (2)$$

where $A_0$ and $A$ are the absorbance's of compound and complex respectively, $E_G$ and $E_{H-G}$ the molar extinction coefficients of compound and complex respectively. From the plot of $A_0/(A-A_0)$ to 1/[DNA], the ratio of the intercept to the slope gave the values of binding constant, $K_b$, Fig. 5.

The Binding constant values were calculated and given in Table 3. The order of magnitude of binding constant $K_b$ at both the pH values ($10^4\ M^{-1}$) for all the investigated compounds with DNA revealed their stronger binding via intercalation and is in agreement with that reported for typical intercalator lumazine ($K = 1.74 \times 10^4\ M^{-1}$) and anthracycline molecules ($K \approx 10^4$–$10^5$ $M^{-1}$) [39,40]. Binding constants at stomach pH (4.7) for all the compounds were evaluated greater, while at pH 7.4 compound APA-3 showed comparatively greater binding constants than that of typical intercalator lumazine, Table 3. Further, the spectral changes in absorption spectra during compound–DNA complex formation in present work inferred intercalation as possible mode of interaction. In addition, the greater binding constants of investigated compounds at both the pH values may be attributed to their structural planarity due to phenyl groups in the investigated compounds.

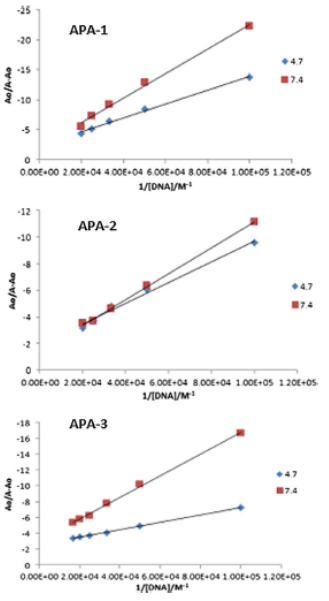



**Fig. 5.** Plots of $A_0/A-A_0$ vs. 1/[DNA] for the application of Benesi-Hildebrand equation for calculation of compound-DNA binding constant at pH 4.7 and 7.4 and at 37 °C.

N. Arshad et al. / Journal of Photochemistry and Photobiology B: Biology 125 (2013) 70–82