

# Metody biofyzikální chemie



## UV-Vis spectra

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1. Basic principles
2. QM of interaction between light and molecules
3. Absorption spectroscopy of electronic states
4. Instrumentation - spectrophotometry
5. Spectroscopic analysis of biopolymers
6. Effect of conformation on absorption

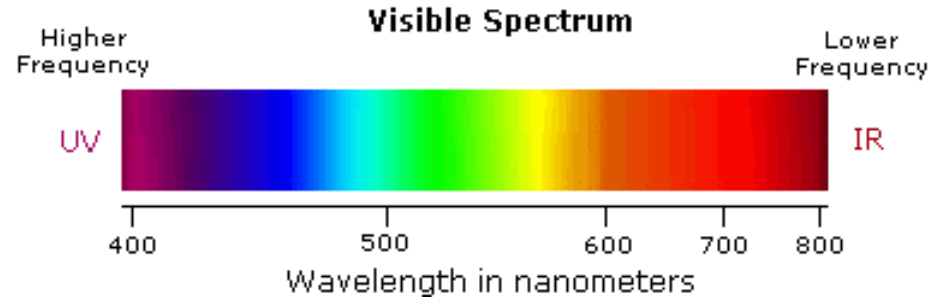
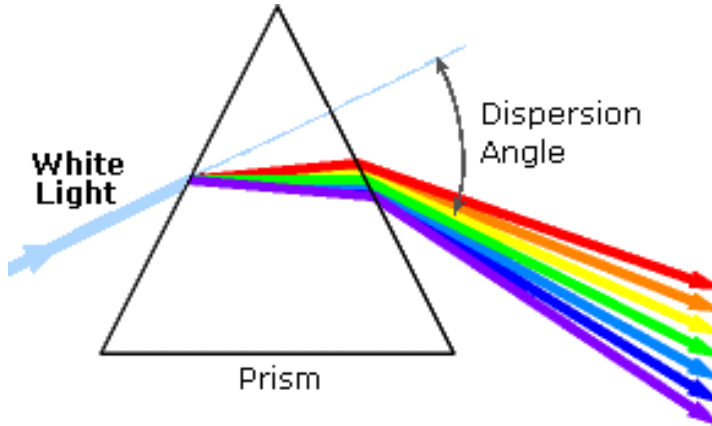
Gauglitz G., Vo-Dinh T.: Handbook of Spectroscopy. Wiley VCH Verlag, Weinheim 2003

Invitrogen Tutorials: [www.invitrogen.com/site/us/en/home/support/Tutorials.html](http://www.invitrogen.com/site/us/en/home/support/Tutorials.html)

# A very brief history of the study of light

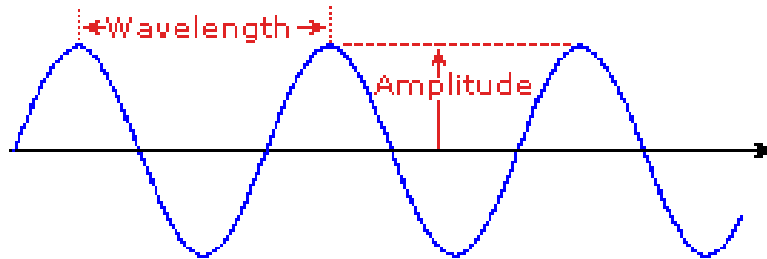
## 1. *Sir Isaac Newton 1672:*

Showed that the component colors of the visible portion of white light can be separated through a prism, which acts to bend the light (refraction) in differing degrees according to the wavelength. Developed a “corpuscular” theory of light.



## 2. *Christian Huygens 1692:*

Developed a wave theory of light



## 3. *Hans Christian Oersted 1820*

Showed that there is a magnetic field associated with the flow of electric current

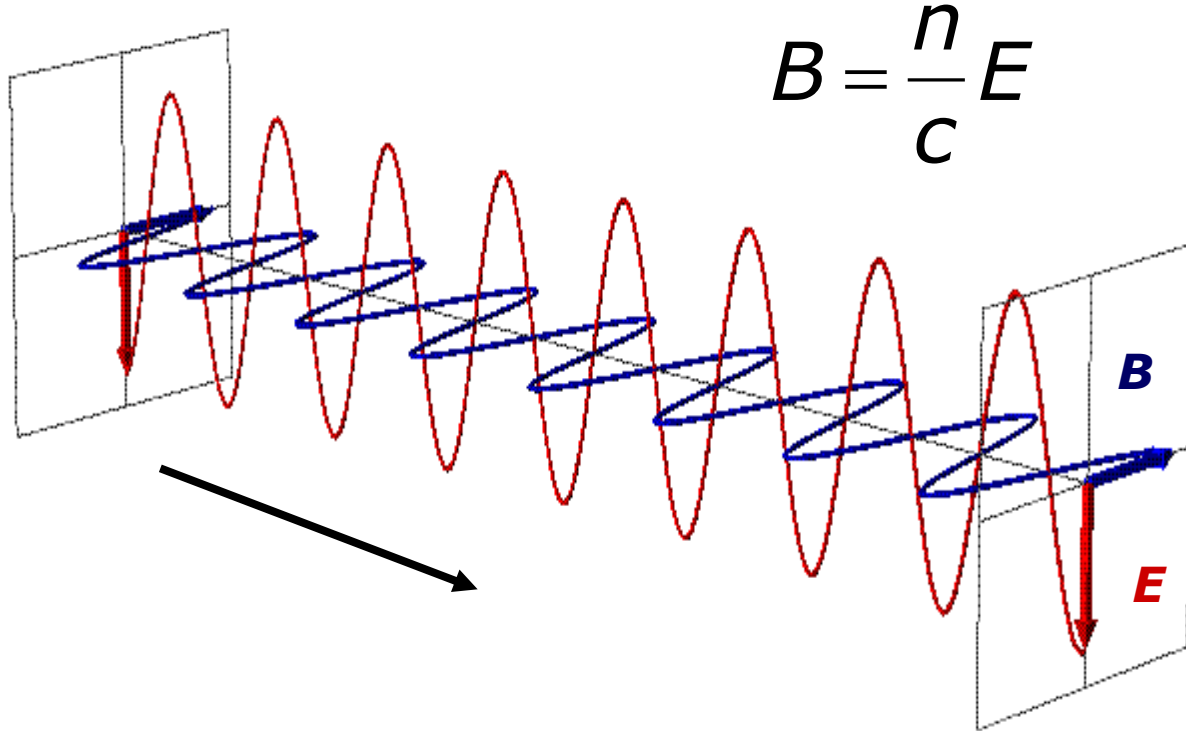
## 4. *Michael Faraday 1831*

Showed the converse i.e. that there is an electric current associated with a change of magnetic field

## 5. James Clerk Maxwell: (1831-1879)

Published his “Dynamical theory of the electromagnetic field” which combined the discoveries of Newton, Young, Foucault, Oersted and Faraday into a unified theory of electromagnetic radiation

Light consists of electromagnetic transverse waves of frequency  $\nu$  and wavelength  $\lambda$  related by  $\lambda\nu = nc$  where  $n$  is the index of refraction of the medium and  $c$  is the speed of the light in vacuum  $c = 3 \times 10^{10}$  cm/s



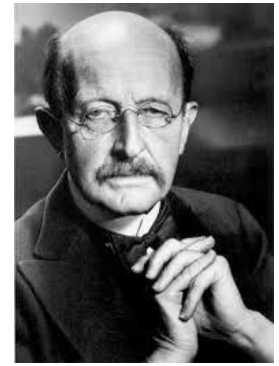
*James Clerk Maxwell.*

we are interested in interactions of the electric field with the matter

**6. Max Karl Ernst Ludwig Planck: (1858-1947)**

**NP: 1918**

Explained the laws of black body radiation by postulating that electromagnetic radiation is emitted at discrete energetic quanta  $E = h\nu$ , where Planck constant  $h = 6.6256 \cdot 10^{-34}$  Js.

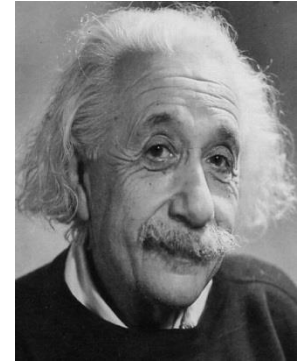


*M. Planck*

**7. Albert Einstein: (1879-1955)**

**NP:1921**

Explained the explained the photoelectric effect by assuming that light is adsorbed at discrete energetic quanta  $E = h\nu$ , photons.



**8. Louis Victor Pierre Raymond de Broglie: (1892-1987)**

**NP:1929**

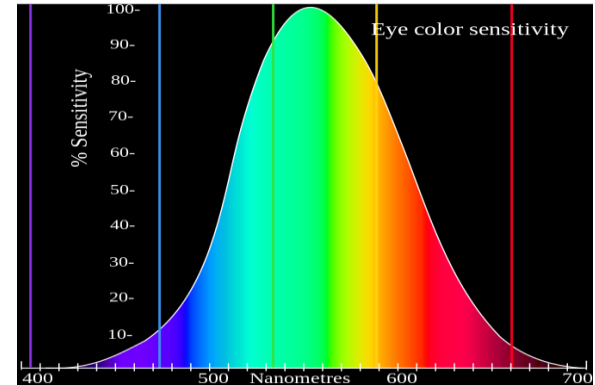
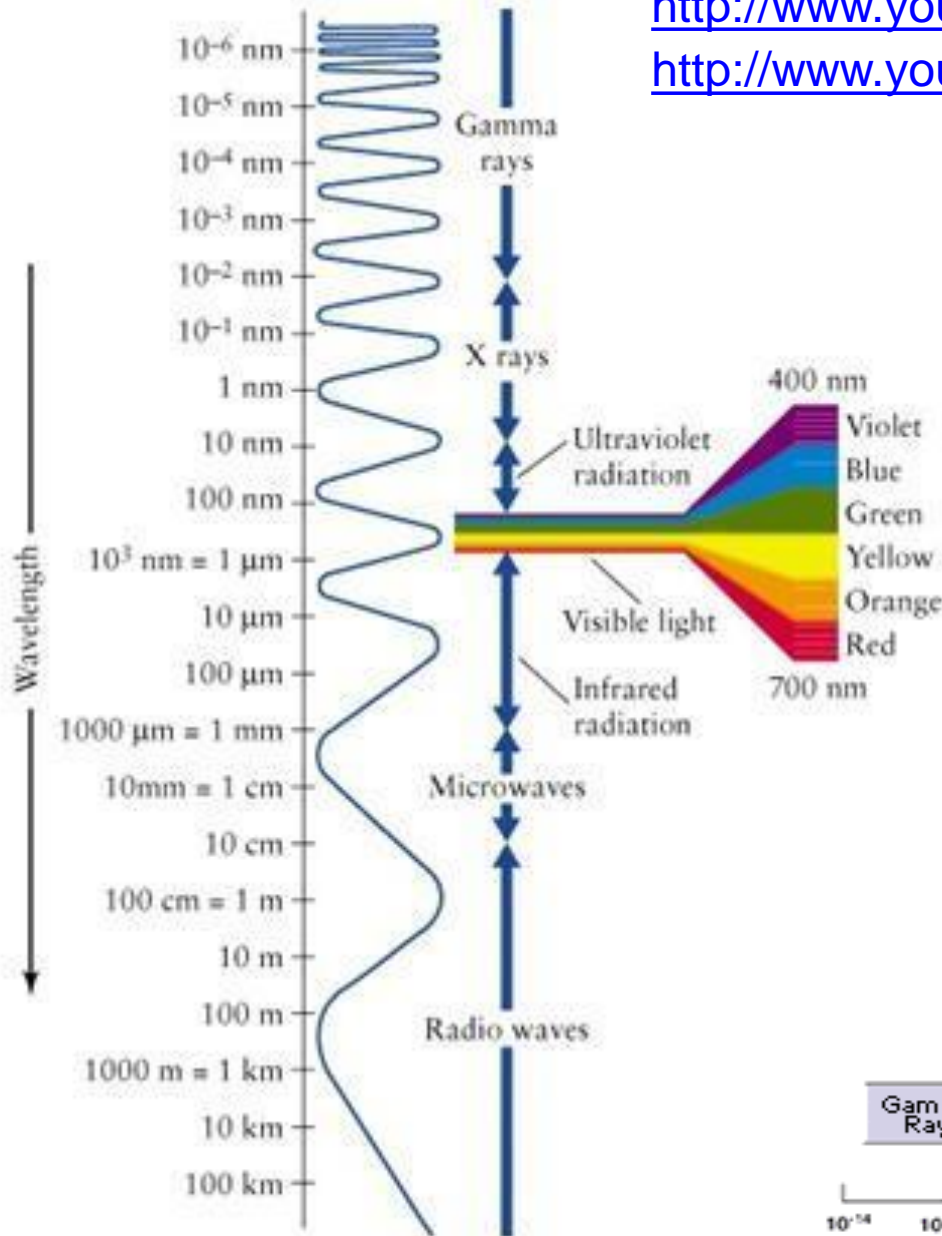
Introduced properties of electromagnetic waves to all particles – the wave-corpusecular dualism of quantum physics. A freely moving particle of momentum  $p$  has wavelength  $\lambda = h/p$ .



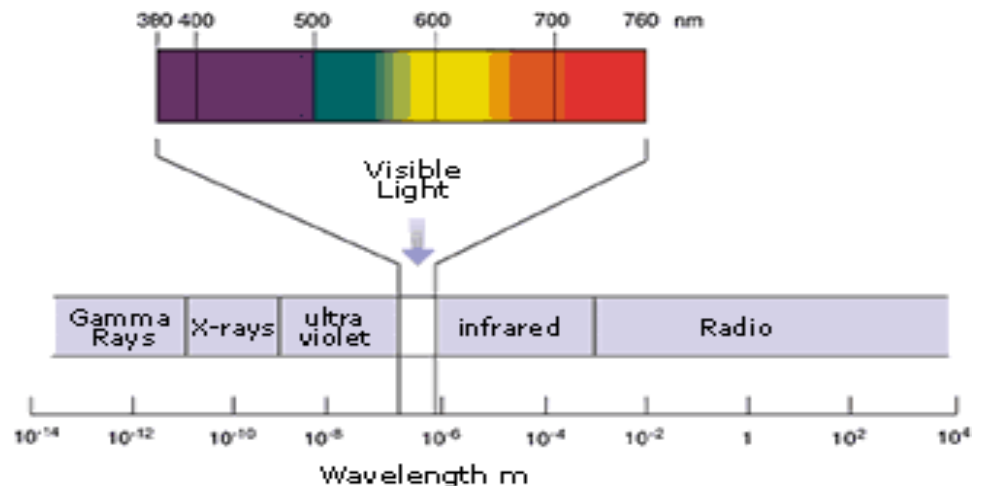
# The electromagnetic spectrum

<http://www.youtube.com/watch?v=cfXzwh3KadE>

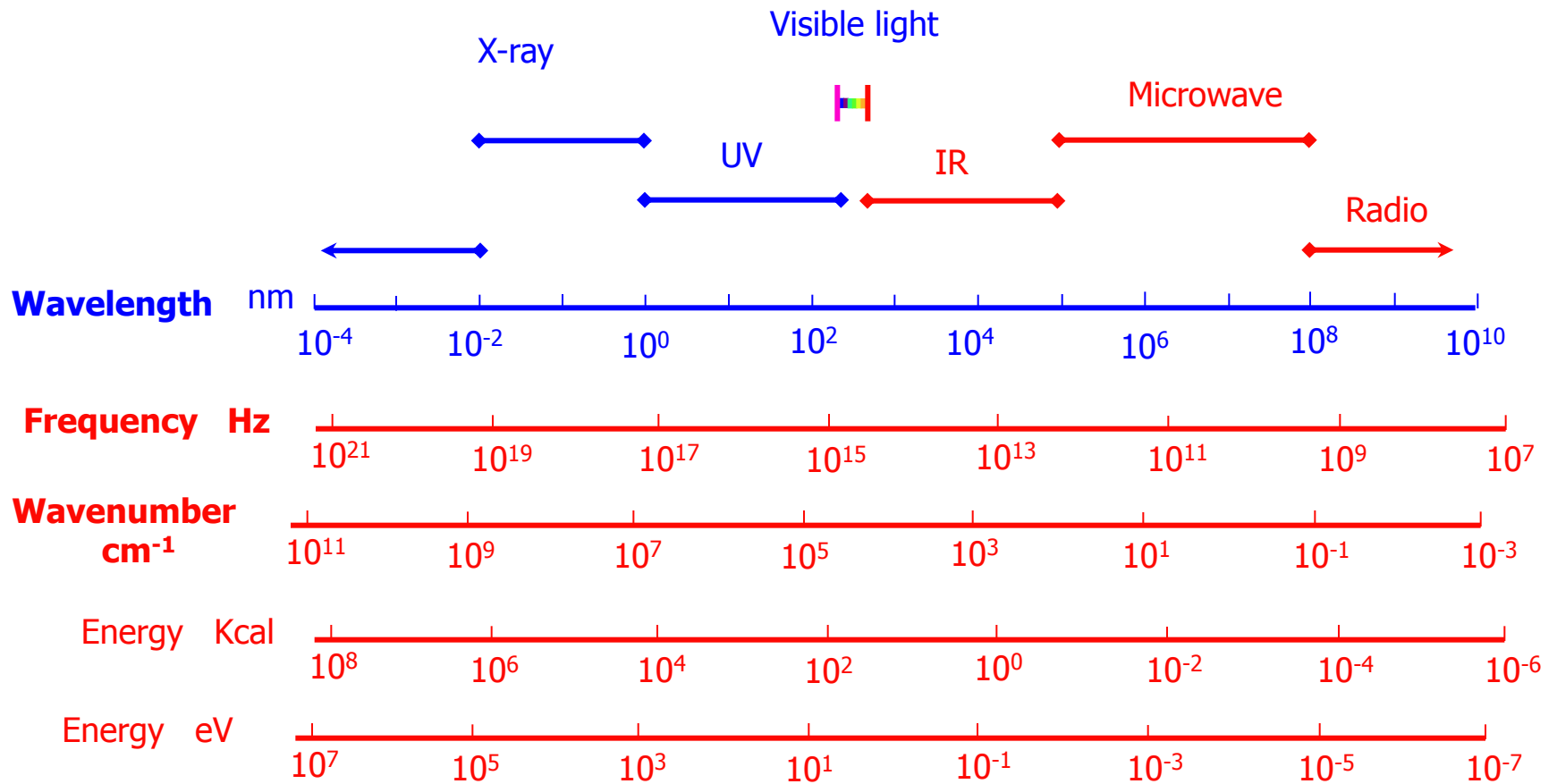
<http://www.youtube.com/watch?v=m4t7gTmBK3g>



Relative brightness sensitivity of the human visual system as a function of wavelength

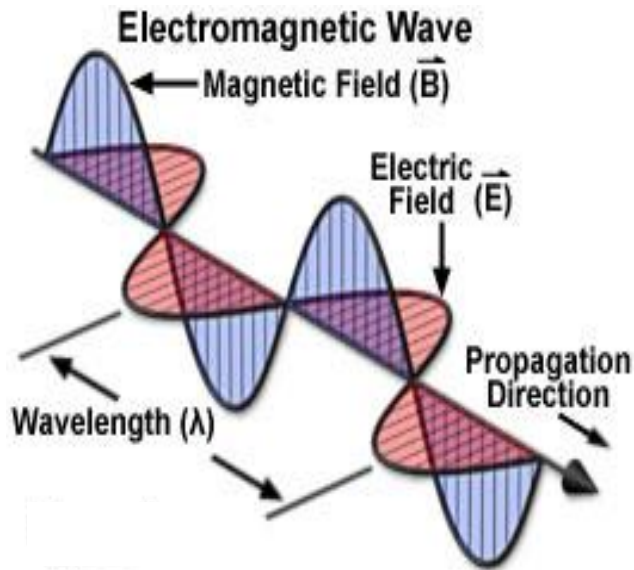


# Wavelength and energy scale, appropriate units



# What is light?

According to Maxwell, light is an electromagnetic field characterized by a frequency  $\nu$ , velocity  $c$ , and wavelength  $\lambda$ . Light obeys the relationships:



induction

intensity

$$\vec{D} = \epsilon \vec{E}$$

$$\vec{B} = \mu \vec{H}$$

electro

magneto

$$\epsilon_0 = 8.859 \cdot 10^{-12} \text{ Fm}^{-1}$$

$$\mu_0 = 4\pi \cdot 10^{-7} \text{ Hm}^{-1}$$

$$\frac{1}{\sqrt{\epsilon_0 \mu_0}} = c$$

$\epsilon \rightarrow$  permittivity

$\mu \rightarrow$  permeability

<http://www.edumedia-sciences.com/en/a185-transverse-electromagnetic-wave>

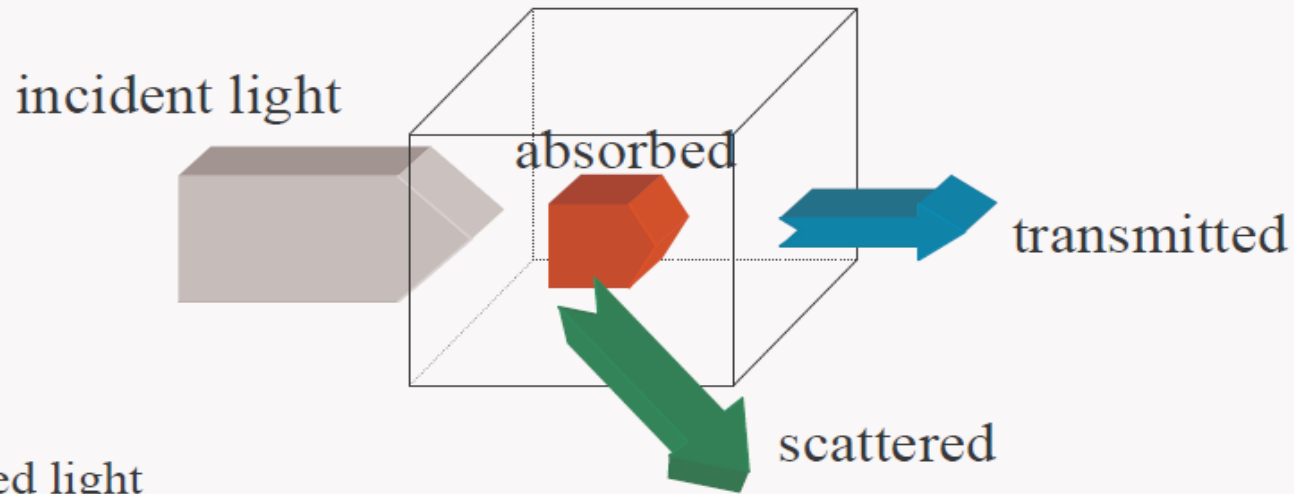
$$E = h\nu = h \frac{c}{\lambda}$$

$$\nu = \frac{c}{\lambda} \quad \tilde{\nu} = \frac{1}{\lambda} \quad \nu = \tilde{\nu} \cdot c$$

h.....Planck constant  $6.63 \cdot 10^{-34} \text{ J.s}$

# Light

- Consider a beam of light on a material
  - It can be scattered, absorbed, or transmitted

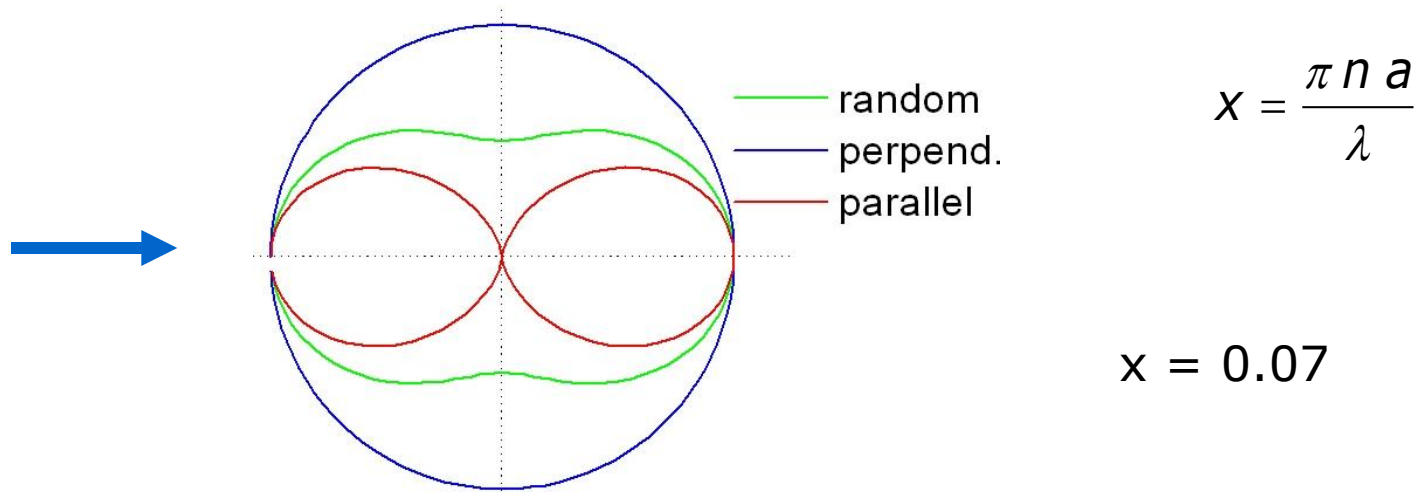


- Transmitted light
  - Light emerges propagating in the same direction as the incident light
- Absorbed light
  - Energy from light is absorbed in the volume of the material
- Scattered light
  - Light emerges in a different direction from the incident light

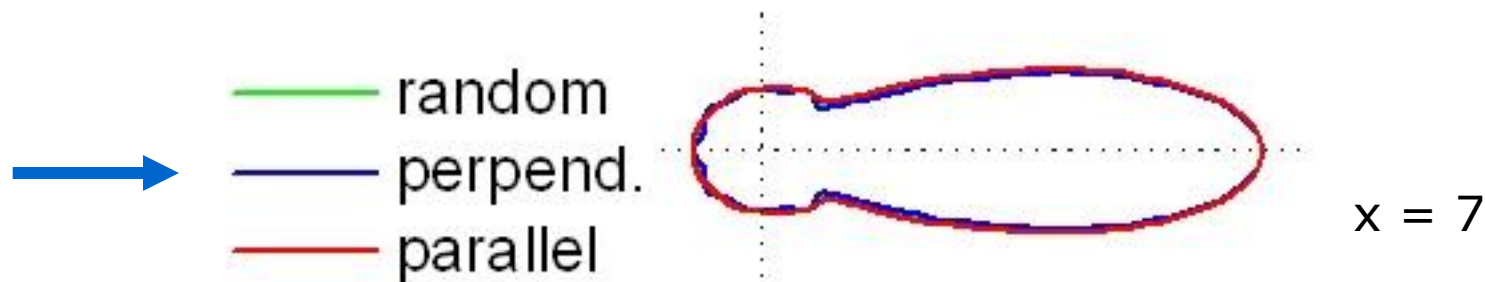


# Elastic scattering of light

- Rayleigh scattering – small molecules ( $x < 0.3$ ) as a “point dipole”,  $I_{sc} \approx \nu^4 \rightarrow$  blue sky, red sunset



- Larger scatterers – macromolecules, cells, Mie theory for spherically symmetrical scatterers

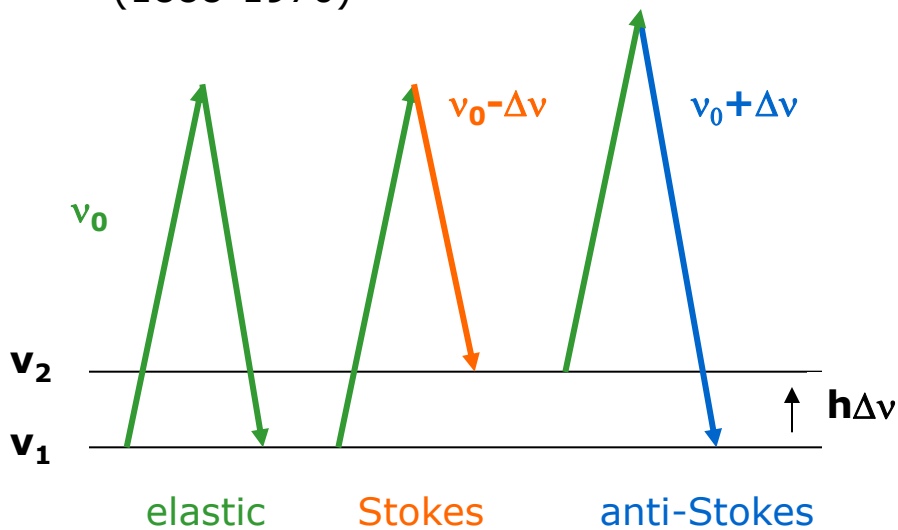


# Raman scattering



C.V. Raman  
(1888-1970)

- 1923 theoretically predicted by Adolf Smekal using classical physics
- 1928 observed by C. V. Raman



branch of Raman spectrum

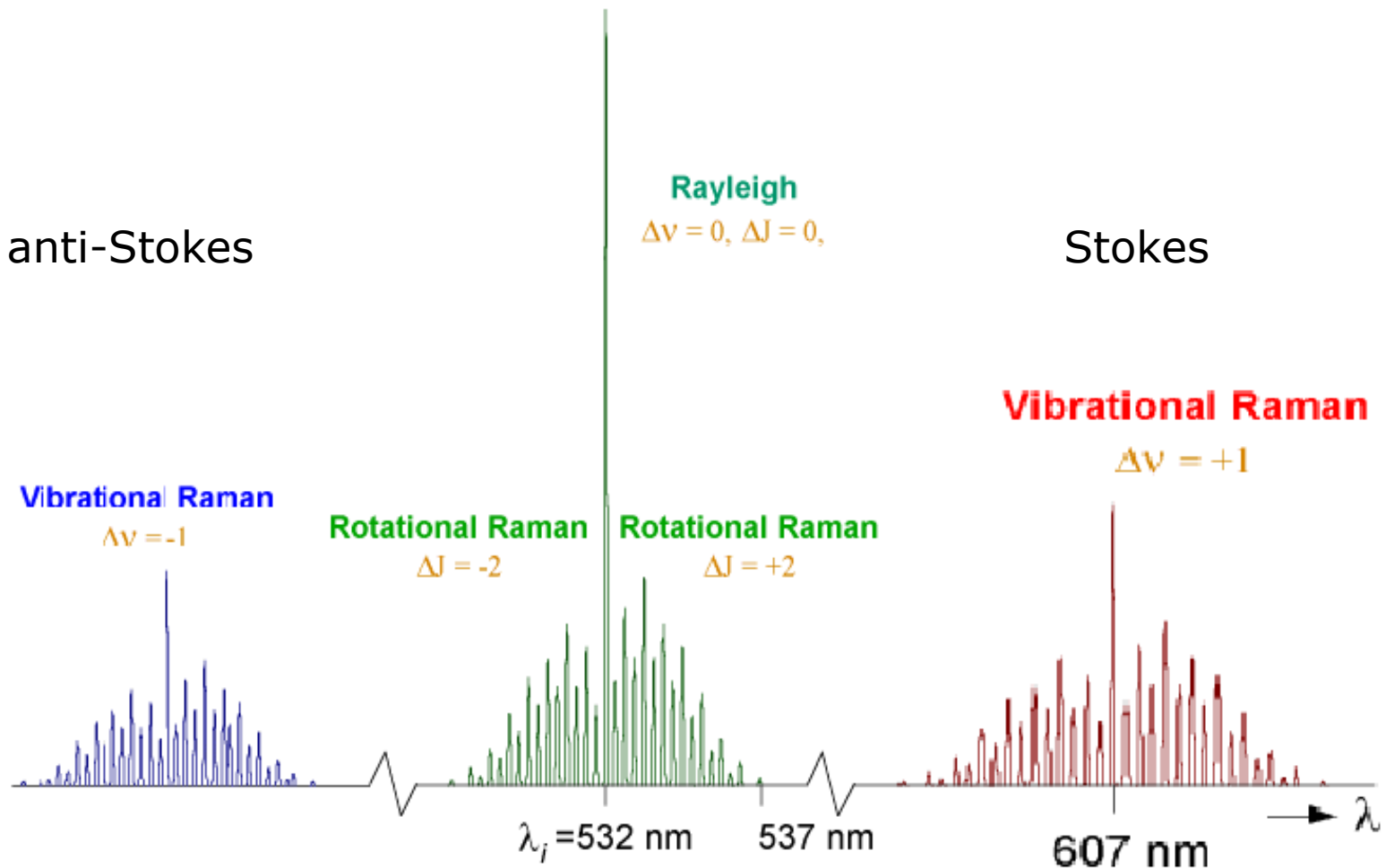
the photon and the molecule exchange energy

the photon is not absorbed:

scattering is an instantaneous and coherent

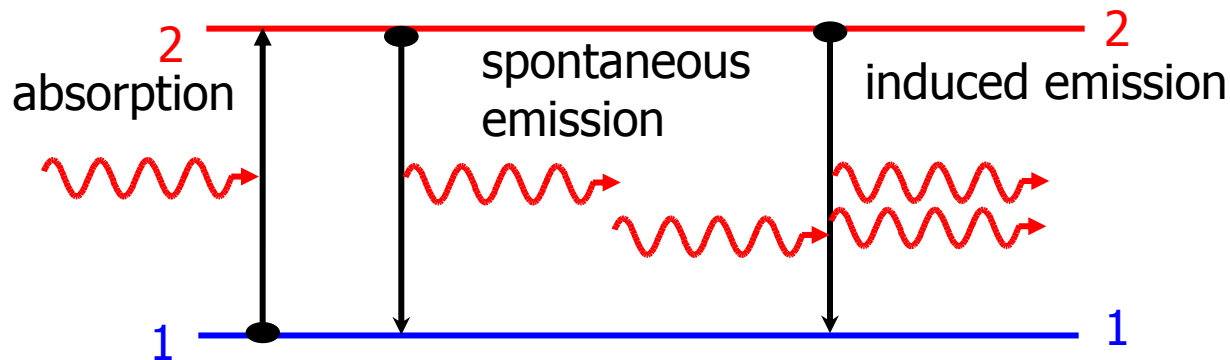
# Raman spectrum

intensity of Stokes branch is higher by a factor  $\left(\frac{2\nu_0 - \Delta\nu}{2\nu_0 + \Delta\nu}\right)^4 \exp\left(\frac{h\Delta\nu}{2kT}\right)$



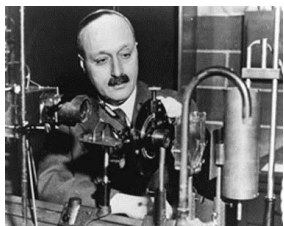
# Interaction of light with matter – overview of processes

- elastic scattering – no exchange of energy between the molecule and the photon
- inelastic (Raman) scattering – the photon either gives a part of its energy to the molecule or vice versa
- absorption or emission of photons by the molecule



- induced emission is coherent with incident light
- spontaneous emission by individual molecules is incoherent
- scattering is **coherent** and **instantaneous**

# Franck-Condon (FC) Principle

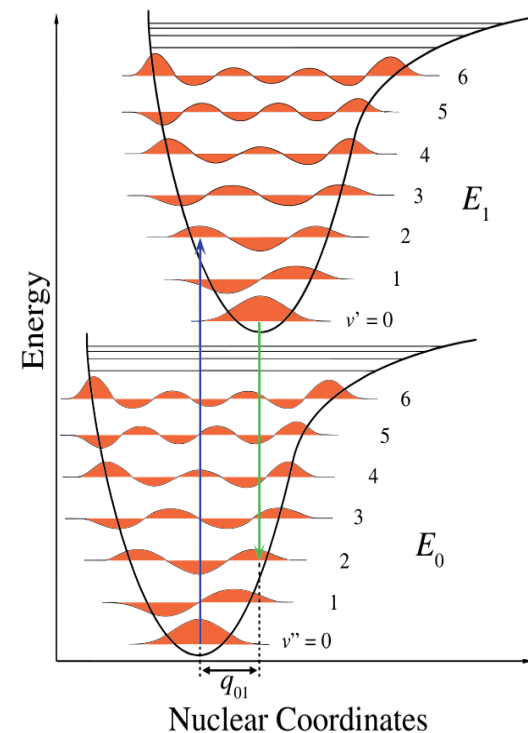


James Franck (1882-1964)



Edward Uhler Condon 1902-1974

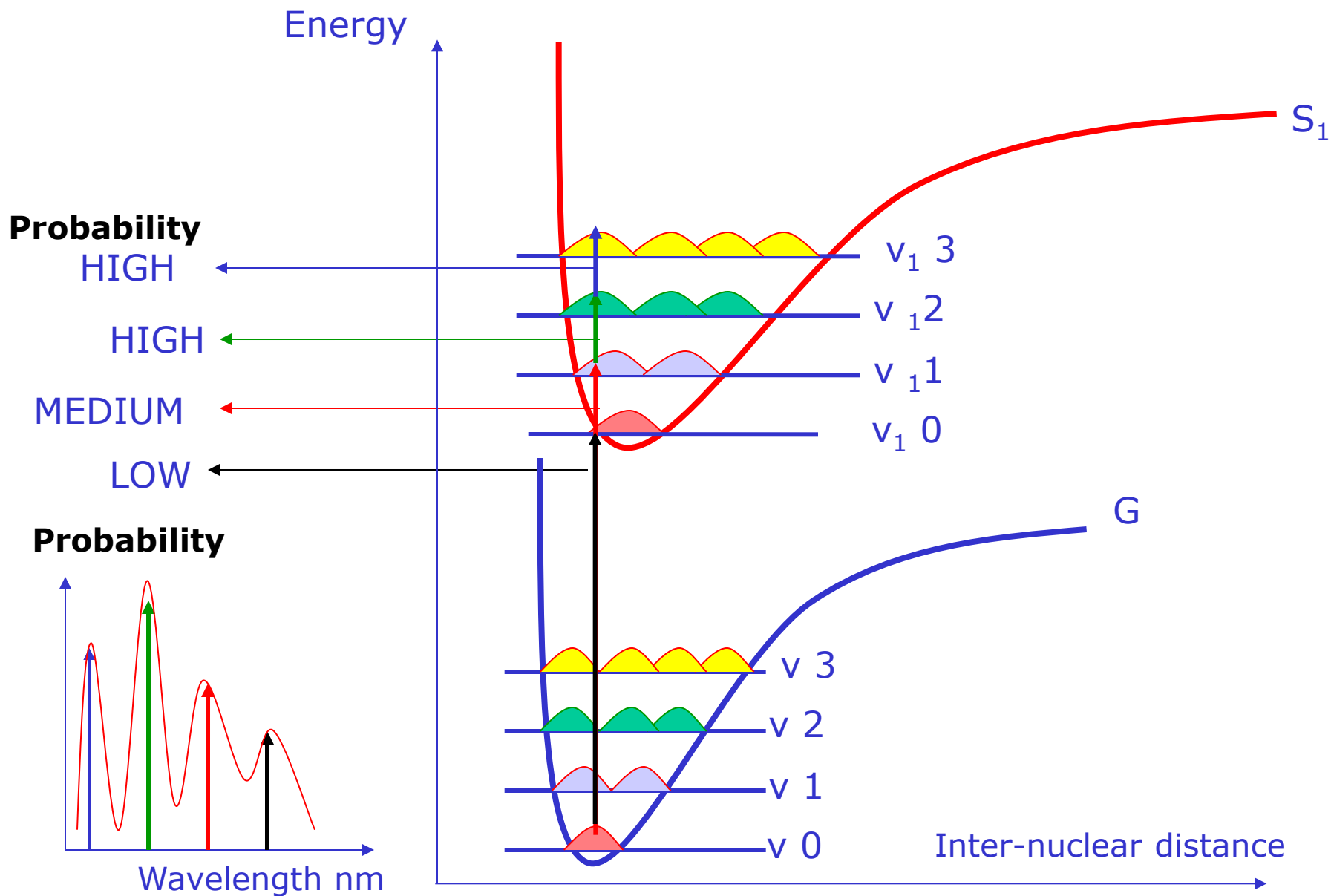
- The FC principle states that during an electronic transition, a change from one vibrational energy level to another will be more likely to happen if the two vibrational wave functions overlap more significantly.
- Classically, the FC principle is the approximation that an electronic transition is most likely to occur **without changes in the positions of the nuclei** in the molecular entity and its environment. The resulting state is called a FC state, and the transition involved, a **vertical transition**.
- The quantum mechanical formulation of this principle is that the intensity of a vibrational transition is proportional to the square of the overlap integral between the vibrational wave functions of the two states that are involved in the transition.



**lazy nuclei**

Since electronic transitions are very fast compared with nuclear motions, vibrational levels are favored when they correspond to a minimal change in the nuclear coordinates. The potential wells are shown favoring transitions between  $v = 0$  and  $v = 2$ .

# Electronic transitions from the ground state to the excited state



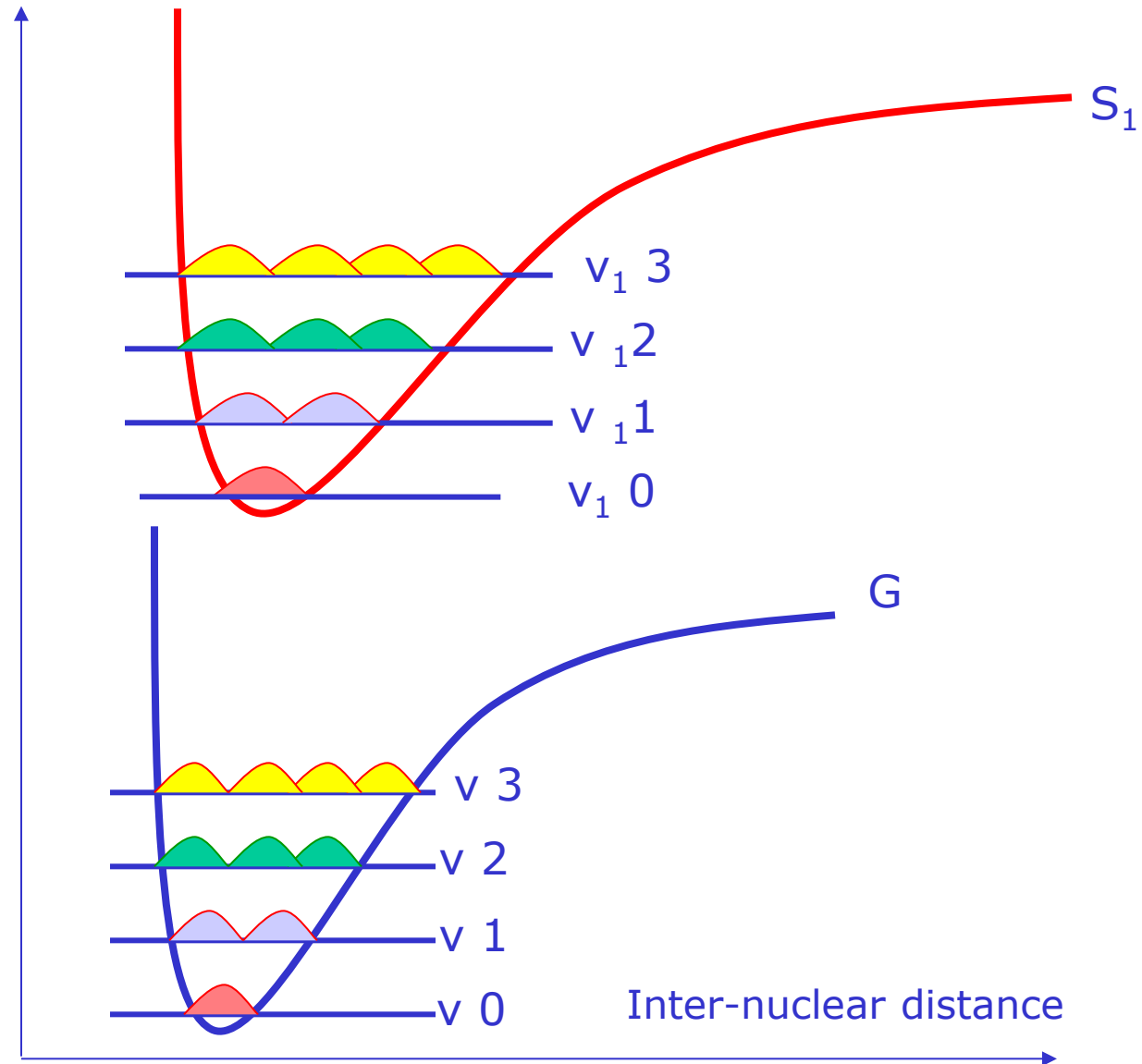
# Electronic transitions from the ground state to the excited state

Shaded areas reflect the probability of where the electron would be if it were in that vibrational band

Most favored transitions occur **From** the

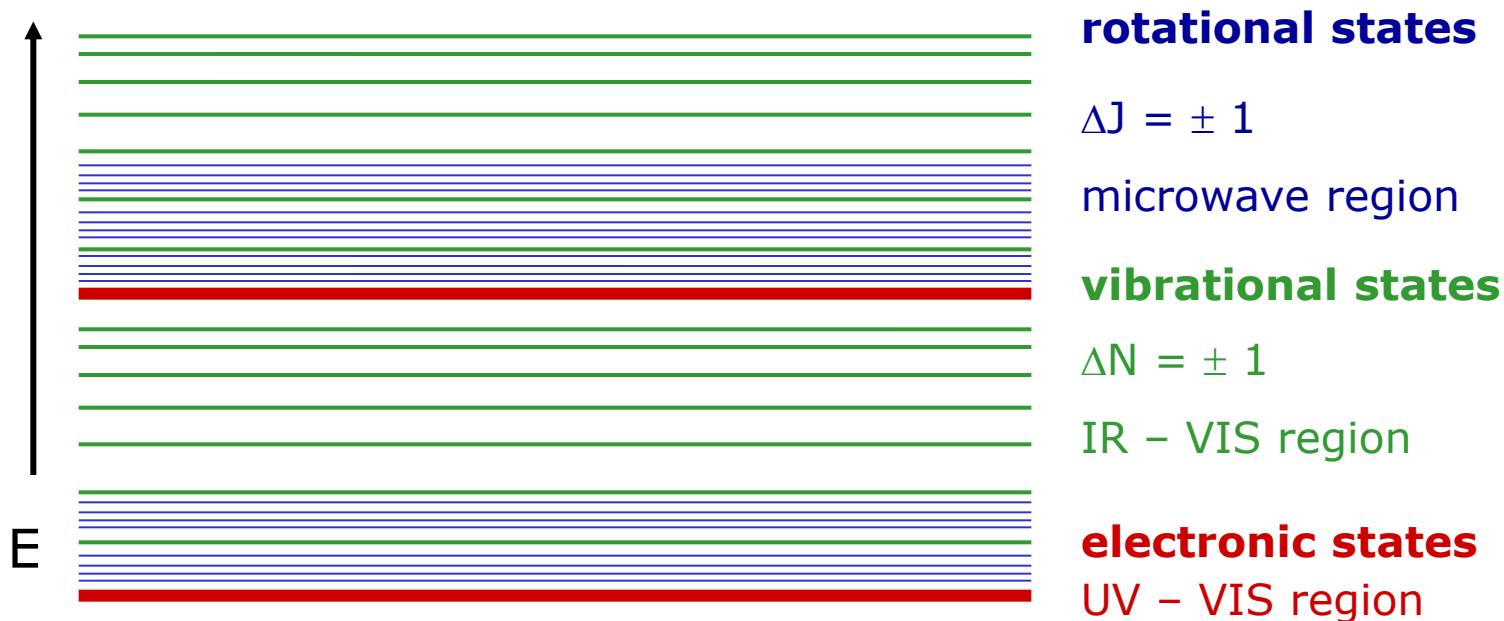
maximum shaded areas of the ground state

**To** the maximum shaded areas of the excited state



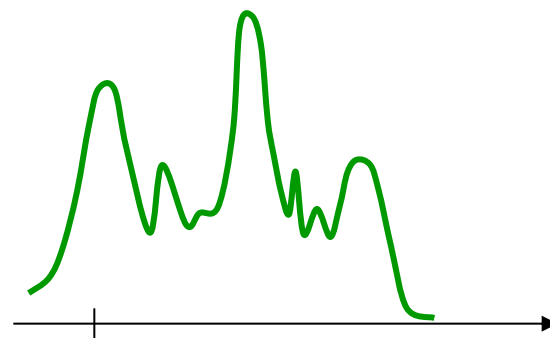
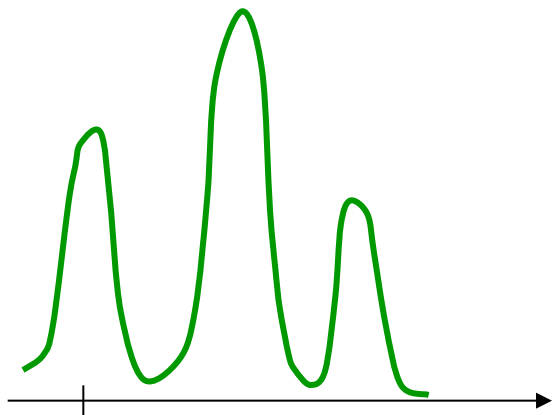
# Interaction of molecules with photons - quantum description

- Light exists in form of discrete quanta – photons  $E = h\nu$
- Atoms and molecules occupy discrete energetic states, which can be found as the solution of Schrodinger's equation.
- Exchange of energy with photons is accompanied by transitions between those states.

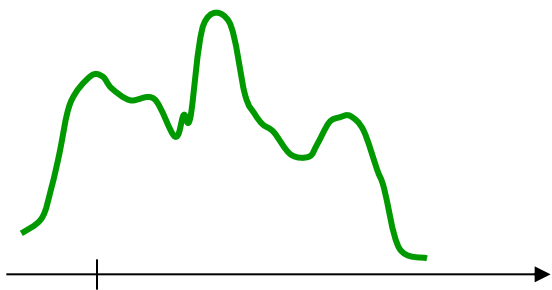




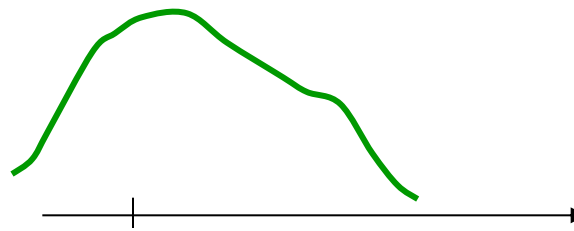
# Electronic – vibrational spectrum



other transitions (other vibrational modes, non-fundamental transitions,...)



effect of room temperature



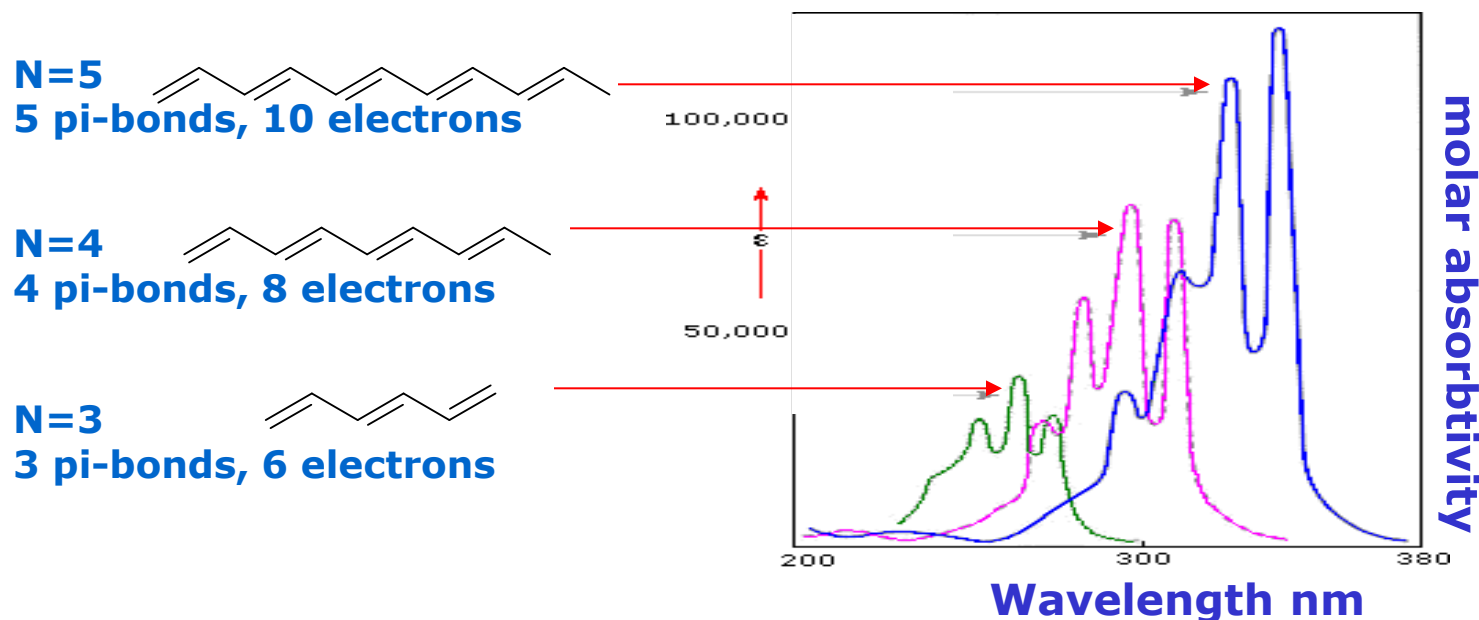
effect of molecular surroundings

# Absorption maxima : The importance of conjugation

The wavelength value of the **absorption maximum**  
and the **molar absorptivity**

are determined by the degree of **Conjugation of  $\pi$ -bonds**

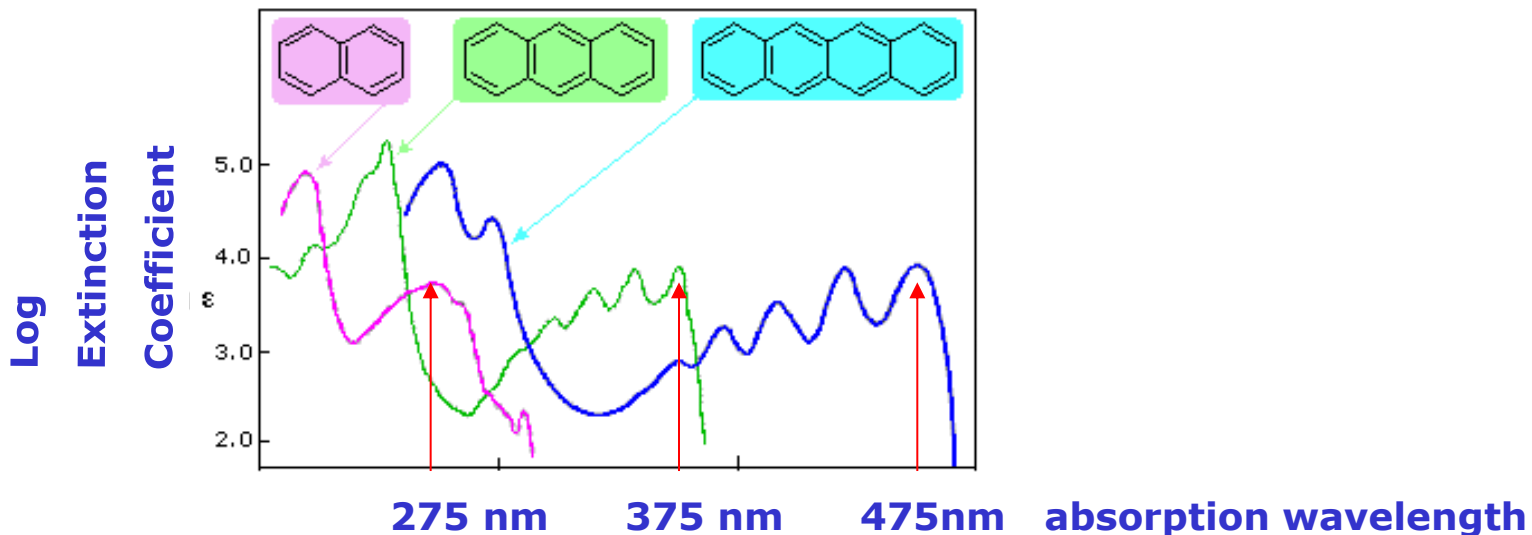
*Increasing the number of double bonds shifts the absorption to lower energy*



Increasing the number of aromatic rings increases the absorption maximum

**Benzene < Naphthalene < Anthracene < naphthacene < pentacene**

Abs. Max	262nm	275 nm	375 nm	475 nm	580 nm
Log $\epsilon$ (Extinction)	3.84	3.75	3.90	4.05	4.20



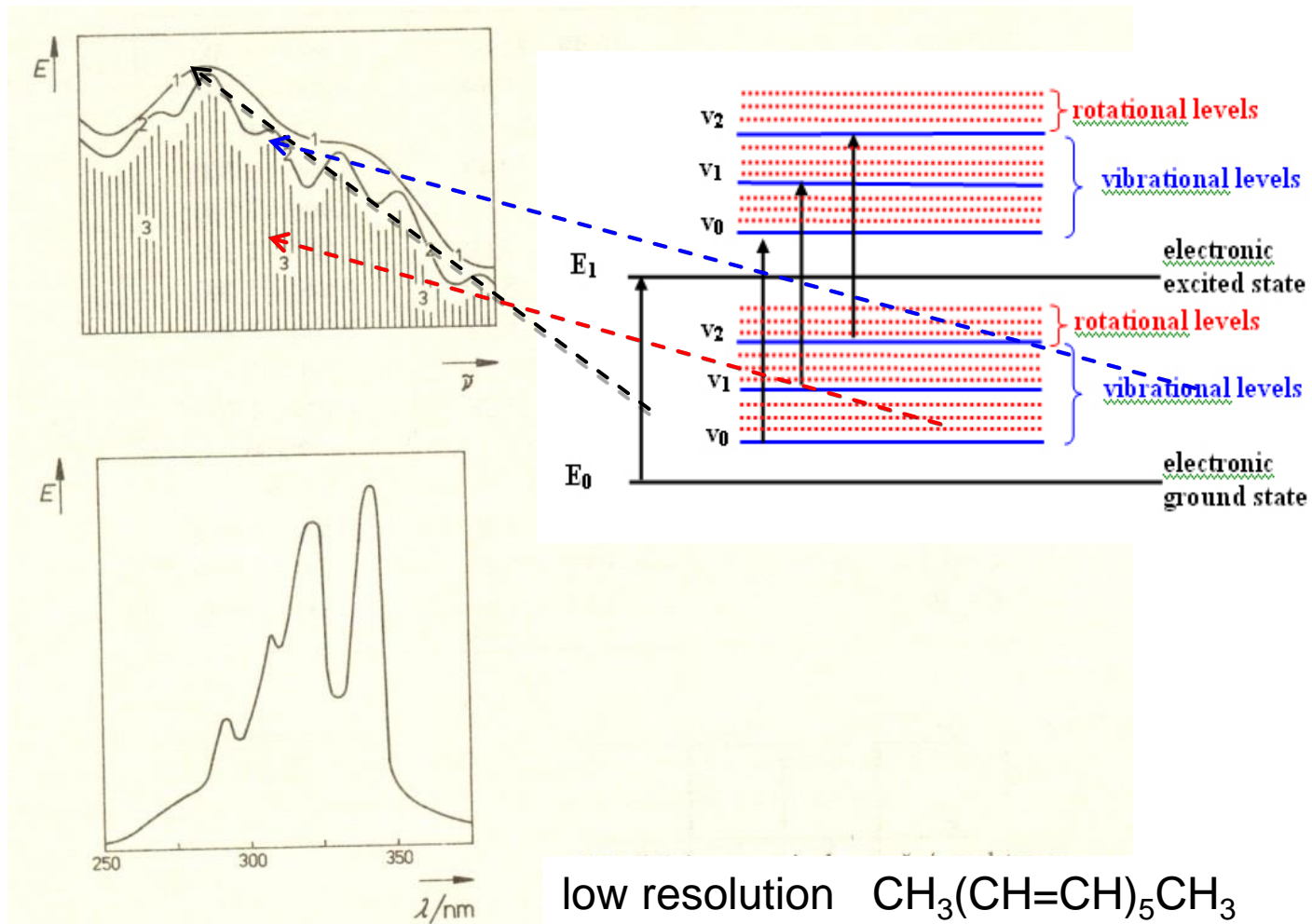
As the **degree of conjugation increases**

(i.e the number of electrons involved in the delocalized  $\pi$ -orbitals)

the **absorption energy decreases** ( $> \lambda$ , the energy between the ground and excited state decreases)

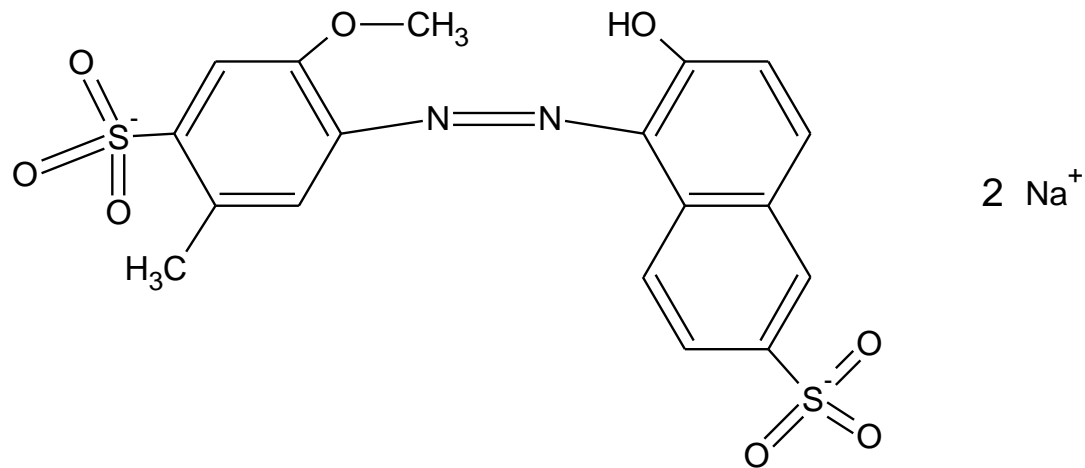
the **absorption becomes more intense** ( $> \epsilon$ , increased probability of absorption)

# Are absorption spectra simple?



# Chromofory:

- charakteristická funkční seskupení zodpovědná za barevnost sloučeniny – obsahují násobné vazby nebo konjugovaný systém násobných vazeb
- $>C=C<$     $-N=N-$     $>C=O-$     $-N=O$     $-C\equiv N$



# Absorpční maxima $\lambda_{\max}$

- Benzen 207 nm
- Naftalen 285 nm
- Antracen 375 nm
- Tetracen 471 nm žluto-oranžový
- Pentacen 580 nm fialový

# Biologically useful spectroscopic regions in UV-Vis-IR

Wavelength (cm <sup>-1</sup> )	Energy (aprox.) (J/mol)	Spectroscopic region	Techniques /Applications
~ 10 <sup>-5</sup>	1250	vacuum UV	electronic spectra
3.10 <sup>-5</sup>	420	near UV	electronic spectra
<b>carbon-carbon bond energy</b>			
6.10 <sup>-5</sup>	200	visible	electronic spectra
~ 10 <sup>-3</sup>	15	IR	vibrational spectra
<b>RT at ambient temperature</b>			
10 <sup>-2</sup>	1.5	Far IR	vibrational spectra

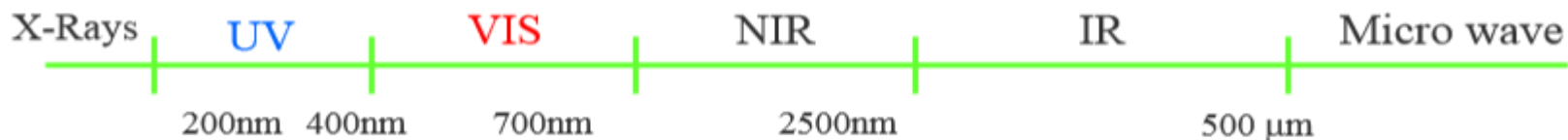
There is no simple way to explain the interaction of light with matter. Why?

*Light is rapidly oscillating electromagnetic field. Molecules contain distribution of charges and spin that have electrical and magnetic properties and these distributions are altered when a molecule is exposed to light.*

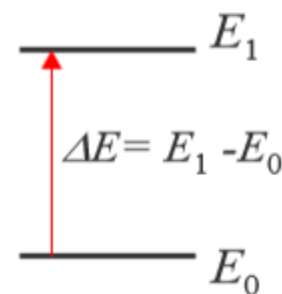
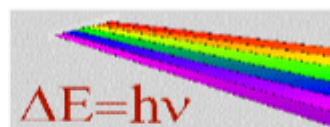
Explanation:

1. The rate at which the molecule responds to this perturbation
2. Why only certain wavelength cause changes in the state of the molecule
3. How the molecule alters the radiation

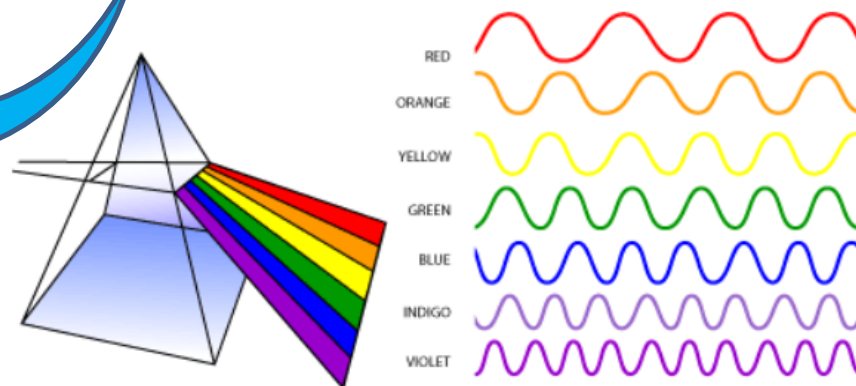
# Type of transition



Type of Radiation	Type of Transition
X-Rays	Inner Electron
UV/VIS	Outer Electron
IR	Molecular vibration
Micro Wave	Molecular rotation



- (1)  $\pi$ ,  $\sigma$ , and  $n$  electrons
- (2)  $d$  and  $f$  electrons
- (3) charge transfer electrons



Each color in a rainbow corresponds to a different wavelength of electromagnetic spectrum.



# INTERACTION BETWEEN LIGHT AND MOLECULES

## Calculation of properties of molecules by QM

1. The state of a system is described by a **wavefunction**
2. An observable quantity ( $E$ ,  $\mu$ , the location in space) is governed by a mathematical device known as an **operator**
3. The result of a measurement on a state can be computed by taking the **average value of operator** on that state
4. **A transition** between two state can be induced by a perturbation which is measured by an operator (deform the initial state - resembling final state)
5. The ability of light to induce transitions in molecules can be calculated according to its ability **to induce  $\mu(s)$**  that oscillate with the light.
6. The preferred directions for **inducing dipole moments  $\mu(s)$**  are fixed with respect to the geometry of the molecule.

# Interaction of light with molecules (chromophores)

For simplicity: I. What can be ignored?

- 1) ... the magnetic vector, only the electric vector
- 2) ... the spacial variation of the electric field of the light within the molecule

Chromophore  $\sim 10 \text{ \AA}$   Wavelength of light  $\sim 3000 \text{ \AA}$

The electric field felt by a molecule:  $E(t) = E_0 e^{i\omega t}$  ( $\omega = 2\pi\nu = 2\pi \frac{c}{\lambda}$ )

3) .... the effect of time?

$\Psi_a$  .....original state  
 $\Psi$  .....final state

What we need to compute?.... the rate at which light causes transitions between  $\Psi_a$  and  $\Psi$

For simplicity: II. What can be ignored? .... other states

$\Psi_a$  .....original state     $\Psi_b$  .....final state

Is the interaction (light – molecule) dependent on time?....  $\hat{H}' = \hat{H} + V(t)$

$$\Psi(t) = C_a(t) \Psi_a e^{-iE_a t/\hbar} + C_b(t) \Psi_b e^{-iE_b t/\hbar}$$

# Interaction of light with molecules

$$\Psi(t) = C_a(t) \psi_a e^{-iE_a t/\hbar} + C_b(t) \psi_b e^{-iE_b t/\hbar}$$

$$i\hbar(\psi_a(t) e^{-iE_a t/\hbar} dC_a/dt + \psi_b(t) e^{-iE_b t/\hbar} dC_b/dt) = \hat{V}(t) [\psi_a e^{-iE_a t/\hbar} C_a(t) + \psi_b e^{-iE_b t/\hbar} C_b(t)]$$

$C_a$  and  $C_b$  calculation: some approaches

1) ... to expand the charge distribution in a multipole series  $\longrightarrow$  the electric dipole

$e_i$  the electronic charge at the position  $\hat{r}_i$   
Born-Oppenheimer approximation

$$\hat{\mu} = \sum_i e_i |\hat{r}_i|$$

The interaction energy:

$$\hat{V}(t) = \hat{\mu} E_0 e^{i\omega t}$$

$\hat{\mu}$  the dipole operator

★ the spatial part from  $-x$  to  $+x$ , from  $-y$  to  $+y$ , from  $-z$  to  $+z$

★  $E_0$  is constant

$$\langle \psi_a | \hat{\mu} | \psi_b \rangle = \langle \psi_b | \hat{\mu} | \psi_a \rangle$$

$$h\nu = \hbar\omega = E_b - E_a$$

spectral bands - light absorption or light-induced transition  
only at certain narrow wavelength (or frequency) interval

# Interaction of light with molecules

$$B_{ab} = (2/3)(\pi/\hbar^2) |\langle \psi_b | \hat{\mu} | \psi_a \rangle|^2$$

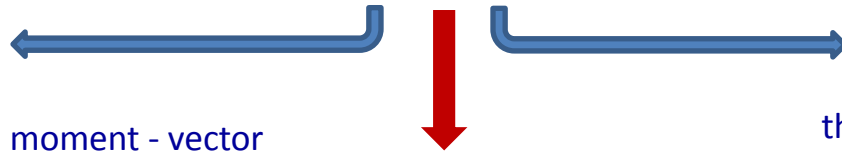
transition dipoles

$$\langle \psi_b | \hat{\mu} | \psi_a \rangle$$

light-induced dipole

$$\mu_{ind} = \hat{\alpha} E$$

the transition dipole moment - vector  
MATRIX



$B_{ab}$  - the transition probability

the ability of light to distort a molecule

not preferred direction, only a preferred orientation

from intensity  $I(\nu)$   $\longrightarrow$   $|\langle \psi_b | \hat{\mu} | \psi_a \rangle|^2$  electronic distribution within the molecule

$I(\nu) = f$  (the relative orientation of the molecule with )

$$\langle \psi_b | \hat{\mu} | \psi_a \rangle \cdot E_0$$

## excitation in phase or out of phase

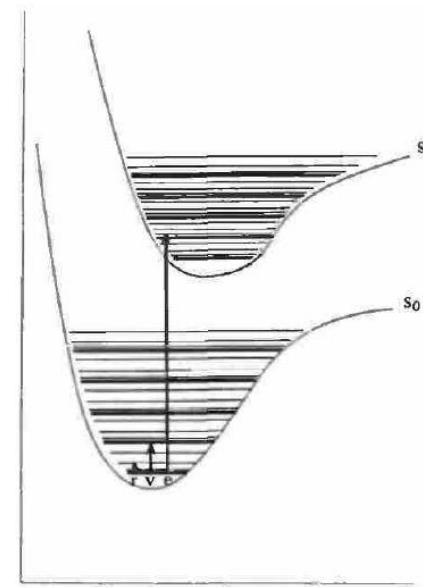
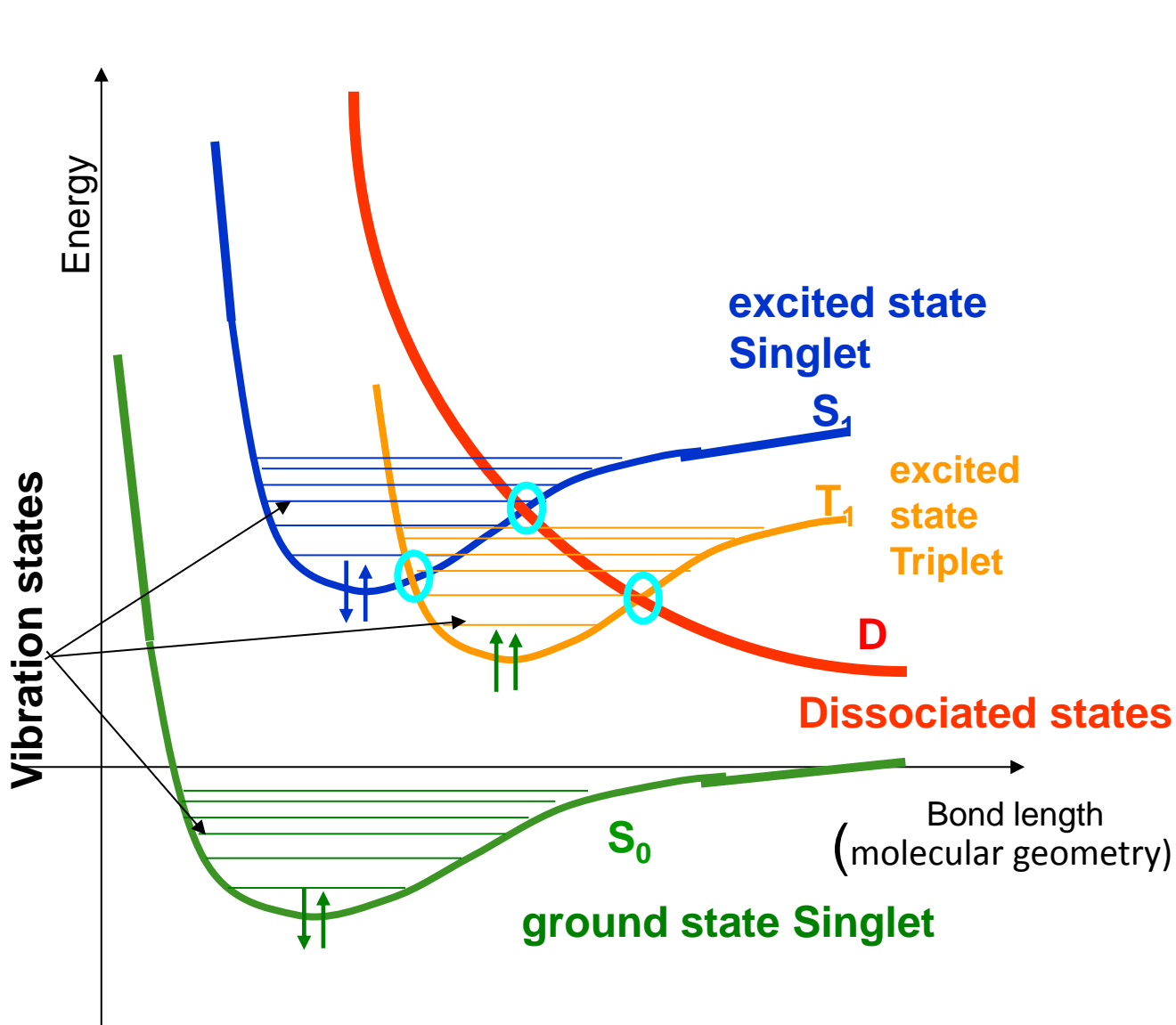
e.g. exempli gratia

	In phase	Out of phase by 180°
Time = t	↑↑	↑↓
Time = t + 1/2 ν	↓↓	↓↑

Induced neighboring chromophores – the interactions can be attractive or repulsive

# ABSORPTION SPECTROSCOPY OF ELECTRONIC STATES

## Electronic structures of simple molecule



Transitions corresponding to:

e – electronic

v – vibrational

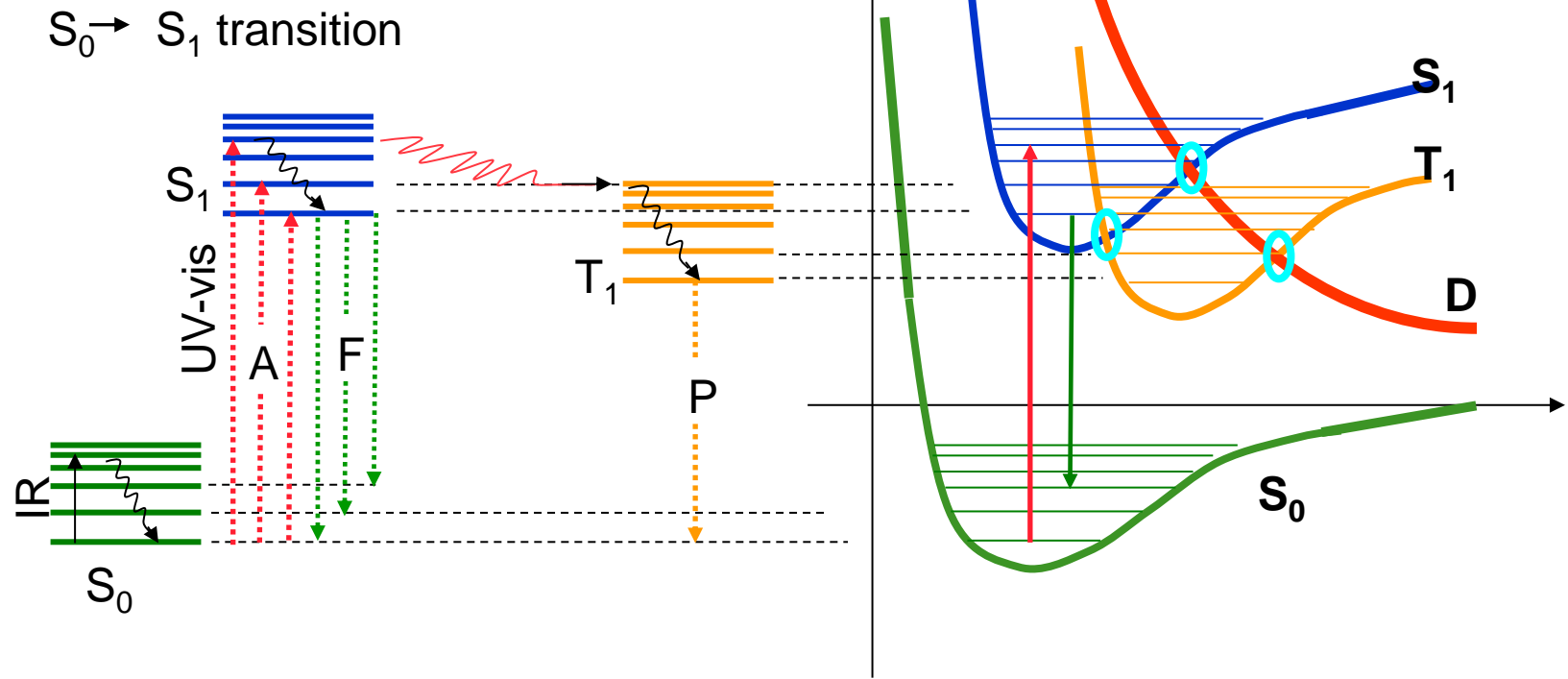
r – rotational

Energy  $S_1 - S_0 \sim 80 \text{ kcal/mol}$   
335 kJ/mol

Energy v  $\sim 10 \text{ kcal/mol}$   
42 kJ/mol

Energy r  $\sim 1 \text{ kcal/mol}$   
4.2 kJ/mol

# Interaction between photon and molecule



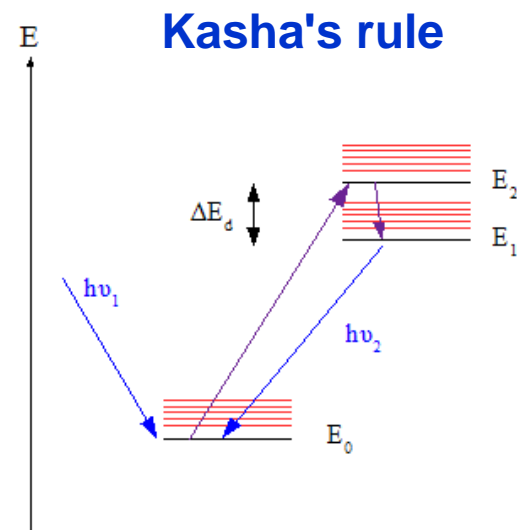
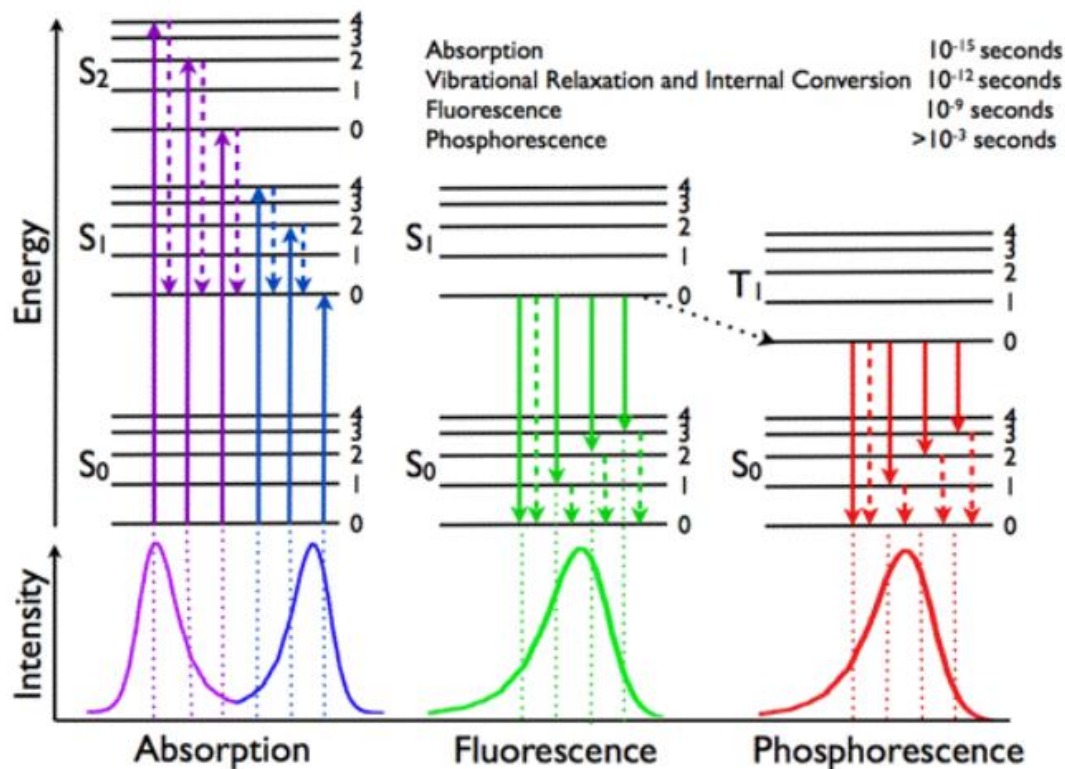
Absorption  
Fluorescence  
Phosphorescence

# Absorpce fotonu



# Interaction between photon and molecule

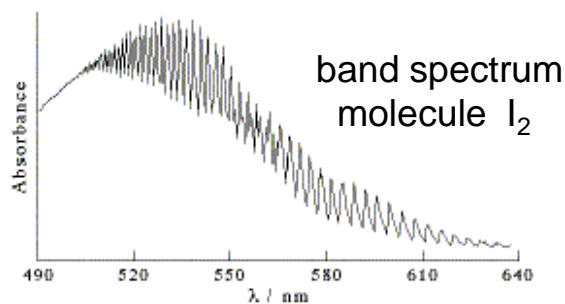
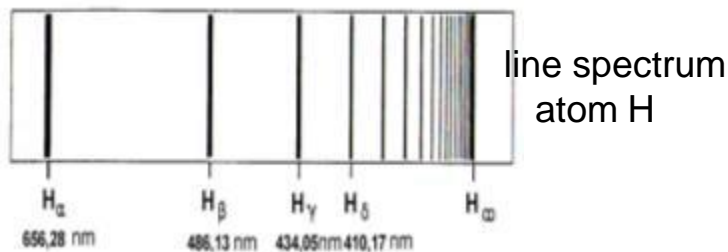
## Jablonski diagram



**Kasha's rule** is a principle in the photochemistry of electronically excited molecules. The rule states that photon emission (fluorescence or phosphorescence) occurs in appreciable yield only from the lowest excited state of a given multiplicity.

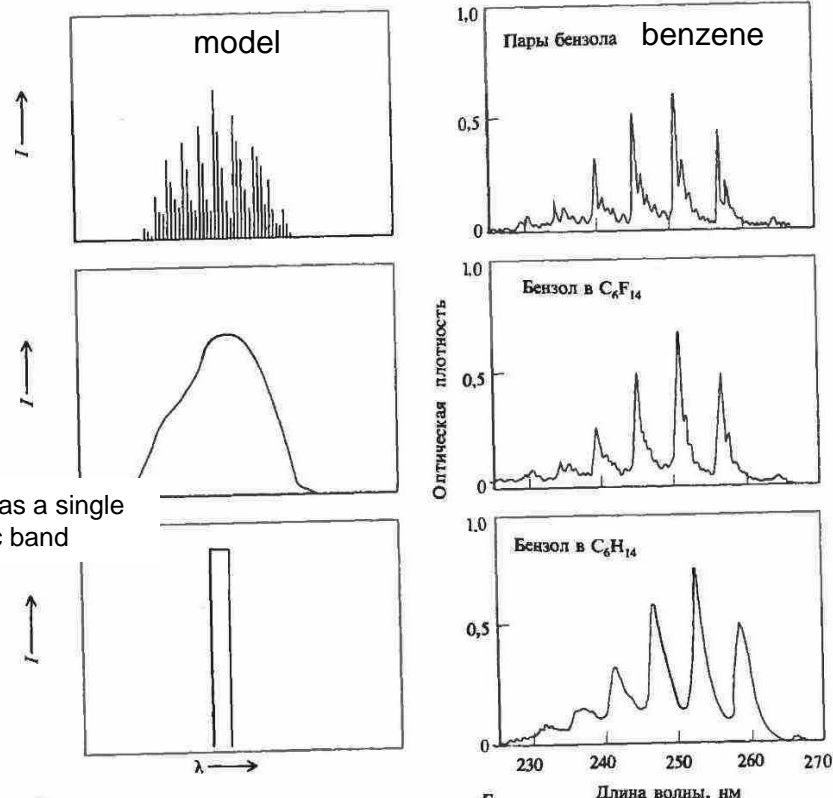


# Electronic absorption spectra of small molecules



magnitudes of transition dipoles

$$\langle S_1^{vib, rot} | \hat{\mu} | S_0 \rangle$$



absorption spectra of benzene showing solvent-induced broadening (gas, in solution, benzen in  $C_6H_{14}$ ).

Band broadening: enviromental heterogeneity, Doppler shifts and other effects

# Absorbing species

## 1. excitation



The lifetime of the excited species:  $10^{-8}$  -  $10^{-9}$  s

## 2. relaxation (conversion of the excitation energy to heat)



The absorption of ultraviolet or visible radiation generally results from excitation of bonding electrons.

## The electrons that contribute to absorption by a molecule are:

- (1) those that participate directly in bond formation between atoms
- (2) nonbonding or unshared outer electrons that are largely localized about such atoms as oxygen, the halogens, sulfur, and nitrogen. The molecular orbitals associated with single bonds are designated as sigma ( $\sigma$ ) orbitals, and the corresponding electrons are  $\sigma$  electrons.

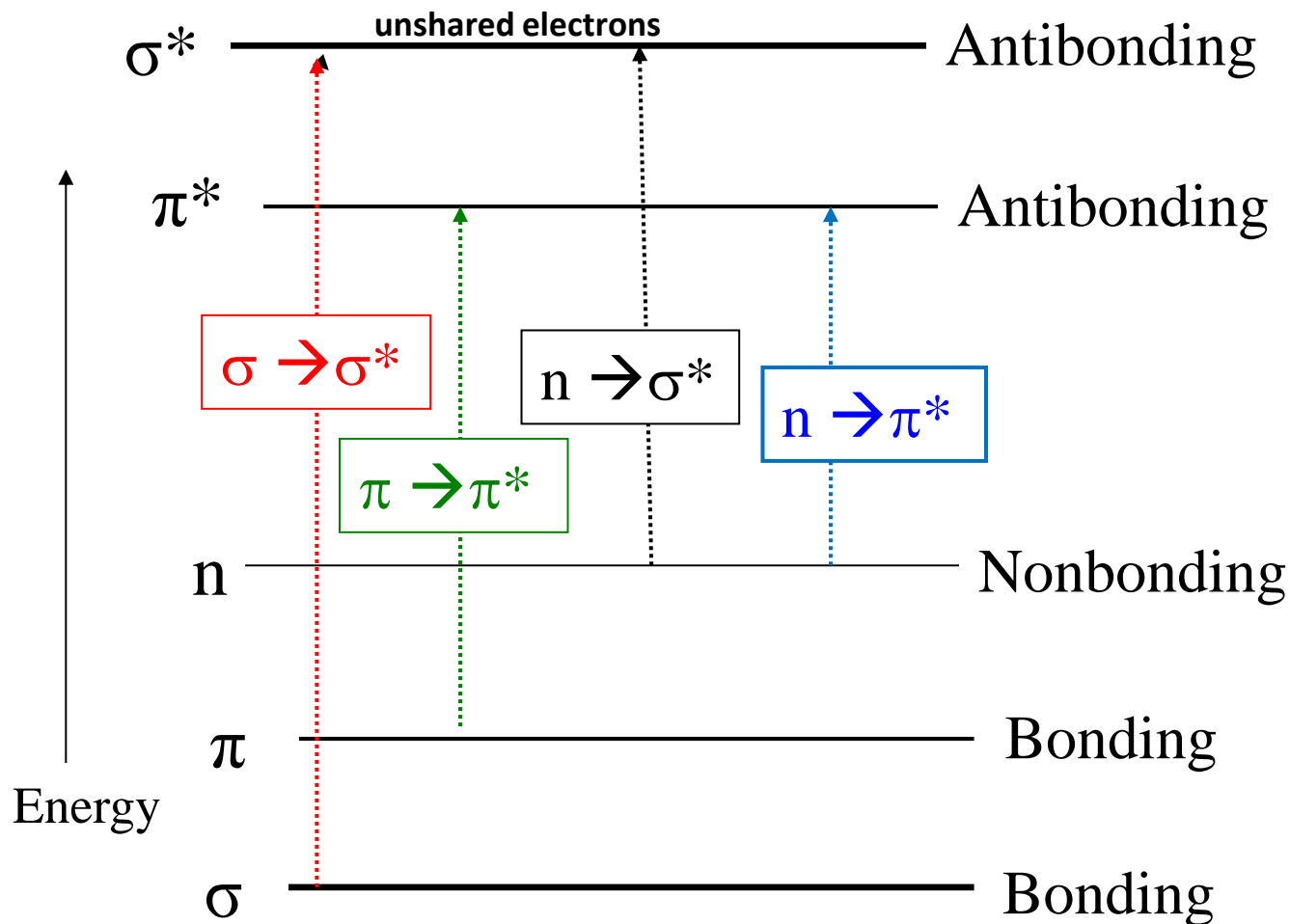
# Energy



Methan 125 nm; 150-250nm ;

most applications; most applications;

200-700nm; 200-700nm



# Chromophores

Compound sample	Transition	$\lambda_{\text{max}}$ (nm)
H <sub>2</sub> O	$\sigma \rightarrow \sigma^*$	183
C-C a C-H, CH <sub>4</sub>	$\sigma \rightarrow \sigma^*$	cca 170, 173
C-X, CH <sub>3</sub> OH, CH <sub>3</sub> NH <sub>2</sub> , CH <sub>3</sub> I	$n \rightarrow \sigma^*$	180-260, 187, 215, 258
C=C, H <sub>2</sub> C=CH <sub>2</sub>	$\pi \rightarrow \pi^*$	160-190, 162
H <sub>2</sub> C=CH-CH=CH <sub>2</sub>	$\pi \rightarrow \pi^*$	217
C=O, H-CH=O	$n \rightarrow \pi^*$ , $\pi \rightarrow \pi^*$	270, 170-200, 270, 185
H <sub>2</sub> C=CH-CH=O	$n \rightarrow \pi^*$ , $\pi \rightarrow \pi^*$	328, 208
C=N	$n \rightarrow \sigma^*$ , $n \rightarrow \pi^*$	190, 300
N=N	$n \rightarrow \pi^*$	340
C=S	$n \rightarrow \pi^*$	500
NO <sub>2</sub>	$n \rightarrow \pi^*$	420-450
N=O	$n \rightarrow \pi^*$	630-700

# Chromophores

## Absorption Characteristics of Some Common Chromophores

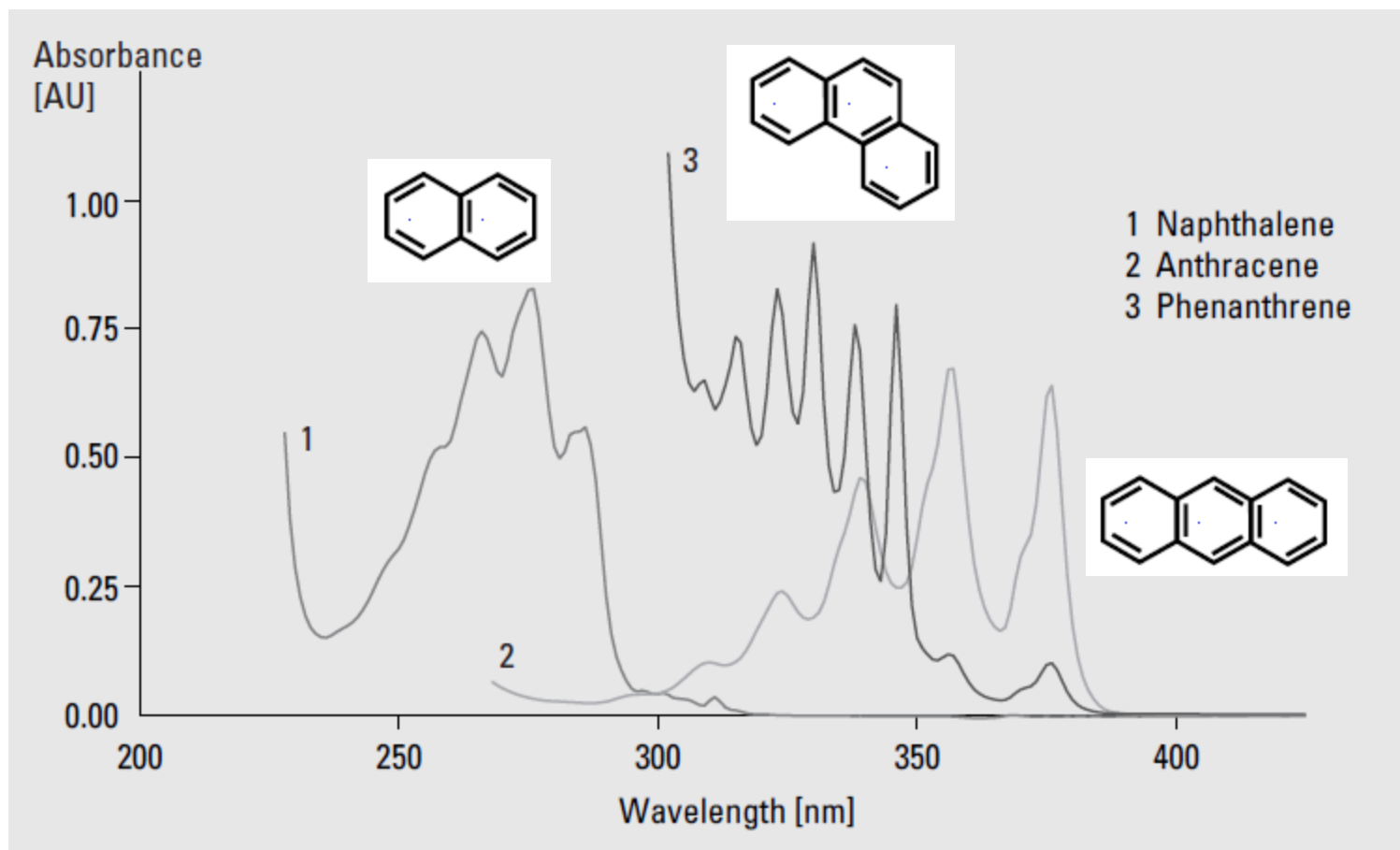
Chromophore	Example	Solvent	$\lambda_{\max}$ , nm	$\epsilon_{\max}$	Transition Type
Alkene	$C_6H_{13}CH=CH_2$	<i>n</i> -Heptane	177	13,000	$\pi \rightarrow \pi^*$
Alkyne	$C_5H_{11}C\equiv C-CH_3$	<i>n</i> -Heptane	178	10,000	$\pi \rightarrow \pi^*$
			196	2000	—
			225	160	—
			186	1000	$n \rightarrow \sigma^*$
Carbonyl	$\begin{array}{c} CH_3CCH_3 \\    \\ O \end{array}$	<i>n</i> -Hexane	280	16	$n \rightarrow \pi^*$
			180	large	$n \rightarrow \sigma^*$
Carboxyl	$\begin{array}{c} CH_3CH \\    \\ O \end{array}$	<i>n</i> -Hexane	293	12	$n \rightarrow \pi^*$
			204	41	$n \rightarrow \pi^*$
Amido	$\begin{array}{c} CH_3CNH_2 \\    \\ O \end{array}$	Ethanol	214	60	$n \rightarrow \pi^*$
Azo	$CH_3N=NCH_3$	Ethanol	339	5	$n \rightarrow \pi^*$
Nitro	$CH_3NO_2$	Isooctane	280	22	$n \rightarrow \pi^*$
Nitroso	$C_4H_9NO$	Ethyl ether	300	100	—
			665	20	$n \rightarrow \pi^*$
Nitrate	$C_2H_5ONO_2$	Dioxane	270	12	$n \rightarrow \pi^*$

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## Effect of Conjugation of Chromophores

**1,3-butadiene,  $CH_2=CHCH=CH_2$ :** a strong absorption band that is displaced to a longer wavelength by 20 nm compared with the corresponding peak for an unconjugated diene

# Chromophores and conjugated double bonds

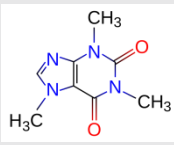


- Not only the kind of chromophore is important; the benzene-rings are chromophores with  $\pi$ - $\pi^*$  transitions but conjugated double bonds have the spectral effect. The environment of the chromophores or the combination with other chromophores also have a strong influence on the position and values of absorbance bands
- From naphthalene to anthracene there is an increasing conjugation of the  $\pi$  bonds. The spectral region of absorbance changes as well as the intensity of absorbance and the form of the absorbance bands
- Anthracene and phenanthrene have the same chemical formula but their spectra are different because their structure and hence the electronic environment of the  $\pi$  bonds are different.

# Energies of Light at the Absorbance Maxima

Compound	$\lambda_{\max}$ [nm]	Absorbance [AU]
Caffeine	205	0.699
Erythrosine	526	0.798

Absorbance [AU]



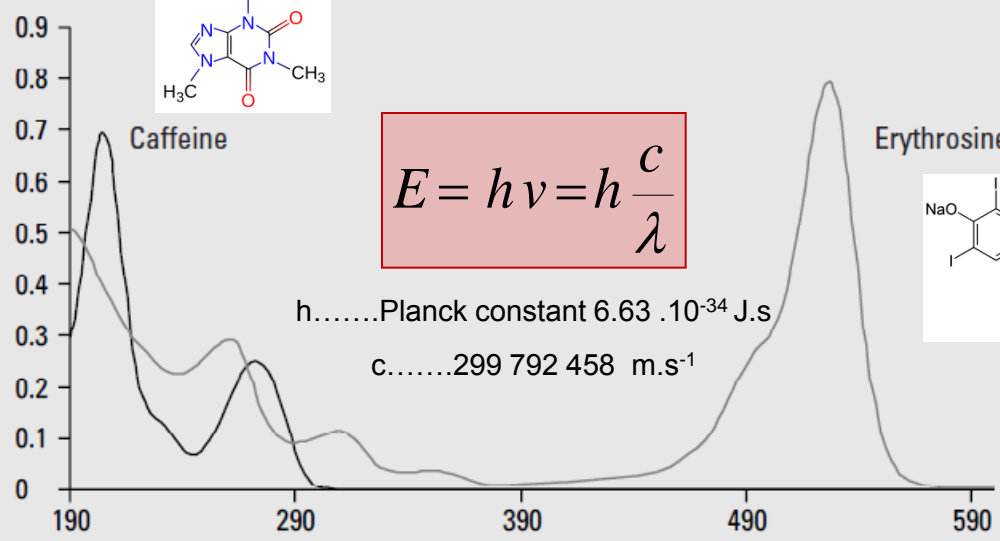
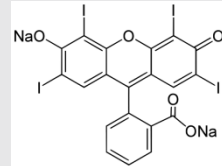
Caffeine

$$E = h\nu = h \frac{c}{\lambda}$$

h.....Planck constant  $6.63 \cdot 10^{-34}$  J.s

c..... $299\,792\,458$  m.s<sup>-1</sup>

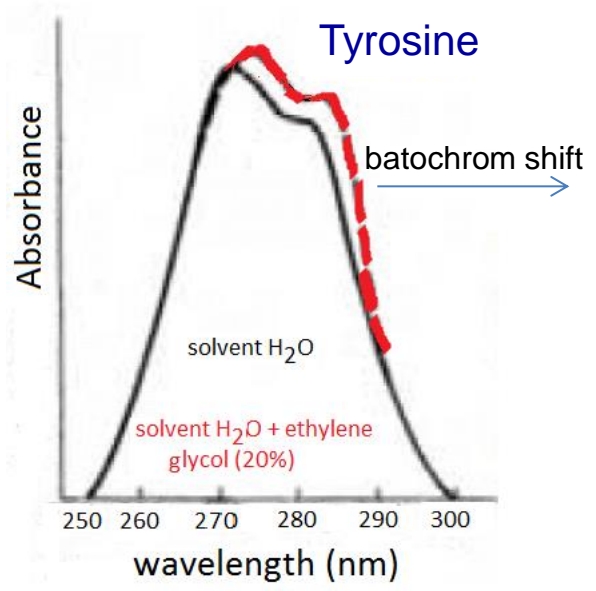
Erythrosine



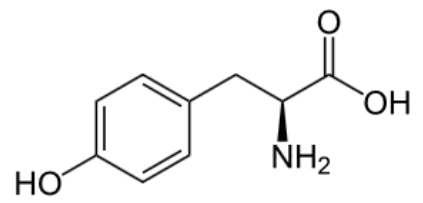
Wavelength [nm]

Compound	$\lambda_{\max}$ [nm]	Energy [J]
Caffeine	205	$9.69 \cdot 10^{-19}$
Erythrosine	526	$3.78 \cdot 10^{-19}$

## Solvation effect

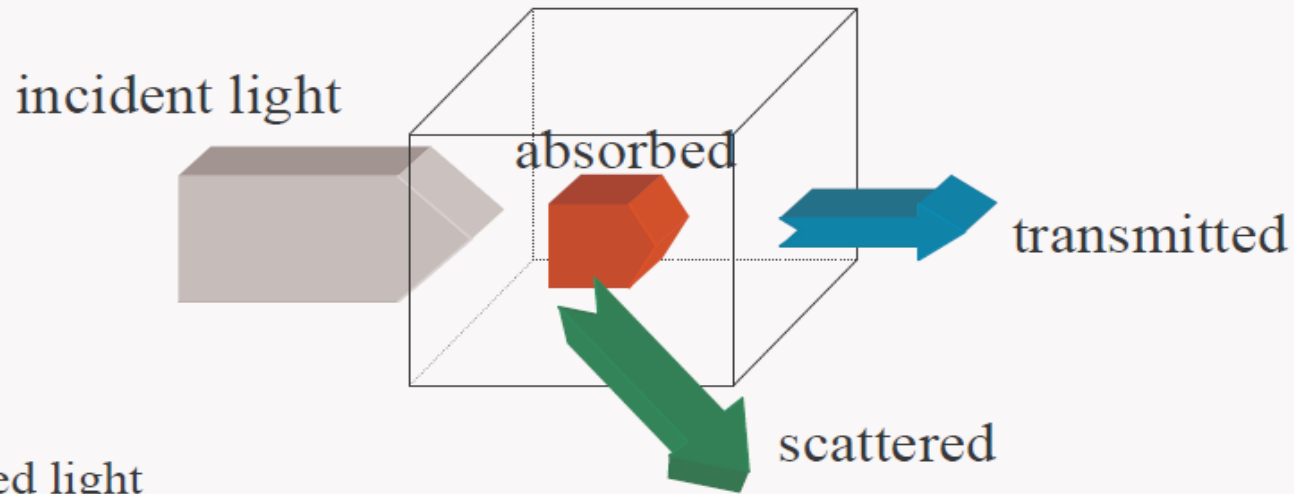


solvation influences the distribution of energy levels of the base and the excited state (effect of surface active substance)  
 There is a relationship between the wavelengths of the absorbance maxima and the polarity of the solvent used (permittivity)  
 similar effect of pH: protonation-deprotonation



# Light

- Consider a beam of light on a material
  - It can be scattered, absorbed, or transmitted

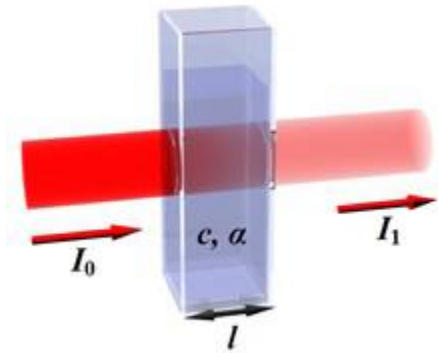


- Transmitted light
  - Light emerges propagating in the same direction as the incident light
- Absorbed light
  - Energy from light is absorbed in the volume of the material
- Scattered light
  - Light emerges in a different direction from the incident light



# Beer–Lambert–Bouguer law

the extinction coefficient



$$-\frac{dI}{I} = C\varepsilon' dl$$

**Absorbance A**

$\varepsilon'$  the molar extinction coefficient = f ( $\lambda$  or  $\omega$ )

$$-\int_{I_0}^I \frac{dI}{I} = \int_0^l C\varepsilon' dl \longrightarrow \ln \frac{I_0}{I} = C\varepsilon' l \longrightarrow$$

$$A(\lambda) \equiv \log \frac{I_0}{I} = C\varepsilon(\lambda) l$$

$$\varepsilon = \varepsilon' / 2.302$$

- low concentrations  $< 10^{-2}$  M
- monochromatic light
- stability of sample

**Transmittance T**

$$T(\lambda) \equiv \frac{I}{I_0}$$

$$I = I_0 e^{-\varepsilon'(\lambda) l}$$

$$A(\lambda) = \log \frac{I_0}{I} = \log \frac{I_0}{I_0 e^{-\varepsilon'(\lambda) l}} = \log e^{\varepsilon'(\lambda) l} = \varepsilon'(\lambda) l \log e = 0.4343 \varepsilon'(\lambda) l = 0.1886 \varepsilon(\lambda) l$$

the most accurate measurements of A

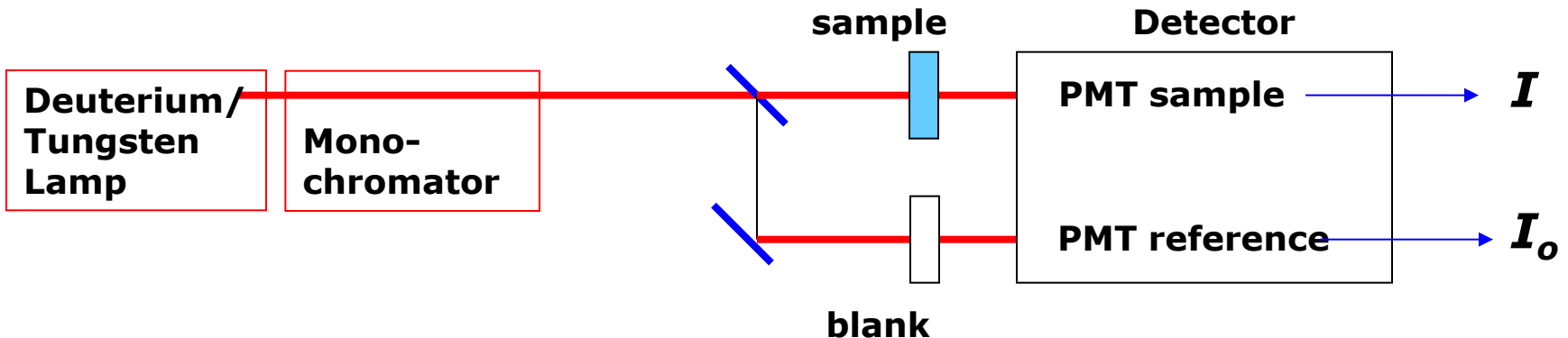
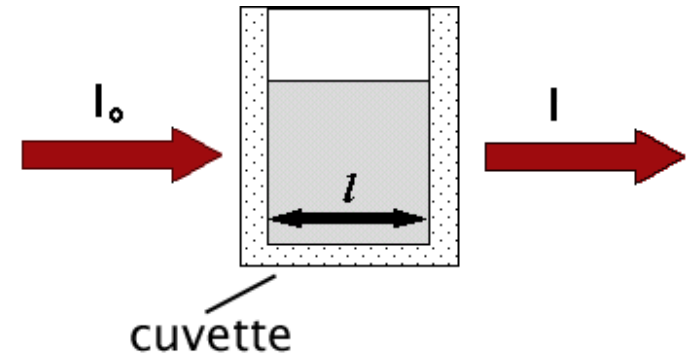
# Absorption: measurement

## The Beer Lambert Law

$$\text{Absorption (Optical Density)} = \log I_0 / I = \epsilon c l$$

$l$  is the path length of the sample (1 cm)

- a typical sample: a solution in a cuvette
- the solvent and the reflection from the cuvette walls contribute to the extinction of light
- relative measurement of absorption



## DNA bases

$$\epsilon_{max} [mol^{-1}cm^{-1}] \cong 10^4$$

## biopolymers

problems with  $\epsilon$ : high and non-correct due to not known molecular weight .....the average  $\epsilon$  per residue, for ODN = phosphate residues

$\epsilon$  molar and residue extinction is often implicit

## biopolymers

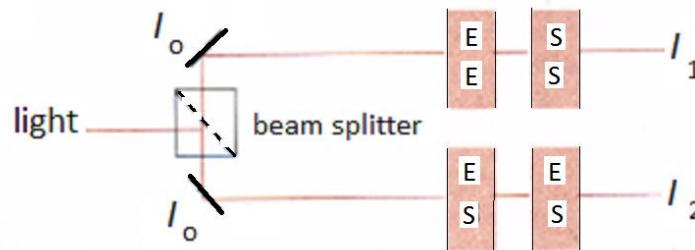
Sample:  $\log I_S = \log I_0 - A_S$

Reference:  $\log I_R = \log I_0 - A_R$

### Double beam spectrophotometer

$$\log \frac{I_R}{I_S} = A_S - A_R$$

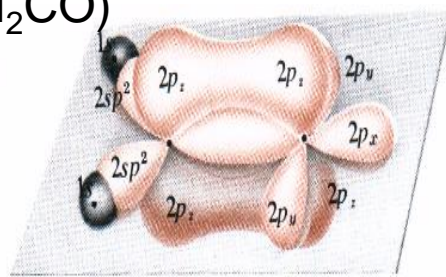
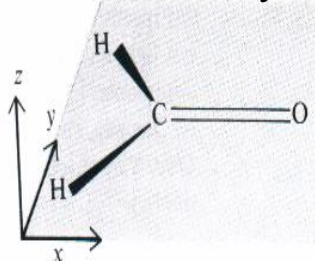
### Differential spectrophotometry



$$\log \frac{I_2}{I_1} = A_1 - A_2 = 2[(\epsilon_E C_E + \epsilon_S C_S) - (\epsilon_E C'_E + \epsilon_S C'_S + \epsilon_{ES} C'_{ES})]l$$

# Spectral properties of a simple molecule

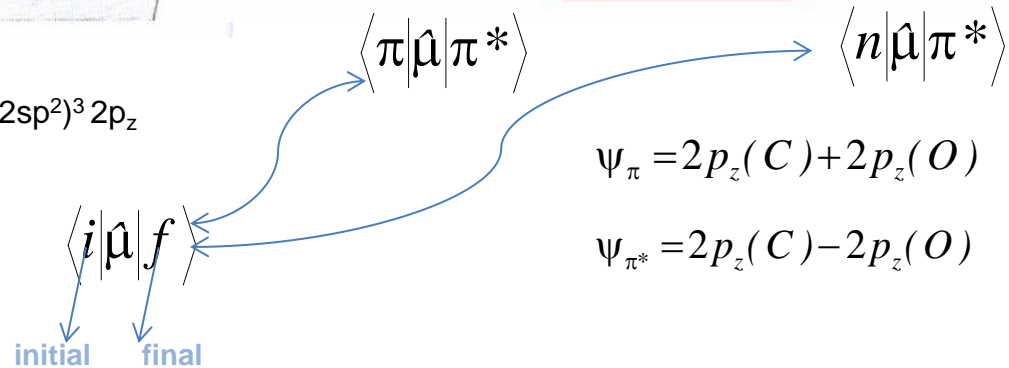
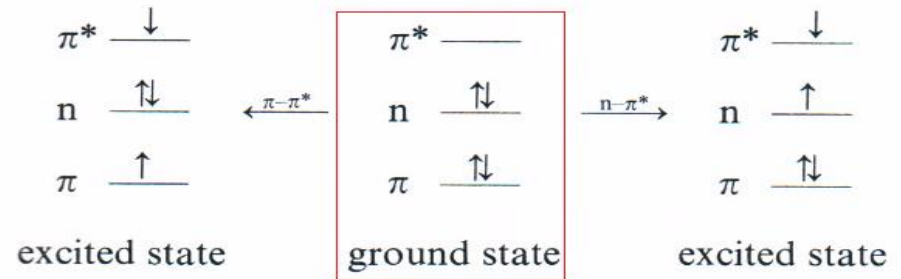
Formaldehyde (H<sub>2</sub>CO)



H.....1s  
 C.....1s<sup>2</sup> 2s<sup>2</sup> 2p<sub>y</sub> 2p<sub>x</sub> hybridization.... 1s<sup>2</sup> (2sp<sup>2</sup>)<sup>3</sup> 2p<sub>z</sub>  
 O.....1s<sup>2</sup> 2s<sup>2</sup> 2p<sub>y</sub><sup>2</sup> 2p<sub>x</sub> 2p<sub>z</sub>

The absorption intensity

the transition dipole moment

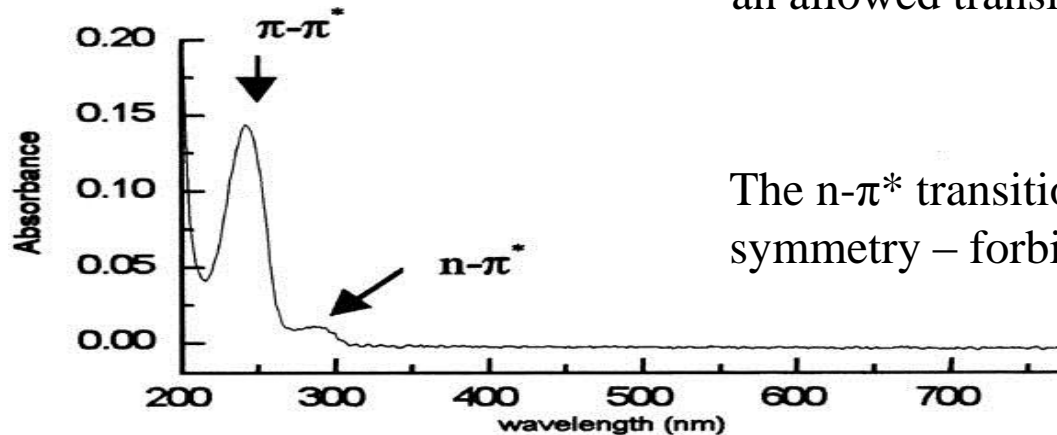


$$\psi_{\pi} = 2p_z(C) + 2p_z(O)$$

$$\psi_{\pi^*} = 2p_z(C) - 2p_z(O)$$

The  $\pi$ - $\pi^*$  transition is called an allowed transition

The  $n$ - $\pi^*$  transition is called a symmetry – forbidden transition



# Instrumentation - spectrophotometry

- **Spectrophotometer**

- **Single** and **double** beam instruments

Components of optical instruments

1. Sources

2. Wavelength selectors (filters, monochromators)

3. Sample containers

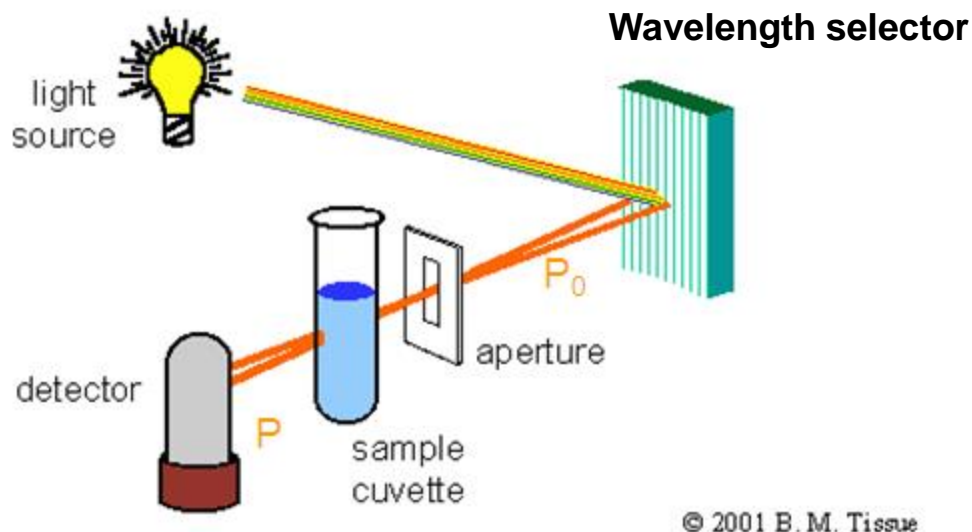
4. Detectors

5. Readout devices

- **Applications of Spectrophotometry**

Spectrophotometry is more suited for quantitative analysis rather than qualitative one

## Instrumentation (Spectrophotometers)

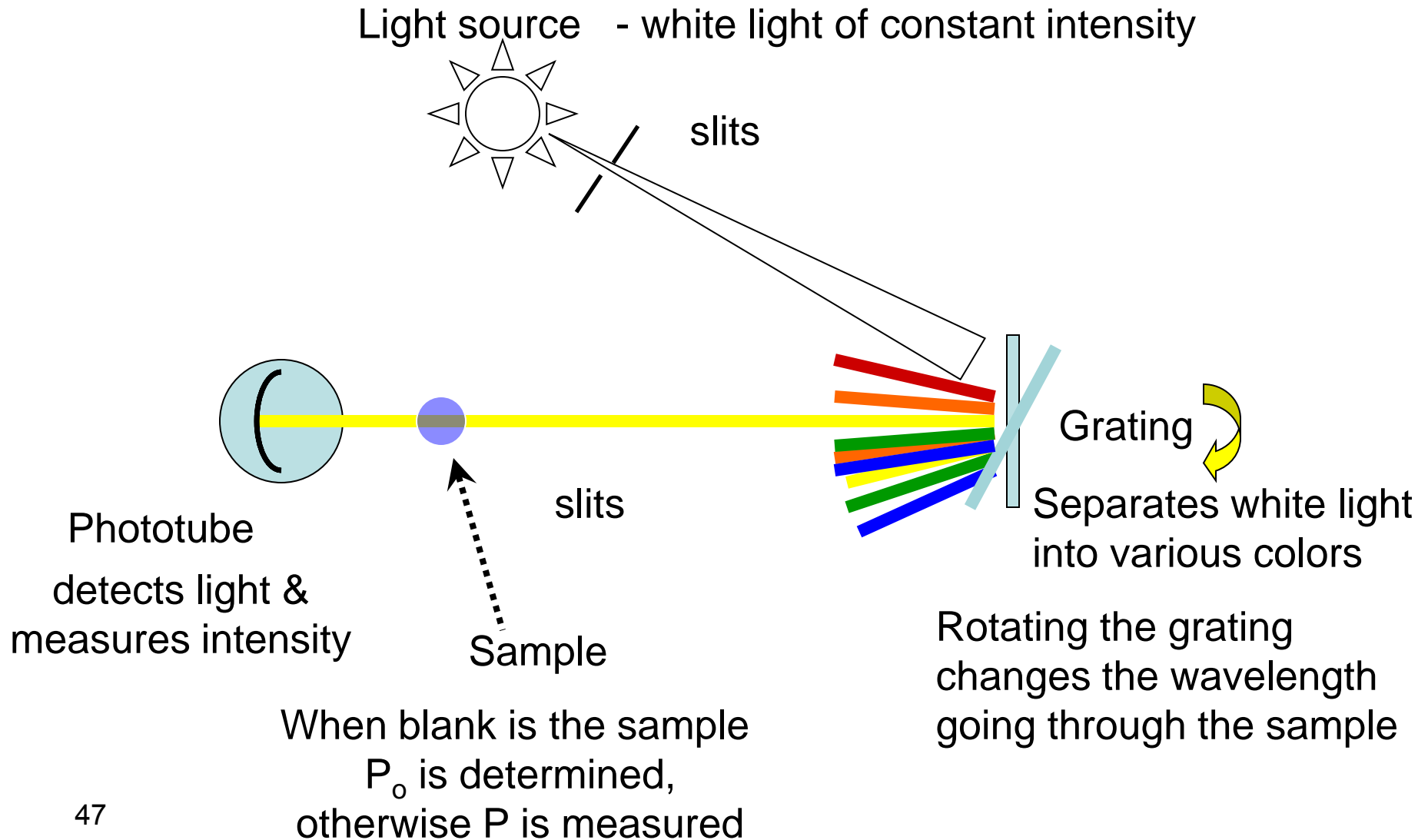


### **A single beam spectrophotometer**

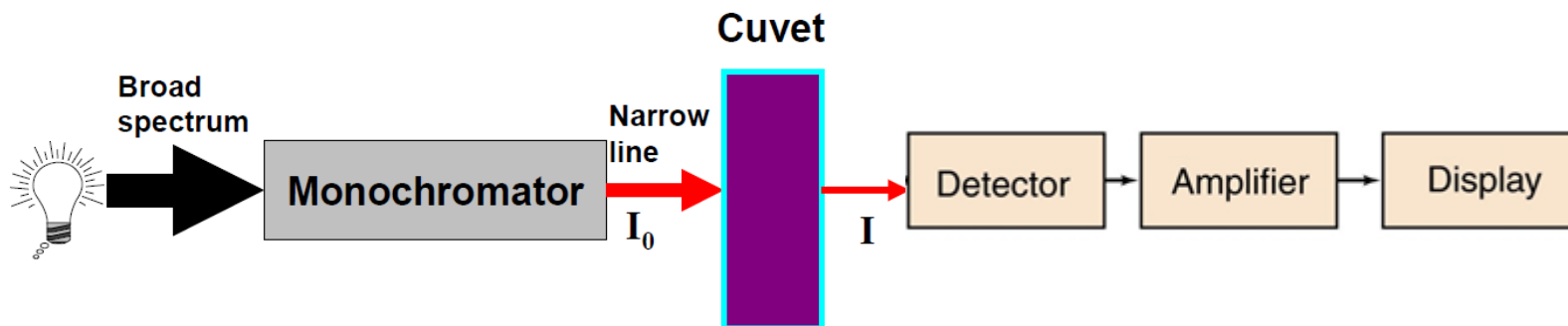
The above essential features of a spectrophotometer shows that polychromatic light from a **source** separated into narrow band of wavelength (nearly monochromatic light) by a **wavelength selector**, passed through the **sample compartment** and the transmitted intensity,  $P$ , after the sample is measured by a **detector**

In a **single beam instrument**, the light beam follows **a single path** from the source, to the monochromator, to the sample cell and finally to the detector

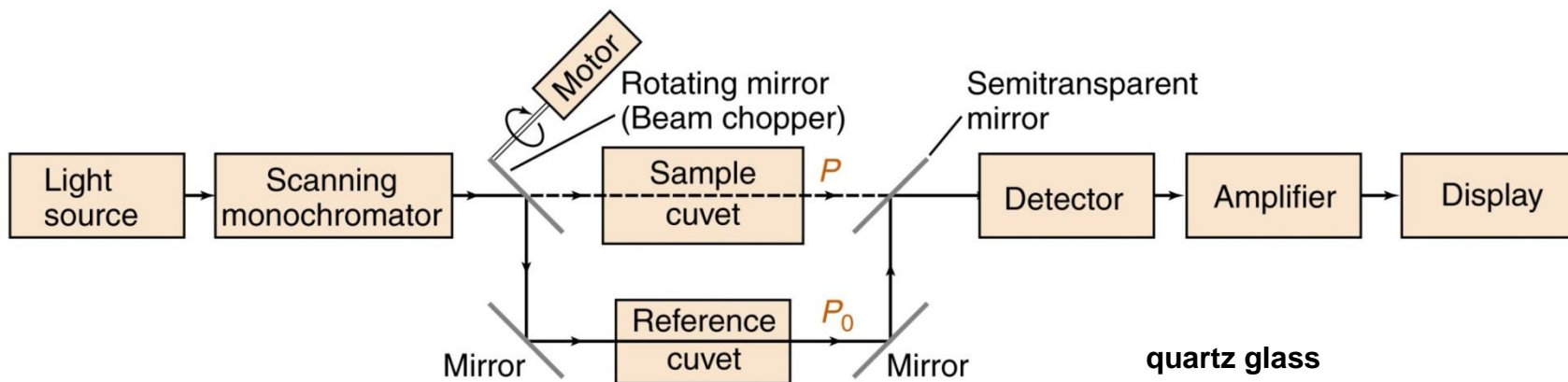
# The components of a single beam spectrophotometer



# Single beam spectrophotometer



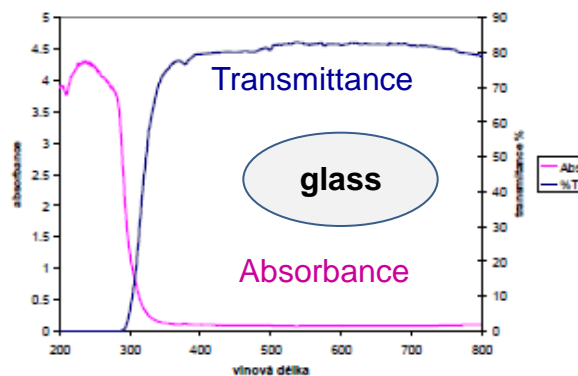
# Double Beam Spectrophotometer



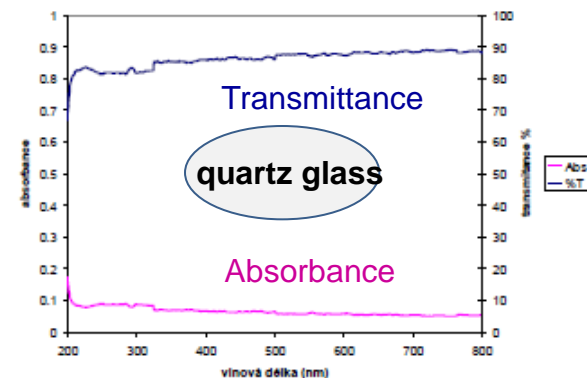
Range: 200 – 1100 nm

Occuracy: 2 nm

Absorption of air <200 nm



quartz glass





# Light sources

Sources used in UV-Vis Spectrophotometers are continuous sources.

- **Continuous sources** emit radiation of all wavelengths within the spectral region for which they are to be used.
- **Sources of radiation** should also be stable and of high intensity.

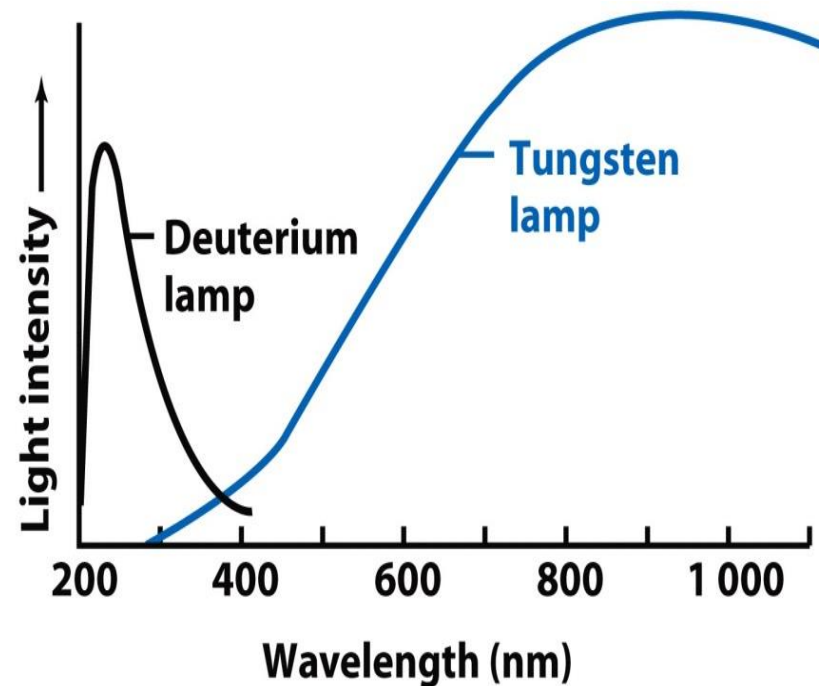
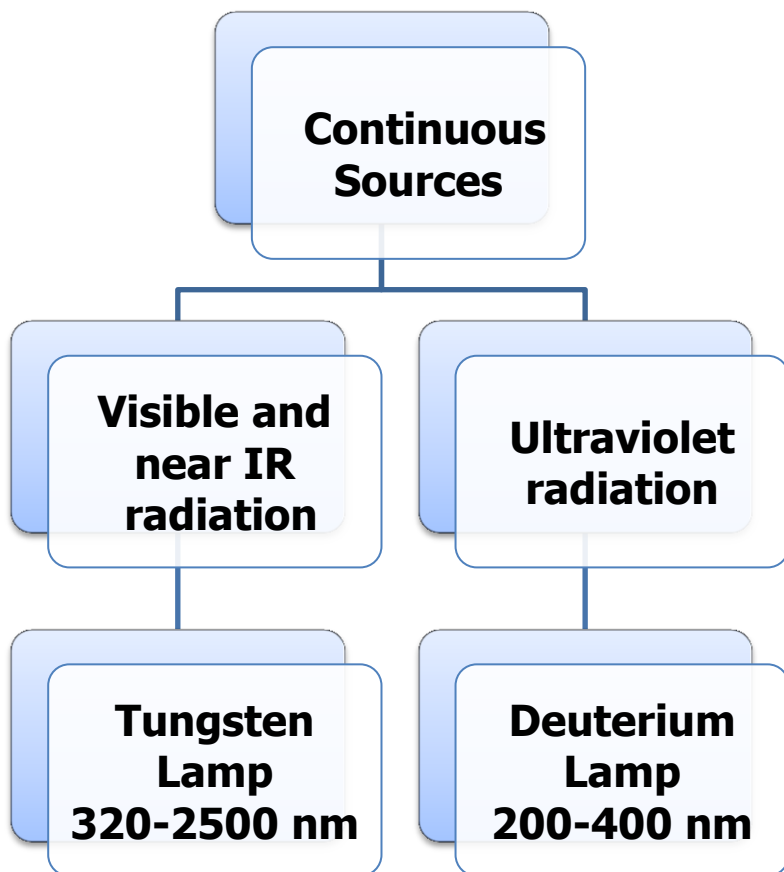
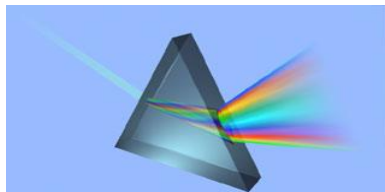


Figure 20-3  
Quantitative Chemical Analysis, Seventh Edition  
© 2007 W. H. Freeman and Company

# Monochromators

Early spectrophotometers used prisms

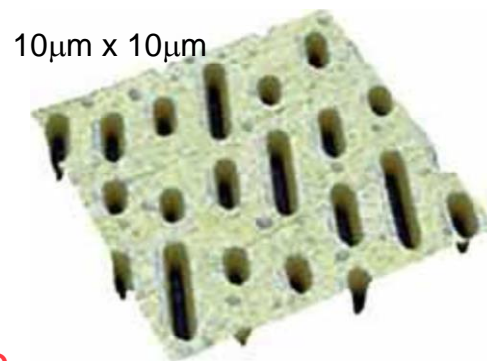
- quartz for UV
- glass for vis and IR



These are now superseded by:

*Diffraction gratings:*

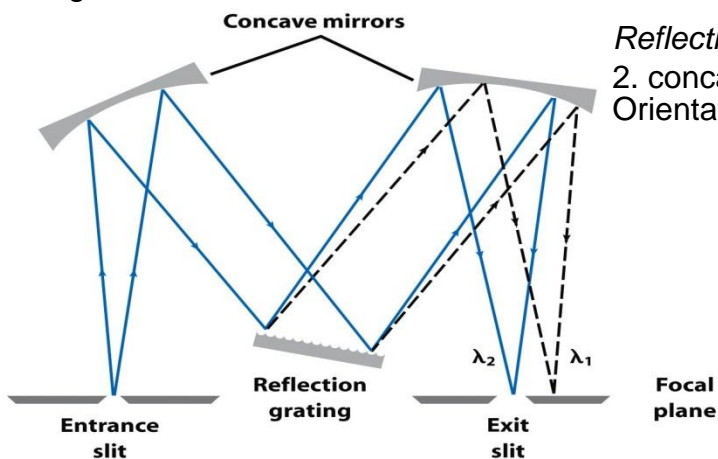
- made by drawing lines on a glass with a diamond stylus
  - ca. 20 grooves  $\text{mm}^{-1}$  for far IR
  - ca. 6000  $\text{mm}^{-1}$  for UV/vis
- can use plastic replicas in less expensive instruments



Think of diffraction on a CD

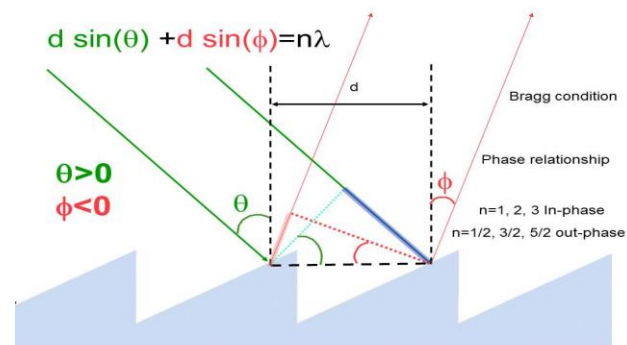
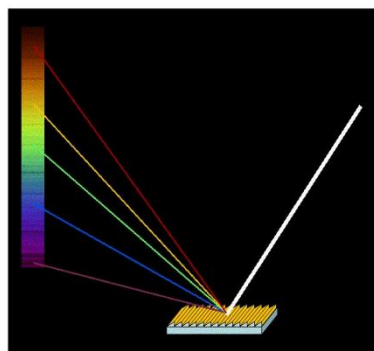
## What is the purpose of concave mirrors?

The light is *collimated* the first concave mirror



1. Reflection grating diffracts different wavelengths at different angles
  2. concave mirror focuses each wavelength at different point of *focal plane*
- Orientation of the reflection grating directs only one narrow band to exit slit

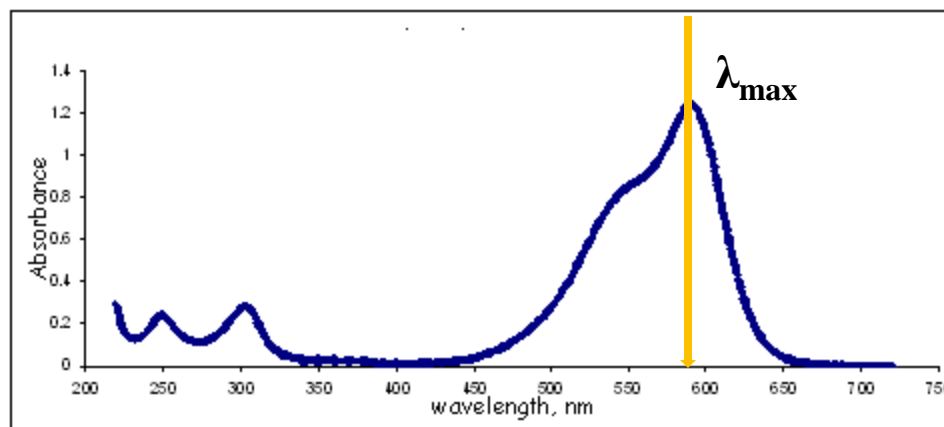
Polychromatic radiation enters



# Selection of wavelength

Absorbance measurements are always carried out at **fixed wavelength** (using monochromatic light). When a wavelength is chosen for **quantitative analysis**, three factors should be considered

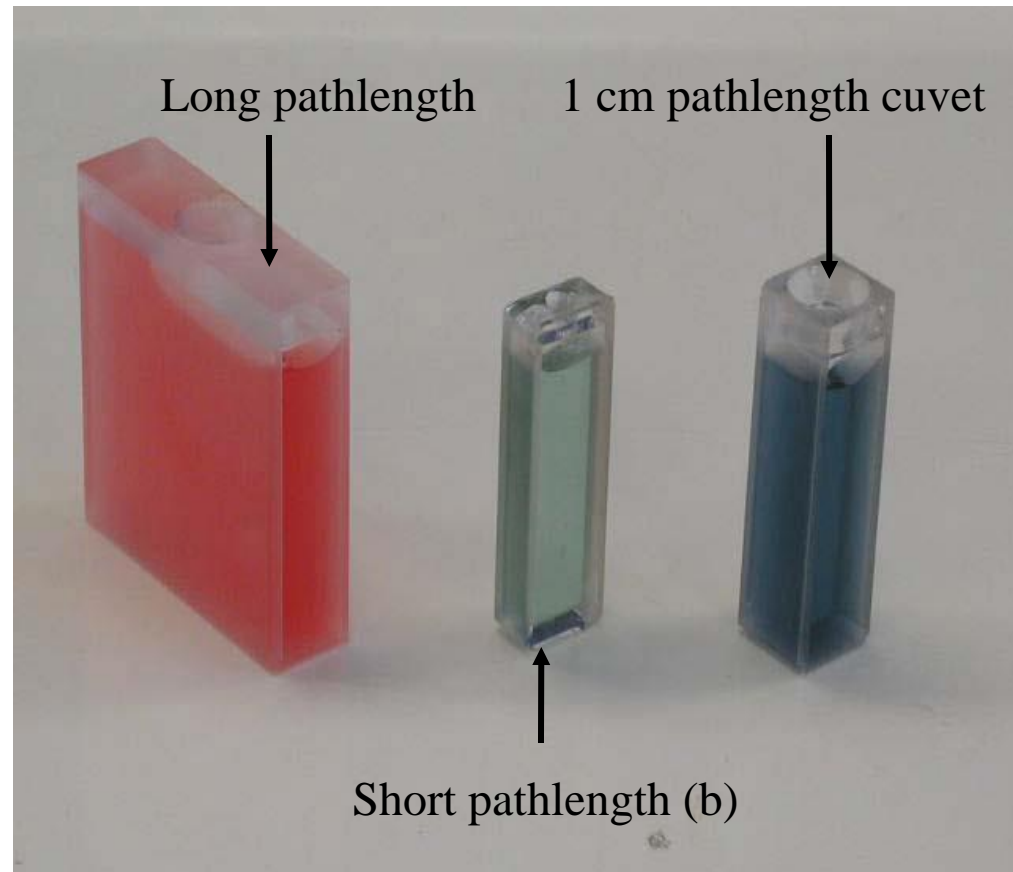
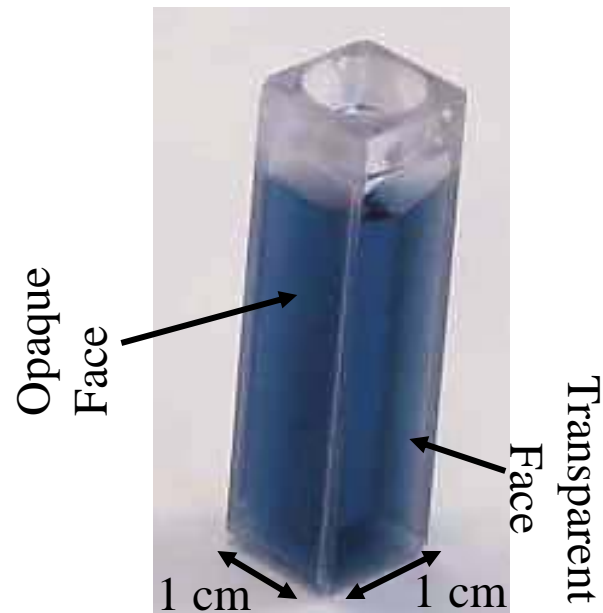
1. Wavelength should be chosen to give the **highest possible sensitivity**.  
This can be achieved **by selecting  $\lambda_{\max}$**  or in general the wavelengths at which the absorptivity is relatively high.



$\lambda_{\max}$  - wavelength where maximum absorbance occurs

# Sample compartment (cells)

- For Visible and UV spectroscopy, a liquid sample is usually contained in a cell called a **cuvette**.
- **Glass** is suitable for **visible** but not for UV spectroscopy because it absorbs UV radiation. **Quartz** can be used in **UV** as well as in visible spectroscopy

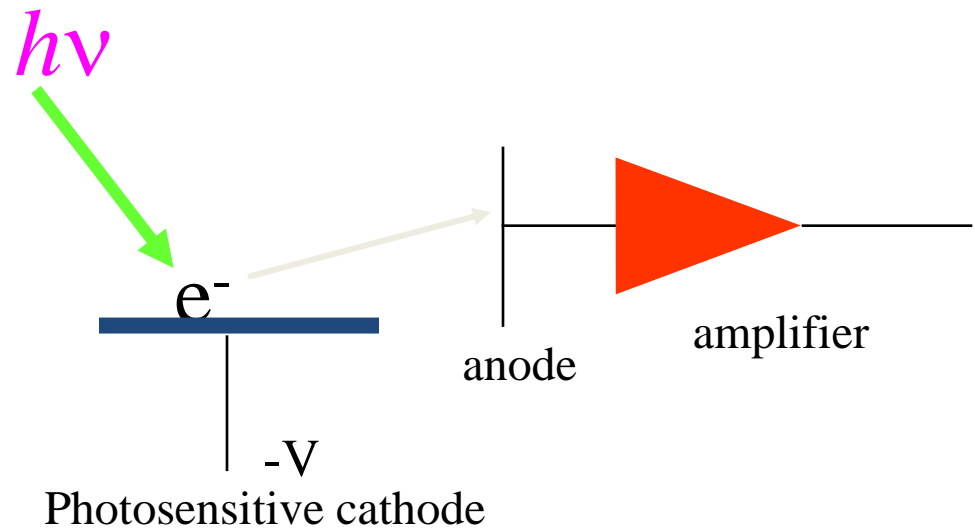


# Detectors

- 👁 The detectors are devices that **convert radiant energy into electrical signal**.
- 👁 A Detector should be **sensitive**, and has a **fast response** over a considerable range of wavelengths.
- 👁 In addition, the electrical signal produced by the detector must be **directly proportional** to the transmitted intensity (**linear response**).

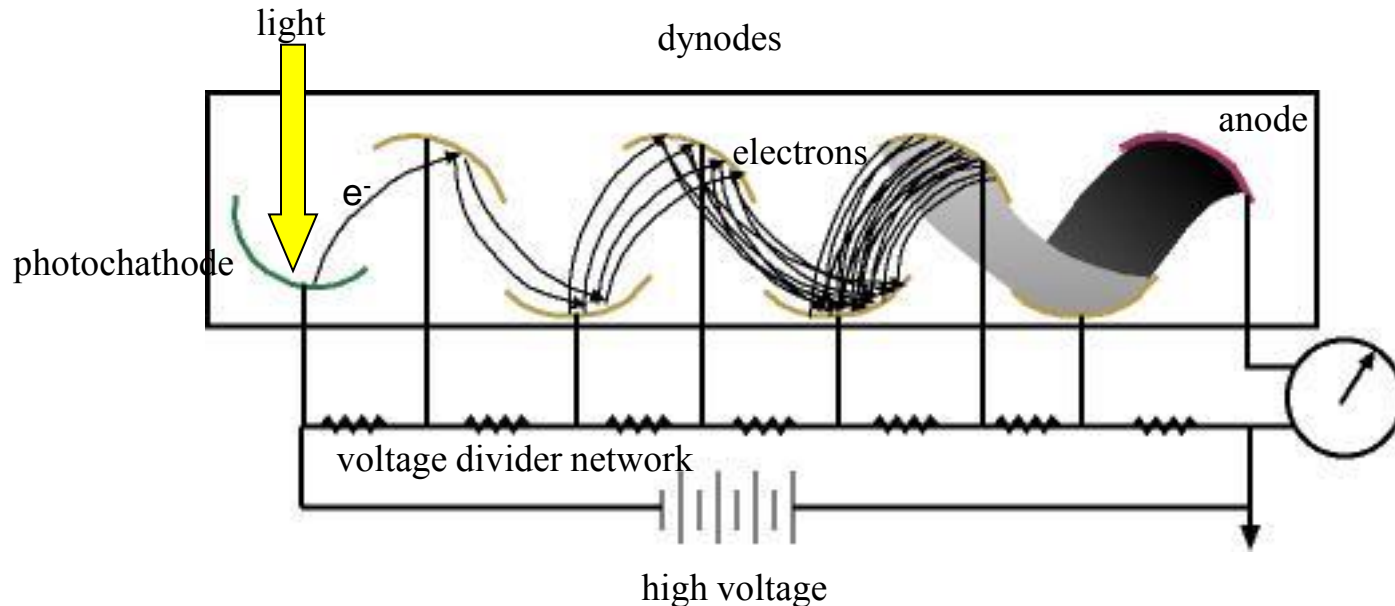
## i- Phototube

- Ⓢ Phototube emits electrons from a **photosensitive, negatively charged cathode** when struck by visible or UV radiation
- Ⓢ The electrons flow through vacuum to **an anode** to produce current which is proportional to radiation intensity.



# Photomultiplier tube

- It is a very sensitive device in which electrons emitted from the photosensitive cathode strike a second surface called **dynode** which is positive with respect to the original cathode.
- Electrons are thus accelerated and can knock out more than one electrons from the dynode.
- If the above process is repeated several times, so more than  $10^6$  electrons are finally collected for each photon striking the first cathode.

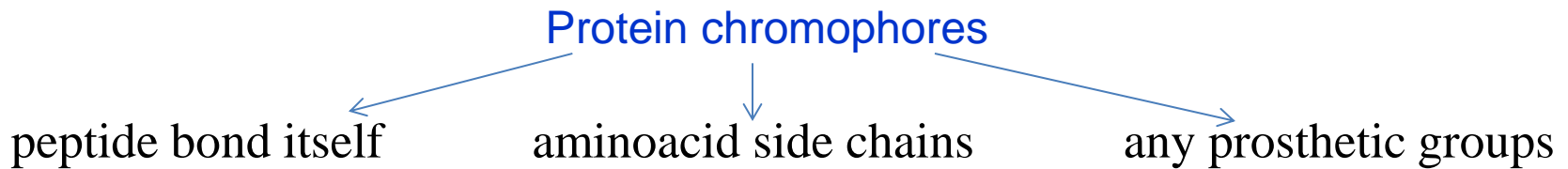


# The important characteristics of Spectrophotometric methods

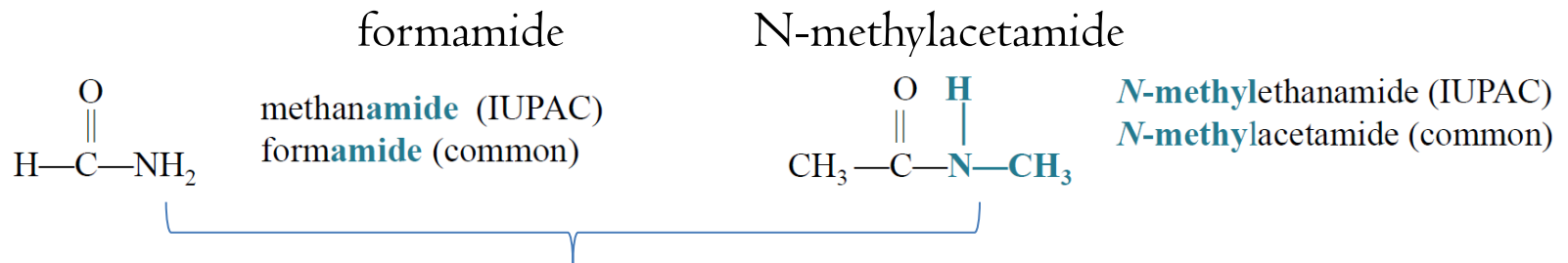
1. *Wide applicability* to both organic and inorganic systems
2. *High sensitivity* of  $10^{-6}$ - $10^{-4}$  M
3. *Moderate to high selectivity*.
4. **Good accuracy** the relative error encountered in concentration lie in the range from **1% to 3%**
5. **Ease and convenience** of data acquisition

# Spectral properties of biological molecules

- Much more complex than FA
- Major constraints – solvents and solvation
- In water experiments at  $\lambda > 170\text{nm}$ , water is strongly polar, electronic absorption bands are broader than in most other solvents (at various orientations and distances), problem of temperature (1 – 100°C)



## MODEL



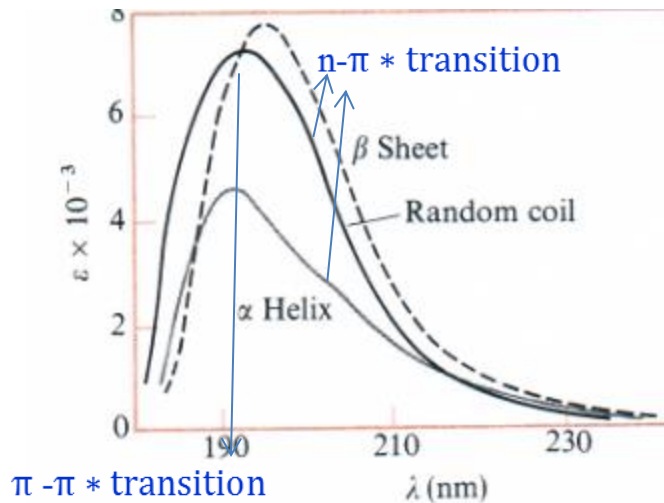
$\pi$  electrons are delocalized over C,O,N

$n\text{-}\pi^*$  transition – the lowest energy of electronic transition is symmetry – forbidden

210-220 nm,  $\epsilon_{\text{max}} \sim 100$

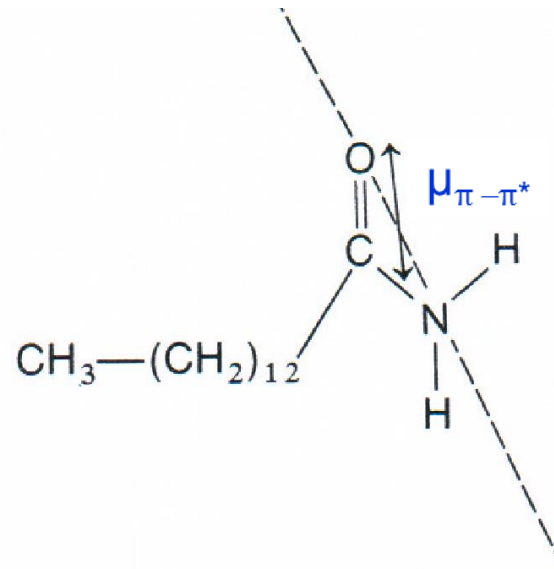


# UV spectra of poly-L-lysine in aqueous solutions



Ultraviolet absorption spectrum of poly-L-lysine in aqueous solution: random coil, pH 6.0, 25°C;  $\alpha$  helix, pH 10.8, 25°C;  $\beta$  sheet pH 10.8, 52°C. [After K. Rosenheck and P. Doty, *Proc. Natl. Acad. Sci. USA* 47:1775 (1961).]

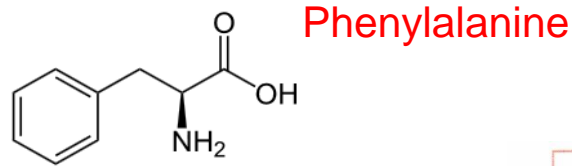
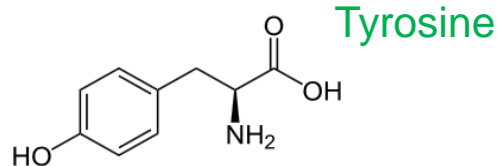
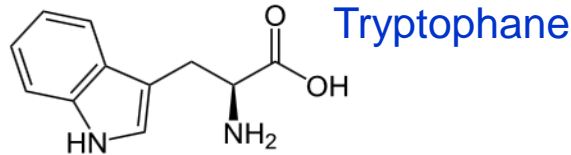
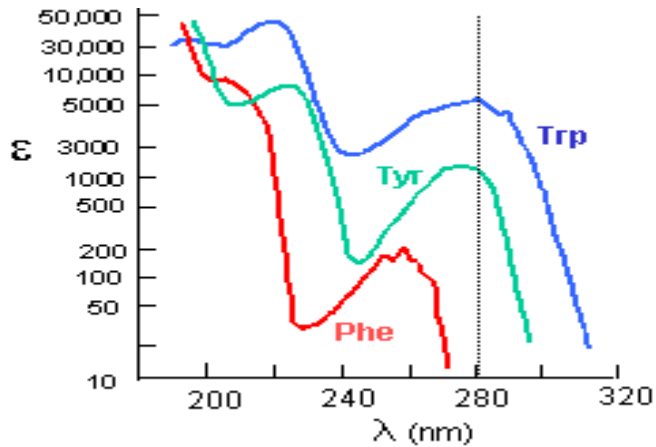
In FA  $\pi - \pi^*$  transition is polarized along the C – O axis, in peptide does not lie along any particular bond



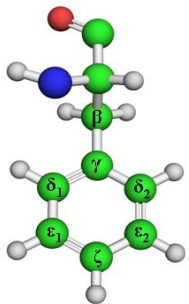
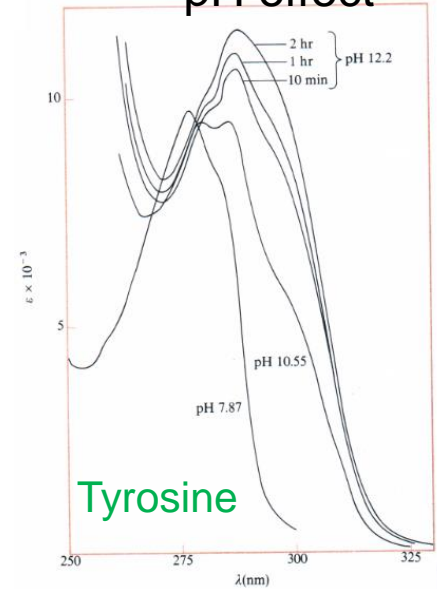
# UV spectra of aromatic amino acid

AA – in the same spectral region – the strong peptide absorption ( $\pi-\pi^*$ )

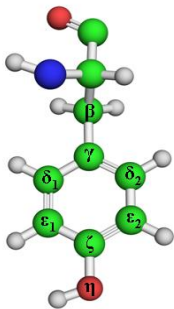
AAA – in pH 7 ( $\pi-\pi^*$ ) symmetry



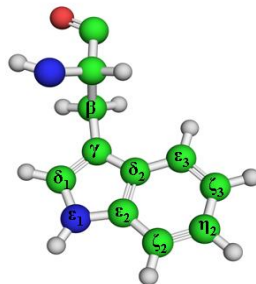
pH effect



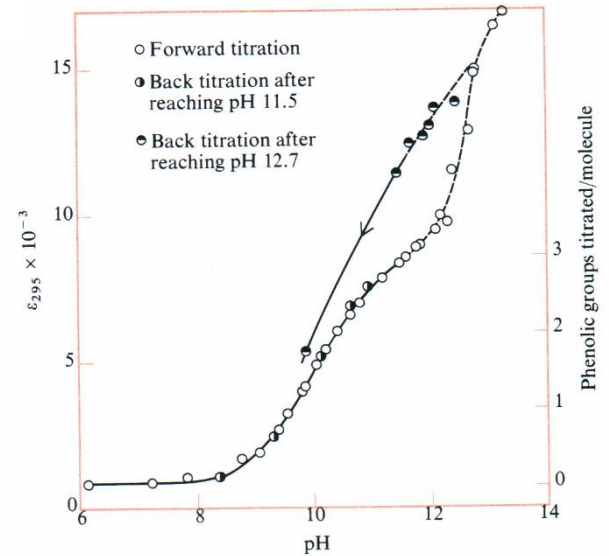
Phenylalanine  
(Phe, F)



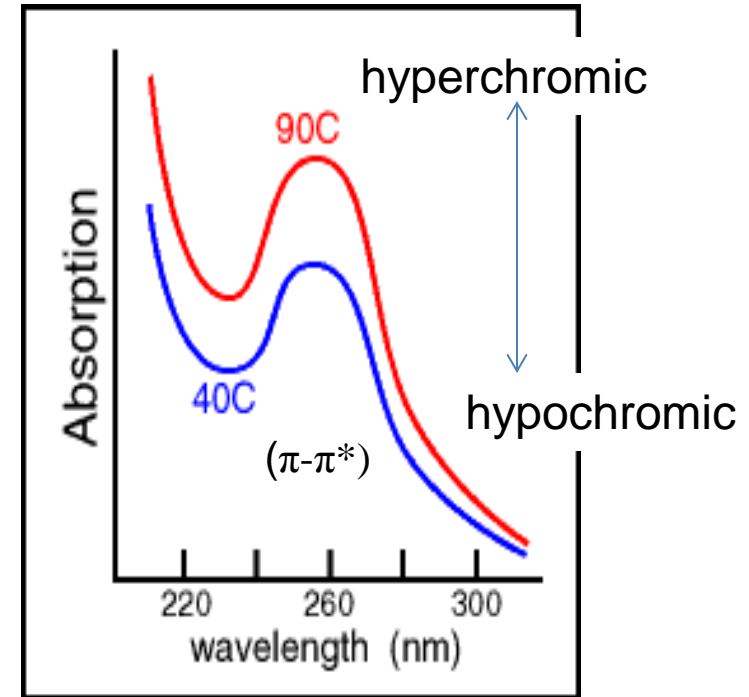
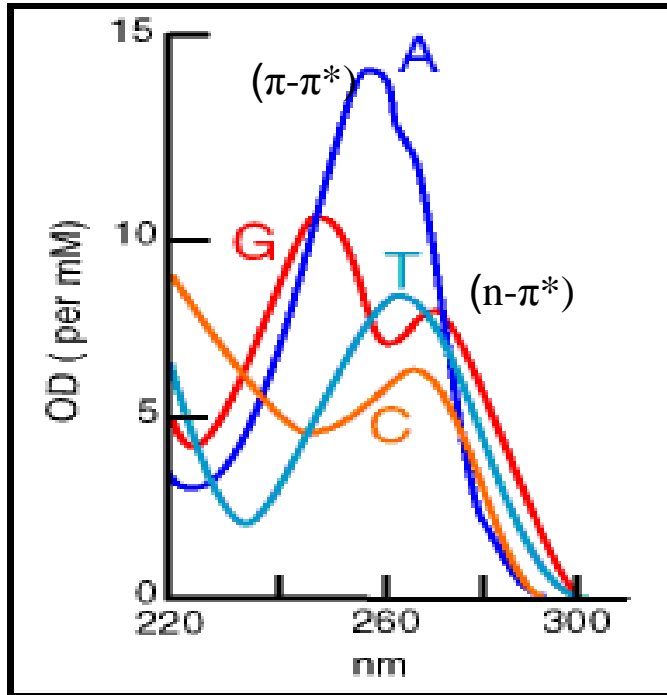
Tyrosine  
(Tyr, Y)



Tryptophan  
(Trp, W)



# UV spectra of DNA and its bases



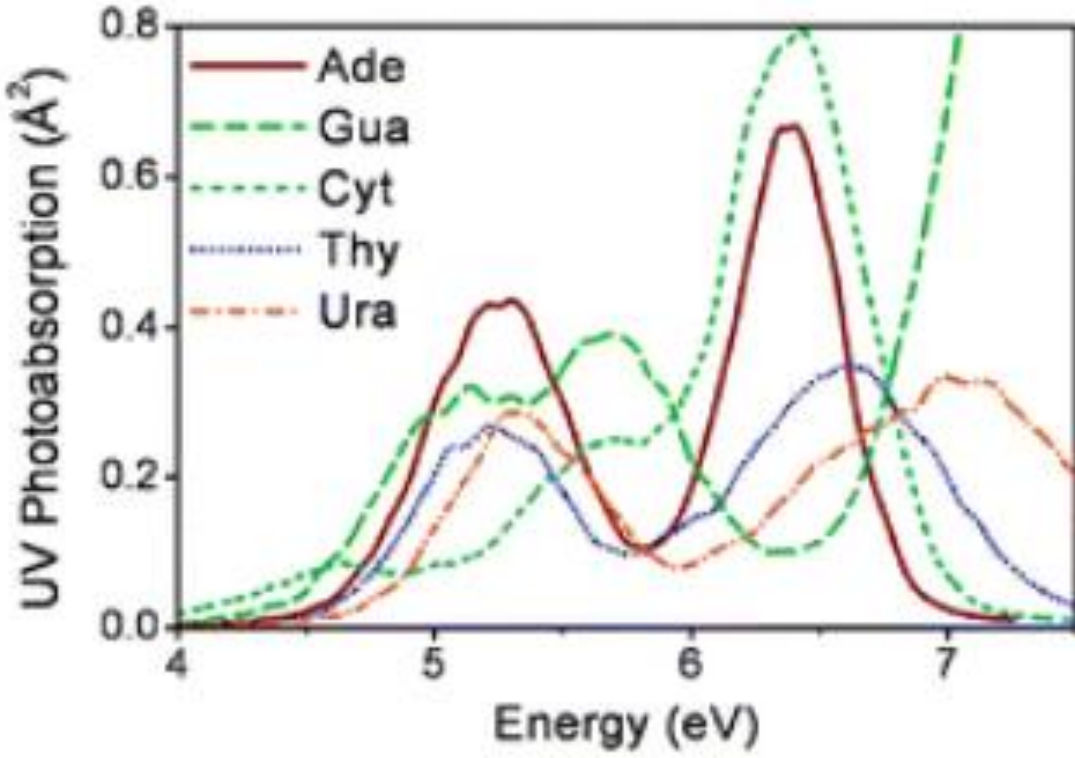
A pure DNA solution appears transparent to the eye, and absorption doesn't become measurable until 320 nm. Moving further into the u.v. region, there is a peak at about 260 nm, followed by a dip between 220 and 230, and then the solution becomes essentially opaque in the far u.v.

When a DNA helix is denatured to become single strands, e.g. by heating, the absorbance is increased about 30 percent. This increase, called the hyperchromic effect, reveals the interaction between the electronic dipoles in the stacked bases of the native helix.

Electronic state of nucleobases is more complex than chromophores of peptides

- low symmetry
- many nonbonded electrons
- several different transition  $(\pi-\pi^*)$   $(n-\pi^*)$

The UV absorption of nucleobases: semi-classical *ab initio* spectra simulations

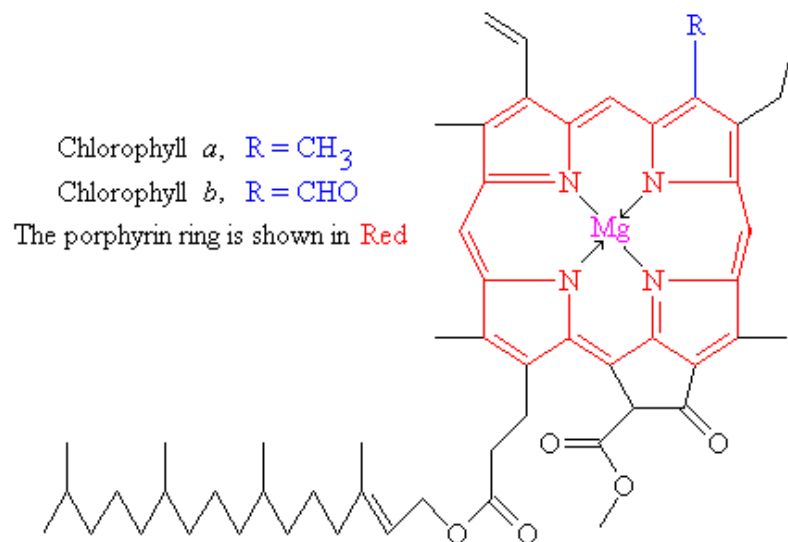


Physical Chemistry Chemical Physics

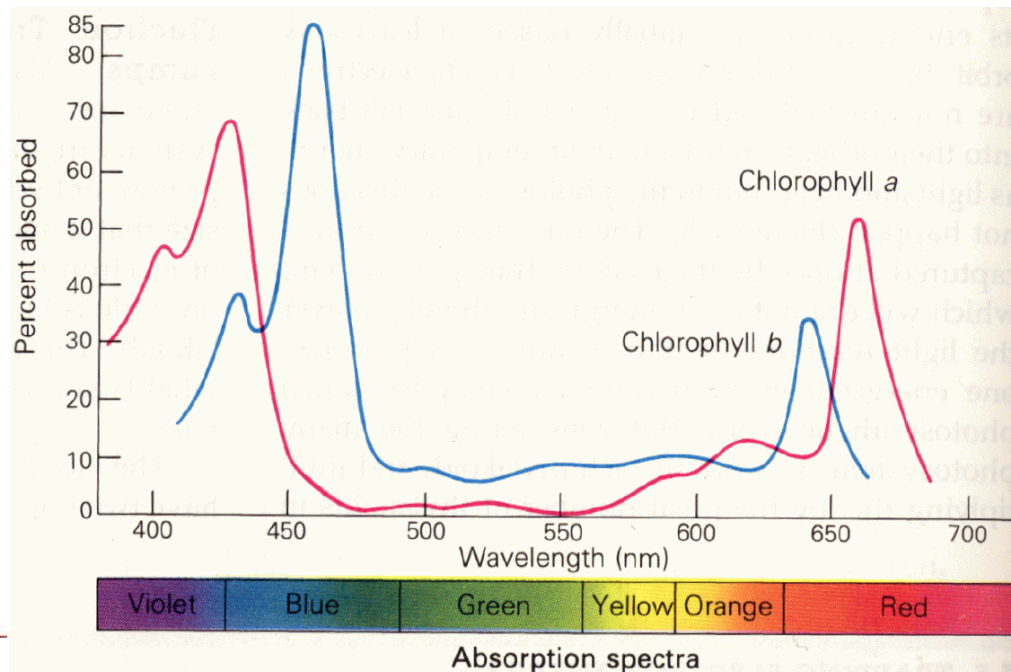
# Effect prosthetic groups

Polypeptide chain – prosthetic group (apoprotein)  $\begin{cases} \text{local environment} \\ \text{oxidation-reduction} \\ 200 - 300 \text{ nm} \end{cases}$

- Prosthetic group must have a high enough molar extinction coefficient to be detectable at typical protein concentrations
- To avoid the formation of intermolecular aggregates

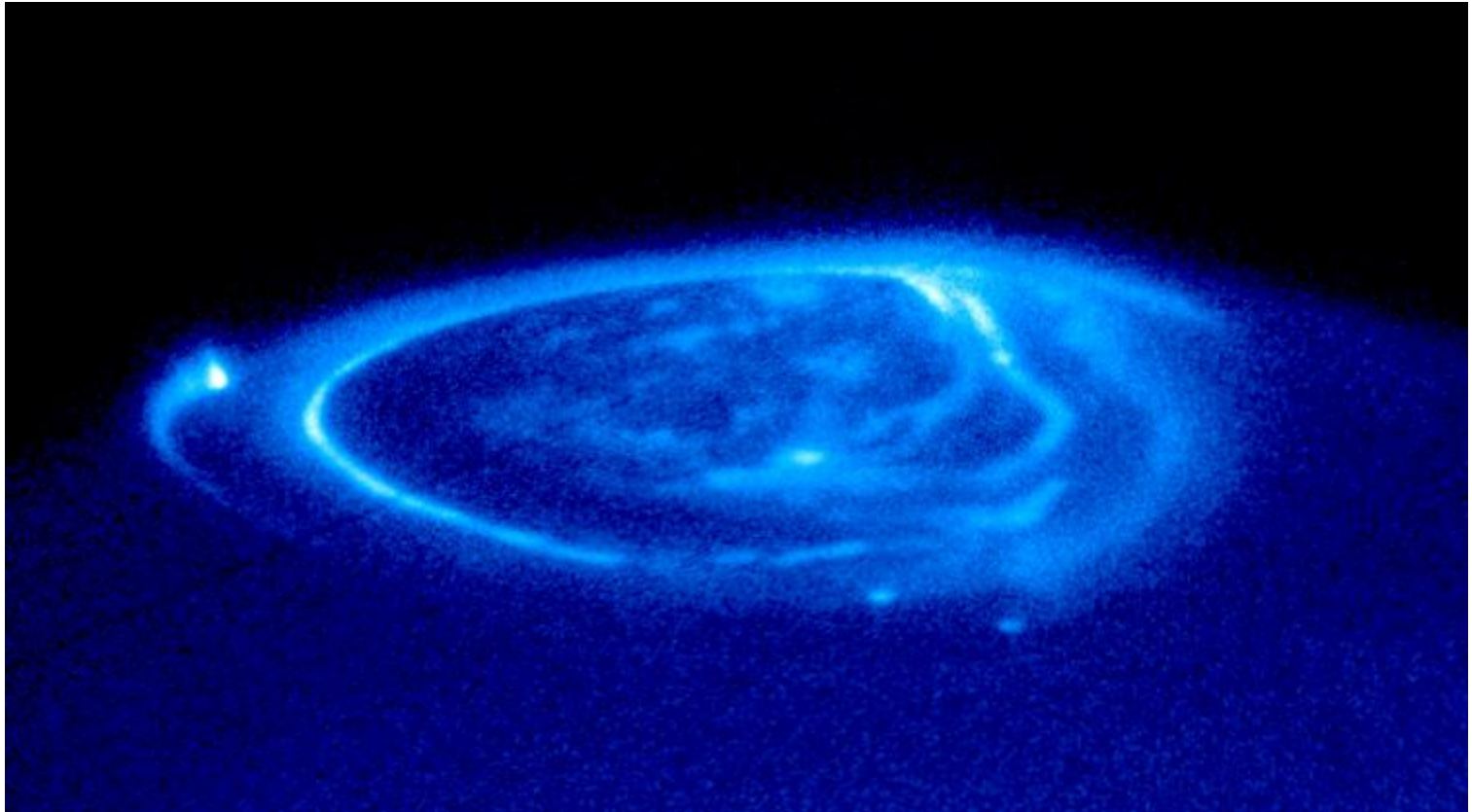


## Absorption spectra of chlorophylls



Spectroscopic properties of proteins containing prosthetic groups

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



Snímek polární záře na Jupiteru, jak ji v ultrafialovém oboru spektra zaznamenal Hubbleův vesmírný dalekohled

Podle moderních modelů evoluce je vznik a evoluce prvotních **proteinů a enzymů** schopných reprodukce připisován právě existenci ultrafialového záření.