Experiment 3: Fluorescence Spectroscopy I (continued)

Last week: Part I.A: Introduction to steady state spectra

Today: Part 1.B: Fluorescence Quenching and the Stern-Volmer Relation

Prelab Lecture 9feb17
P. Callis

All life appears to be nurtured by the excitation of electrons by light in photosynthesis.

The vision enjoyed by higher life forms begins with the electronic excitation of a conjugated polyene.

All life appears to be nurtured by the excitation of electrons by light in photosynthesis. The vision enjoyed by higher life forms begins with the electronic excitation of a conjugated polyene.

1. Introduction

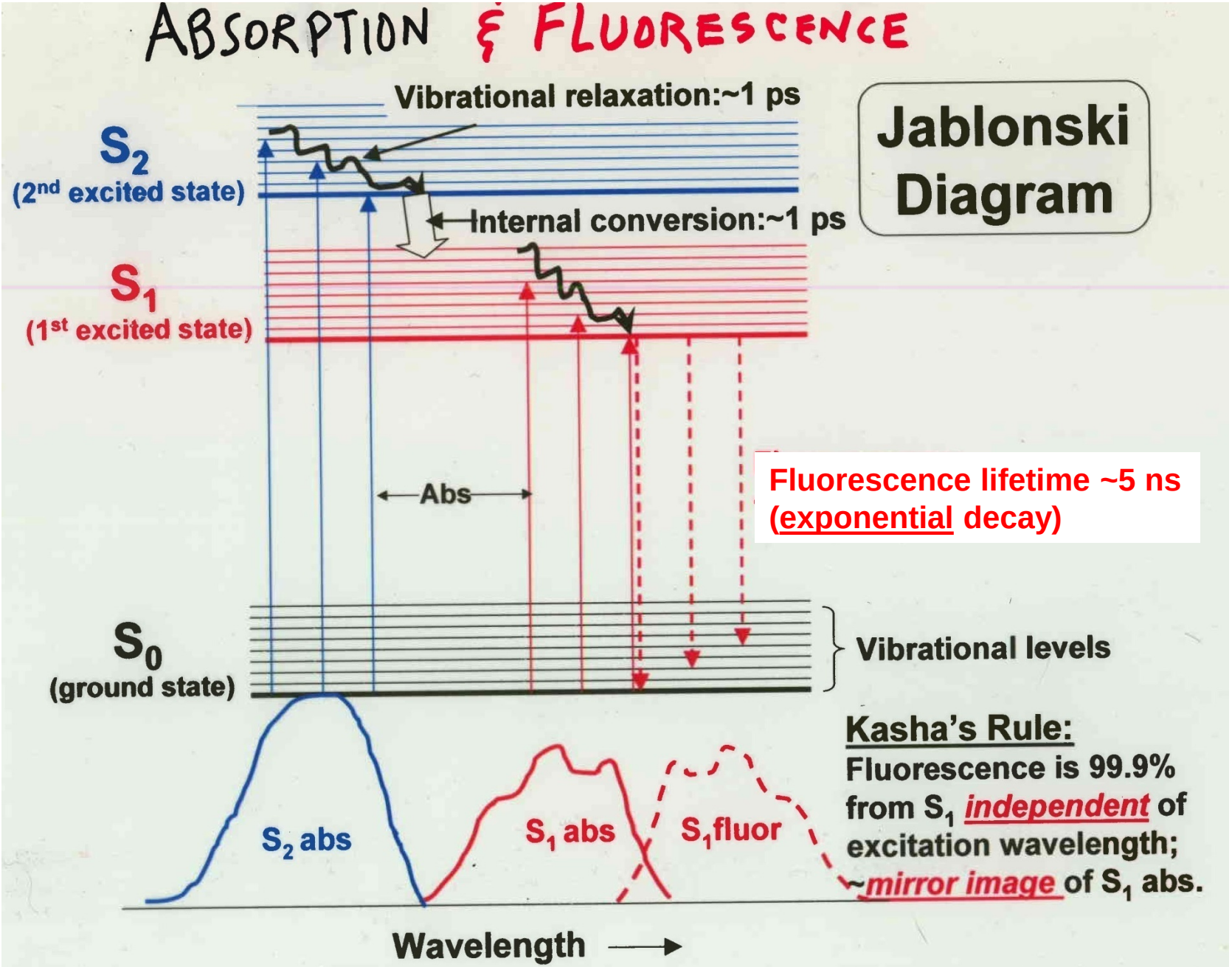
Motivation

- **To understand our very existence we must understand the absorption and emission of light.**

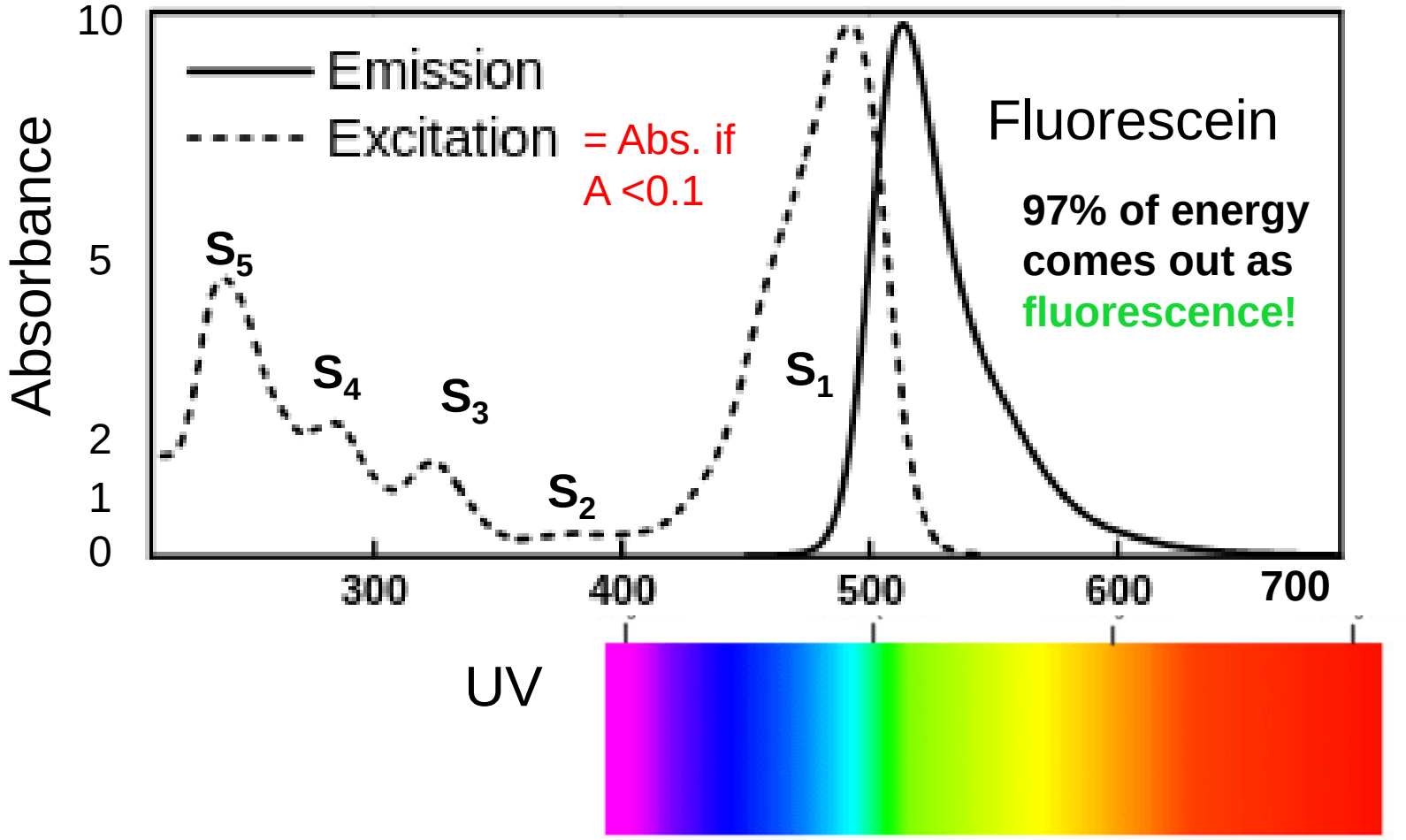
- **From a practical standpoint, there is an explosion in the use of fluorescent dyes to visualize processes in living cells and other materials at unprecedented detail, and in breathtaking color.**

- **These images are a new art form. Fluorescence-based assays are also playing a rapidly increasing role in areas traditionally served by radioactive assays. Major reasons for this are the high sensitivity of fluorescence detection and its environmental friendliness.**

- **In addition, light emitted within 100 fs following pulses of light as short as 10 fs represent the current frontier for the observation of fast chemical events.**


ABSORPTION & FLUORESCENCE
Vibrational relaxation:~1 ps
Jablonski Diagram
S2
(2nd excited state)
Internal conversion:~1 ps
S1
(1st excited state)
Abs
Fluorescence lifetime ~5 ns
(exponential decay)
S0
(ground state)
Vibrational levels
Kasha's Rule:
Fluorescence is 99.9%
from S1 independent of
excitation wavelength;
~mirror image of S1 abs.
S2 abs
S1 abs
S1fluor
Wavelength

https://en.wikipedia.org/wiki/Fluorescein

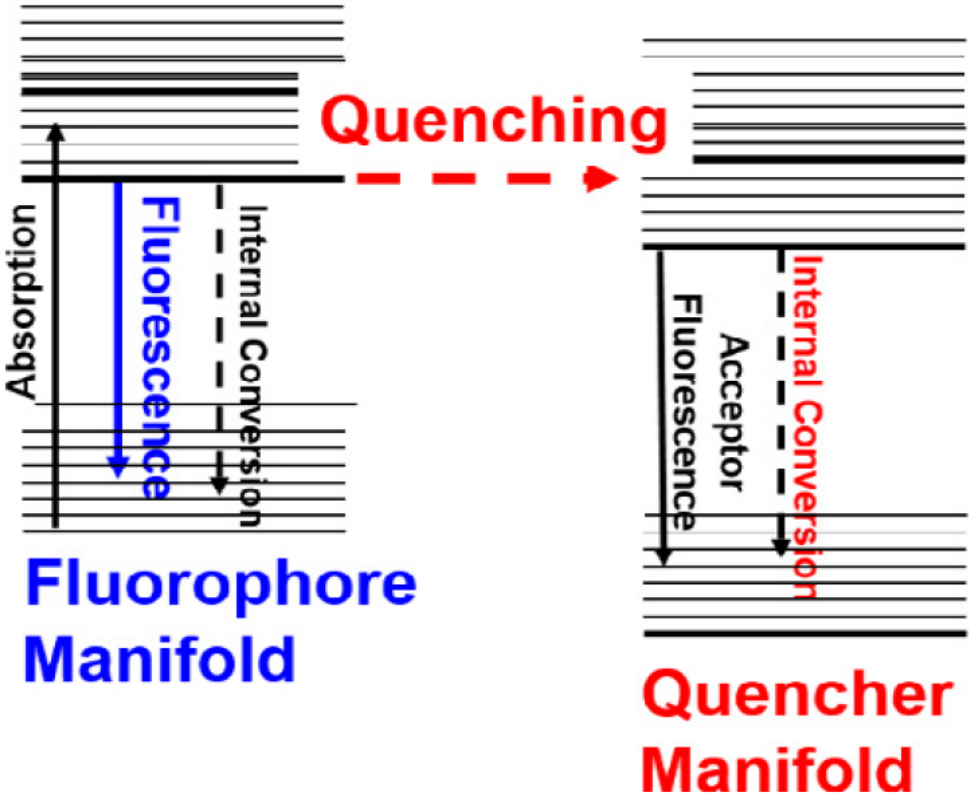


“Quenching”

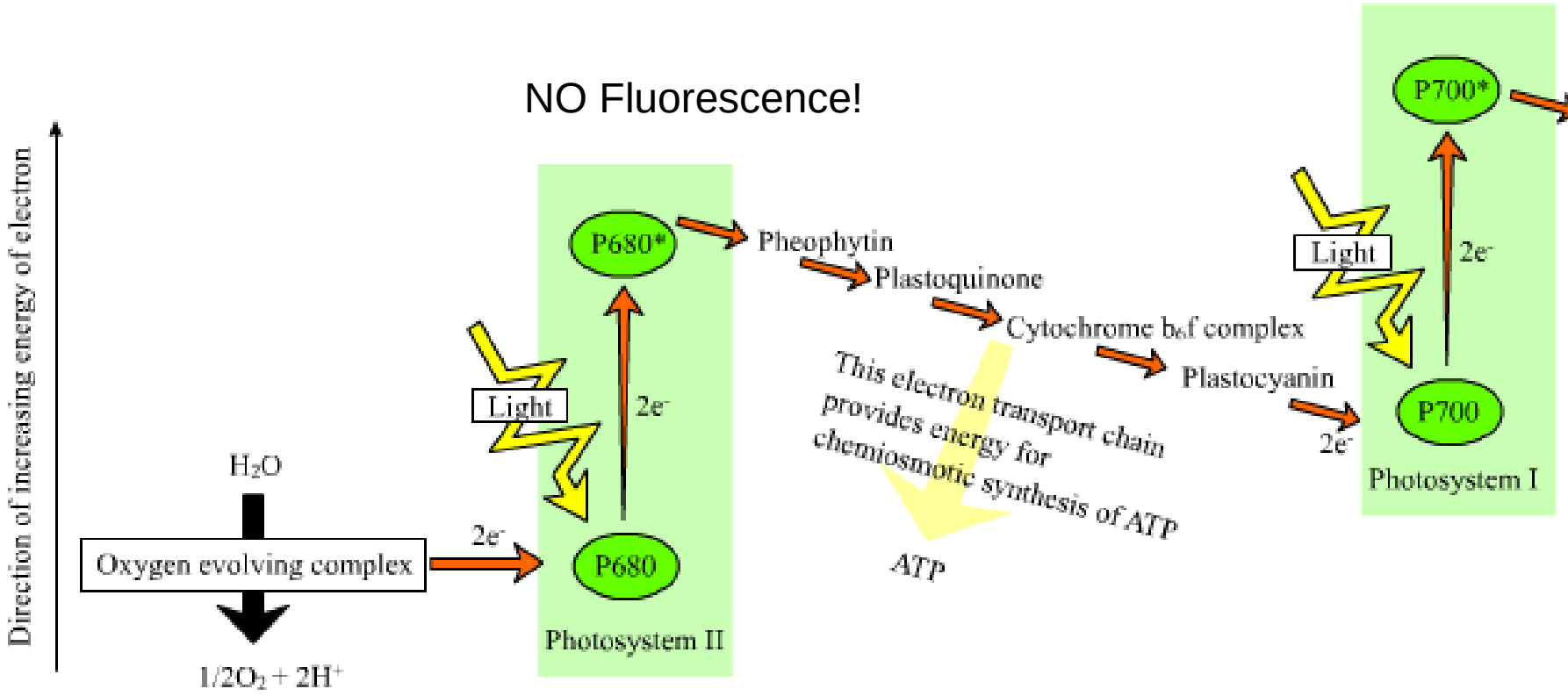
IN NATURE

"Quenching" is a generic term usually referring to a process that leads to a decrease in fluorescence.... (***quenching is therefore technically a form of internal conversion***)

Supermolecule Jablonski Diagram (two molecules close together)



The Second Step of Photosynthesis: is quenching of the fluorescence of chlorophyll



By w:User:Bensaccount - http://en.wikipedia.org/wiki/Image:Z-scheme.png, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=3461098

Binding phenomena and fluorescence quenching. I: Descriptive quantum principles of fluorescence quenching using a supermolecule approach ☆

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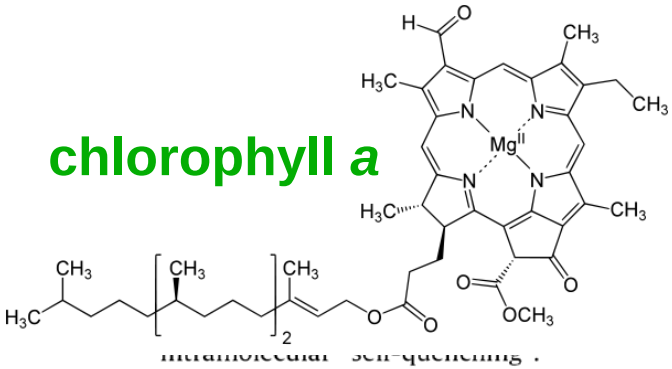
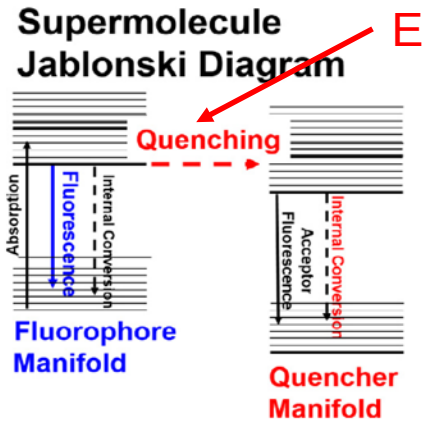
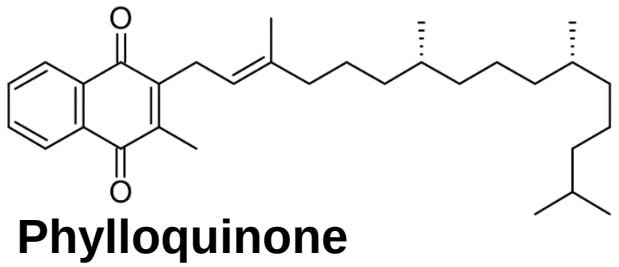
HIGHLIGHTS

GRAPHICAL ABSTRACT

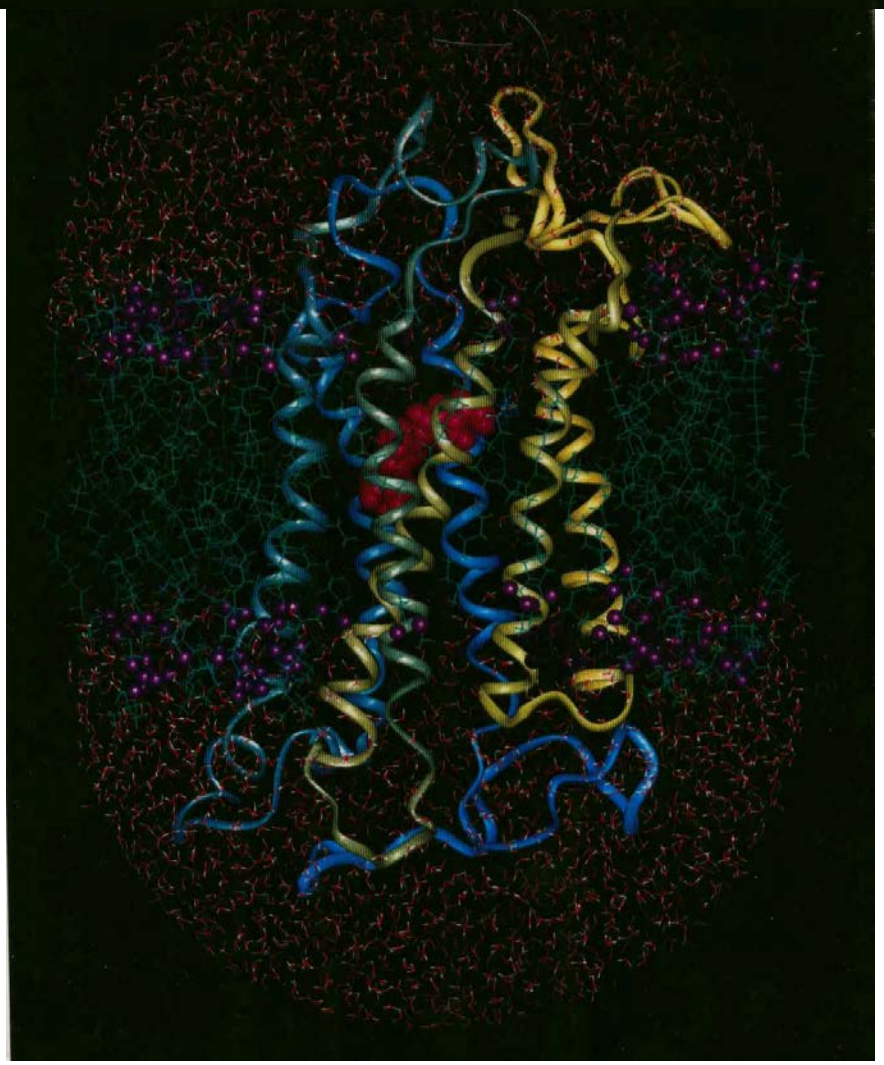






The Second Step of Photosynthesis:
is quenching of the fluorescence of chlorophyll

Rhodopsin, harboring the visual pigment retinal (pink) is a member of the large class of signal transducing proteins called G-protein coupled receptors.



The vision enjoyed by higher life forms begins with the electronic excitation of a conjugated polyene.

This is actually a form of INTERNAL CONVERSION not quenching

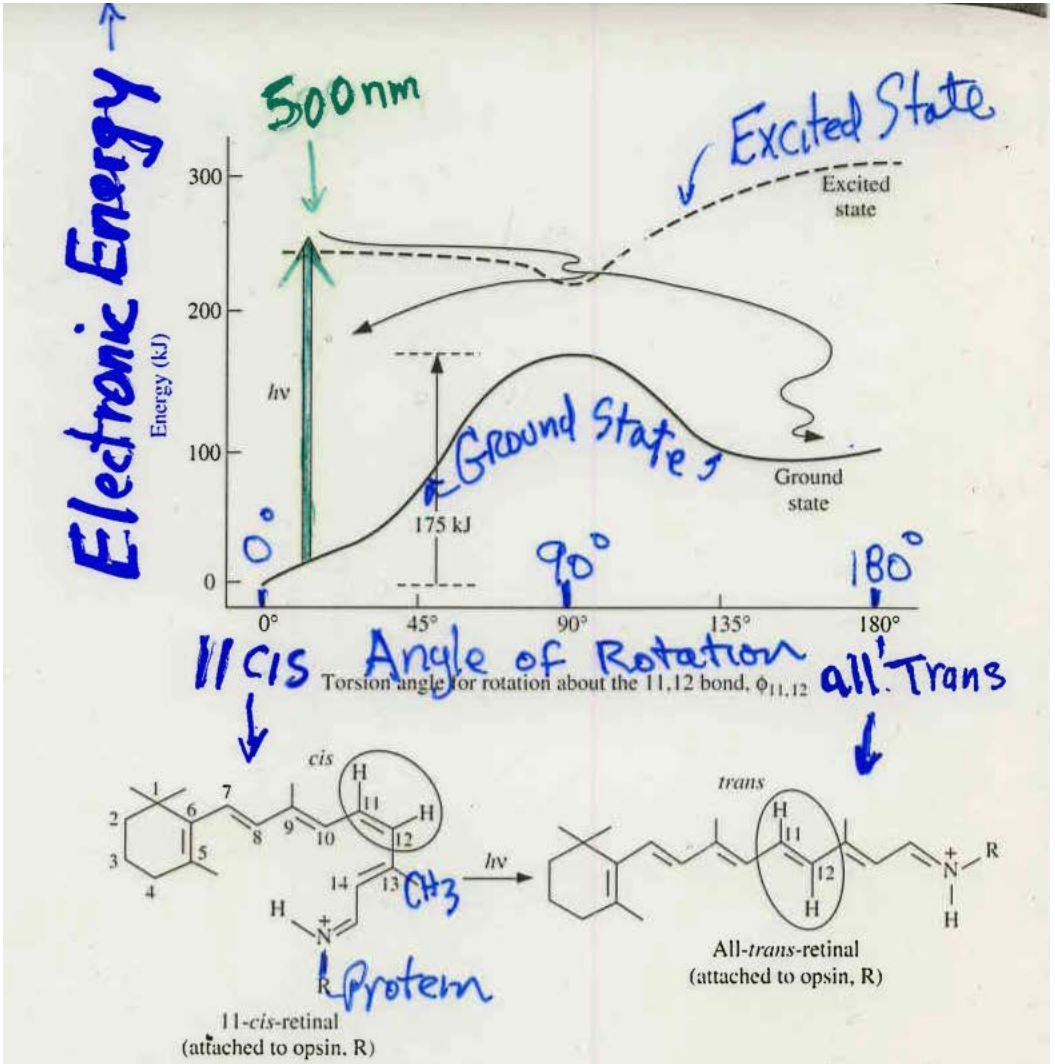
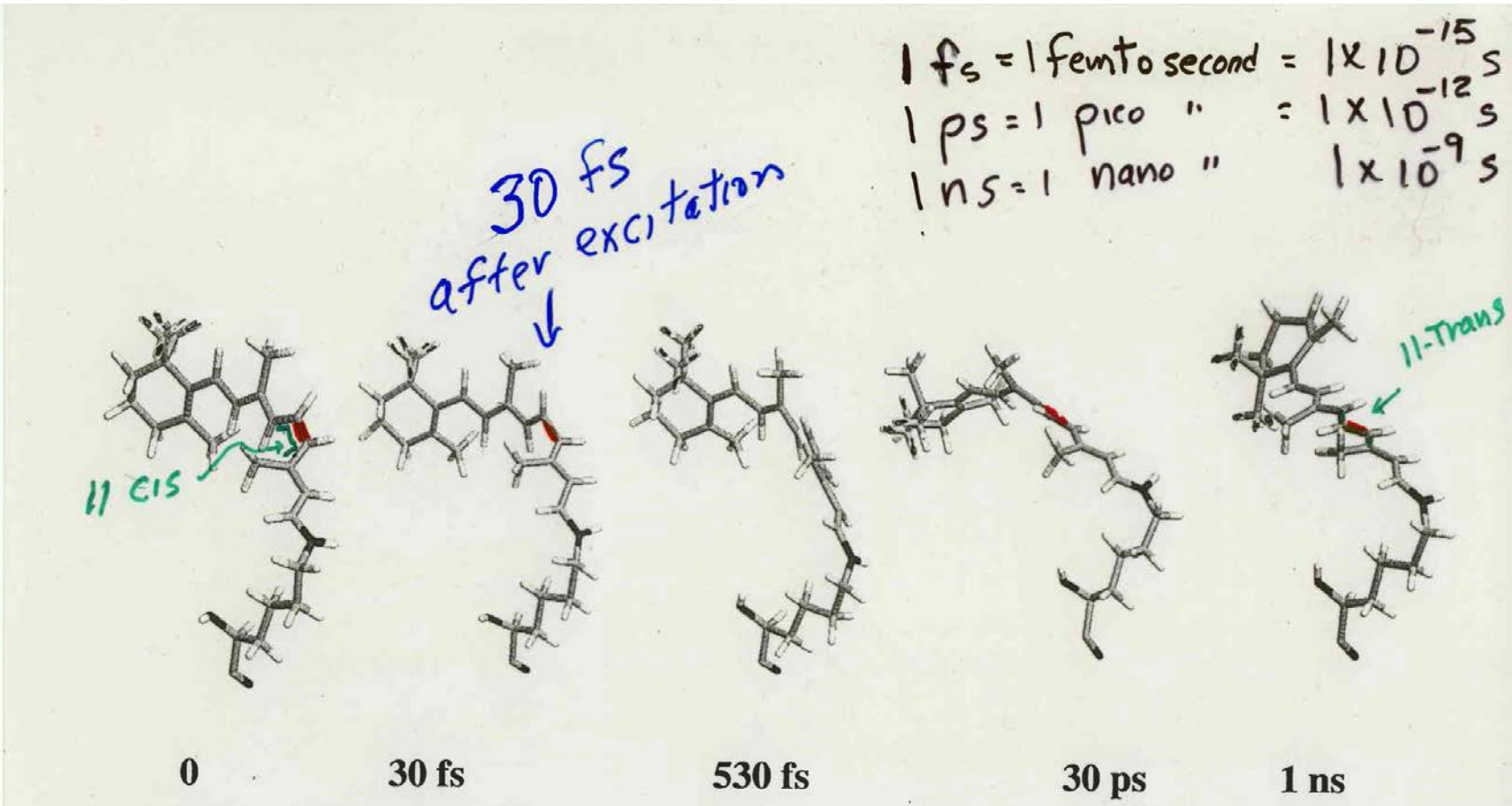


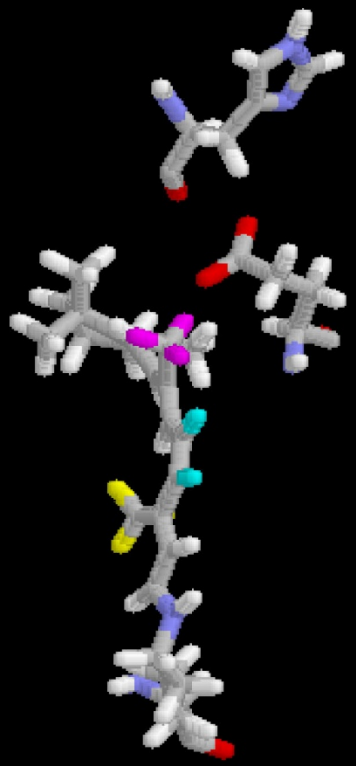
Fig. 9.1 Light-driven isomerization of 11-*cis*-retinal attached to opsin (R) by a Schiff's base linkage. Theoretical calculations of the energy of both the ground state and an excited electronic state suggest a possible path whereby absorption of a photon will lead to conversion from the *cis* to the *trans* configuration about 60% of the time. The angle of rotation about the 11,12-double bond is plotted as the horizontal axis.

Figure 11.3
page 410

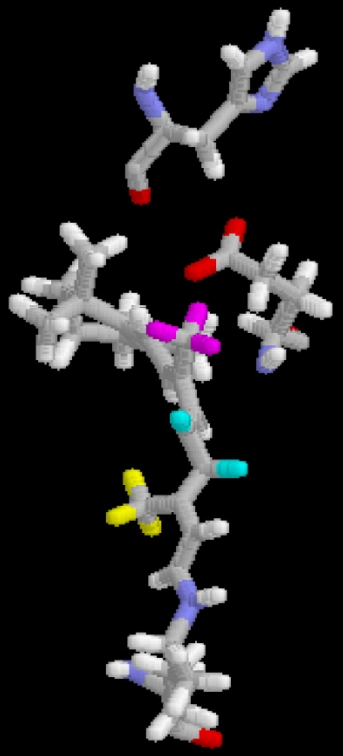


Snapshots of simulated isomerization of retinal Schiff base of rhodopsin (1hzx)

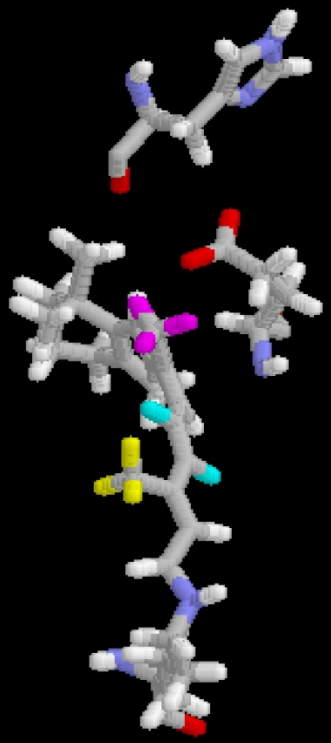
Retinal: 11-cis



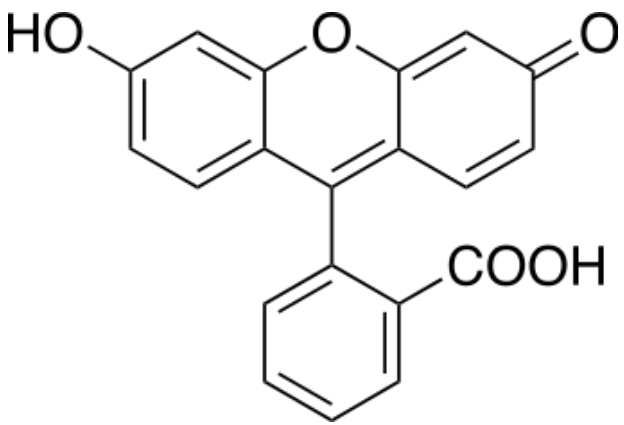
excited state



all-trans

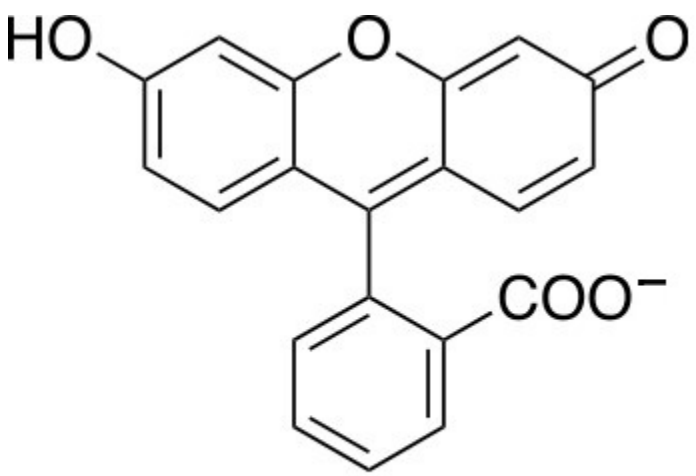


Fluorescein at different pH



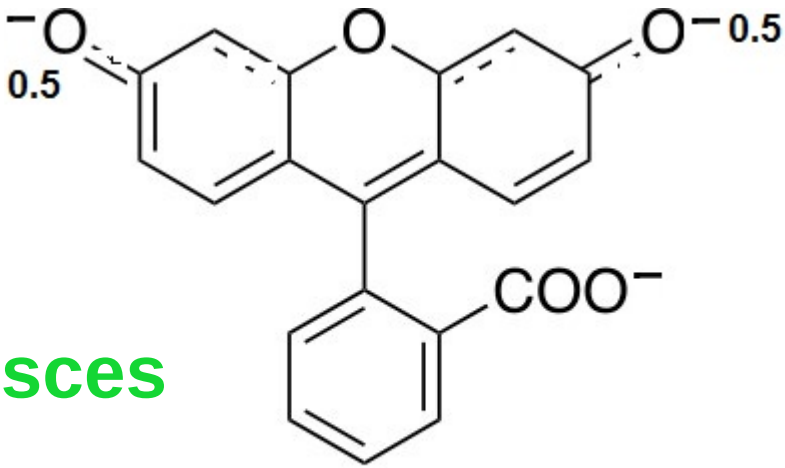
neutral (NOT soluble)
(low pH)

→



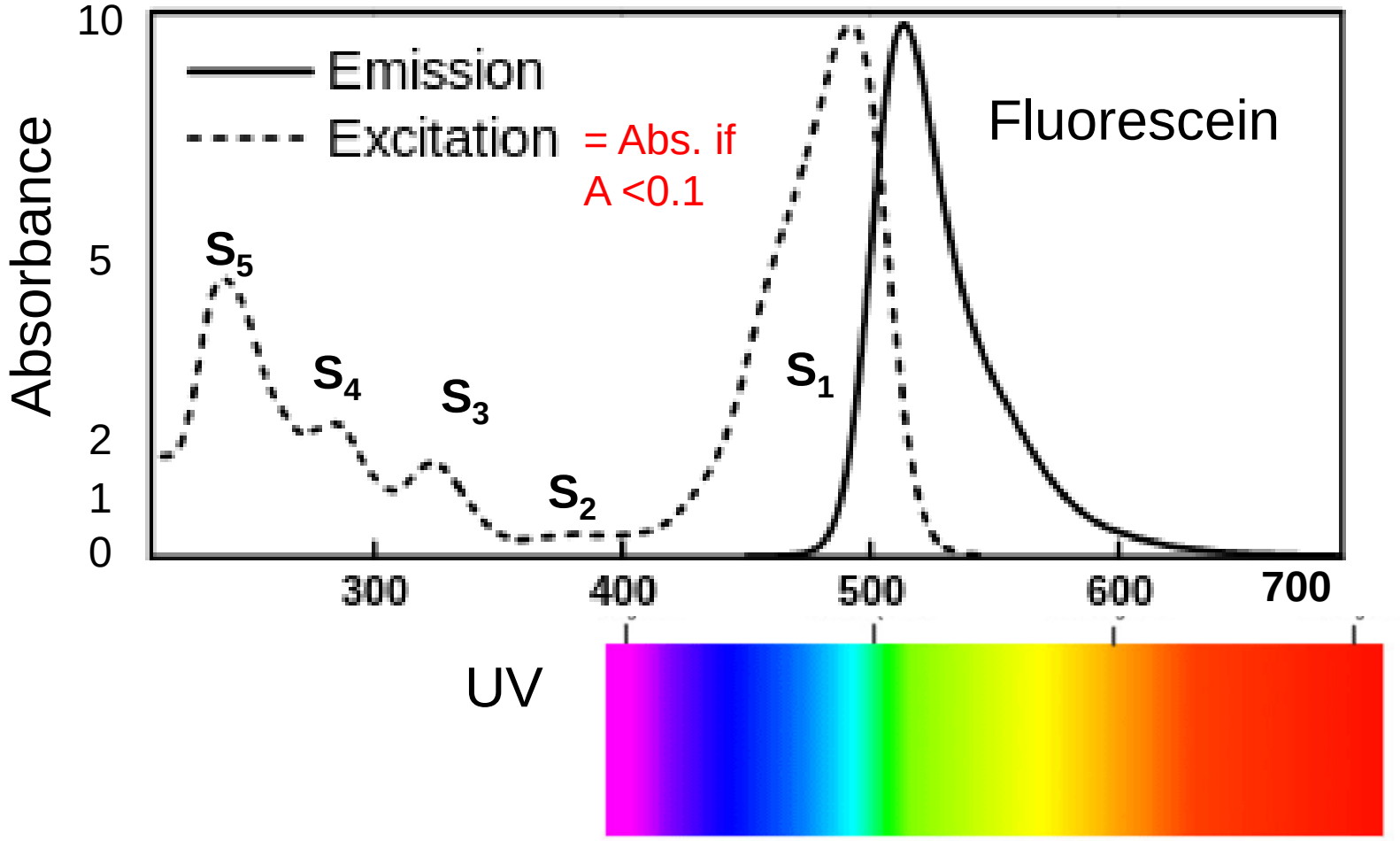
mono-anion (pH 7)

↓

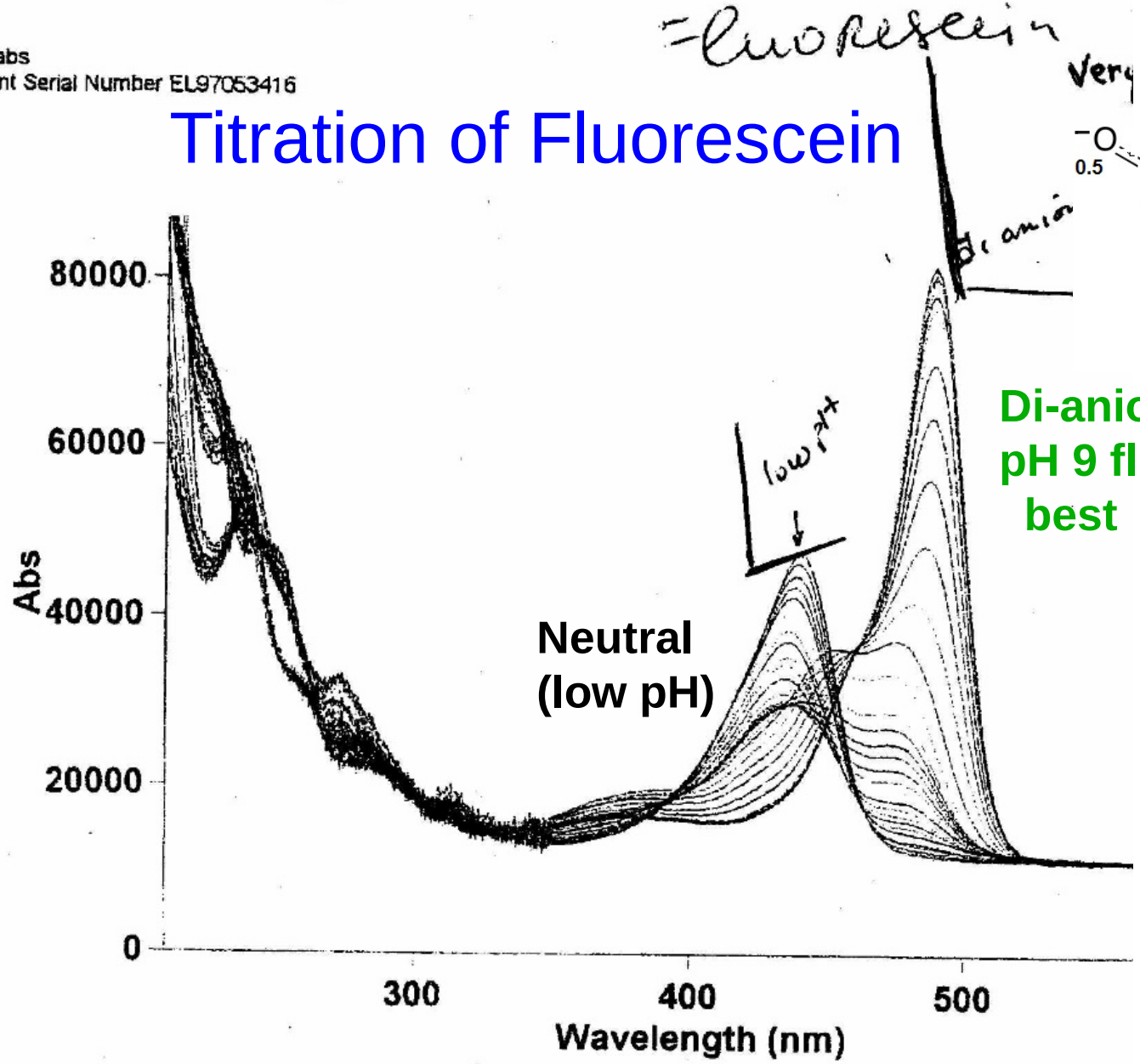


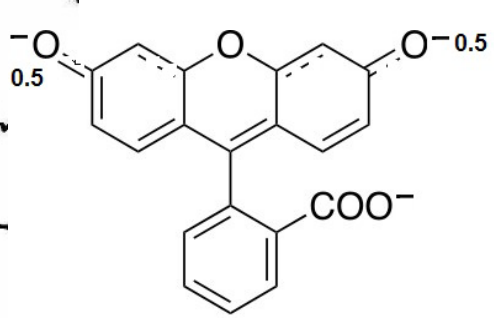
di-anion
pH 9 fluoresces
best

https://en.wikipedia.org/wiki/Fluorescein



Titration of Fluorescein





Neutral (low pH)

Di-anion pH 9 fluoresces best

Courtesy Sergey Tetin, Abbott Labs

Spectral "BUMPS and HUMPS"

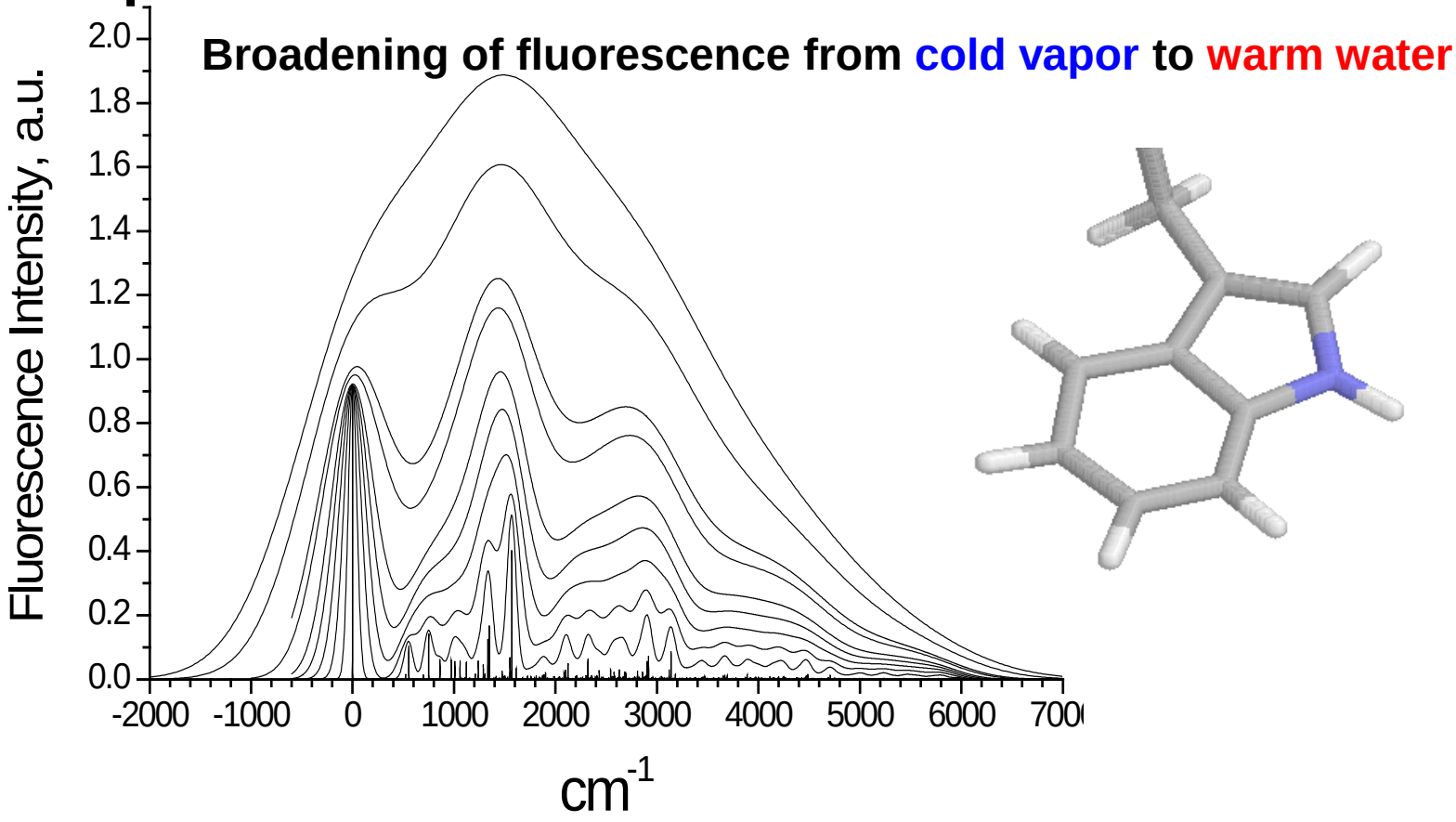
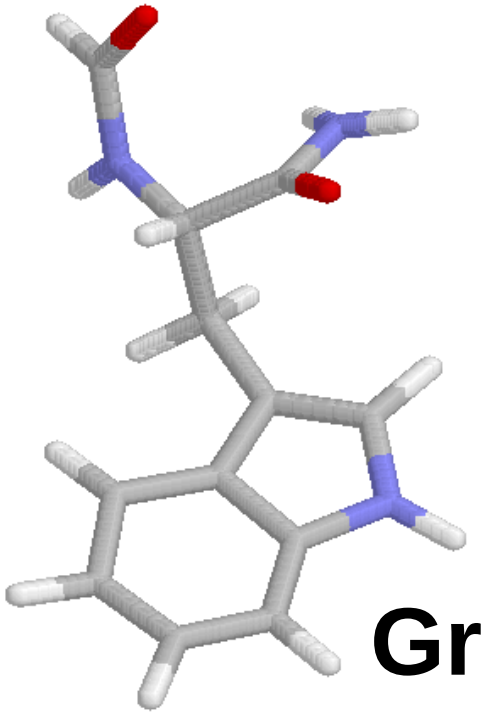


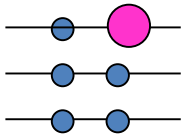
Fig. 21. Broadened calculated spectra. The spectral width of each line of the upwardly displaced spectra is determined by a Gaussian with width of 3, 100, 200, 300, 400, 500, 700, 800, 1200, and 1500 cm^{-1} respectively. The area under each curve is proportional to the line width. (Agrees well with experiment)

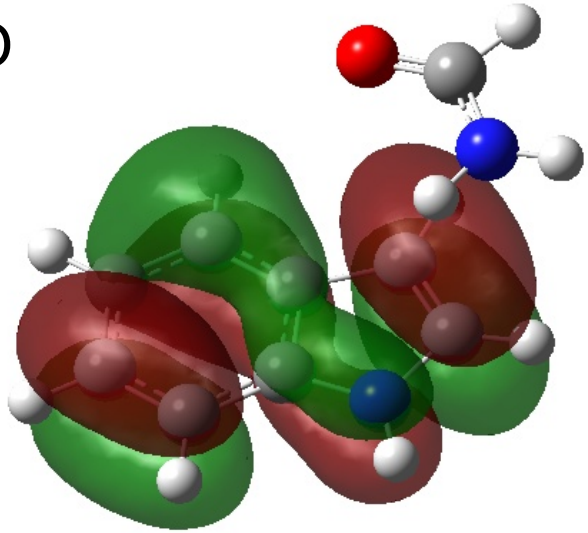
“Quenching”

with iodide ion



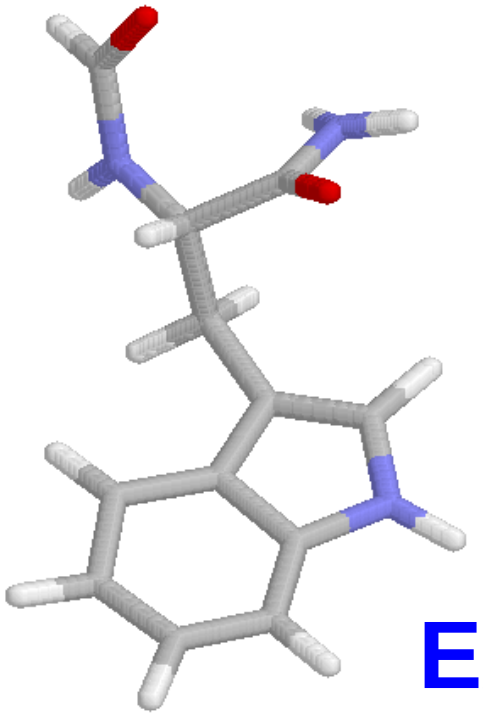
LUMO

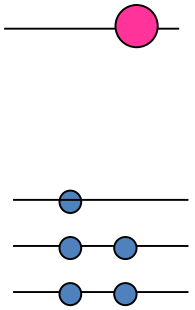


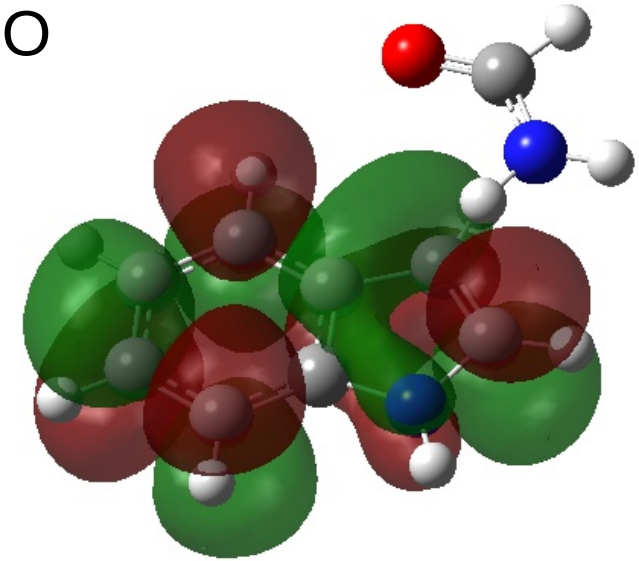


Ground State

Highest Occupied Molecular Orbital



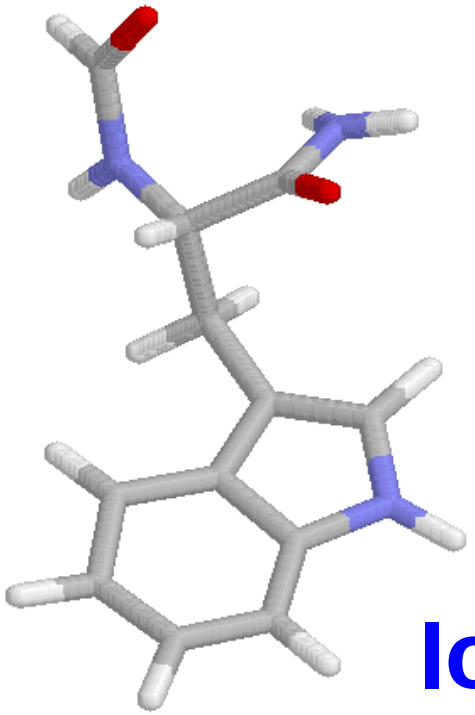


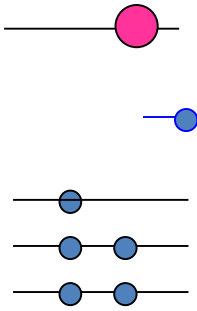


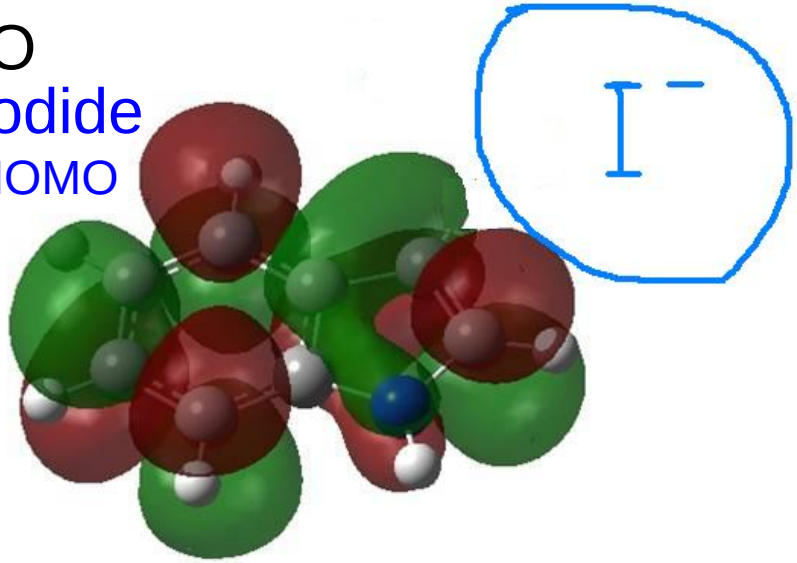
Excited State (fluorescing state)

Lowest Unoccupied Molecular Orbital (electron excited)

Quenching by iodide ion

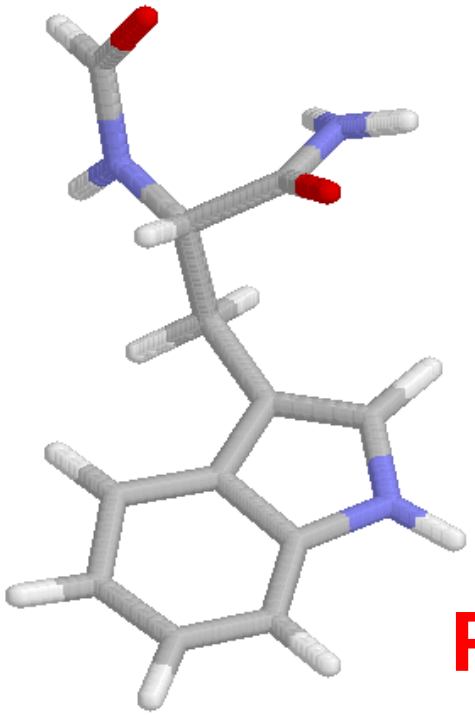


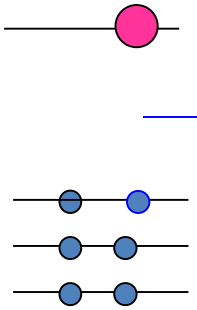


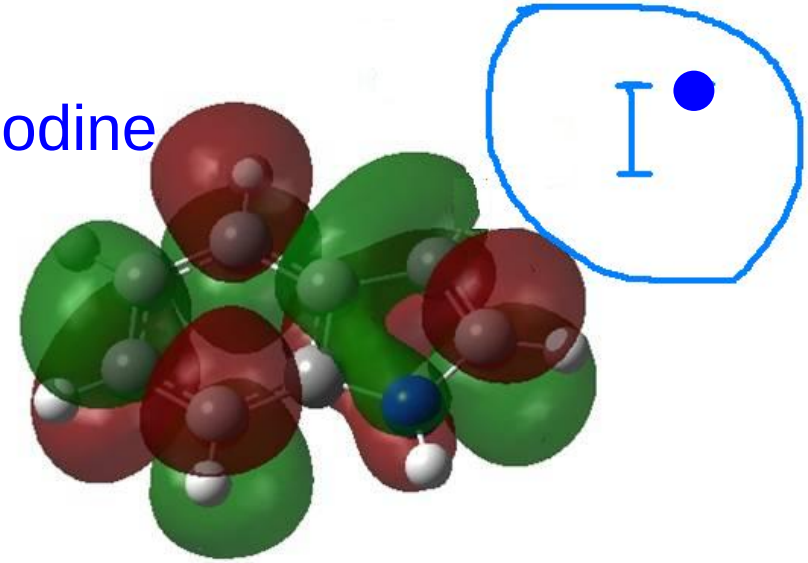


Iodide ion collides;
(has higher HOMO)
will quench fluorescence

Electron transfer from I^- to indole makes a radical pair that cannot fluoresce. (would violate Pauli exclusion)



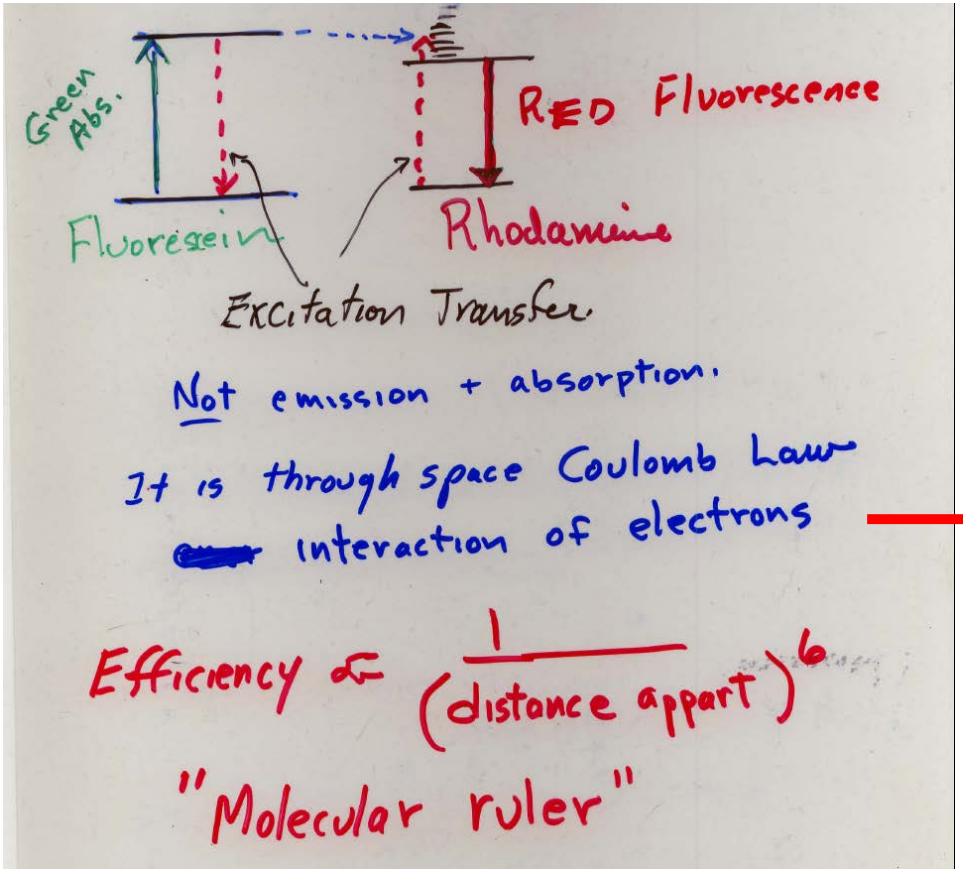




Radical Pair (can't fluoresce)

Electron transferred from iodide to vacancy in HOMO of ring
i.e., <u>QUENCHING</u>

Another kind of quenching:
Forster Resonance Energy Transfer (FRET)



London dispersion force (the very same force that holds liquid nitrogen together.

The FIRST Step of Photosynthesis:
is FRET between "light harvesting" chlorophylls funneling the energy of any absorbed photon to the reaction center chlorophyll.

Binding phenomena and fluorescence quenching. I: Descriptive quantum principles of fluorescence quenching using a supermolecule approach ☆

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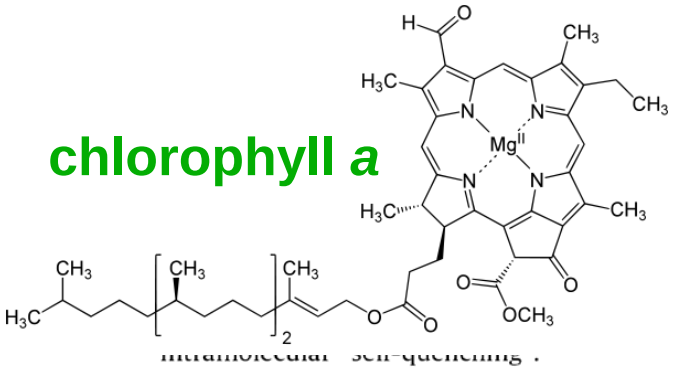
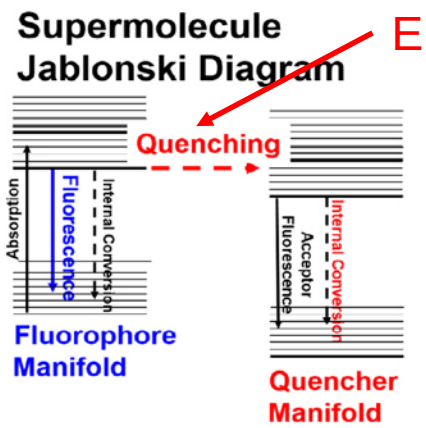
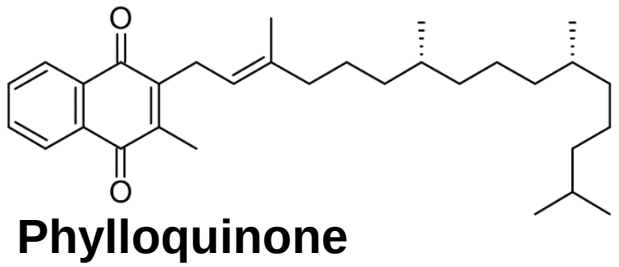
HIGHLIGHTS

GRAPHICAL ABSTRACT







The Second Step of Photosynthesis:
is quenching of the fluorescence of chlorophyll

2.6 Fluorescence Quenching (From Theory Handout)

In fluid solutions, QUENCHING involves a *diffusion controlled*, collisional interaction between the fluorophore and quencher.

What can happen following absorption of a photon?

Absorption: M + hν (photon) → **M*** rate: = ε(λ) [M] x (Intensity of light)

Fluorescence: **M*** → M + hν' rate = k_{rad} [M*]

Internal conversion: **M*** → M + heat rate = k_{ic} [M*]

Intersystem crossing M* → M(triplet) + heat rate = k_{isc} [M*]

Quenching: **M* + Q → M + Q + heat rate = k_q[Q][M*]**

Fluorescence Quantum yield = Φ_f = the rate of fluorescing divided by the total rate of leaving the excited state:

$$\Phi_f = \frac{k_{rad}[M^*]}{(k_{rad} + k_{ic} + k_{isc})[M^*] + k_q[Q][M^*]} = \frac{k_{rad}}{k_{rad} + k_{ic} + k_{isc} + k_q[Q]}$$

For fluorescein, Φ_f = 0.97, τ_{rad} = 4.74 ns so krad= 2.1 x 10^8 k_{ic} + k_{isc} =7 x 10^6 s^{-1}

For quenching by iodide ion, k_q=2 x 10^9 $M^{-1}s^{-1}$.

maximum molar decadic extinction coefficient, ε_{max} = 92,300 $M^{-1}cm^{-1}$ to find the concentration A= εCx

We will measure k_q from the Stern-Volmer Eq.

Rate of collisions = k_q [M*][Q]

Assume every collision quenches. (Diffusion Controlled)

<u>Memorize</u> that this k_q for small molecules is on the order of 10^9 M^{-1} s^{-1} in water.

Depends only on the frictional coefficient (size)

Diffusion coefficient = k_BT/frictional coefficient

We will also make use of the maximum molar decadic extinction coefficient,

ε_{max} = 92,300 $M^{-1}cm^{-1}$ to find the concentration A= εCx

4.2.3 Fluorescence Quenching by Iodide

1. Calculate he quantum yield for each concentration of the iodide, [Q], using numbers from the Theory document and :

Fluorescence Quantum Yield

(a measure of fluorescence ***brightness***)

Quantum Yield = $$\Phi_f = \frac{k_{rad}}{k_{rad} + k_{ic} + k_{isc} + k_q[Q]}$$

Fluorescence Lifetime = 1/(sum of rate constants)

$$= 1/(k_{rad} + k_{ic} + k_{isc} + k_q[Q]) = \tau_f$$

What is fluorescence lifetime?

d[excited molecules]/dt = -k [excited molecules]

d(Intensity)/dt = -k (Intensity) **1st order reaction**

Solution to this differential equation?

Fluor. intensity at time t = (Fluor. Intensity at time 0) x e^{-kt}

or = (Fluor. Intensity at time 0 x) $e^{-t/\tau}$

τ = "lifetime" = 1/k

τ = inverse of 1st order rate constant

4.2.3 Fluorescence Quenching by Iodide

1. Calculate he quantum yield for each concentration of the iodide, [Q], using numbers from the Theory document and

Compare the measured ratio of the peak height of the quenched cases to the unquenched peak with the calculated values.

$$\Phi_f([Q]) = \frac{k_{rad}}{k_{rad} + k_{ic} + k_{isc} + k_q[Q]} = \text{const x Intensity of fluor} = I$$

$$\Phi_f \text{ no quencher} = \frac{k_{rad}}{k_{rad} + k_{ic} + k_{isc}} = \text{const x Intensity of fluor} = I_0$$

$$\frac{I_0}{I} = \frac{(k_{rad} + k_{ic} + k_{isc}) + k_q[Q]}{(k_{rad} + k_{ic} + k_{isc})} = \frac{(k_{rad} + k_{ic} + k_{isc}) + k_q[Q]}{(k_{rad} + k_{ic} + k_{isc})} = \frac{(k_{rad} + k_{ic} + k_{isc})}{(k_{rad} + k_{ic} + k_{isc})} + \frac{k_q[Q]}{(k_{rad} + k_{ic} + k_{isc})}$$

$= 1 + k_q\tau_0[Q]$, where τ_0 is the unquenched fluorescence lifetime

2., the resulting equation is the widely used Stern-Volmer equation:

where τ_0 is the lifetime in the absence of the quencher, $(k_{rad} + k_{ic} + k_{isc})^{-1}$, I and I_0 are the intensities in the presence and absence of quencher, respectively, and k_q is the diffusion-controlled quenching rate. The product $k_q\tau_0$ is known as the Stern-Volmer constant, K_{sv}.

3. Plot ~~I/I$_0$~~ I_0/I vs. [Q]. Do you get a straight line? What is the Stern-Volmer constant determined from your plot?