INTERACTIONS BETWEEN LIGHT AND MATTER

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The scope of photobiophysics

The present chapter being an introduction to a biophysics course on light and life, let us first look at the light-source reaching the Earth : the Sun.

Table 1 - Average light-energies incident on the Earth, per year

Location	Energy, W m^{-2}
Red Sea	300
Australia	200
U.S.A.	185
Belgium	105

The solar spectrum (Fig. 1) shows clearly that photobiology - including photobiophysics - proceeds within a rather narrow window of radiation, roughly between 250 nm (nucleotide photochemistry) and 900 nm (bacterial photosynthesis). A very general description of photobiological phenomena could be the following.
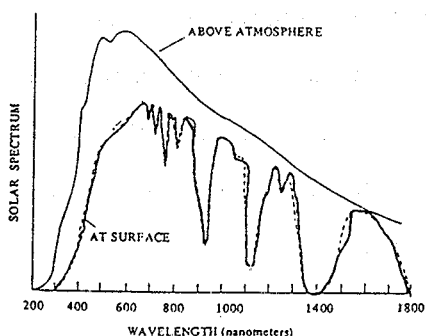
A light *stimulus* is perceived by

Fig. 1 - Incident sunlight

photoreceptors (pigments or *chromophores*). Via *primary processes* the stimulus is *transducted* to a hugely amplifying system bringing about the *response*. The latter can be observed and measured. Think of the light reaching our eyes : it yields nervous impulses that can be easily measured in millivolts (it really means an important amplification). For such an audience it certainly is redundant to go into details of the energy carried by radiation quanta (photons). Such energies – very small indeed – are usually measured in Joules (J) or in electron-volts (eV). $E = h c / \lambda$ with $h = 6.63 \ 10^{-34}$ J s $= 4.14 \ 10^{-15}$ eV s, $c = 3 \ 10^8$ m s^{-1} and λ is in metres. Hence the following table.

Type of radiation	Wavelength range, nm	Energy of a photon	
		J 10^{-17}	eV
X (mean value)	1	20	1240
ultraviolet (uv)	< 350	> 0.06	> 3.5
visible	350 to 700	0.03 < E < 0.06	1.7 < E < 3.5
infrared (ir)	> 700	< 0.03	< 1.7
microwaves	> 50 000	< 0.0004	< 0.02
radiowaves	$> 10^9$		

The two following spectra (Fig. 2 and 3) are so-called photobiological spectra (P.S. Song, personal communication). They show the biological effects of several wavelengths of light and the photoreceptors which are involved in those effects. Obviously living beings have efficiently utilized most of the light available on the surface of our planet. Note that photosynthesis, the topic of two of the following talks, uses indeed a very small part of the spectrum.
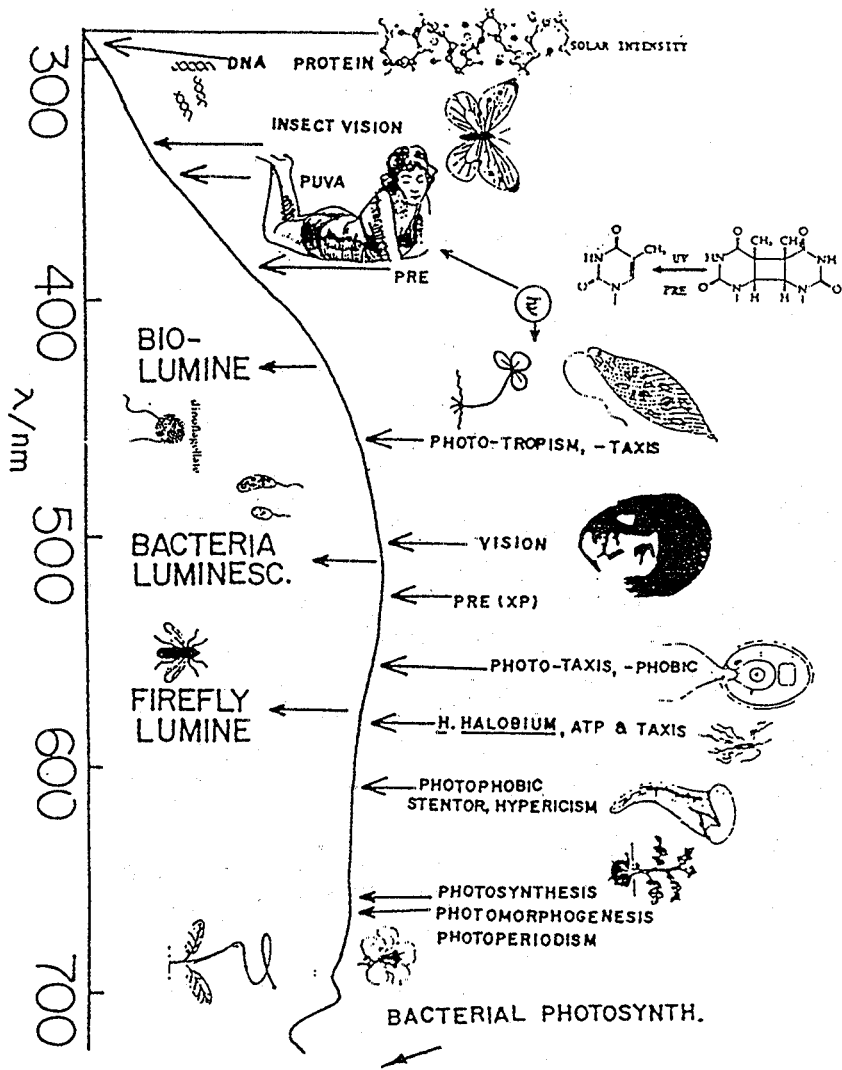
Fig. 2 - Photobiological spectrum - The biological effects produced by light
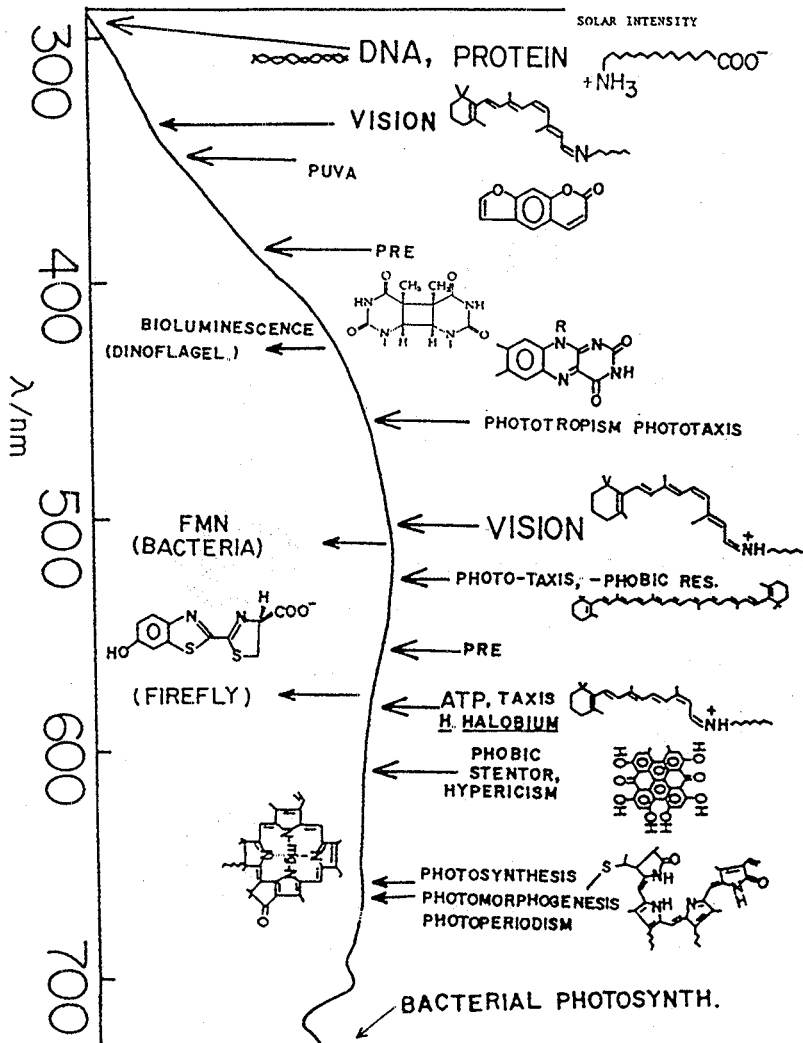
300

DNA, PROTEIN

VISION

PUVA

400

PRE

BIOLUMINESCENCE
(DINOFLAGEL.)

λ/nm

PHOTOTROPISM PHOTOTAXIS

500

FMN
(BACTERIA)

VISION

PHOTO-TAXIS, -PHOBIC RES.

PRE

(FIREFLY)

ATP, TAXIS
H. HALOBIUM

600

PHOBIC
STENTOR,
HYPERICISM

PHOTOSYNTHESIS
PHOTOMORPHOGENESIS
PHOTOPERIODISM

700

BACTERIAL PHOTOSYNTH.

Fig. 3 - Photobiochemical spectrum - The photoreceptor molecules involved

How does light interact with molecules (matter) ?

The electrons of an organic molecule are located by pairs, with antiparallel spins, in zones of the molecule called *orbitals*. Each orbital corresponds to a quantified energy level (Fig. 4).

The occupied levels are, all of them, topped by an equal number of unoccupied orbitals. Photo(bio)chemistry usually deals only with the *highest occupied level* (HO) and the *lowest unoccupied one* (LU).

Let a light-ray hit perpendicularly the plane of a molecule (Fig. 5). The vector of its electric field can excite an electron from a HO orbital to a LU orbital provided the energy (E or hν) of the exciting photons is at least equal to the energy difference $\Delta E = E_{LU} - E_{HO}$. ΔE also represents the energy difference between the excited molecule (M*) and the molecule in its ground state (M_0).

In its M* (excited) state, the molecule is unstable. The "excited" electron must fall back to its original orbital and give back the energy that excited it, as heat or as light (fluorescence, phosphorescence). But in some cases it can also, induce photochemical processes involving other chemical species. On Fig. 6 the excited electron of molecule D (donor) can fall back to the LU orbital of molecule A (acceptor) which is close by and whose LU orbital is slightly lower (in terms of energy) than the former LU orbital of D. From there, the excited electron will fall back
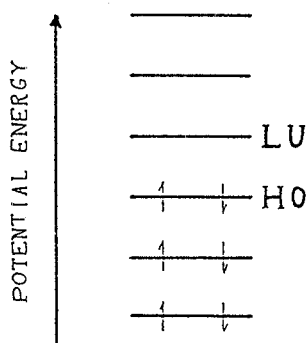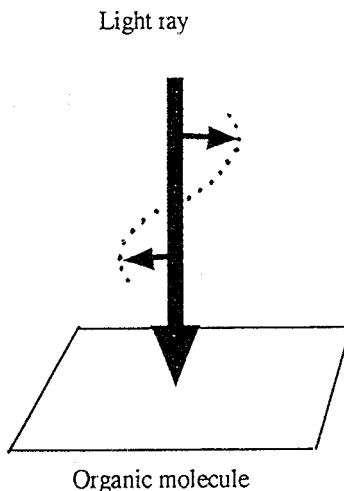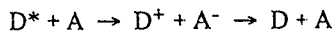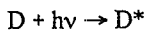
Fig. 4 - Molecular orbitals

Light ray

Organic molecule

Fig. 5 - Light on a molecule

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to its original HO orbital. The whole process can be written

$$D + h\nu \rightarrow D^*$$

$$D^* + A \rightarrow D^+ + A^- \rightarrow D + A$$

Note that (i) in the absence of light (hν) D^* cannot donate electrons to A; (ii) when A is reduced it becomes a donor molecule to D. **Light induced a reversible charge separation between the two molecules.**
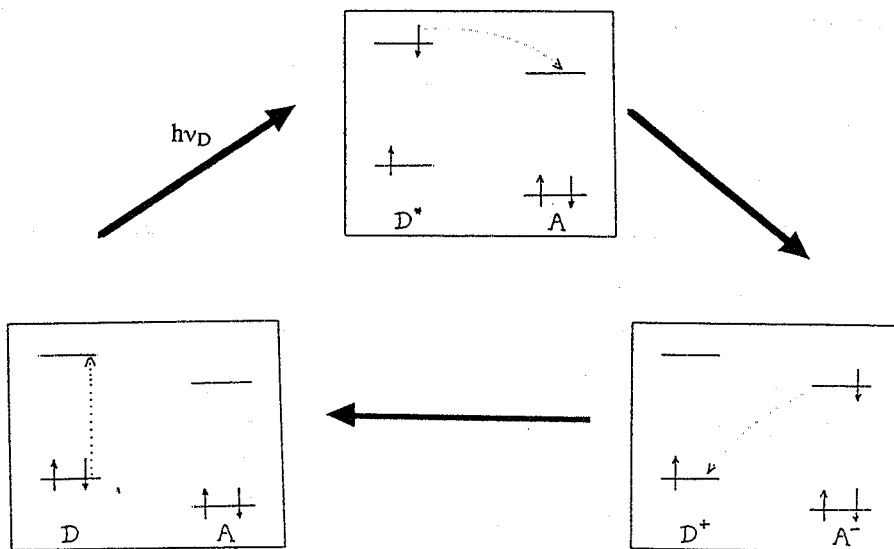

Fig. 6 - A photochemical reaction. Reversible charge separation between D and A

Such a sequence of reactions can be an amusing merry-go-round but it can hardly have

any physical or biological significance. It will have one if, on the A side, some secondary acceptor molecule A' (an electron *sink*) steals the excited electron from A_{LU} and if a "secondary" donor molecule D' (an electron source) is able to replenish the D_{HO} orbital. The charge separation goes then from D' to A' *irreversibly* : it is a *vectorial separation charge* which can be illustrated by the family drama of fig. 7. An example is the way photosynthesis is considered nowadays. The green leaf chloroplast was and, up to a certain degree still is, a black box. 150 years ago it was filled on one side with CO_2 and H_2O and, in the light, it produced O_2 and carbohydrates $(CH_2O)_n$ on the other side. Nowadays, on one side of the black box, light creates a positive charge which oxidizes water to O_2 and on the other, negative, side, it reduces CO_2 to $(CH_2O)_n$. There is, indeed, an irreversible charge separation across the green black box.
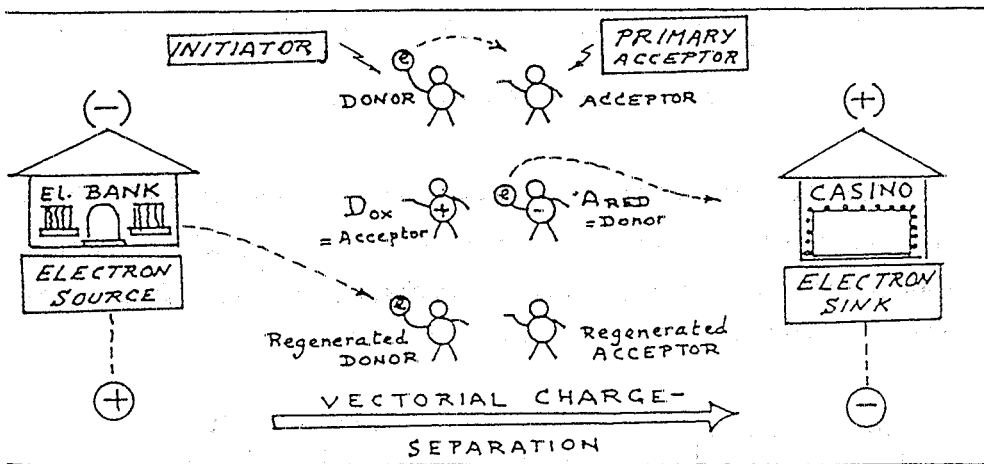

Fig. 7 - A family affair illustrating vectorial charge separation (Van Dormael, 1981)
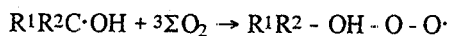
Thus one can accept the idea that light promotes oxidation-reduction reactions. Look for instance at the following sequence of events : sensitised abstraction of hydrogen from an

alcohol[1] . The product of the reaction is a hydroxyhydroperoxide which, in the presence of water, decomposes to a ketone and H_2O_2.
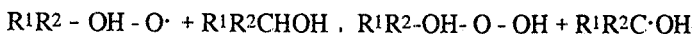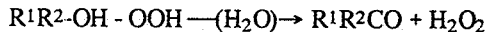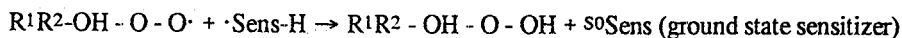
1st step, *Excitation*

$$Sensitizer \overset{(hv)}{\longrightarrow} {}^{T1}Sensitizer \text{ (triplet state)}$$
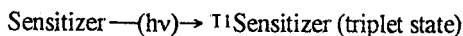
2nd step, *Initiation and incorporation of ground state oxygen*

$${}^{T1}Sens + R^1R^2CHOH \rightarrow \cdot Sens\text{-}H + R^1R^2C\cdot OH$$

$$R^1R^2C\cdot OH + {}^{3\Sigma}O_2 \rightarrow R^1R^2 - OH - O - O\cdot$$

3rd step, *Propagation*

$$R^1R^2 - OH - O\cdot + R^1R^2CHOH \rightarrow R^1R^2\text{-}OH\text{-} O - OH + R^1R^2C\cdot OH$$

4th step, *Termination*

$$R^1R^2\text{-}OH - O - O\cdot + \cdot Sens\text{-}H \rightarrow R^1R^2 - OH - O - OH + {}^{S0}Sens \text{ (ground state sensitizer)}$$

$$R^1R^2\text{-}OH - OOH \overset{(H_2O)}{\longrightarrow} R^1R^2CO + H_2O_2$$

O_2 acts on the radical derived from the alcohol. Such a sequence is called a Type I mechanism of oxidation. A type II mechanism is illustrated by the oxygenation of fluorescein or Bengal Red : it involves direct reaction of the oxidizable organic compounds with oxygen instead of the reaction of oxygen with a photoinduced radical

$$\longrightarrow (hv, O_2) \longrightarrow$$
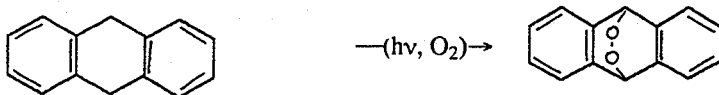
Light can promote other types of reactions such as *cis-trans* isomerism, addition reactions and substitutions. Let us look at *cis-trans* isomerism which has direct biological implications (vision).

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[1] Such examples can be found in any organic photochemistry textbook.

The visual pigment located in the cells of the retina is a trans-membrane protein, *rhodopsin* (28 000 < M_r < 35 000) bearing *retinal* (a half-β-carotene) as a chromophore (linked by a protonated Schiff-base to a lysine residue of the protein). Thanks to the retinal, rhodopsin behaves as a *photochromic* reagent. In the dark retinal is in the 11-cis configuration and rhodopsin has maximum absorbance at 500 nm. Light promotes the isomerisation of 11-cis to an all-trans structure and bleaching of the pigment (λ_{max} = 387 nm).

In this configuration retinal cannot be bound to the apoprotein (*opsin*) any more. In the dark retinal reverts slowly to its 11-cis form and it recombines with opsin, spontaneously[2].
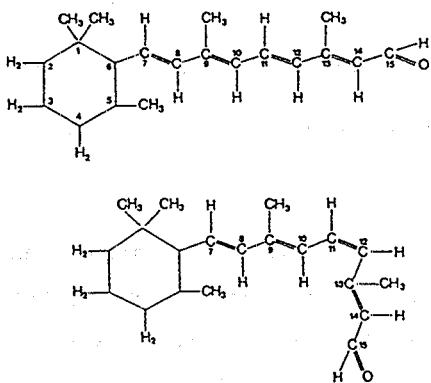
Fig. 8 - All-trans and 11-cis retinal

It is not the aim of this introductory chapter to deal with detailed photochemistry. Let it be known that light (uv and visible) indeed induces all kinds of chemistry in biomolecules – one can think of photo-polymerisation or -depolymerisation of proteins but it is only one of very many photoreactions which are well documented[3]. The rest of this chapter will be devoted to some aspects of uv-visible spectroscopy of organic biological molecules and objects, without treading on the flower-beds of the other lecturers in this meeting.

Absorbance spectroscopy of an electronic transition such as seen in Figs. 4 and 6, at any wavelength, depends on the probability P of the transition and on the size of the absorbing molecule. In other words, the absorbance maximum corresponds to the highest probability of transition in that region of the spectrum

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2 When you look at bright light you are partially blinded (bleaching of rhodopsin). In the dark you recover slowly your vision.

3 See, for instance, the several editions of N. Turro's *Molecular Photochemistry* , W.A. Benjamin Inc. publisher.

$$\varepsilon = k\,P\,\alpha$$

where ε is the extinction coefficient (in the usual and well-known Beer, Lambert and Bouguer formalism $\varepsilon = A\,/\,c\,\ell$ with A the value of absorbance, c the molar concentration of the absorbing molecule and ℓ the path length), k is a constant of the order of 10^{20} and α is the cross-section (area) of the molecule. Supposing $P = 1$ and $\alpha = 0.1$ nm$^2 = 10^{-15}$ cm^2, which is an average for organic molecules, ε is of the order of $1\ 10^5$. Experimentally the highest measures of ε are indeed $1\ 10^5$.

P is proportional to the square of the transition moment. This moment depends on the change of electronic charge distribution when the molecule is excited (by light or another stimulus). An immediate consequence is that a transition between states where the symmetry is the same (no change) is "forbidden" because $P \to 0$. Experimentally the intensity of such a transition is much smaller than that of an "allowed" one. $\varepsilon_{allowed}$ is of the order of $1\ 10^5$ as noted above but $\varepsilon_{forbidden}$ is usually less than $1\ 10^3$ ($P < 0.01$).

Infrared and Raman spectroscopy

Up to now ultraviolet and visible absorbance spectroscopy was dealt with. Infrared (ir) absorbance and Raman technologies can give impressive information on molecular bonds by measuring transitions between vibrational levels (stretching and / or bending) in an electronic state. It happens that several bonds can thus be identified (the table gives some of them).

Bonds	Stretching vibration cm^{-1} + 100 cm^{-1}
$\equiv$ C-H	3300
- O-H	3680
= N-H	3500
= C=O	1700
= C=N	1650
- C$\equiv$C-	2050

A big polyatomic molecule can be seen as an assembly of many diatomic oscillators : the smallest amino-acid, glycine $^-OOC\text{-}CH_2\text{-}NH_3^+$ displays up to 24 such oscillators. It is readily understandable, therefore, that ir spectroscopy cannot be very useful in identifying a molecule as a whole but it can give practical information on several bonds, provided they are not too close to the bulk of the molecule, i.e. mainly bonds in end-groups.

The vibrational states of molecules can also be measured as follows. Shine a bright monochromatic light of frequency ν on a sample which is transparent for this light. Most of the light will pass through the sample as such, without change. A small part of it will be scattered and keep the same frequency ν (Rayleigh scattering). A very small part will be scattered, though with different frequencies. The shifts to those "new" frequencies are in close relationship with the vibrational frequencies of the molecules. This is called the *Raman effect*, on which a spectroscopy was founded. For small and medium-sized molecules, Raman and ir spectroscopies can be used in addition to one another : fortunately they give similar results. In biology and biological chemistry, Raman spectroscopy is much more useful than ir spectroscopy because water has a negligible Raman spectrum whereas it absorbs ir intensely. Therefore Raman spectroscopy can be performed with biological samples and with aqueous solutions ... it is impossible with ir spectroscopy which must necessarily be done in the absence of even minute amounts of water (see note below).

Optical rotatory dispersion and circular dichroïsm

Before dealing with emission spectroscopy, let us take a fast look at optical rotatory dispersion (ORD) and circular dichroïsm (CD). Most biological molecules have *optical activity* : they rotate the polarization plane of polarized light mainly because they possess asymmetric carbon atoms.

As was shown above (Fig. 5) *plane polarized light* can be represented by its electric vector E which oscillates along a sinusoïd in a plane. Once such light passes through an optically active substance, its electric vector travels along an elliptical helix with a minor axis so much smaller than the major axis that it is as if the polarization plane were rotated. The rotation depends on the wavelength λ of the incident light. ORD measures this rotation as a

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function of λ.

But if the light is *circularly polarized* (instead of in a plane) it appears that absorbance of left-polarized and right-polarized lights are different (Cotton effect). This phenomenon is called CD. Whole books have been written on theory and applications of ir and Raman spectroscopies, on ORD and on CD. The interested readers will consult them.

Light scattering (see Feynman et al, 1972)

The index of refraction of a substance results from the fact that an incident ray of light will cause its atoms to radiate again : the electric field of the incoming beam drives the electrons up and down. Their acceleration causes them to radiate in various directions (see previous comments) and behave like microscopic antennae.

The total scattered energy is proportional to the square of the incident field, i.e. to the energy per unit area (usually m^2) coming in. It is why the brighter the sunlight, the brighter the sky looks.

What fraction of the incoming energy is scattered ? The scattering is proportional to the 4th power of the frequency. Light which is of a higher frequency by a factor of 2 is scattered 16 times as much : this explains that blue light ($\nu \cong 75 \ 10^{15} \ s^{-1}$) is scattered much more than red light ($\nu \cong 45 \ 10^{15} \ s^{-1}$).

Emission spectroscopy

Molecular electronic transitions can be produced by absorption of light – let us limit ourselves to ultraviolet, visible and near infrared regions of the electromagnetic spectrum, where $200 < \lambda$, nm < 1000, roughly. Their relaxation is accompanied by light-emission.

When light is emitted from a $\pi-\pi^*$ state to the ground state it is called *fluorescence* . In the vocabulary defined above, fluorescence characterizes "allowed" transitions between states with different symmetries. The lifetime of fluorescence is usually of the order of or smaller than $1 \ 10^{-8}$ second for molecules like chlorophylls. Some states have a much longer life (over 10 000 times longer) and light emitted by their relaxation to the ground state is called *phosphorescence* . Fluorescence is emitted from the first excited *singlet state* and

phosphorescence from the first of the so-called metastable *triplet states* . Because of the lifetimes of singlet and triplet states – before their falling back to the ground state – most photobiochemistry is brought about by triplet states. Few if any biochemical reactions indeed last less than $1\ 10^{-6}$ second.

Fluorescence and phosphorescence are very much used as indexes of the "photochemical status" of absorbing molecules, to check their relations with their chemical environment for instance. Thus chlorophylls *in vivo* emit much less fluorescence than when they are completely dispersed, solvated in acetone. When they are dispersed in dry benzene however, they are not fluorescent at all. They are really solvated in acetone or alcohols with which they can form hydrogen bonds – essentially not with water however. In dry benzene such bonds are not possible either a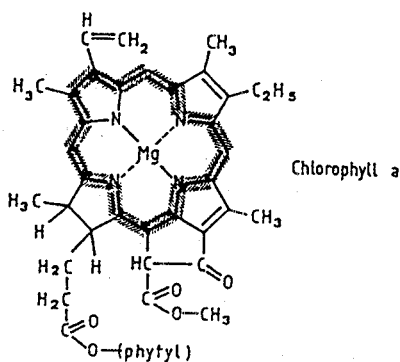nd chorophylls aggregate. With traces of water, fluorescence is recovered. Keeping the example of chlorophylls (Fig. 9) the conjugation of their porphyrin "heads" forms electronic clouds on each side for it. When such molecules are aggregated one can imagine that their π-electron clouds are mixed, fused with those of neighoring molecules. Excited electrons lose their potential energy by migrating from one cloud to the other and back and emit very little or no fluorescence.

Fig. 9 - A chlorophyll molecule

Fluorescence spectra are usually mirror-images of absorption spectra. It would be more accurate, for biomolecules, to state that the fluorescence band of the first excited singlet state is a mirror-image of its absorption band. It is not exactly true in practice because of temperature and solvent effects as well as of the spacing of the vibrational levels of the absorbing molecules. Fig. 10 shows this clearly (Walker and Straw, 1962).

Qualitative analysis and occasionally as a help in structural determinations are two important applications of fluorescence. Seldom is fluorescence used for determining

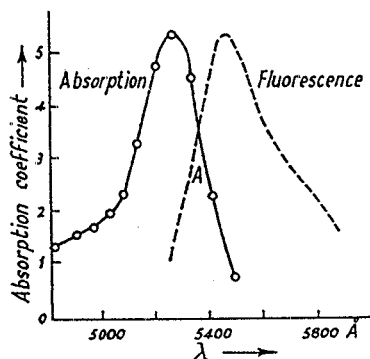concentrations. Fig. 10 shows why. In order to emit fluorescence, a sample must be illuminated and some of the light must be absorbed. Since in most of the cases that interest biophysical chemists, the absorption and emission spectra overlap more or less, it is clear that some of the emitted light can be reabsorbed by the emitting molecules thus adulterating the measures of the quantity of emitted light.

Fig 10 - Absorption and emission of eosine in water

In many cases the quantum yield of fluorescence (number of light quanta emitted per 100 quanta absorbed) is very high, therefore fluorescence can be used to detect the presence of minute amounts of molecules in a sample. Also, and for the same reason, it can be used for dosage only when the samples are extremely dilute. Then the absorption is negligible.

Phosphorescence has a much longer lifetime than fluorescence, to the point that it can still be observed as an afterglow after the incident light is cut off. Actually, the lifetime of phosphorescence can be $1 \cdot 10^{-4}$ second as mentioned earlier but it can be as long as 10 seconds : the longest lifetimes characterize aromatic compounds usually.

It is easy to make a difference between fluorescence and phosphorescence based on time. The fundamental difference, however is that fluorescence occurs between two electronic states (S_1 and S_0, first excited singlet state and ground state) whereas phosphorescence needs at least three : $S_1 \rightarrow T_1$ and $T_1 \rightarrow S_0$ where T_1 is the first excited triplet - metastable - state. In the electronic states S_0, S_1, ... the electron spins are paired. In T_1, T_2, ..; they are parallel. $S_1 \rightarrow T_1 \rightarrow S_0$, two "forbidden" transitions are improbable therefore long lived.
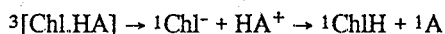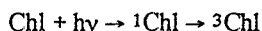
Phosphorescence peaks almost always at much longer wavelengths than fluorescence. If fluorescence of solvated chlorophyll a peaks about 680 nm (absorbance maximum about 660 nm), its phosphorescence lies way beyond 700 nm.

How can one know that a particular photosensitized reaction proceeds through a triplet or through a singlet state ? It is not readily determined but it is well established, by now that three types of reactions (at least) surely occur thanks to a triplet state of one of their reagents.

(a) Photoreduction of porphyrins and phtalocyanins

(b) Photooxidations (see above) It must be added that oxygen quenches several triplet states therefore turns phosphorescence off.

(c) Reversible photoreduction of chlorophyll *in vitro* (Krasnovsky's reaction, see Rabinowitch 1956. The superscripts 1 and 3 refer to singlet and triplet states respectively. HA is the solvent, ascorbate and pyridine)

$$Chl + h\nu \rightarrow {}^1Chl \rightarrow {}^3Chl$$

$${}^3Chl + HA \rightarrow {}^3[Chl.HA]$$

$${}^3[Chl.HA] \rightarrow {}^1Chl^- + HA^+ \rightarrow {}^1ChlH + {}^1A$$

and the color of the chlorophyll solution shifts from bright blue-green to bright ... pink !

Supramolecular structures and absorption

Consider what happens to the absorbance spectrum (uv-visible) of solvated chlorophylls when they are in such an environment that they crystallize (Fig. 11). The absorption of the first singlet excited state (about 660 to 670 nm) diminishes considerably and a new band appears and increases about 740 nm. In this case it could be called *hyperchromism* because the new absorbance band absorbs about 1.5 times more than the original one.

In order to understand what can happen in such a case let us suppose that chlorophyll (with absorption at frequency ν) begins by forming dimers on its way to forming crystals. Let us further suppose that in the dimers the interaction between the molecules has energy U. The single monomer band will be split in 2 bands with frequencies

$$\nu' = \nu \pm (U / h)$$

and the relative absorbing intensities will depend on the geometric relations between the transition moments of the 2 monomers (in the present example the broadening of the bands due to vibrational transitions prevents the observer from seeing the two bands, there is just a shift in the wavelength of the absorption maximum).
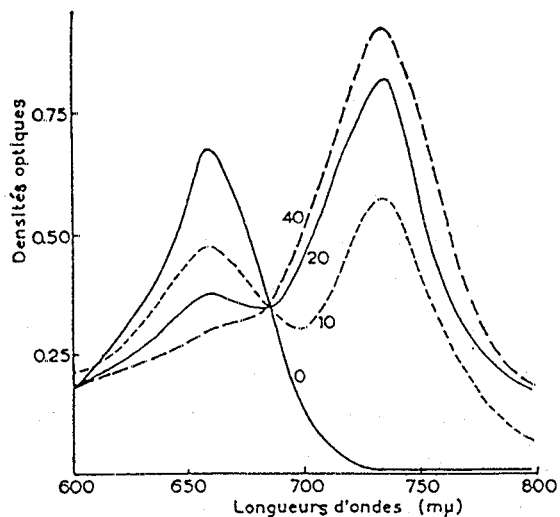
Fig. 11 - Absorption spectra of chlorophylls in 75 % methanol (v/v). Note the shift of the red absorbing band and the hyperchromic effect. Note also the isosbestic point (see text). The numbers on the curves are the time (minutes) after beginning of reaction (Aghion 1964)

A very general rule states that configurational changes cannot change the total absorption "integrated over all the bands" if they don't change the fundamental electronic structure. Indeed when the red band shifts and its absorbance increases, the absorption in the blue band (about 430 nm) decreases accordingly. The *isosbestic point* where all the curves meet at 685 nm is a serious hint that the electronic structures are conserved.

A more traditional example is the *hypochromism* of nucleotides : Adenosine-Mono-Phosphate (AMP) absorbs at 260 nm (A = 0.8 in an experimental setup). Single-stranded polyAdenosine absorbs at the same wavelength with A = 0.55. Double-stranded polyAdenosine absorbs at 257 nm (a slight blue hift) with A = 0.45. If the abovementioned rule still holds, there surely is a hyperchromic effect somewhere in the ultraviolet region of the spectrum, which cannot be or has not been seen.

Concluding remarks

Much more could have been said about the interactions of light - or electromagnetic radiations - with matter : we have not mentioned the spectral features of charge-transfer complexes whereby charges are transferred between loosely bonded aromatic separate molecules. Nothing was said about magnetic resonance spectroscopy which can give really

many structural informations about organic molecules, or about X-ray spectroscopy and so on and so forth, all techniques which are more and more in use in biophysics laboratories.

It is to be hoped that the audience feels now a little more familiar than before with such subjects, but just a little : the whole topic was seen, here, as intuitively as possible, never were mathematical developments even evoked. Such developments can be found in specialized books and articles. Not necessarily very modern ones : the references at the end of this paper are certainly not very recent : the most "modern" one is almost 20 years old !

The main conclusion to be drawn from all this – and after having read the next chapters of this meeting – is that spectroscopy is a very useful tool which must necessarily be helped by other tools. But this is nothing new in experimental research.

Note added in extremis.

Several research groups are now developing multidimensional vibrational techniques that will potentially complement multidimensional Nuclear Magnetic Resonance methods. How is it done ? In usual vibrational spectroscopy, the frequency of radiation is changed and the spectrum is plotted against the frequencey. There is only one coordinate that is changed. In multidimensional spectroscopy, several radiative fields are utilized and changed simultaneously. Then it is possible to see and study the effect of one frequency on another and to draw correlation plots which are very informative.

Multidimensional ir and Raman spectroscopies can address different chemical problems. Technologically, ir experiments are restricted to higher frequencies (lower wavelengths) and are best used to study dilute solutes such as proteins and peptides. Raman methods are excellent for lower frequencies and the study of neat liquids to study collective molecular structure and dynamics.

For details and references, look up Chemical and Engineering News *7 8 (Feb. 7 2000) 41-50.*

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