

Photobiology in Medicine

Edited by
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Michael A. J. Rodgers and
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NATO ASI Series

Series A: Life Sciences Vol. 272

Photobiology in Medicine

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Series A: Life Sciences

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PREFACE

Man has recognised an association of light with life and medicine for over 3000 years. Today the major challenges to this topic include the elucidation of photochemical reactions involved in photobiology at the molecular level. This includes the use of a variety of modern probing techniques that directly measures the reactivity of excited states and free radicals involved in biological reactions.

This text-book is based on such an approach and has arisen from some of the lectures delivered at the NATO ASI held at Hotel Capo Caccia near the Centre for Advanced Research in Photobiology (CARP) in Sardegia, Italy. The ASI took place from 30 September - 13 October 1993 and involved a total membership of 90.

The book, like the NATO ASI itself, is divided into four themes starting with fundamental aspects and ending with complex medically related systems. Thus Theme 1 covers aspects of the underlying photophysics and photochemistry with particular emphasis on modern experimental techniques to study molecular mechanisms of biological processes. Theme 2 applies many of these fundamental studies to the chemical reactions of most relevance to photobiology and photomedicine such as photo-addition, -isomerization, -sensitization and -pigmentation. The third and fourth Themes deal with the deleterious and therapeutic aspects of light with particular emphasis on the use of Photo-Dynamic Therapy (PDT) to treat cancer and on viral and microbial infections.

Photobiology in Medicine is a wide-ranging subject that obviously cannot be totally covered comprehensively in one book alone. Rather, chosen topics of current interest in photomedicine were focused upon and these are linked to the major underlying themes and techniques in photophysics and photochemistry.

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October, 1993

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PHOTOPHYSICAL TECHNIQUES USED IN PHOTOBIOLOGY AND PHOTOMEDICINE

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INTRODUCTION

This chapter describes some of the photophysical techniques applied to the study of light-induced reaction processes in biological systems. It concerns experimental details and applications of these methods in determination of photophysical parameters such as quantum yields of formation, lifetimes, absorption coefficients and spectral properties of transient species. The aim is to provide a general understanding of the techniques rather than a rigorous description of each individual method and establish the information which can be obtained. Given the general nature of the chapter a bibliography of suggested background material is listed rather than specific references.

All of the techniques described (with the exception of pulse radiolysis) give a means to study processes initiated by light absorption in the uv-visible region to produce electronically excited states. Following the absorption of a photon relaxation processes such as fluorescence, internal conversion and intersystem crossing may occur in addition to chemical reactions such as photoionization or bond cleavage. The following chapter will outline the means for study of the deactivation and reaction pathways of important biomolecules in the condensed phase.

ABSORPTION MEASUREMENTS

Absorbance (A) is directly proportional to the molar concentration (c) of a compound and the optical path length (ℓ) of the sample through the Beer-Lambert relationship.

$$A = \epsilon \cdot c \cdot \ell \quad (1)$$

The proportionality constant is the molar (decadic) absorption coefficient (ϵ , in $M^{-1}cm^{-1}$) which is a measure of the probability that a photon of a particular wavelength (and energy through $E = hv = hc/\lambda$) will be absorbed by the compound. The ϵ value at a given wavelength can be determined from the slope of a plot of A vs. $c \cdot \ell$. One must ensure that such a plot is linear as many compounds display a tendency to aggregate at higher concentrations resulting in a deviation from linearity. The absorption spectrum of a compound is normally displayed in terms of a plot of ϵ (or A) vs. λ and is measured using a spectrophotometer which compares the irradiance of an analyzing beam transmitted

through a solution with (**I**) and without (**Io**) the compound of interest. The absorbance is then determined from;

$$A = \log\left(\frac{I_0}{I}\right) \quad (2)$$

Commercial spectrophotometers often consist of a broadband analyzing beam which interrogates the sample with a scanning monochromator used for calibrated wavelength selection before the photodetector. A double beam arrangement is often used where the analyzing beam is split with one fraction interrogating the sample (**I**) and the other an identical sample minus the compound (**Io**). This approach minimizes effects of variation in light intensity of the analyzing source. Diode array spectrophotometers are convenient alternatives for rapid measurements where a spectrograph projects the dispersed spectrum to a diode array detector.

FLUORESCENCE MEASUREMENTS

Fluorescence denotes a radiative relaxation process between electronic states of like multiplicity. The most common type is that corresponding to S_1-S_0 transitions. Fluorescence emission competes with other non-radiative relaxation processes. In rigid media or at low temperatures fluorescence tends to increase as collisional deactivation processes become less effective. A spectrofluorimeter typically consists of a broadband source and a photodetector with monochromators inserted in both the excitation and emission paths. Excitation and detection is performed at 90° for optically dilute solutions whereas for optically thick solutions or solid samples a front-face arrangement is normally used where the sample is at 45° to both excitation and detection. A fluorescence emission spectrum is recorded by scanning the emission monochromator at a constant excitation wavelength. The emission spectrum should be corrected for any wavelength dependence of the detection system and instability in the excitation source output. A fluorescence excitation spectrum is obtained by detecting at a constant wavelength while scanning the excitation monochromator. The excitation spectrum is analogous to an absorption spectrum and is useful for identifying species responsible for fluorescence in multicomponent systems. A corrected excitation spectrum is obtained through normalization for the spectral output of the excitation source. Quantitative evaluation procedures for other fluorescence parameters are outlined below.

Quantum Yield of Fluorescence (Φ_F)

The quantum yield of fluorescence is determined by the relative rate constants of radiative emission (k_r) and all other processes deactivating the state from which fluorescence occurs through;

$$\Phi_F = \frac{k_r}{\sum k} \quad (3)$$

where the observed fluorescence lifetime (τ_F) is equal to $1/\sum k$. The radiative lifetime ($\tau_r = 1/k_r = \Phi_F / \tau_F$) can be estimated from the absorption spectrum using the Strickler-Berg relationship;

$$\tau_r = \frac{3.417 \times 10^8}{\bar{v}_{\max}^2 n^2 A} \quad (4)$$

where v_{\max} is the frequency of maximum emission, n is the refractive index of the solvent and A is the integrated absorption coefficient over the entire absorption band. Φ_F values are normally measured by a comparative approach using secondary reference fluorophores with well-defined fluorescence parameters. The reference should be excited at the same wavelength as the sample and should possess a similar quantum yield and emission

spectrum for experimental reasons. Solutions should be optically dilute ($A < 0.05$) to avoid inner filter effects and experimentally the integrated fluorescence over the emission spectrum is measured as a function of sample absorbance. From the slopes (F) of these linear plots Φ_F of the unknown can be determined from;

$$\Phi_F = \frac{F_u n^2}{F_s n_o^2} \bullet \Phi_F(s) \quad (5)$$

where u and s subscripts denote unknown and standard, $\Phi_F(s)$ is the quantum yield of fluorescence of the standard and n and n_o are the refractive index values of the solvents for the solutions of unknown and standard, respectively.

An attractive alternative for absolute measurements is the application of calorimetric techniques (*vide infra*), especially for compounds emitting in the red where few suitable standards exist.

Singlet Energy

Under certain conditions the singlet energy ($E_s = E_{0-0}$) of a fluorescent molecule can be obtained by comparison of the absorption and fluorescence emission spectra. If the molecule displays no large Stokes shift and a mirror image relationship between spectra is exhibited the singlet energy can be obtained from the overlap of the normalized spectra. This is demonstrated in Fig. 1 for the photosensitizer, benzoporphyrin derivative (BPD) where a singlet energy of ~ 176 kJ mol $^{-1}$ is determined.

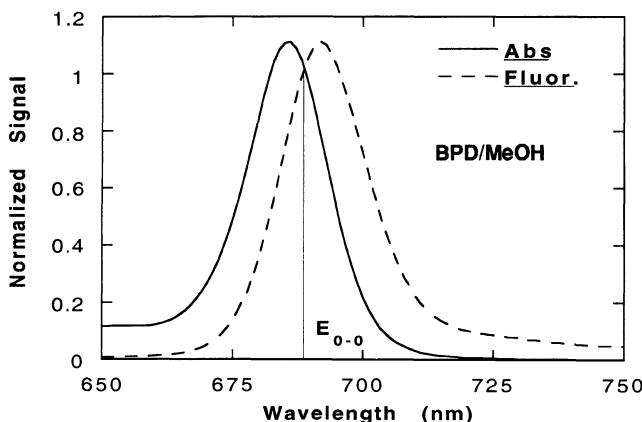


Figure 1 - Mirror image relationship of absorption and fluorescence emission spectra for benzoporphyrin derivative (BPD) in methanol.

Energy Gap Law From the study of non-radiative processes it is well known that the rate constant for a radiationless process is dependent on the energy difference between the states involved, the rate constant decreasing exponentially with increasing energy gap. Such a relationship was shown to be true for iodopropane quenching of a series of aromatic singlet states proceeding through a heavy-atom-induced intersystem crossing mechanism. From the rate constant for fluorescence quenching the energy difference between singlet and triplet states in a molecule can be determined. Provided the singlet state energy is known (from fluorescence) the triplet state energy can be calculated. The energy difference obtained in this way corresponds to the states involved in the transition which may not always be the S_1 and T_1 states. Intersystem crossing between S_1 and T_2 states will lead to false estimation of the T_1 state.

Singlet Lifetime

Various methods are employed to measure or estimate singlet lifetimes through fluorescence measurements. Some are outlined below

Real Time Detection In real time detection the time-dependence of the fluorescence emission is monitored directly using a photodetector with a response time which is short with respect to the excitation pulse duration and fluorescence lifetime, *e.g.*, fast photomultipliers in the nanosecond and streak cameras in the picosecond domain. The fluorescence lifetime is extracted by kinetic analysis of the time-dependent signal generated by the detector.

Time Correlated Single Photon Counting (SPC) This is the most commonly used method for determination of fluorescence lifetimes. It is based on the fact that the temporal fluorescence distribution must follow the statistical distribution of all emitted photons. Accurate statistical counting of single-photon emission events collected from a very large number of excitation pulses allow the construction of the time profile of fluorescence emission. The experiment consists of a high repetition rate pulsed source in combination with sensitive fast response detection and accurate timing electronics. A schematic diagram of a general SPC apparatus is shown in Fig. 2. Suitable excitation sources include flash lamps ($\tau \sim 2$ ns) and mode-locked, cavity dumped argon ion lasers which can also be used to pump tunable dye lasers. The sample is excited at low intensity (to ensure single photon conditions) in a light-tight compartment and the emitted photon is detected by a highly sensitive, fast response detector such as a photomultiplier tube (PMT), avalanche diode or microchannel plate.

Of critical importance is the accurate timing of the delay between the excitation pulse and the emitted photon. The start PMT generates an electrical signal correlated to the excitation pulse. The pulse travels to a time-to-amplitude converter (TAC) where charging of a capacitor in a linear voltage ramp is initiated. The emitted photon is detected by the stop PMT which then halts the ramp at a voltage corresponding to the delay. The voltage is then read by the analog to digital converter and a count is added to the appropriate channel of a multichannel analyzer (MCA). Discriminators are used to ensure accurate timing. With high repetition rates the time profile of the fluorescence profile is quickly constructed.

In cases where the lifetime to be measured is on the same time scale as the response function, the actual decay may be obtained by deconvolution of the measured signal using an instrumental response function (IRF) generated from a sample where the emission is known to occur with a much shorter time constant than the response function. Lightscattering samples such as non-dairy creamer or silica are often used to generate the IRF.

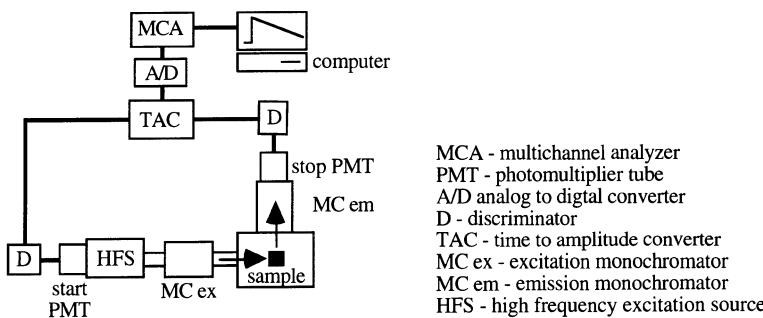


Figure 2 - Schematic of time-correlated SPC experiment.

Phase Shift Methods

An alternative to direct measurement of fluorescence lifetimes is the phase-shift method. Instead of a short laser pulse the sample is excited by a sinusoidally modulated continuous wave (CW) source where the modulation frequency, $\omega = 2\pi f$, where f is in Hz. As the fluorescence has a finite lifetime the signal at the detector will be shifted (lagging) in phase by an angle ϕ and demodulated (lower amplitude) by a factor m with respect to the excitation source, as shown in Fig. 3. From measurements of these parameters the phase (τ_p) and modulation lifetimes (τ_m) can be determined from;

$$\tan \phi = \omega t_p \quad (6)$$

$$m = \left[1 + \omega^2 \tau_m^2 \right]^{-\frac{1}{2}} \quad (7)$$

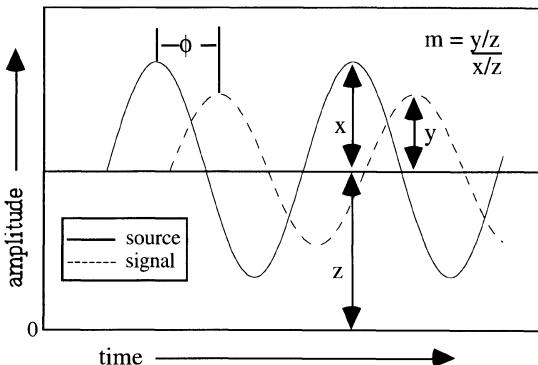


Figure 3 - Phase shift and demodulation due to fluorescence emission. (adapted from Lakowicz, 1983)

An increase in the fluorescence lifetime results in a increase in ϕ and decrease in m . The experimental measurement of τ_p and τ_m requires a modulation frequency similar to the fluorescence decay rate. Measurement of these parameters at a single frequency only allows analysis in terms of a single exponential decay ($\tau_s = \tau_p = \tau_m$). A more rigorous approach is to repeat the experiment at multiple frequencies and apply statistical least-squares fitting methods to obtain fluorescence decay kinetics of more complex systems with greater precision.

Fluorescence Quenching

Fluorescence emission can often be quenched by bimolecular reactions involving a quencher molecule, Q . In such cases the fluorescence intensity is correlated to the quencher concentration [Q] through the Stern-Volmer relationship;

$$\frac{\Phi_F^0}{\Phi_F} = 1 + k_q \tau_F [Q] \quad (8)$$

where Φ_F^0 is the fluorescence yield in the absence of quencher, Φ_F in the presence of Q and k_q is the bimolecular quenching rate constant. A plot of Φ_F^0 / Φ_F vs. $[Q]$ yields a straight line with slope equal to the product $k_q \tau_F$ ($= K_{sv}$, the Stern-Volmer coefficient). This method can be applied for reactions where k_q can represent rate constants for energy

transfer and electron transfer reactions. In the former information on the singlet energy levels can be obtained while the latter can be used to determine redox potentials.

Fluorescence in Biological Material

Fluorescence has found widespread use in biology and medicine as a useful analytical and diagnostic probe. Time-resolved fluorescence has been extensively used in the investigation of early events in photosynthesis, for example. In tissue, measurements may be made using fiber optic couples through which the excitation light and the fluorescence emission are directed. Spectral information is gained by using spectrograph/diode array detectors. This type of information has been of use in tumor diagnostics using fluorophores targeted toward the malignancy. Spatial resolution of the fluorophore distribution is also possible using highly sensitive CCD cameras to image the fluorescence emission. Quantitative measurements are complicated by tissue optics but fluorescence detection has been utilized to estimate drug concentrations and measure pharmacokinetics of drug uptake *in vivo*.

PHOSPHORESCENCE MEASUREMENTS

Phosphorescence denotes radiative emission between states of different multiplicity. The transition is spin-forbidden and is consequently weaker than fluorescence as most of the excited species decay by collisional deactivation. T₁-S₀ phosphorescence provides information on lowest excited triplet states. Phosphorescence emission can be separated from fluorescence by its red-shifted spectrum and by its much longer decay time. Conventional phosphorescence detection involves gated detection such that the detector only monitors the sample at a suitable delay after excitation when the fluorescence has already decayed to zero. The weak nature of phosphorescence dictated the use of rigid media at low temperatures for early phosphorescence measurements but with current technology it is often possible to measure phosphorescence emission at room temperature. A topical example is the study of phosphorescence from low energy red-absorbing photosensitizer molecules using apparatus outlined in Fig. 4. The excitation source is modulated by the chopper and the emission is spectrally resolved by the monochromator and generates a signal at the germanium detector. The use of a lock-in amplifier with detection at the frequency of the chopper vastly increases the S/N and the integrated emission is plotted as a function of wavelength to generate the spectrum. An additional application of such apparatus is the measurement of phosphorescence from relaxation of excited singlet molecular oxygen, ¹O₂, formed by energy transfer from triplet state sensitizers. Photosensitization of ¹O₂ is thought to be of great importance in photobiology and photomedicine. The study of such a photosensitizer in the absence and presence of oxygen allows measurement of the phosphorescence emission from the sensitizer triplet state and ¹O₂, respectively. Phosphorescence can be used to estimate the triplet energy level (E_T) although care has to be taken for broad emission spectra. Ideally, one would combine the T₁-S₀ phosphorescence emission spectrum with the S₀-T₁ absorption spectrum, which is observable in some cases through the use of heavy atom solvents, and determine E_T from the overlap of the spectra, as outlined for fluorescence above.

Time-Resolved IR Phosphorescence Detection

This technique has been applied extensively in the investigation of the sensitized formation and bimolecular reactions of singlet oxygen, ¹O₂. It depends on the use of sensitive IR detectors such as germanium photodiodes to monitor the weak radiative relaxation of ¹O₂ to its triplet ground state. This phosphorescence emission displays a maximum in the near-IR at 1268 nm. A schematic of the apparatus in the Wellman Laboratories, which is fairly representative, is shown in Fig. 5.

Phosphorescence from the sample solution in a fluorescence cuvette is monitored at 90° to the excitation laser beam. A silicon filter is usually placed between the sample and detector to remove stray laser light or sample fluorescence which can interfere with the phosphorescence. Alternatively, an interference filter with maximum transmission in this spectral region may be used. The larger the active area of the Ge photodiode the larger the amplitude at the cost of the rise time of the signal. A compromise such as a 5 mm diameter

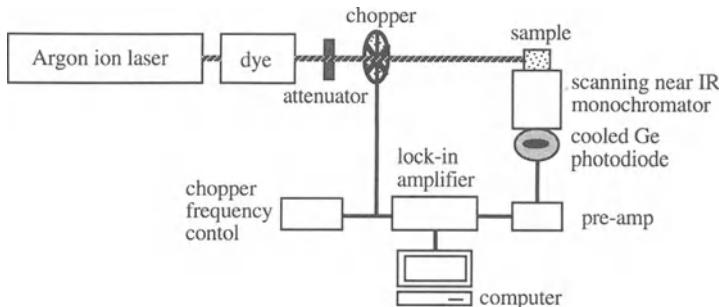


Figure 4 - Apparatus for near-IR phosphorescence measurements

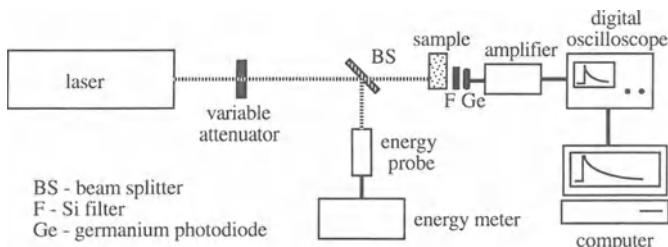


Figure 5 - Schematic for time-resolved IR phosphorescence detection apparatus

photodiode results in good sensitivity and a rise time of the order of 1 μ s. An interesting new proposal is the use of up-conversion of IR phosphor materials, which emit in the visible region on absorption of the IR emission from the sample, in conjunction with PMT's to provide sensitive detection with good S/N and the possibility of gated detection, which can remove any intense 'spikes' at the laser pulse due to fluorescence emission from the sample.

The lifetime (τ_Δ) of $^1\text{O}_2$ is solvent dependent and can be determined from kinetic analysis of the phosphorescence decay. Under conditions where $^1\text{O}_2$ reacts with a substrate (S) a plot of the observed rate constant for singlet oxygen decay (k_{obs}) against [S] yields the bimolecular quenching rate constant k_q from the slope. It should be noted that k_q determined in this fashion is actually the sum of rate constants for all processes through which the substrate reacts with $^1\text{O}_2$, both physically and chemically. Independent product studies are required to obtain the chemical component alone.

Quantum yields of singlet oxygen formation (Φ_Δ) are often measured by a comparative method using excitation of optically matched samples of a reference with known Φ_Δ and the sample of interest in the same solvent system. The Φ_Δ for the unknown is then obtained by direct comparison of the slopes of the linear plots of the initial phosphorescence amplitude (L_0) against incident laser pulse energy for both samples. The Φ_Δ values can then be used along with the intersystem crossing yield for the same compound to give the quantum efficiency (S_Δ) of generation of $^1\text{O}_2$ from;

$$S_\Delta = \frac{\Phi_\Delta}{\Phi_T} \quad (9)$$

FLASH PHOTOLYSIS (TRANSIENT ABSORPTION SPECTROSCOPY)

Flash photolysis is a method whereby a large population of transient metastable species can be produced in a very short time such that their spectral characteristics and intramolecular relaxation and intermolecular reaction processes can be studied in a time-resolved manner. Flash photolysis is commonly used to investigate species with lifetimes encompassing the picosecond to second domain, including electronic excited states, free radicals, radical ions, biradicals, photoisomers, phototautomers, carbenes, nitrenes, and a variety of more exotic species. This section will outline the types of apparatus required to study the photobehavior of such species in the different time domains.

General Principles

The flash photolysis experiment consists of an intense pulsed light source, normally a laser, which irradiates the sample in a spectral region where the ground state exhibits absorption to form a relatively large concentration of primary transient intermediates and the resultant change in the intensity of an analyzing beam transmitted through the sample to a light sensitive detector is monitored. In reality, this simple outline covers a diverse array of experimental approaches and equipment.

Typically, the detection consists of a broadband analyzing beam, a monochromator to tune the detection wavelength and a photodetector, usually a photomultiplier tube. In this way time-resolved optical changes in the sample at a particular wavelength are measured, allowing transient kinetics to be determined. In order to obtain spectral information the measurement is repeated at a series of increments over the spectral range of interest and a point-by-point spectrum is constructed.

Alternatively, the analyzing beam can be resolved using a dispersive element such as a polychromator or diffraction grating to project the whole spectrum onto a light-sensitive detector such as a diode array in a gated optical multichannel analyzer (OMA). This approach has the benefit that the whole spectrum can be obtained in a single measurement. However, it provides no time resolution, although kinetics can be estimated by repetitive measurements where the delay of the gate on the OMA is varied allowing time-dependent spectra to be constructed. The S/N of diode arrays is typically worse than PMT's which makes them less suitable for applications to systems where weak transient absorptions are displayed. Unfortunately, it is not trivial to obtain both full kinetic and spectral information from a single measurement although a few approaches are possible.

Time Resolution

The time resolution of a flash photolysis experiment will be determined by the detection response time and the excitation pulse duration. On going to shorter time scales a pump-probe approach may be preferred to real time detection, as described later.

Microsecond Resolution For this resolution a short pulse laser is not required and flashlamp discharges of a few μs duration suffice as excitation sources. Such was the case in early experiments before the application of lasers and is often termed *conventional* flash photolysis. In the simplest set-up a sample solution contained in a long path length cuvette is excited by a flashlamp in close proximity. A low power CW analyzing beam is transmitted through the sample to the detection system, often consisting of a monochromator / PMT / oscilloscope combination. Flash-induced changes in the sample absorption and related light intensity at the detector can then be followed in a time-resolved manner. In addition to the pulse duration a further disadvantage of this system is the polychromatic nature of the excitation pulse which imparts a lack of selectivity in sample excitation, especially important where one is not dealing with a completely pure compound or a multicomponent system. This problem is somewhat alleviated by the use of filters between lamp and sample to limit the excitation bandwidth. One advantage is that flashlamps emit high energies which can be effectively coupled into the whole absorption profile of the sample.

This resolution is limited to the detection and study of transient species of μs existence or longer. Thus, it is possible to study relatively long-lived species such as photoisomers, free radicals, radical ions, excited triplet states and phototautomers.

Nanosecond Resolution

Nanosecond laser flash photolysis is the most common type of flash photolysis and will therefore be described in most detail. The advent of nanosecond pulsed lasers in the 1960's initiated a tremendous advance in the ability of flash photolysis to study transient intermediate behavior with approximately three orders of magnitude improvement in time-resolution, as pulse duration caught up with detector technology. An important advantage of laser excitation sources is the monochromatic nature of the emitted light which allows high selectivity in excitation and has the practical consequence of simplifying actinometry in the measurement of quantum yields and absorption coefficients of transient species.

Early pulsed lasers such as Q-switched ruby or Nd/YAG produced laser pulse energies of ~ 1J in a 10-30 ns pulse at their fundamental lasing wavelengths of 694 and 1064 nm, respectively. The emission wavelength can be varied by non-linear harmonic generator crystals through which double, triple and quadruple (in YAG lasers) frequencies are obtained. Gas lasers such as nitrogen or excimer lasers are useful high power, nanosecond pulsed lasers for monochromatic excitation in the uv region. Limited continuous tunability was subsequently obtained with the introduction of dye lasers pumped either by flashlamps or other lasers. One drawback with dye lasers is the limited tunability which requires that for extended spectral ranges a combination of dyes have to be exchanged and the laser realigned to some extent. Conversion efficiency is also low, a 15% conversion of the pump pulse energy to dye output would be considered good.

The lasers mentioned above have been the workhorses in flash photolysis in the past but some exciting new technological developments should further extend the scope of the technique. Tunable solid state lasers are now becoming increasingly more powerful and reliable. The Ti-sapphire laser (pumped by a Nd/YAG laser) has useful tunability in the 700 to 1000 nm range which can be extended to shorter wavelengths by frequency doubling. Perhaps the most exciting development is the optical parametric oscillator which is capable of efficient conversion of a high power uv laser pulse to longer wavelengths. A non-linear crystal converts the pump pulse frequency to two distinct output frequencies such that $v_{in} = v_{out}^1 + v_{out}^2$. By tuning the angle of the crystal one can change the output frequencies with the practical result that the emission wavelengths are almost continuously tunable from 400-2,000 nm with a relatively flat power spectrum. With frequency doubling one can extend the emission to ~ 200 nm such that one laser system is capable of high power, continuously tunable, monochromatic emission in the uv-vis-near IR region.

Apparatus The nanosecond laser flash photolysis facility in our laboratory is shown schematically in Fig. 6. The basic outline is as follows. Excitation is achieved through the use of a Nd/YAG, excimer or excimer-pumped dye laser. The analyzing beam from a 75W CW xenon arc lamp passes through an aperture and overlaps the excitation beam at the first mm of the sample to maximize the signal intensity (*vide infra*). The arc lamp may also be pulsed to give a more intense beam for a few ms when S/N is a limiting factor. The light transmitted through the sample is focused on the input slit of a monochromator for wavelength selection and finally impinges a photomultiplier tube (for uv-vis) or Si diode (for near IR) detector. The signal generated at the detector is then acquired by a digital oscilloscope and ultimately transferred to a Macintosh computer.

The timing sequence for the experiment is shown in Fig. 7. The shutters protect the sample and detection system from overexposure and open immediately prior to the laser pulse. After the fast shutter opens the light reaches the photomultiplier and a signal voltage is developed across a load resistor which is directly proportional to the incident light intensity. The back-off is a sample-and-hold device which samples the signal voltage and then applies an equal but opposite voltage such that the output level is set to zero. The digitizer then triggers just before the laser and the energy monitor and a signal showing the pre-pulse region, laser-initiated change and kinetic decay is acquired. This signal is then read by the computer for conversion to absorbance, signal averaging and data analysis.

The back-off serves a dual purpose. The pre-pulse signal generated by the PMT (I_0) is sampled and held and can be read by the computer. The resulting pre-pulse level on the digitizer is thus set to zero volts and the laser-induced change in voltage (x) can be measured as a function of time on a sensitive scale. From the Beer-Lambert relationship the absorbance change (ΔA), at any time after the laser pulse, can be calculated from;

$$\Delta A(t) = \log \frac{I_0}{I_0 - x(t)} \quad (10)$$

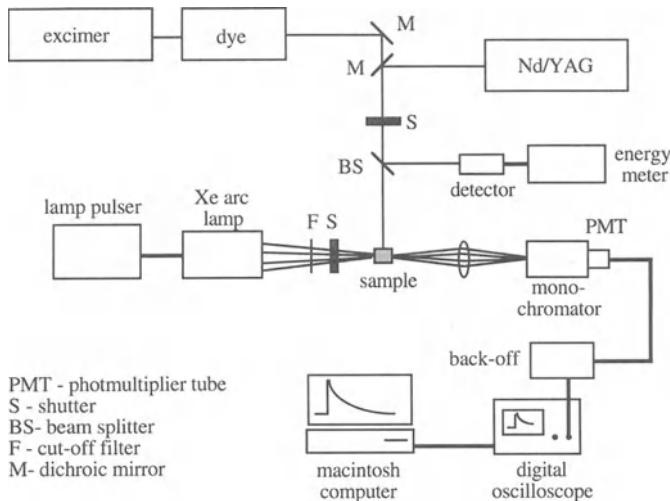


Figure 6 - Schematic of the nanosecond laser flash photolysis facility at Wellman Laboratories.

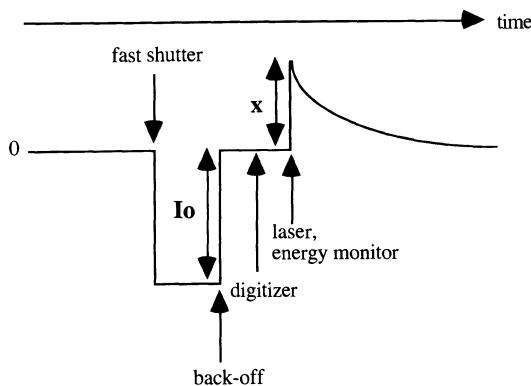


Figure 7 - Timing sequence for nanosecond laser flash photolysis experiment

Experimental Aspects

Several commonly encountered experimental problems and their solutions are outlined here.

Fluorescence Correction. In the study of fluorescent compounds the burst of fluorescence at the laser pulse can saturate the PMT for some time before the signal recovers. The signal developed at the PMT depends not only on the amount of incident

light but on the voltage supplied to the PMT itself. Fluorescence interference on the absorption signal can be minimized by increasing the analyzing light intensity (by pulsing for example) with concomitant decrease in the voltage supplied to the PMT such that the I_0 in effect is unchanged but the proportion of fluorescent light to analyzing light which reaches the sample is reduced. In cases where significant interference remains, a correction for the fluorescence "spike" can be made by acquiring a pair of traces, one of which has both laser and analyzing beam incident on the sample and the second where the shutter on the analyzing beam remains closed (fluorescence only reaches the PMT). Subtraction of the second signal from the first removes the fluorescence component from the signal profile.

Baseline Correction. Another common problem is that the baseline (I_0) of the signal drifts with time, making the correct evaluation of ΔA problematical. Such a scenario can arise when a pulsed lamp source or an AC coupled detection system is used. Providing the drift is reproducible, a simple correction may be applied by collecting a pair of signals one of which has both laser and analyzing beams incident on the sample and the second where the shutter on the laser remains closed such that only the analyzing beam reaches the detector. Subtraction of the second signal from the first corrects for the baseline drift in the transient absorption profile.

Beam Geometry. The geometry of the overlap of both excitation and analyzing beams merits some discussion. When measuring the absorbance of a solution in a spectrophotometer the chromophore is homogeneously distributed within the sample volume. This is not always the case in flash photolysis experiments, as illustrated in Fig.8 below.

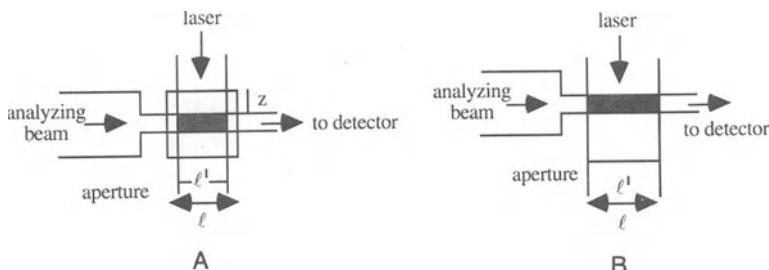


Figure 8 - Overlap of pump and probe beams in flash photolysis experiment

For purposes of discussion Figure 8 shows a plan view of two different excitation and probe beam overlap geometries. In both conditions the solution is contained in a $\ell \times \ell$ cm cuvette and the analyzing beam passes through an aperture (~1mm wide) before traversing the sample with path length ℓ . In A the laser beam diameter ℓ' is less than the path length ℓ of the sample. In addition the overlap region shown in heavy shading is in the central portion of the sample. Such a geometry presents the following considerations.

- (1) The path length of the solution through which the absorbance is measured is greater than the path length of the sample irradiated by the laser pulse. This creates a problem in equating transient absorbances, concentrations and absorption coefficients as the concentration of transient across the entire path length in the z direction is not constant. Care therefore has to be taken when quantitative evaluation of parameters such as quantum yields and absorption coefficients (*vide infra*) are attempted.
- (2) The sample cross-section which is interrogated by the analyzing beam is offset by a distance z from the incident surface of the laser beam. As the laser intensity is attenuated exponentially by the sample absorbance as it travels through the sample the concentration of transient species formed follows the same distribution and decreases with distance from the front surface. This is especially important when dealing with samples with high absorbance at the laser wavelength and has the result of reducing the sensitivity of the

experiment by probing the sample at a point where the transient concentration is less than maximum.

The geometry shown in **B** addresses the above points. The laser beam covers the entire sample path length ℓ and the sample is probed in the fraction nearest to the surface where the laser beam is incident.

Photodegradation of Sample. When irradiation causes some irreversible chemical change in the sample problems may arise due to appreciable degradation which can reduce signal intensity with time. Absorption of the exciting light by photolysis products acting as filters or to produce additional transient absorption can be problematic. If the extent of degradation is relatively small a static sample with continuous stirring using a micro-stirrer may suffice. If not, a continuously flowing solution is required to ensure irradiation of a fresh portion of sample with each laser shot, with the flow rate adjusted to the repetition rate of the laser excitation. Flow-through experiments have the disadvantages of requiring large sample volumes (which sometimes becomes unfeasible for biological material) and presenting more difficulty in removing and excluding oxygen from the system.

Picosecond Resolution

Laser flash photolysis with picosecond resolution was dependent on the development of lasers with ps pulse duration and suitable detection systems which conserve the resolution inherent in the shorter laser pulse. Picosecond pulsed lasers became generally available in the 1970's with the advent of mode-locking techniques. A non-mode-locked laser will support a multitude of oscillating modes which satisfy the standing wave condition but possess no phase relationship. A mode-locking device fixes the relative phases of oscillating modes within the laser cavity which modulates the gain of the laser with a repeating duration of the laser cavity round-trip time and a train of short duration ps pulses is emitted.

In order to be of practical use in flash photolysis experiments single pulse selection from the whole train leaving the cavity is required. This can be achieved through use of an extracavity Pockel Cell / polarizer arrangement timed to act as an open shutter for a duration shorter than the cavity round-trip time to allow one pulse, usually the maximum intensity pulse, to be emitted.

Picosecond Detection Methods. There are two common methods for picosecond resolution in flash photolysis experiments.

(a) **Real-time detection** - Monitoring an absorbance variation in the picosecond time domain is a difficult proposition for an electronic device. The fastest response PMT currently available has a response time of the order of a few 100 ps. The fastest digital oscilloscopes have around 5 Giga-samples per second sampling rate. The combination gives a response time of a few hundred ps. The fastest real-time detection is provided by streak cameras which have not been widely applied in absorption measurements due to a combination of sensitivity, experimental complexity and cost.

(b) **Pump-probe detection** - In this approach the time resolution is provided by the optical set-up of the apparatus. The analyzing or probe light is not a CW source but is a pulse generated by the excitation source. In a Nd/YAG laser a fraction of the output pulse from the laser is removed for use in generating the probe pulse (see below) and is delayed in time with respect to the excitation or pump pulse by using a variable optical delay line. The probe beam overlaps the irradiated region of the sample and is incident on a detector from which the integrated intensity is measured. Various detection strategies are employed, as outlined below.

(i) **Laser Harmonic Generation** - For samples with suitable absorption characteristics two different harmonics of the fundamental laser emission can be used to pump and probe the sample. Frequency doubling and tripling of the Nd/YAG emission to 532 nm and 355 nm is useful when the uv wavelength can be used to excite samples to produce transient species which can be probed by the delayed 532 nm wavelength using a simple photodiode or phototube detector combined with a boxcar integrator.

(ii) **Dye Laser** - A portion of the pump laser emission can be used to pump a tunable dye laser, resulting in similar duration output at wavelengths which are determined by the dye used and laser optics. This is more versatile as it provides limited tunability in the probe

wavelength, although the stability of dye lasers may be a problem. Again, a simple detector-boxcar integrator detection system can be employed.

(iii) *Continuum Generation* - This is the most commonly used method and relies on the non-linear effect of focusing a high power laser pulse into a solvent to produce a broadband continuum emission of similar duration to the pump pulse. The spectral profile depends on the solvent used and covers the visible-near IR region. For example, 532 nm pumping of CCl₄ produces a useful continuum between 400-900 nm. A disadvantage is the difficulty in achieving efficient continuum generation in the uv region. A fraction of the pump laser beam is removed for continuum generation delayed with respect to the pump pulse by means of an optical delay line. As the probe beam in this case is broadband in nature some wavelength resolution is required in the detection end. A common approach is to use an OMA after the sample where a dispersive element such as a grating projects the resolved light on a calibrated diode array detector. Through calibration, each diode corresponds to a particular wavelength and the intensity of the signal generated at the diode is proportional to the amount of light incident at the diode. This approach has the decided advantage that full spectral information can be obtained in a single measurement although the OMA is expensive and the S/N may be a problem in the absence of extensive signal averaging. Such a set-up as in Wellman Laboratories is shown in Fig. 9. For stability reasons the optical path of the probe beam is often fixed and the pump laser beam is varied to produce the required delay between the pulses.

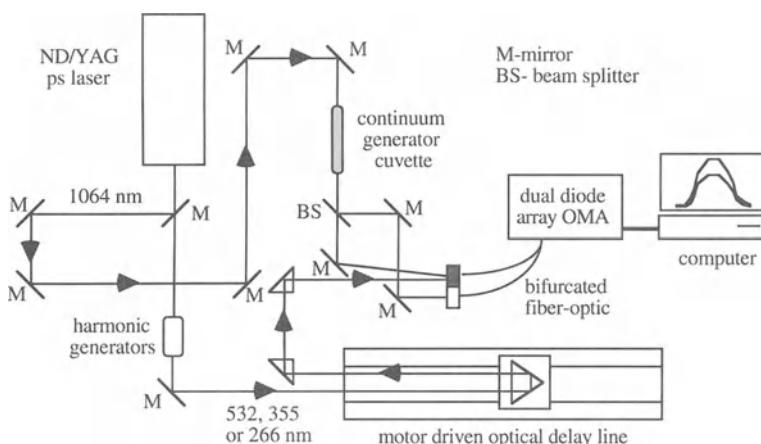


Figure 9. - Picosecond laser flash photolysis apparatus in Wellman Laboratories.

(iv) *Stimulated Raman Scattering* - A common problem in the above approaches is the difficulty in obtaining emission wavelengths for probing the uv region. Stimulated Raman scattering of a uv picosecond pulse of frequency ν_{ex} when focused in a cell containing a material with vibrational frequency ν_{vib} produces a line emission spectrum where $\nu = \nu_{\text{ex}} \pm n\nu_{\text{vib}}$ ($n=\text{integer}$) consisting of Stokes and anti-Stokes emission lines. The exact wavelengths produced are dependent on the compound in the Raman cell. For example using 266 nm from a Nd/YAG laser to excite hydrogen, anti-Stokes emission lines at 199.8, 217.8 and 239.5 nm are produced. By using various pump wavelengths in combination with a variety of compounds a probe wavelength spectrum can be constructed.

(v) *Stimulated Fluorescence* - In methods (i-iv) the probe pulse duration will approximately equal that of the pump-pulse. A method of producing variable duration and wavelength probe pulses is to pump stimulated emission from fluorescent dyes. The spectral output and duration of the probe pulse depends on such factors as the dye, solvent, concentration, pump intensity and path length. In effect, this is an inexpensive method of producing a limited broad spectral output for absorption measurements.

In all the above approaches the transient absorption changes (ΔA) are again calculated using the expression in equation 10. In the OMA experiment the absorption change at each diode, and hence wavelength, is calculated using the signal generated in the presence (I) and absence (I_0) of the pump pulse. An elegant approach is to employ a dual diode array detector, as shown in Fig. 9, where the continuum is split into two equal fractions one of which is transmitted through a volume of the sample which overlaps the region irradiated by the pump pulse and the other which traverses the same sample but in an unirradiated region. Both components are then projected on to their respective diode arrays using a bifurcated fiber optic cable and the normalized signals used to determine the transient absorbance change.

CHARACTERIZATION OF TRANSIENT INTERMEDIATES

In flash photolysis experiments either time-dependent (kinetic) or spectral information on the transient absorption can be obtained. In addition, both quantitative or qualitative information can be derived. A description of commonly adopted practices for such experiments is given in this section.

Measurement of Transient Kinetics

In time-resolved experiments the absorption of the sample is followed as a function of time. In nanosecond experiments this information can be obtained in a single pulse, although signal averaging is commonly employed to improve S/N. In pump-probe measurements however, the nature of the experiment is that the time-dependence of the absorption must be constructed by repetitive measurement of signal as one changes the delay between pump and probe beams. Transient absorption kinetics can take on a variety of forms depending on the system under study and give valuable information on the mechanisms by which transient species relax or react. Some of the more typical kinetic forms are described below.

First-Order Decay Single Exponential Decay. This is the simplest form observed in kinetic experiments and is described by;

$$A(t) = A_0 \bullet e^{-kt} + A_\infty \quad (11)$$

where $A(t)$ is the time dependent transient absorbance, A_0 is the absorbance observed immediately following the laser pulse, k is the rate constant for decay of the species in s^{-1} ($= 1/\tau$, where τ is the lifetime of the species in s) and A_∞ is the residual absorbance, if any, remaining after decay of the species. A first-order, single exponential growth has the same form as the above except that the signal grows rather than decays with time. The trace is reversed as A_0 is now minimum and A_∞ maximum. The only difference is in the sign of the exponent.

Sum of Exponential Decays This case is observed when more than one transient species decaying by first or pseudo-first order kinetics contributes to the overall trace. The time-dependent absorption change for n species will be defined by the following expression;

$$A(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + \dots + A_n e^{-k_n t} + A_\infty \quad (12)$$

For practical reasons the number of exponentials which can be resolved is determined by the S/N ratio of the signal, the relative values of k (should be well separated) and the resolution of the absorbance measurement. In simple experiments one should be content with sum of two exponentials. Mixed growth and decay kinetics are often the case in bimolecular reactions such as energy or electron transfer where the initial pseudo-first order formation of a transient species is then followed by its own decay.

Second Order Decay. This case applies in bimolecular reactions where both reactants are present in approximately equal amounts. For a bimolecular reaction where the partners are identical such as triplet-triplet annihilation or radical-radical recombination the absorbance change is given by;

$$A(t) = \frac{A_0}{1 + (ktA_0)} \quad (13)$$

and a plot of $1/A$ vs. t gives a straight line with slope of $2k / \epsilon\ell$, where k is the second-order rate constant and ϵ is the molar absorption coefficient of the transient species.

Kinetic Analysis

From the above, it is clear that in many cases the lifetimes of transient species may be derived. For example, for a first order decay the rate constant, k , can be obtained simply from the slope of a plot of $\ln A$ vs. time. For statistical reasons the best fit of the data to a particular function is required. A variety of procedures exist for extracting the best fit of an experimental trace to a chosen function. These are normally based on iterative least-squares regression analysis. In our laboratory a modified Levenberg-Marquardt non-linear least-squares approach is used to extract k and A values from the integrated rate equations. A more complex approach is to utilize a global analysis where many traces at different wavelengths are considered in an attempt to extract common k values as A varies with wavelength.

Care is required in interpretation of simple exponential decays. In many cases the decay will be pseudo-first order from reaction of the observed transient with another species which is present in excess concentration, rather than a true first order unimolecular reaction. For triplet states possible reactant partners include the ground state (self-quenching), oxygen and other substrates. The pseudo-first order rate constant (k_{obs}) is determined by;

$$k_{obs} = k_1 + k_q [Q] \quad (14)$$

where k_1 is the true unimolecular rate constant and k_q is the bimolecular rate constant for reaction with quencher Q . A plot of k_{obs} vs. $[Q]$ allows determination of k_q (in $M^{-1}s^{-1}$) from the slope and k_1 from the intercept.

Spectral Analysis

Most transient absorption spectra are straightforward to measure using either point-by-point or diode array approaches outlined earlier. The transient absorption spectrum is measured as a function of time to ascertain if one or more species is contributing to the measured spectrum. This is useful in the study of bimolecular reactions where an initially formed transient species (X) reacts to form a secondary intermediate (S). In such a case the decay of X will be correlated to the formation of S and the observed rate constants for both processes should be equal. In many cases where time-dependent absorption spectra are observed the individual contributions can be separated by simple subtraction of spectra recorded at different time delays if the lifetimes are significantly different, or by repeating the experiment in the presence of a specific quencher for one of the components.

Transient Absorption Coefficients

There are a number of methods for measuring absorption coefficients for transient species. Flash photolysis measurements are of *differential* absorption between the intermediate and ground state precursor, *i.e.* ΔA . From the Beer-Lambert relationship the absorbance change is given by

$$\Delta A = c \cdot \Delta \epsilon \cdot \ell \quad (15)$$

where c is the concentration of the intermediate and $\Delta \epsilon$ is the difference in absorption coefficient between intermediate and precursor. The difficulty in measuring an absorption

coefficient for any intermediate is to ascertain its concentration. Three possibilities to provide this information and $\Delta\epsilon$ are outlined below.

Sensitization This is perhaps the most suitable method and is based on a bimolecular reaction where one of the reactants, either donor or acceptor, has a well characterized $\Delta\epsilon$ value. The assumption made here is that the efficiency of the process is 100%, i.e., every donor excited state on quenching gives rise to an acceptor excited state, thus, their concentrations are equal. For a reaction where excited donor (D_o^*) sensitizes the formation of excited acceptor (A_c^*), the measurement of excited state absorption (ΔA_{D_o}) in the absence of the acceptor (A_c) and the sensitized acceptor absorption (ΔA_{A_c}) under conditions where all D_o^* is quenched to give A_c^* allows the determination of the unknown $\Delta\epsilon$ from;

$$\frac{\Delta A_{D_o}}{\Delta A_{A_c}} = \frac{\Delta\epsilon_{D_o}}{\Delta\epsilon_{A_c}} \quad (16)$$

This method depends on the above conditions being fulfilled and that formation of A_c^* should only come from the sensitization route, not from direct excitation. Some kinetic corrections may also have to be applied for factors such as incomplete quenching and decay of A_c^* during its formation.

Complete Conversion Commonly used for triplet states, this method consists of measuring the dependence of the transient absorption on the laser pulse energy with the aim of converting all the ground state molecules to intermediate. This method requires cycling of those molecules which when excited come back to the ground state by internal conversion or fluorescence. Each cycle bleeds off a certain fraction of the excited singlet molecules to intermediate (given by Φ_{INT}) until all molecules are in the intermediate state. This can only be achieved when the laser pulse duration is much longer than the singlet lifetime, and a significantly high Φ_{INT} is exhibited by the molecule, such that enough cycling takes place for complete conversion to occur. If so, then the concentration of intermediate is simply equal to that of the ground state before the pulse and $\Delta\epsilon$ is obtained from the saturated value of ΔA . The value obtained in this manner is a lower limit.

Ground State Depletion This method is dependent on the assumption that there will be negligible contribution from the intermediate in a region of strong absorption exhibited by the ground state, i.e. a strong transient bleaching will occur and as ΔA is directly proportional to $\Delta\epsilon$, which at this wavelength is equal to $-\epsilon$ (ground state), the values of $\Delta\epsilon$ at all wavelengths can be easily obtained. This method gives an upper limit for $\Delta\epsilon$ and suffers from being very subjective.

Quantum Yields

Under some conditions the measurement of quantum yields of formation of transient species through flash photolysis measurements is possible.

Comparative Technique This method involved measurement of the transient absorbances (ΔA) from solutions of the compound under study (U) and a reference compound (R) when excited by absorption of exactly equal numbers of photons. The transient absorbances (ΔA) of both samples are measured and the following relationship applies;

$$\frac{\Delta A_R}{\Delta A_U} = \frac{\Delta\epsilon_R \cdot \Phi_R}{\Delta\epsilon_U \cdot \Phi_U} \quad (17)$$

This allows evaluation of the product of $\Delta\epsilon \cdot \Phi$ and providing $\Delta\epsilon$ is known, then Φ can be determined. For greater accuracy the slope of the dependence of transient absorption on laser pulse energy should be used in place of ΔA from a single measurement.

Heavy-Atom Fluorescence Quenching Method This approach developed by Medinger and Wilkinson applies to the evaluation of intersystem crossing yields of

fluorescent compounds which exhibit an enhancement in intersystem crossing induced by heavy atoms or paramagnetic species. The reduction of the fluorescence yield can be correlated with the increased triplet absorbance yielding the intersystem crossing yield (Φ_T) from;

$$\frac{F^o}{F} = \left(\frac{\Delta A_T F^o}{\Delta A_T^o F} - 1 \right) \bullet \Phi_{isc} + 1 \quad (18)$$

where F^o and F are fluorescence quantum yields and ΔA_T^o and ΔA_T are triplet state absorbances in absence and presence of heavy atom quencher, respectively.

Triplet State Energies (E_T)

The triplet state energy of a compound can be measured from phosphorescence, heavy atom-induced S₀-T₁ absorption or photoacoustic and photothermal calorimetry techniques (*vide infra*). Flash photolysis experiments also give the possibility to estimate triplet energies.

Energy Transfer Method The interaction of a given molecule of unknown E_T with a variety of molecules encompassing a wide range of triplet energies allows rate constants for triplet energy transfer to be measured. The Sandros equation shown below gives the relationship between the rate constant (k_{ET}) for energy transfer and the energy difference (ΔE) of the two triplet levels.

$$k_{ET} = \frac{k_{diff}}{1 + e^{-\frac{\Delta E}{RT}}} \quad (19)$$

where k_{diff} is the diffusion controlled rate constant in the solvent used. This approach will give an estimate but an accurate value can only be obtained where the components have almost isoenergetic levels and a reversible energy transfer is observed. ΔE_T can then be determined from the equilibrium concentrations using,

$$\Delta G = -RT \ln K \quad (20)$$

where K is determined from the equilibrium concentrations of the triplet partners (knowledge of ϵ values are required) and ΔE_T is correlated to ΔG .

Thermodynamic Properties

Various thermodynamic properties of excited intermediates can be established by flash photolysis experiments. From temperature dependence of the excited state parameters such as lifetime and quantum yields it is possible to demonstrate the existence of energy barriers to photophysical processes from which ΔE_a , the activation energy, can be calculated from the Arrhenius relationship.

$$k = A \bullet e^{-\frac{E_a}{RT}} \quad (21)$$

Plots of $\ln k$ for some unimolecular and bimolecular reactions against $1/T$ yields the activation energy from the slope ($= -E_a/R$) with intercept equal to the natural log of the pre-exponential factor A . This is an important application to the study of processes like isomerizations which are thermally activated.

DIFFUSE REFLECTANCE LASER FLASH PHOTOLYSIS

The predominant use of laser flash photolysis measurements has been to probe compounds of biological or medical relevance in homogeneous solution or simple model systems such as water, organic solvents, micelles, vesicles, etc. The problem with direct study of *in vivo* or *in vitro* biological systems is of detection of analyzing light. Cellular material or tissue is either opaque or highly scattering such that an alternative to transmission based methods is required. An attractive variant is the so-called diffuse reflectance laser flash photolysis method pioneered by Wilkinson and co-workers.

When light is incident on a scattering material some light is directly reflected at the surface (*specular reflectance*) while some penetrates into the material and is attenuated by scattering and/or absorption events before a fraction, termed the *diffuse reflectance*, is remitted. As long as no mechanical or thermal changes in the sample occur, changes in the intensity of the diffuse reflectance induced by a laser pulse can be equated to absorption and can be used in a fashion similar to normal transmission measurements.

The diffuse reflectance laser flash photolysis apparatus in our laboratory is shown in Fig. 10.

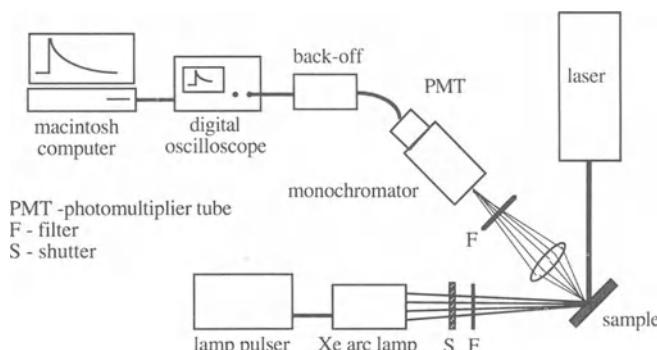


Figure 10 - Diffuse reflectance laser flash photolysis set-up

The excitation and analyzing beams are perpendicular and the diffuse reflectance is collected at 45° to these beams. Crossed polarizers inserted before and after the sample reduce the contribution from specular reflectance. As the efficiency of collecting diffuse reflectance is lower than transmission mode a more intense analyzing source is required and the lamp must be operated in pulsed mode.

The technique can be applied to any biological material such as cell pellets, excised tissue, skin and even intact animals. Spectral and kinetic information may be obtained although the following points should be mentioned concerning the use of the diffuse reflectance technique.

(1) As light scattering is a wavelength dependent phenomenon the white light analyzing beam does not interrogate the same volume of the sample for all wavelengths. The practical consequence is that uv absorption bands tend to be underestimated with respect to bands in the red. An additional problem occurs when the transient absorption spectrum displays a bleaching in an area of ground state absorption. The extent of transient bleaching is underestimated as the reduction in absorbance in the near-surface area only results in an increased depth of penetration and has little effect on the remitted diffuse reflectance. The consequence of the above is that spectral profiles of the same transient species recorded in transmission and diffuse reflectance experiments are similar but not superimposable.

(2) Although kinetic measurements may be made quantitative parameters such as quantum yields and absorption coefficients are not trivial to obtain due to uncertainties in factors such as number of photons absorbed, overlap of sample volume irradiated by pump and probe beams and local concentrations.

Notwithstanding the limitations of the diffuse reflectance method it is potentially a very powerful tool in ascertaining whether the photoproperties of biological molecules in dilute solution also apply in the complex heterogeneous, compartmentalized world of living cells and tissue.

PHOTOCAPACITIVITY

A useful additional detection method is that of measuring photo-induced conductivity in a sample. Production of charged molecules can be used to confirm the nature of species observed in transient absorption experiments. Indeed, it is fairly trivial to measure both transient absorption and conductivity simultaneously in the same sample using a cell such as the one shown in Fig. 11, as used in our laboratory.

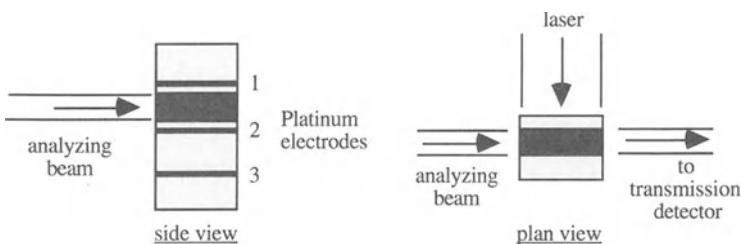


Figure 11 - Schematic of differential conductivity cell and beam arrangement

The geometry for the transient absorption detection is similar to before but the cell contains 3 platinum electrodes. Electrode 2 is common and only the volume between electrodes 1 and 2 is irradiated. A high voltage pulse is applied across the electrodes and the conductivity is measured between electrodes 1 and 2 and electrodes 2 and 3, the unirradiated region. The latter acts as a background for the conductivity in absence of the laser pulse. The signals from the electrode pairs are fed into a differential amplifier the output of which constitutes the laser-induced *change* in conductivity associated with production of transient species. The time-resolution of conductivity measurements depends on the nature of the detector but can be as short as nanoseconds. Thus, correlation of kinetic behavior in transient absorption and conductivity detection measurements is a useful tool in identification of some transient species. The signal amplitude from conductivity measurements depends not only on the yield but the nature of the ions formed. Highest conductances are seen for small ions such as e^-_{aq} , H^+ and OH^- whereas larger ions produce 3-4 times less signal.

TWO-COLOR LASER FLASH PHOTOLYSIS

Another variant is the two-laser or two-color laser flash photolysis method. This technique is designed to study the effects of higher excitation processes, particularly excited transient intermediates. A two pulse approach is used; the first pulse is used as a *synthesis* pulse to generate the intermediate of interest and the second or *photolysis* pulse is used to excite the intermediate and the resulting effect is observed using transient absorption detection. Under some conditions similar effects can be seen with one laser, high power multiphoton processes but the advantage of this approach lies in the ability to *selectively* excite the ground state precursor and the intermediate under conditions where the wavelengths of the lasers and time delay between the pulses can be arranged to maximize both absorption processes. Indeed, the development of this method has arisen out of the recognition that photochemical processes induced by low and high power excitation can exhibit substantial differences. Comparison of the difference absorption spectrum before and after the second pulse gives information on reaction mechanisms of excited intermediates.

Although the technique appears complex quantitative measurements are possible. By adoption of the geometry shown in Fig. 8B where the lasers are collinear the system reverts to a single photon process as the absorbance of the homogeneous distribution of intermediate in the volume probed by the analyzing beam is measured at the exact delay and laser wavelength of the second pulse by transient absorption in the absence of the second pulse. A "one-laser" reference sample can then be used which is adjusted to the same absorbance as the unknown at the second pulse wavelength. Changes in absorbance produced by both samples on irradiation of the second pulse can then be compared and providing absorption coefficients are known, quantum yields can be obtained from equation 17.

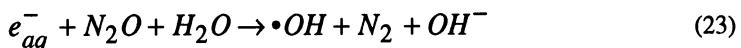
Possible applications include the study of biphotonic absorption processes by biomolecules such as DNA and amino-acids and intensity dependent behavior of photosensitizers. The transient absorption changes observed in two-color experiments can be correlated with product studies to determine the exact mechanism of reaction. The method presents a means of producing a highly energy-rich molecule, which can then undergo photoionization or bond cleavage, without the requirement of deep uv photons, which have poor penetration in scattering media. The total energy (E_{tot}) introduced to the molecule is given by;

$$E_{tot} = E_{int} + h\nu_2 \quad (22)$$

where E_{int} is the energy content of the intermediate and ν_2 is the frequency of the second pulse.

PULSE RADIOLYSIS

Pulse radiolysis, like flash photolysis, is a powerful technique for the generation and study of transient intermediates. The excitation source in pulse radiolysis is ionizing radiation. The experimental set-up is very similar to flash photolysis except that a source such as a linear accelerator (LINAC) or van de Graff generator is used to produce a short pulse (typically in the ns domain) of high energy electrons. In flash photolysis, selectivity in solute excitation is achieved through choice of wavelength; in pulse radiolysis the reaction of the electrons is rather indiscriminate and depends only on the concentration of species in solution. By definition, the solvent is present in highest concentration and the primary reactions are between electrons and solvent molecules. Solute transient intermediates are then produced from secondary reactions between solute and solvent-derived intermediates. The overall reaction of solute is dependent on the nature of the solvent-derived intermediates with which it interacts. Radiolysis of water rapidly produces both reducing (e^{-}_{aq}, H^{\bullet}) and oxidizing ($\cdot OH$) species. In order to impart some selectivity to the solute intermediates formed, additives are used to suppress one of the possible redox reactions. Exclusively reducing conditions can be obtained by addition of formate which reacts with oxidizing $\cdot OH$ to form reducing $CO_2^{\bullet-}$. Alternatively, alcohols such as *t*-butanol can be used to remove $\cdot OH$. Predominantly oxidizing conditions can be achieved by adding nitrous oxide (N_2O) which reacts with e^{-}_{aq} to give further oxidizing $\cdot OH$.



The $\cdot OH$ radical is a highly reactive species which can undergo addition reactions in addition to promoting one-electron oxidation. In order to generate only the latter species $\cdot OH$ is often quenched by addition of halide or pseudo-halide ions to give milder oxidizing agents which then interact with the solute of interest.

The most powerful aspect of pulse radiolysis is its ability to generate radicals and radical ions in high yields, which is often not possible by flash photolysis. It can also be used to generate electronic excited states by processes such as ion recombination and energy transfer although such reactions are limited to non-polar solvents where the initially produced ions are not readily solvated. As a result pulse radiolysis and laser flash photolysis are highly complementary techniques as the former is preferred for generation and study of radicals and radical ions while the latter is preferred for electronic excited

states. Pulse radiolysis can provide an unambiguous identification of transient species such as radicals which can be used as a fingerprint in laser flash photolysis studies.

Pulse radiolysis has proved very useful in biological applications as a means not only for studying radical behavior but in the ability to produce biologically relevant oxidants such as $\cdot\text{OH}$ and the superoxide anion radical, $\text{O}_2^{\bullet-}$, produced by reaction of oxygen with reducing species such as e_{aq}^- and $\text{CO}_2^{\bullet-}$. The reactions of these highly important oxidative species with biologically relevant compounds can then be monitored.

Pulse radiolysis has also been important in the study of electron transfer reactions as experimental conditions can be arranged such that formation of one half of an electron transfer couple is generated by the pulse and the kinetics of the subsequent electron transfer can be followed as a function of time and reactant concentration. When the reduction potentials of both components are similar an equilibrium is obtained and the difference in reduction potentials (E) of the couple can be obtained by calculating K from equilibrium concentrations of both species from transient absorbance measurements and using;

$$E(D / D^{\bullet-}) - E(A / A^{\bullet-}) = 2.303 \frac{RT}{F} \log K \quad (24)$$

Experimental Aspects Pulse radiolysis detection is similar to flash photolysis as both involve transient absorption measurements. The pulse duration is typically in the ns domain and a useful characteristic of sources such as LINACs is the ability to vary the pulse duration. Picosecond measurements have been carried out taking advantage of the structure of the electron pulse and using broadband Cerenkov radiation, produced as electrons slow down on passing from materials of lower to higher refractive index, as probe beam in pump-probe measurements.

Pulse radiolysis is a destructive technique as the sample is irreversibly changed by the absorption of the electron pulse. A fresh sample is required for each pulse requiring the use of a flow-through cell as described previously. Sample changing is controlled remotely by the operator since the potentially lethal effects of ionizing radiation require the operator to be outside a lead or concrete shielded laboratory containing the source and sample. Shielding of the detection electronics is also imperative (for LINAC sources) and isolation can be provided by a Faraday cage.

In experimental terms pulse radiolysis can produce a homogeneous distribution of intermediates in solution, thereby reducing the importance of beam geometry considerations. One important factor to consider is sample purity, especially solvent purity, as the concentration dependent nature of subsequent reactions means that small fractional impurities can be present in high enough concentration to affect the desired reaction sequences.

Quantitative measurements using pulse radiolysis pose a different problem from flash photolysis. Instead of measuring a quantum yield as the number of intermediates as a function of absorbed photons, yields in pulse radiolysis are expressed as the number of intermediates produced per 100 eV of absorbed radiation. These so called G-values are measured using dosimeters where the G-value (and molar absorption coefficient) is accurately known, such as the generation of thiocyanate ion (CNS^-) $_2^{\bullet-}$ from irradiation of aqueous solutions of CNS^- with a G-value of 2.9. During an experiment the relative energy of the electron pulse is monitored using a secondary emission chamber which monitors the number of electrons in the beam. The combination allows quantitative evaluation of factors such as molar absorption coefficients.

PHOTOACOUSTIC TECHNIQUES AND PHOTOTHERMAL TECHNIQUES

Following absorption of light energy radiationless relaxation processes determine that a fraction of the absorbed energy is released by the chromophore to its surroundings as heat. As a result, changes occur in temperature, density, pressure and refractive index of the medium. Under high laser intensities more extreme effects such as vaporization or ablation may occur. The localized heating of the sample gives rise to thermoelastic expansion which launches propagating acoustic waves in the sample, the so-called photoacoustic effect. In photochemistry there has been an increasing realization of the complementary nature of these techniques to established methods such as flash photolysis.

The basis of interpretation of most photoacoustic and photothermal techniques is calorimetric and lies in the use of a simple energy balance equation relating energy absorbed with heat evolved through;

$$E_{abs} = \alpha E_{abs} + E_{em} + E_{st} \quad (25)$$

where E_{abs} is the absorbed energy, α is the fraction given up as 'prompt heat', E_{em} is the energy lost through emission, usually fluorescence, and E_{st} is the stored energy. The prompt heat component is the energy given up as heat within the heat integration time of the detection which is determined by the following; laser pulse duration, the detector response time and the acoustic transit time (τ_a), the time taken for the acoustic wave to traverse the beam diameter r ($\tau_a = r/v$, where v is the speed of sound in the medium). E_{st} denotes energy which is not given up within the heat integration time and depending on experimental conditions can be equated to energy contents of metastable species, heats of chemical reaction and bond energies.

Photoacoustic Spectroscopy and Calorimetry

The photoacoustic effect (transduction of light into acoustic energy) can be probed by the use of piezoelectric detectors (which transduce the acoustic impulse into an electrical signal) to generate signals proportional to the amount of pressure and heat evolved. A non-CW light source such as a pulsed laser or modulated CW laser or lamp is used to produce a transient effect which can be picked up by the detector.

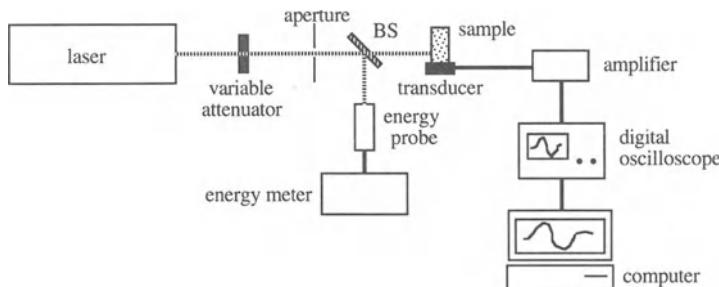


Figure 12 - Apparatus for pulsed excitation with photoacoustic detection.

The basic pulsed calorimetry experiment is a simple measurement of the amplitude of the transducer signal as a function of absorbed energy. This is a comparative technique requiring the use of a reference compound with known photophysical properties, and thus, α value. Ideal reference systems are compounds which decay exclusively by non-radiative means in a time scale shorter than the time window of the measurement, *i.e.*, $\alpha = 1$. Sample and reference solutions, in exactly the same solvent system, due to the solvent dependent variation of thermoelastic properties determining signal amplitude, are prepared with identical absorption (typically < 0.2) at the laser wavelength and the slope of the plots of signal amplitude vs. laser energy are measured. The α value for the sample of interest is obtained by the simple ratio of the slopes. Equation 25 can be written;

$$(1 - \alpha)E_{abs} = E_{em} + E_{st} \quad (26)$$

which can be expanded and reorganized to;

$$(1 - \alpha)v_\ell = \Phi_F v_F + \frac{\Phi_{st} E_{st}}{N_A h} \quad (27)$$

where v_L is the laser frequency, Φ_F and v_F are the quantum yield and weighted average fluorescence frequency, and $\Phi_{st} E_{st}$ is the product of the quantum yield and the energy content of the storing process. Measurement of α and knowledge of the fluorescence parameters allows evaluation of the product $\Phi_{st} E_{st}$. If one of these values can be independently measured the other can be evaluated. This approach has been used to measure triplet state energies, quantum yields of triplet state and singlet oxygen formation, heats of reaction, bond dissociation energies and photoisomerization enthalpies.

Experimental Aspects

Solvent Dependence

The amplitude of photoacoustic signals depend not only on the amount of energy deposited as heat in the sample but on the thermoelastic properties of the medium, usually solvent. Signal amplitudes are proportional on the value of β / C_p where β is the thermal expansion coefficient and C_p is the specific heat capacity of the solvent. The signal magnitude varies widely with solvent and unfortunately for biological studies this value is lowest of all in water, making studies in aqueous media less sensitive. One way around this problem is to locate the compound of interest in the water core of a reverse micelle. As the magnitude of the signal is dependent on the bulk properties of the medium, in this case predominantly hydrocarbon, considerable enhancements may be observed over pure aqueous systems.

Time resolution

Piezoelectric transducers can be resonant or non-resonant in nature. Non-resonant materials such as polyvinylidene difluoride (PVF_2) foils or highly-damped ceramic transducers are more accurate in following the time course of the evolution of the heat within the sample and a degree of time resolution becomes possible. In the last decade the development of time-resolved photoacoustic detection schemes has been initiated. A heat evolving process which takes place within the detection time window will alter the amplitude and cause a phase shift of the signal with respect to a system where heat release is very rapid. The latter can be used as an instrument response function for use in deconvolution of the response from the time-dependent heat evolution process.

The time resolution depends on the detection scheme employed. In the simple set-up (Fig. 12) the excitation beam passes through an aperture, such as a slit or pinhole, before passing through the sample parallel to the detector surface. The acoustic wave generated in the irradiated volume travels through the solution to the cuvette surface in contact with the transducer where it is detected. Alternatively, a front-face geometry can be used in conjunction with a high frequency transducer to give high sensitivity and very fast time resolution of a few nanoseconds. Using these approaches lifetimes of transient species such as triplet states and biradicals have been obtained.

Sensitivity

PAC is a highly sensitive method to monitor very low sample absorption ($< 10^{-6} \text{ cm}^{-1}$) and can be used in analytical applications to monitor very low concentrations of trace materials. Low probability events such as simultaneous biphotonic absorption can be measured allowing determination of two-photon absorption cross-sections, important in high power excitation. The technique has also been extended to two-pulse, two-color experiments where calorimetric information can be obtained following excitation of transient intermediates by the second pulse (*vide supra*).

Photoacoustic Spectroscopy (PAS)

PAS is a variation whereby the amount of heat evolved and photoacoustic signal amplitude is measured as a function of excitation wavelength in a manner analogous to fluorescence excitation spectra. The resulting spectrum is an action spectrum for the heat evolution and can be interpreted as equivalent to an absorption spectrum. The apparatus is very similar to that shown in Fig. 4 except that a transducer or a microphone is used as the detector. Lock-in amplification and integration of the signal amplitude as a function of wavelength gives a spectral profile. This spectrum must be corrected for the lamp emission profile. This is conveniently carried out using a totally absorbing sample such as carbon black where the spectral profile of the PAS signal correlates with the lamp emission profile.

This method has an advantage that it can be used to study opaque samples of biological interest. The acoustic detector in this case is a microphone which senses the periodic pressure changes in the gas phase above the sample induced by the modulated excitation. Absorption and deactivation processes in biological material such as tissue and leaves, demonstrate the usefulness of the technique. The method can also provide information on spatial distribution of chromophores in a heterogeneous sample (depth profile) as the fraction under the surface which is actually probed depends on the modulation frequency. For relaxation processes which occur on time scales much shorter than the modulation frequency the oscillation of the photoacoustic signal follows the modulation of the source. Heat which is generated in the sample diffuses to the surface to cause gas heating and generate the photoacoustic signal. The depth to which heat evolution can contribute to the signal is determined by the thermal diffusion time and the modulation frequency; the higher the frequency the shallower the depth below the surface where heat evolution will contribute to the sample. Variation in the action spectrum as a function of modulation frequency will reflect the chromophore distribution.

Time-Resolved Thermal Lensing (TRTL)

The same calorimetric information from PAC can be obtained by photothermal methods such as Time-Resolved Thermal Lensing (TRTL) or the related Photothermal Beam Deflection (PBD) calorimetry. Excitation of the sample with a gaussian pulse and subsequent non-radiative relaxation causes changes in temperature, density and refractive index such that the solution acts as a divergent lens (as dn/dT is generally negative) in the irradiated region. Any beam which passes coaxial through this volume will be defocused. This thermal blooming is observed in single beam experiments and is a problem in laser design where telescope optics have to be introduced to correct for thermal blooming as the lasing medium heats up. In TRTL the pump and probe beam are aligned almost coaxial, under a small angle, with maximum overlap within the sample and the probe beam is defocused by the thermal lens causing a reduction in the irradiance of light passing through the aperture and striking the photodiode in the far field. In order to avoid absorption effects the probe beam should be of a wavelength which is not absorbed by either ground state of the sample or transient species formed on excitation by the pump beam. CW lasers such as helium-neon and diode lasers are commonly used for this purpose. An experimental apparatus for TRTL is outlined in Fig. 13A.

The power of the lens is directly proportional to the heat evolved in the sample. The temporal behavior of the TRTL signal follows the time dependent release of heat in the sample allowing kinetic measurements to be made. TRTL and PBD can provide absolute experimental data and do not necessarily require the use of external calorimetric standards. A simulated TRTL signal is shown in Fig. 14. The signal displays a rapid initial decrease (U_1) due to non-radiative relaxation processes (e.g. internal conversion, intersystem crossing) occurring within the time resolution of the experiment determined by τ_a (*vide supra*). Heat release on time scales longer than τ_a constitute the further slow decrease in signal (ΔU) related to the stored heat component. The energy balance can be rearranged to give;

$$\frac{\Delta U}{U_{tot}} = \frac{\Phi_{st} E_{st}}{N_A h(v_\ell - \Phi_f v_f)} \quad (28)$$

A plot of ΔU vs. U_{tot} is carried out to establish linearity with laser pulse energy and providing the fluorescence parameters are known the product $\Phi_{st} E_{st}$ can be evaluated. Quantum yields of metastable species such as triplet states and singlet oxygen have been evaluated in this way.

These techniques can also be used in conjunction with a calorimetric reference to obtain fluorescence quantum yields. In these experiments the ratio of the slopes of U_{tot} vs. pulse energy from optically matched solutions gives the α value for the system under study. In samples where the missing energy is solely due to fluorescence emission ($\Phi_{st} E_{st} = 0$) the fluorescence yield can be obtained from;

$$(1 - \alpha) v_\ell = \Phi_f v_f \quad (29)$$

From S/N considerations this approach is only really accurate when one is dealing with compounds which exhibit relatively high quantum yields ($\Phi_f > 0.2$).

In addition to calorimetric information transient kinetics can be obtained directly from the time-dependence of the signal. It is useful to measure transient lifetimes as a function of pulse energy to further ensure that multiphoton effects do not contribute to the signal. Kinetic analysis is simple and follows the same approach as outlined for flash photolysis. The useful window for kinetic measurements is approximately 1 μs - 1 ms. The short end is determined by the acoustic transit time τ_a and the upper limit by heat diffusion which degrades the lens.

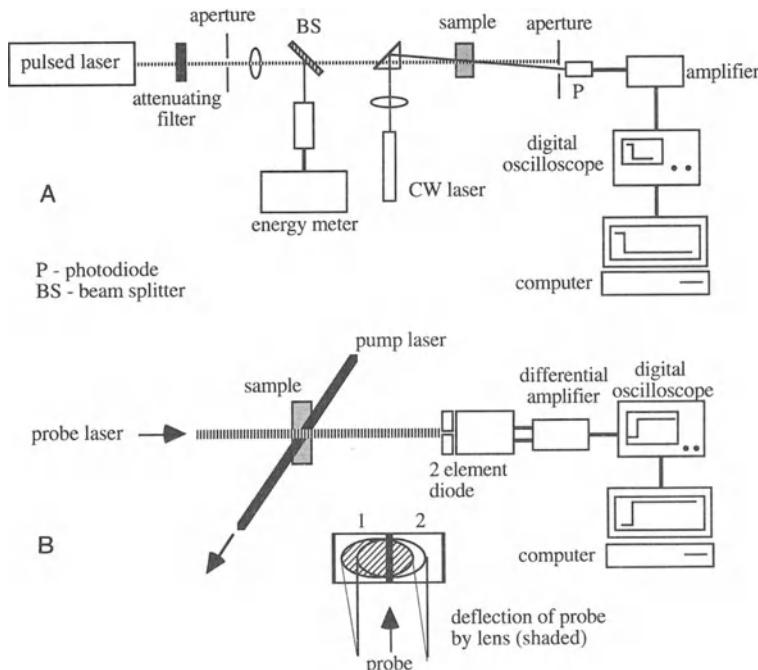


Figure 13 - (A) TRTL apparatus. (B) PBD apparatus.

Photothermal Beam Deflection (PBD)

This method is very similar to TRTL but instead of using coaxial beams and monitoring a decrease in irradiance caused by passage of the probe beam through the center of the lens, the probe beam is aligned to probe the edge of the lens resulting in spatial deflection of the probe beam rather than defocusing. A schematic of a PBD apparatus is shown in Fig. 13B. Instead of the pinhole used in TRTL a position sensing detection approach is used. A two component photodiode can be used for this purpose. The detector is aligned such that in the absence of the pump beam both elements receive equal probe irradiance and the differential signal is zero. On formation of the lens the probe beam is deflected by an angle proportional to the heat evolved and more light is incident on one of the elements (shown as shaded cross-section in inset) and a corresponding signal is obtained from the differential amplifier. The signal has the same form as the TRTL signal and at low deflection angles the signal can be analyzed in exactly the same fashion to produce calorimetric and kinetic information.

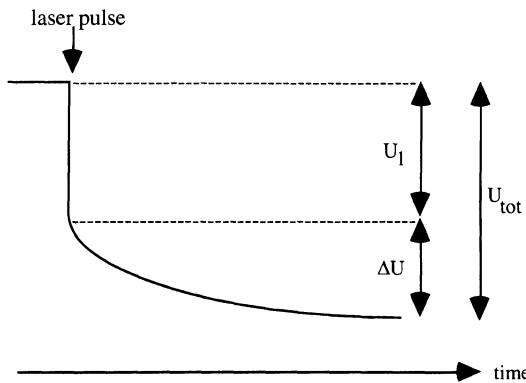


Figure 14 - Simulated TRTL signal showing fast and slow heat evolving processes.

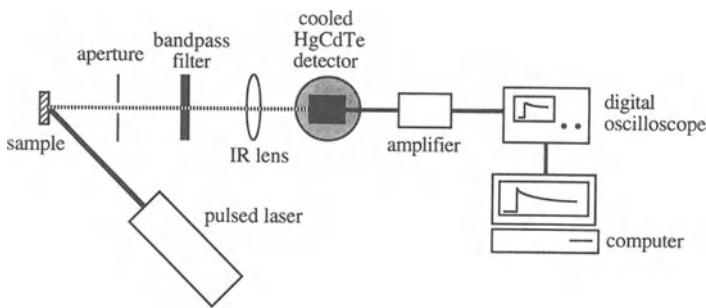


Figure 15 - Pulsed Photothermal Radiometry apparatus

Pulsed Photothermal Radiometry (PPTR)

This method is an IR sensing method which monitors the change in blackbody emission due to temperature rise induced by absorption of light. The signal amplitude is proportional to the temperature at the surface of the material and the IR detector can be calibrated for temperature using a standard blackbody source. The time evolution of the signal is dependent on optical absorption and thermal diffusion processes. Using a pulsed laser source, as shown in Fig. 15, the two effects can be separated in time. Spatial resolution of chromophore distribution (depth profiling) is possible as the time delay between the laser pulse and the maximum temperature evolved at the surface, and signal is a function of thermal diffusion time from lower layers in the material. This method has direct relevance to investigation of biological material and has been applied in skin to transient temperature measurements following pulsed irradiation and evaluation of pigment distribution and exogenous dye concentration. It is also a useful tool in determining tissue optics where the consequences of both absorption and scattering must be considered.

SUMMARY

All of the above techniques are commonly used in the investigation and characterization of the photoproperties of biologically relevant chromophores. An example which aptly demonstrates the complementary nature of these methods is shown below where the photophysical properties of benzoporphyrin derivative (BPD) in methanol are summarized with reference to the techniques used to obtain the individual parameters.

Table 1

PARAMETER	VALUE	TECHNIQUES USED
λ_{\max} (ground state)	428, 686 nm	absorption spectroscopy
ε_{\max} (ground state)	75,700 (428), 34,000 (686)	Beer-Lambert plot
λ_F (max)	692 nm	fluorescence spectroscopy
τ_s	5.2 ns (O_2)	time -correlated single photon counting
E_s	176 kJ mol ⁻¹	0-0 overlap of fluorescence emission and absorption spectra
Φ_F	0.105 (N_2) 0.030(O_2)	fluorescence spectroscopy
Φ_T	0.68 (N_2) 0.71 (N_2) 0.79 (O_2)	PAC Medinger-Wilkinson
λ_{\max} (T-T)	720 nm	Flash photolysis
ε_{\max} (T-T)	26,500 M ⁻¹ cm ⁻¹	Flash photolysis (energy transfer method)
τ_T	> 25 μ s	Flash photolysis
E_T	109 kJ mol ⁻¹ 119 kJ mol ⁻¹	Sandros behavior Energy gap
Φ_Δ	0.77 0.76	Time resolved IR phosphorescence PAC
S_Δ	~1	

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REACTIVE OXYGEN SPECIES

Santiago Nonell

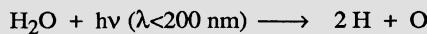
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INTRODUCTION

It has been known for over a century that the combination of light, oxygen, and several dyes leads to the oxidation of organic substrates. Photooxygenation reactions are known to involve active forms of oxygen, formed as a direct consequence of the absorption of light. There are several such active forms, each with its own properties and reactivity. Much of the present knowledge has been gained during the last three decades and several excellent reviews covering each of the individual species can be found in the literature. This chapter intends to give an overview to the present status of knowledge.

THE EVOLUTION OF ATMOSPHERIC OXYGEN

The chemical composition of the atmosphere in the prebiotic era was by no means equal as the one we know today. In particular oxygen was absent and it is generally accepted that the major components were ammonia, methane, and carbon monoxide and water. The sole route for oxygen production must have been water photolysis by UV light from the sun, which, in the absence of oxygen and ozone, could reach the earth's surface:



This mechanism alone cannot account for the present concentration. Two other major events have determined the present composition of the atmosphere: the first one is the appearance of photosynthetic organisms about 3.5×10^9 years ago, which essentially oxidized water to oxygen producing a steep increase in the atmospheric oxygen concentration. In addition, oxygen filtered the UV component from the sun light, thus preventing further water photolysis. The second event is the development of respiration as an oxygen-consuming process, which leveled off the concentration-time curve yielding an approximately steady state in which oxygen accounts for *ca.* 21% of the atmosphere contents.

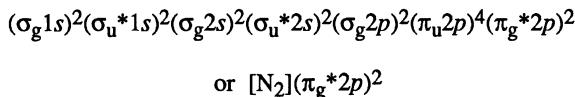
It is our common experience that oxygen is reactive, as any combustion process shows. Thermodynamics explain this observation in terms of the spontaneity of the process, as the well-known methane combustion reaction exemplifies::



A question immediately arises: How can living organisms containing plenty of organic compounds survive in an atmosphere with 21% of oxygen? Shouldn't they - we - burn immediately? The word "immediately" gives a clue to the answer to this question: it is not thermodynamics but kinetics that is responsible for the metastability of organic compounds and living organisms in the earth atmosphere. Combustion reactions need an activation energy to occur. A fire does not light spontaneously but once lighted it can be kept burning as long as oxygen and fuel are supplied. It must be concluded that ground-state molecular oxygen (O_2) is not a very reactive species and some kind of activation is needed to make it react with organic compounds. The term "active oxygen" has been created to refer to its more labile forms and this chapter attempts to summarize the photochemical pathways leading to oxygen activation as well as the main physical and chemical properties of the active oxygen species.

THE ELECTRONIC STRUCTURE OF OXYGEN

The non-reactivity of oxygen can be readily understood by examining its electronic structure. O_2 has 16 electrons distributed among its molecular orbitals with the following configuration:



Molecular Orbital theory thus predicts that O_2 contains a closed-shell structure identical to that of nitrogen *plus* two electrons in antibonding orbitals π_g^* . Using the Pauli principle it is possible to combine 2 π^* orbitals and 2 spin wave functions to form six different spinorbital wave functions which are antisymmetric with respect to exchange of electrons:

$$\begin{aligned} {}^1\Delta_g: \quad & \left\{ \begin{array}{l} \psi_1 = \pi_+ \pi_+ (\alpha\beta - \beta\alpha) \\ \psi_2 = \pi_- \pi_- (\alpha\beta - \beta\alpha) \end{array} \right. \\ {}^1\Sigma_g^+: \quad & \psi_3 = (\pi_+ \pi_- + \pi_- \pi_+) (\alpha\beta - \beta\alpha) \\ {}^3\Sigma_g^-: \quad & \left\{ \begin{array}{l} \psi_{4a} = (\pi_+ \pi_- - \pi_- \pi_+) \alpha\alpha \\ \psi_{4b} = (\pi_+ \pi_- - \pi_- \pi_+) (\alpha\beta + \beta\alpha) \\ \psi_{4c} = (\pi_+ \pi_- - \pi_- \pi_+) \beta\beta \end{array} \right. \end{aligned}$$

The ground state configuration of oxygen therefore gives rise to three electronic states which are spectroscopically termed ${}^1\Delta_g$, ${}^1\Sigma_g^+$, ${}^3\Sigma_g^-$. For our present discussion it is enough to concentrate on the number appearing as a superscript. Two terms have a "1" which is read "singlet". This means that the spin multiplicity of these states is singlet or that their total spin quantum number S is 0, corresponding to two paired electrons. Most ground-state organic compounds have closed-shell configurations with totally-paired electrons and thus are also singlets. This will have a severe influence on the reaction rate constants between oxygen and organic compounds as discussed below. The third state, named ${}^3\Sigma_g^-$, is read triplet, *i.e.*, has a spin quantum number $S=1$ corresponding to two unpaired electrons. Hund's Rule predicts that the ${}^3\Sigma_g^-$ state is the lowest in energy and therefore ground-state oxygen is a triplet. The paramagnetism of ground-state oxygen nicely confirms this prediction. The three electronic states are illustrated in Figure 1.

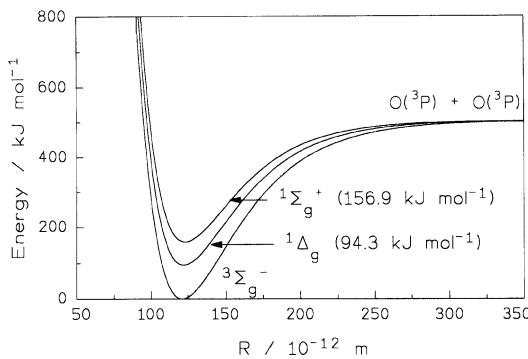


Figure 1. Electronic states of molecular oxygen.

A key result from quantum mechanics concerning chemical reactivity is the requirement that total spin angular momentum be conserved, *i.e.*, a chemical transformation in a system of molecules cannot modify its total spin angular momentum. This is known as **Wigner's spin rule** and for bimolecular reactions such as those between oxygen and a substrate the following rules can be deduced:



The reactions of organic substrates R with ground-state, non-activated molecular oxygen fall in the first category: most R are singlets and O₂ is a triplet thus if the reaction product is also a singlet (and we expect ground-state RO₂ products to be singlets) Wigner's spin rule tell us that these reactions are forbidden, that's to say, they will occur with a low rate constant. This is the fundamental reason which explains the relative inertness of organic materials (and living organisms) towards atmospheric oxygen. The second rule offers a way to improve oxygen reactivity. If somehow oxygen spin multiplicity could be changed from triplet to singlet there would be no spin objections to its reaction with organic substrates. States of singlet multiplicity are available for molecular oxygen and we will see later that light can induce the required spin multiplicity change. This will be the basis for one of the mechanisms of photochemical activation of oxygen.

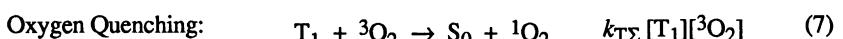
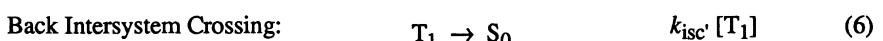
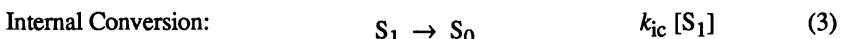
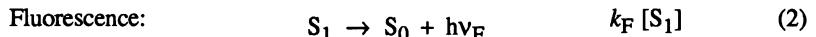
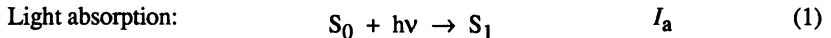
MECHANISMS OF PHOTOCHEMICAL OXYGEN ACTIVATION

For a photochemical process to occur the very first step must be absorption of light. This may seem a trivial remark but it gains some significance when it is realized that oxygen does not absorb the sunlight components reaching the earth's surface. Visible and infrared optical transitions have been observed but only when oxygen pressure is increased to above 100 bar. Thus, in the framework of photobiological processes it can be safely stated that oxygen cannot be activated by direct absorption of light. Photoactivation of oxygen requires the absorption of light by a suitable chromophore and the subsequent interaction of the photoexcited chromophore with oxygen to produce an active oxygen species. The latter is a bimolecular event and two possible mechanisms can be put forth for this interaction: energy transfer and electron transfer. Energy transfer leads to the production of singlet molecular oxygen ¹O₂ (either ¹Δ_g or ¹Σ_g⁺) while electron transfer results primarily in the formation of the superoxide radical anion O₂^{·-} and eventually of HO₂[·], H₂O₂, and HO[·]. Superoxide can also be formed in a more elaborate process: the photoexcited chromophore may react with a

substrate to form an ion pair; the radical anion then reacts with oxygen to produce the superoxide radical anion. The following sections will specifically treat each of these processes and species.

ACTIVATION BY ENERGY TRANSFER

In its simplest form, the mechanism which accounts for the formation of singlet molecular oxygen (either $^1\Delta_g$ or $^1\Sigma_g^+$) can be expressed by the following set of elementary reactions:



Singlet oxygen is formed in the elementary reaction (7) by a spin-allowed energy transfer process from the sensitizer triplet state. A closer look reveals that in order to produce singlet oxygen efficiently it is required (1) that the sensitizer undergoes intersystem crossing to its triplet state; and (2) that oxygen quenching of the sensitizer triplet state be the major deactivation channel for this species. The first condition is quantified by the **intersystem-crossing quantum yield** (Φ_{isc}) or **triplet quantum yield** (Φ_T):

$$\Phi_{isc} \equiv \Phi_T = \frac{k_{isc}}{k_F + k_{ic} + k_{isc}} = \tau_S k_{isc}$$

where τ_S stands for the lifetime of the first excited singlet state. The second condition is quantified by $f_{T\Sigma}$, the **fraction of triplets trapped by oxygen**:

$$f_{T\Sigma} = \frac{k_{T\Sigma}[{}^3O_2]}{k_P + k_{isc} + k_{T\Sigma}[{}^3O_2]} = \tau_T k_{T\Sigma}[{}^3O_2] = \frac{\tau_T^0 k_{T\Sigma}[{}^3O_2]}{1 + \tau_T^0 k_{T\Sigma}[{}^3O_2]}$$

where τ_T and τ_T^0 stand for the lifetime of the triplet state in the presence and absence of oxygen respectively. The **quantum yield for the production of singlet oxygen**, $\Phi_{{}^1O_2}$, is then the product of the two efficiencies:

$$\Phi_{{}^1O_2} = \Phi_{isc} \times f_{T\Sigma}$$

Thus $\Phi_{{}^1O_2}$ depends not only on the unimolecular photophysical properties of the sensitizer, namely Φ_{isc} , but it must be also a function of oxygen concentration, of the triplet state natural lifetime τ_T^0 , and of the medium viscosity which is embedded into $k_{T\Sigma}$ (*vide*

infra). Figure 2 illustrates this dependence for various products $\tau_T^0 k_{T\Sigma}$. The upper-limit value $\Phi^{1\text{O}_2} = \Phi_T$ is reached only for the favorable combination of high oxygen concentration, long natural triplet lifetime and high $k_{T\Sigma}$. In hypoxic or very rigid environments singlet oxygen production may be very inefficient for short-lived triplet states.

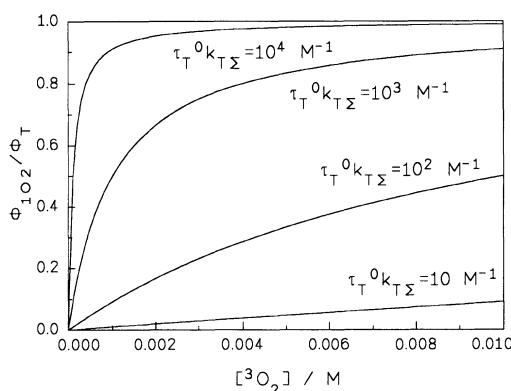
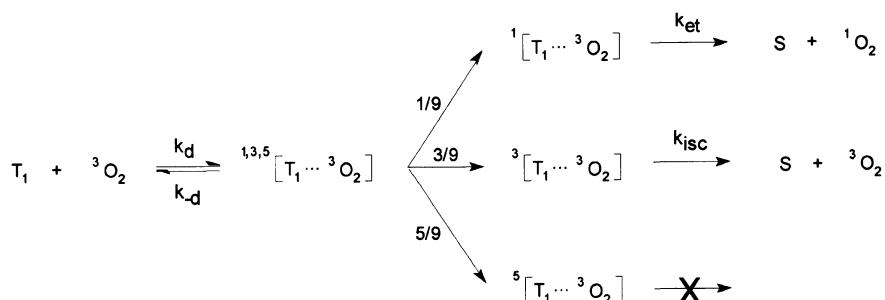


Figure 2. Dependence of singlet oxygen production quantum yield on oxygen concentration.

The preceding discussion has pointed out the major factors governing the production of singlet oxygen by energy transfer from a photoexcited sensitizer. Studies conducted in several laboratories have permitted to draw a more complete picture which includes other factors such as spin-statistics, charge-transfer interactions (as demonstrated by solvent and oxidation potential effects), triplet-state energy, and the presence of heavy atoms.

Other Factors Governing the Formation of Singlet Oxygen.

Oxygen quenching of triplet states can be envisioned as a two-step process: In the first stage a triplet oxygen molecule and the triplet sensitizer diffuse towards each other and form an encounter pair in a solvent cage. The two reactants then undergo several collisions before diffusing away. The larger the number of collisions per encounter the greater the probability that the encounter pair evolve into products rather than dissociate back into reactants. Diffusion theory predicts a rate constant for the formation and separation of the encounter pair inversely proportional to the solvent viscosity, *i.e.*, the more viscous the solvent the more difficult is for pairs of molecules to diffuse together but the greater the number of collisions per encounter.



Scheme 1. Spin-statistical factors in the triplet quenching by oxygen

According to Wigner's spin rule, the collision pairs have spin multiplicities of 1, 3 and 5 and are in statistical proportions, *i.e.*, one out of nine is a singlet, three are triplets and five are quintets. The dissociation of the singlet collision complex produces the singlet ground-state sensitizer and singlet oxygen. This is essentially the energy-transfer step. The dissociation of the triplet collision complex produces the singlet ground-state sensitizer and triplet oxygen, *i.e.*, oxygen catalyzes the sensitizer's back intersystem-crossing. This is a key result since it implies that not all quenching events will lead to the production of singlet oxygen, a complication that we have ignored so far. The parameter S_Δ has been introduced to describe the fraction of triplet quenching events leading to the production of singlet oxygen.

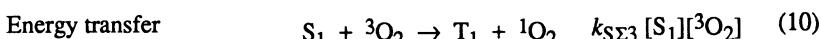
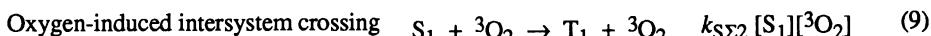
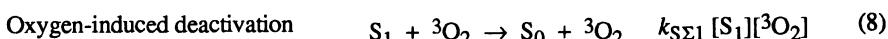
$$S_\Delta = \frac{\frac{1}{9} k_d \left(\frac{k_{et}}{k_{-d} + k_{et}} \right)}{k_{T\Sigma}}$$

No stable products can arise from the quintet collision complex and this implies that the maximum rate constant for oxygen quenching of triplet states, $k_{T\Sigma}$, will be 4/9 of the diffusional value.

$$k_{T\Sigma} = \frac{1}{9} k_d \left(\frac{k_{et}}{k_{-d} + k_{et}} \right) + \frac{1}{3} k_d \left(\frac{k_{isc}}{k_{-d} + k_{isc}} \right)$$

Scheme 1 predicts that when $k_{et} \gg k_{-d}$ and $k_{et} \gg k_{isc}$ then $k_{T\Sigma} = 1/9 k_d$ and $S_\Delta = 1$. It also predicts that upon increasing the participation of the triplet deactivation channel both an increase of the quenching rate constant to an upper limit $k_{T\Sigma}(\text{max}) = 4/9 k_d$ and a concomitant decrease in S_Δ to a minimum value of 0.25 should be observed. Although this framework is qualitatively correct, experimental evidence has made clear that spin statistical factors *alone* cannot account for the observed values for $k_{T\Sigma}$ and S_Δ . A generally accepted refinement is to postulate the formation of charge-transfer states which favor the intersystem crossing between the singlet and triplet collision complexes. This has been successful in explaining why some sensitizers show $S_\Delta < 1$ despite being quenched by oxygen with rate constant close to 1/9 of the diffusional value. The role of charge-transfer states in the mechanism of triplet quenching by oxygen is becoming an area of very active research.

A more realistic picture should also take into account that for sensitizers having sufficiently long singlet lifetimes, oxygen may quench the singlet state as well. It is possible to write three additional elementary reactions:

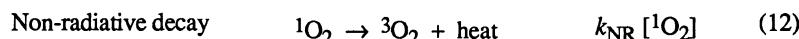
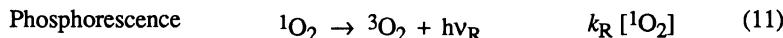


Reaction (8) is thought to be of minor importance. Oxygen quenching of the singlet state then contributes to a $\Phi {}^1\text{O}_2$: (1) it enhances intersystem-crossing (equation 9), hence a higher population of triplet states is achieved; (2) for sensitizers having an energy gap between their singlet and triplet states larger than 94.6 kJ mol⁻¹, the energy contents of $\text{O}_2({}^1\Delta_g)$, the quenching event produces *both* singlet oxygen *and* triplet sensitizer (equation 10). When singlet quenching occurs, $\Phi {}^1\text{O}_2$ depends in a more complex way on the concentration of oxygen and may reach an upper limit of two. The former discussion about the efficiency of oxygen trapping of the triplet state applies as well to the singlet and a similar parameter $f_{S\Sigma}$ can be introduced. Owing to the normally shorter lifetime of the singlets compared to the triplets, $f_{S\Sigma}$ is unlikely to be close to 1 in most situations. In fact, oxygen quenching of the sensitizer singlet state needs only to be considered when the singlet lifetime exceeds *ca.* 1 ns.

It is now time to address the question of the existence of two singlet states, namely ${}^1\Delta_g$ and ${}^1\Sigma_g^+$. The latter will be formed only if the sensitizer triplet state lies above 157 kJ mol $^{-1}$. Moreover, it is expected to quickly deactivate to the ${}^1\Delta_g$ state in a spin-allowed, energetically favorable step. So, for most practical purposes, only the ${}^1\Delta_g$ needs to be considered. We will accordingly restrict our discussion to O₂(${}^1\Delta_g$).

DEACTIVATION OF O₂(${}^1\Delta_g$) IN CONDENSED PHASES.

Bimolecular reactions involving excited singlet states are rather uncommon since singlet states are usually short-lived. Remarkably, singlet oxygen is an exception to this rule and lives long enough for doing bimolecular chemistry. The lifetime of an isolated O₂(${}^1\Delta_g$) molecule in the upper atmosphere is *ca.* 45 minutes. The explanation is provided by the electronic transitions selection rules: the transition ${}^1\Delta_g \rightarrow {}^3\Sigma_g^-$ is extremely forbidden since (1) the spin multiplicity is not conserved, singlet $\leftarrow X \rightarrow$ triplet, (2) the angular momentum along the bond axis changes in more than 1 unit, $\Delta \leftarrow X \rightarrow \Sigma$, and (3) parity is conserved, $g \leftarrow X \rightarrow g$. In condensed phases solvent interactions reduce the lifetime. Thus, τ_Δ° ranges from 3.3 μ s in water to *ca.* 0.1 s in C₂F₃Cl. The decay occurs through two competitive channels:



and the natural lifetime is determined by the rate constants of the two processes. In most solvents, the non-radiative channel dominates and determines τ_Δ° :

$$\tau_\Delta^\circ = (k_R + k_{NR})^{-1}$$

Singlet oxygen phosphoresces and its emission spectrum shows maxima at 1270 nm, corresponding to the (0,0) transition, and at 1580 nm, the (0,1) transition. This is shown in Figure 3:

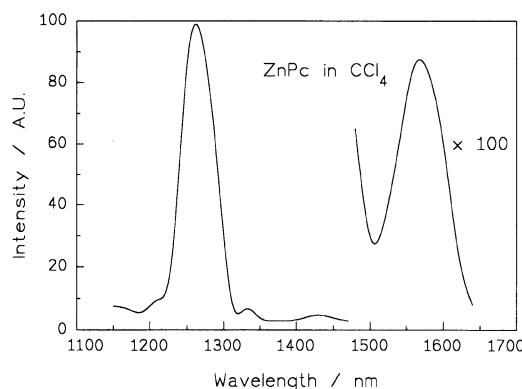


Figure 3. Phosphorescence spectrum of O₂(${}^1\Delta_g$) sensitized by zinc(II)-phthalocyanine (ZnPc).

It is now well established that k_R is solvent dependent and good correlations have been found between this rate constant and the solvent polarizability (*cf.* Figure 4). It is remarkable that water is one of the solvents with the lowest k_R . This will have negative implications for the detection of singlet oxygen in biological media.

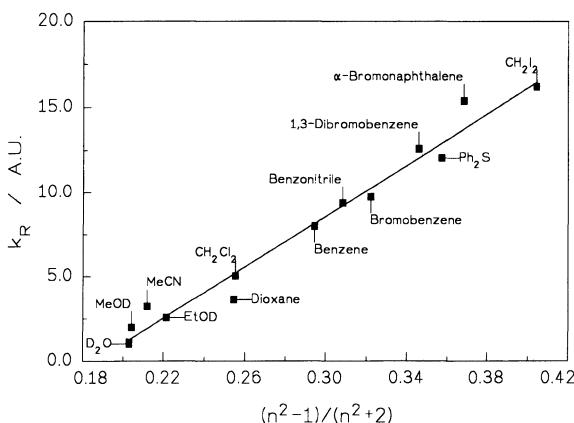


Figure 4. Relationship between k_R and the solvent polarizability, $\frac{n^2 - 1}{n^2 + 2}$, where n is the solvent refraction index.

The solvent dependence of the non-radiative channel follows some trends that can be summarized as follows:

- The longest τ_Δ° is observed in perhalogenated solvents.
- τ_Δ° decreases on increasing the number of H atoms in the solvent molecule.
- The shortest values are observed in solvents having hydroxyl groups. Water, with 3.3 μ s, sets a lower limit for τ_Δ° .
- The presence of heavy atoms reduces τ_Δ° .
- Solvent deuteration invariably increases τ_Δ° .

These observations are explained in terms of an electronic-to-vibrational energy transfer, occurring by coupling of the highest fundamental vibrational mode of the solvent oscillator X—Y with a (0,m) ${}^1\Delta_g \rightarrow {}^3\Sigma_g^-$ transition. k_{NR} can be expressed as the sum of the individual contributions of all oscillators present in the molecule:

$$k_{NR} \approx [M] \times \sum N_i k_i$$

where $[M]$ is the molar concentration of the solvent, N_i is the number of times a particular bond group "i" is present in the molecule, and k_i is the contribution of this group. Oscillators such as O—H, O—D, C—H, and C—D are strongly deactivating, while C—Cl, C—F, and C=S are less efficient in this respect. This model also accounts for the deactivation rate constants in the gas phase. Except for perhalogenated solvents, the natural decay of singlet oxygen is mostly determined by the non-radiative rate constant. Table 1 illustrates the solvent dependence of τ_Δ° . The lifetime of singlet oxygen in condensed phase determines the distance that it can diffuse from the site of formation. Making use of the expression:

$$\langle r^2 \rangle = 6D\tau_\Delta^\circ,$$

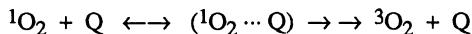
where $\langle r^2 \rangle$ stands for the mean square distance traveled by a diffusing molecule in a time τ_Δ° and D for its diffusion coefficient, singlet oxygen is calculated to travel *ca.* 100 nm in neat water, *ca.* 600 nm in hexane, and *ca.* 30 μm in $\text{C}_2\text{F}_3\text{Cl}$. This will have important implications for the capacity of singlet oxygen for damaging remote sites.

Table 1. Lifetime of singlet oxygen in selected solvents

Solvent	$\tau_\Delta^\circ / \mu\text{s}$	Solvent	$\tau_\Delta^\circ / \mu\text{s}$	Solvent	$\tau_\Delta^\circ / \mu\text{s}$
Acetone	51	Cyclohexane	23	Methanol-d ₄	230
Acetone-d ₆	770	Dichloromethane	95	Tetrachloromethane	28000
Acetonitrile	68	Diethylether	34	Tetrahydrofuran	25
Acetonitrile-d ₃	600	Ethanol	15	Toluene	29
Benzene	32	Hexane	30	Toluene-d ₈	320
Benzene-d ₆	700	Methanol	10.4	Water	3.3
Chloroform	250	Methanol-Od	37	Water-d ₂	68

Physical Quenching

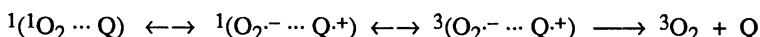
Singlet oxygen can be deactivated by a number of molecules without chemical reaction. In most cases the first step is the reversible formation of an exciplex between singlet oxygen and the quencher, which later evolves into products.



The electronic to vibrational energy transfer pathway by which solvent molecules deactivate singlet oxygen is just one of the mechanisms of physical deactivation. Two additional mechanisms are known to contribute: electronic to electronic energy transfer and reversible charge transfer. In the electronic to electronic energy transfer process singlet oxygen transfers its energy to a ground-state singlet quencher Q and both molecules end up in their triplet states. For the process to be irreversible it is necessary that the triplet energy level of the acceptor lies well below that of singlet oxygen. This seems to be the mechanism by which polyenes such as β -carotene and diamagnetic Ni(II) and Co(II) complexes deactivate singlet oxygen.



Charge-transfer quenching involves the formation of a complex with charge-transfer character that favors oxygen intersystem crossing. In a subsequent step, electronic energy is dissipated thermally in a back electron-transfer process:



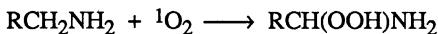
This is the main mechanism by which amines quench singlet oxygen as evidenced by the correlations found between the quenching rate constant and their ionization potential: the lower the ionization potential the higher the quenching rate constant.

Chemical Reactivity

Singlet oxygen is an electrophilic compound and as such reacts with electron-rich substrates: amines, olefins, aromatic hydrocarbons, heterocycles, sulfur compounds and phenols. As in the case of physical quenching, the first step is the reversible formation of an exciplex. Evidence supporting this model comes from the fact that most observed rate constants are well below the diffusion limit, activation enthalpies are close to zero, and activation entropies are negative.

Amines

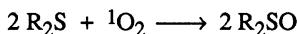
Reaction of amines with singlet oxygen produces the oxygenation of the carbon α -to nitrogen if a hydrogen atom is in this α position.



As already mentioned, amines quench and react with singlet oxygen *via* a charge-transfer mechanism.

Sulfides

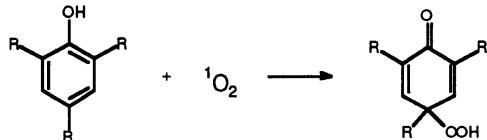
Sulfides react with singlet oxygen to produce mainly sulfoxides, although in some cases sulfones and/or fragmentation products are formed:



Aprotic solvents favor physical quenching while reaction is the main deactivation channel in protic solvents. The reaction mechanism seems to involve also a charge-transfer complex.

Phenols

Phenols also quench and react with singlet oxygen. The main product is a hydroperoxycyclohexadienone as shown in the following equation:



The reaction mechanism seems to proceed again *via* a charge-transfer complex, although for particularly electron rich phenols reaction takes place *via* formation of an endoperoxide (*vide infra*).

Olefins

Unsaturated π -systems (simple olefins, conjugated dienes and aromatic hydrocarbons) usually undergo three different types of reactions with singlet oxygen, namely (1) dioxetane production by [2+2] addition to an activated double bond, where D is an electron-donor substituent, (2) allyl hydroperoxide production by an ene reaction with a C=C double bond which contains at least one hydrogen atom, and (3) endoperoxide production by 1,4-cycloaddition to *cis*-1,3-diene systems, a process formally resembling the Diels-Alder reaction:

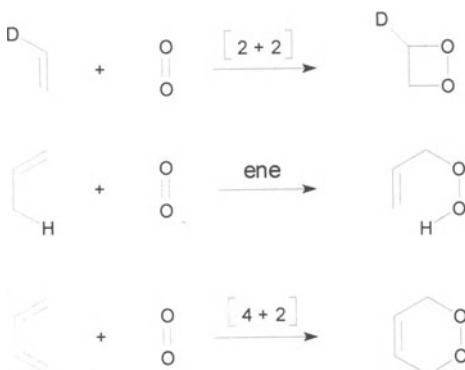
Values for quenching rate constants for several molecules of biological interest are collected in Table 2:

MONITORING OF SINGLET OXYGEN

Singlet oxygen deactivates radiatively emitting a photon in the near-IR, non-radiatively through interactions with the solvent, and may react with a suitable acceptor to form oxidation

Table 2. Total (physical + chemical) quenching rate constants for biological compounds

Substrate	$k / M^{-1} s^{-1}$	Substrate	$k / M^{-1} s^{-1}$
Cysteine	3.7×10^8	Methyl Linoleate	5×10^4
Histidine	3×10^7	Cholesterol	6.6×10^4
Methionine	1×10^7	Ascorbic acid	8.3×10^6
Tryptophan	5×10^7	Chlorophyll- α	7×10^8
Tyrosine	8×10^6	Bilirubin	1.5×10^9
β -Carotene	1.3×10^{10}	Deoxyguanosine	5×10^6
α -Tocopherol	7×10^8		



Scheme 2. Addition of singlet oxygen to double bonds

products. These three distinct decay channels offer alternative ways to detect singlet oxygen. The monitoring of singlet oxygen phosphorescence in the near-IR is becoming the most popular method since it is direct and fairly specific. The present technology is based on the use of germanium photodiodes as detectors, since they are sensitive in the 600-2000 nm region where singlet oxygen emits. Steady-state and time-resolved spectrophotometers have been developed, and their main possibilities and limitations are briefly discussed.

Steady-State Phosphorescence Detection

The emission spectrum of singlet oxygen has been shown in Figure 3. It is important to realize that, under CW irradiation, the signal intensity, I_Δ , is proportional to the steady-state singlet oxygen concentration (and hence to Φ_Δ as the key parameter determining the rate of production and τ_Δ as the key parameter determining the rate of decay) and to the rate constant for radiative decay, k_R .

$$I_\Delta \propto I_a \times k_R \times \Phi_\Delta \times \tau_\Delta$$

Thus, the measurement of the steady-state phosphorescence intensity enables the determination of any of the three parameters provided the other two and the proportionality

constant are known. The latter is eliminated by comparison of the signal with that of a standard measured under the same optical and geometrical conditions. In particular the absorbances at the excitation wavelength should be carefully matched. The most popular use of the technique, however, is for singlet oxygen identification purposes. The detection of an emission signal with maximum at 1270 nm that disappears when oxygen is removed from the system is very convincing evidence for the presence of singlet oxygen in the system. The detection in aqueous media is very challenging owing to the low k_R and τ_Δ values for this solvent. In most cases, the detection limit is not determined by the detector sensitivity but by the interference of other emissions, I_{BG} , at the same wavelength range. This is illustrated in Figure 5. The interference signal originates most often on the sensitizer fluorescence which, specially for red-absorbing sensitizers, tails into the near-IR.

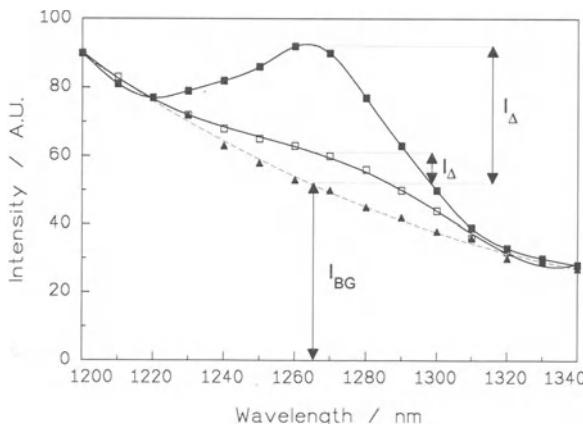


Figure 5: Emission spectra with different singlet oxygen (I_Δ) / background (I_{BG}) ratios: (■—■) $I_\Delta / I_{BG} \approx 1$; (□—□) $I_\Delta / I_{BG} \approx 0.2$; (▲—▲) $I_\Delta / I_{BG} < 0.01$.

Time-Resolved Phosphorescence Detection

The technique is based on the irradiation of the sensitizer with a short pulse of laser light and the monitoring in real time of the subsequent rise and decay of singlet oxygen phosphorescence. Obtaining the kinetic parameters governing this process is straightforward. It is usually found that the signal rises and decays monoexponentially. When the rate of formation is much faster than the rate of decay the decay portion can be expressed as:

$$I(t) = I(0) \exp(-t/\tau)$$

and both constants can be obtained by fitting this equation to the data points (*cf.* Figure 6).

Furthermore, in this case $I(0)$ is related to Φ_Δ :

$$I(0) \propto E_a \times k_R \times \Phi_\Delta$$

where E_a is the energy absorbed by the sensitizer. Measurement of $I(0)$ enables the determination of Φ_Δ values but a standard is again needed to eliminate the proportionality constant. The standard should be measured in the same solvent as the sample since k_R is strongly solvent dependent (see above). Given the inconsistency among the large number of Φ_Δ values that have been published, it is a good practice to measure Φ_Δ using at least two standards. Unfortunately, there are not many sensitizers for which there is general agreement

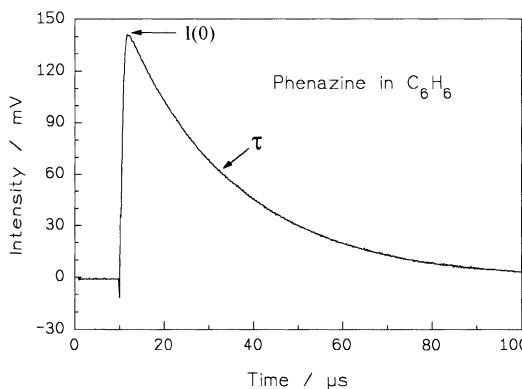


Figure 6. Rise and decay of singlet oxygen phosphorescence at 1270 nm.

on the Φ_Δ value. They are collected in Table 3, together with the solvent where the value holds. Another safe procedure is to check for oxygen concentration effects and linearity of the signal with the laser energy.

The second parameter, τ , contains information on the total decay rate constant:

$$1/\tau = 1/\tau_\Delta^0 + k_q \times [Q],$$

where k_q is the rate constant (physical + chemical) for quenching with an acceptor Q. Thus, measurement of τ for a series of concentrations of Q permits to obtain the quenching rate constant in a straightforward way.

Table 3. Quantum yields of singlet oxygen formation by selected sensitizers

Sensitizer	Solvent	Φ_Δ
Phenazine	Benzene	0.88
2-Acetonaphthone	Benzene	0.71
Benzophenone / Naphthalene 0.1 M	Cyclohexane	1.0
Tetraphenylporphine	Tetrachloromethane	0.62
Rose Bengal	Methanol	0.80
Rose Bengal	Water	0.76

The sensitivity of the method is limited mostly by singlet oxygen lifetime. In situations where its decay rate constant is comparable to or even higher than the formation rate constant, the phosphorescence intensity, *i.e.*, its concentration, may be undetectable.

Photothermal Methods

It is also possible to monitor singlet oxygen through its non-radiative decay. The mostly used techniques are time-resolved thermal lensing (TRTL) and laser induced optoacoustic spectroscopy (LIOAS). The radiationless processes involved in singlet oxygen formation and decay heat locally the solvent and as a result its refraction index changes and a pressure wave develops. The time profile of the refraction index can be monitored with a CW

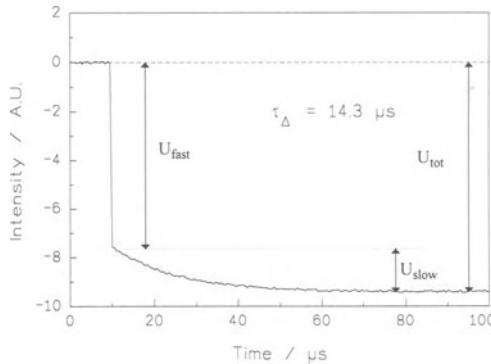


Figure 7. TRTL signal

laser to which the medium is transparent since it will cause a defocusing of this probe beam. A typical TRTL signal is shown in Figure 7.

All radiationless process occurring leading to the formation of singlet oxygen contribute to the early fast component of the signal (U_{fast}) while singlet oxygen decay results in a slower component (U_{slow}). The latter part affords the lifetime of singlet oxygen and the energy released during its decay. The total signal amplitude ($U_{\text{slow}} + U_{\text{fast}}$) is proportional to the fraction of the absorbed energy which is eventually released as heat. Thus, the ratio $U_{\text{slow}} / (U_{\text{slow}} + U_{\text{fast}})$ contains information on Φ_{Δ} :

$$\frac{U_{\text{slow}}}{U_{\text{slow}} + U_{\text{fast}}} = \frac{\Phi_{\Delta} E_{\Delta}}{E_{\text{exc}} - \Phi_F E_F} \quad (13)$$

where E_{Δ} is the energy content of singlet oxygen, E_{exc} is the excitation photon energy, E_F is the fluorescence photon energy, and Φ_F is the fluorescence quantum yield. The key advantage of this technique is that it provides *absolute* values for Φ_{Δ} , *i.e.*, no standards are needed. The main limitations are the need for precise knowledge of Φ_F and the fact that the measurement of heat is non-specific. Thus, analysis of the data in terms of equation (13) is valid only if there are no other heat sources or sinks. In particular the calculations will contain severe errors if a chemical reaction with unknown yields and energetics (exo- or endothermic) is occurring.

Optoacoustic spectroscopy or calorimetry monitors the pressure wave produced by the local heating of the solvent using a piezoelectric transducer. A typical signal is shown in Figure 8 and, as far as this discussion is concerned, it suffices to point out that the amplitude of the first maximum is proportional to the heat released in the fast radiationless processes, *i.e.*, those leading to the production of singlet oxygen.

A *photocalorimetric reference* is a substance that releases all the absorbed energy as fast heat. Thus, when the amplitudes of the signal for both a sensitizer and a reference are compared, the difference is due to the energy "stored" by singlet oxygen (released slowly) and/or released as fluorescence, to which the detector is blind. This is mathematically expressed as:

$$\frac{H_{\text{sample}}}{H_{\text{reference}}} = \frac{E_{\text{exc}} - \Phi_F E_F - \Phi_{\Delta} E_{\Delta}}{E_{\text{exc}}}$$

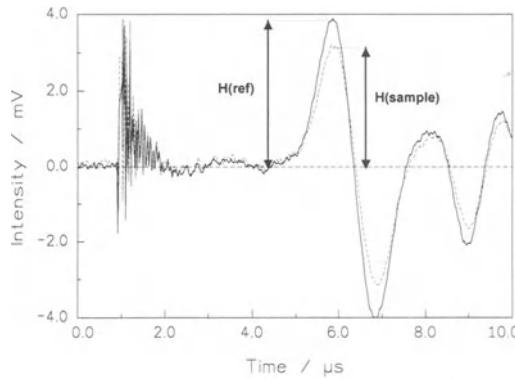
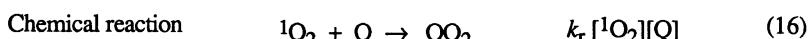
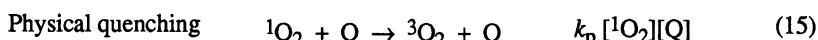


Figure 8. Typical optoacoustic signal for a sensitizer and a photocolorimetric reference.

The main information provided by LIOAS is the value of Φ_Δ . It is also possible to obtain lifetimes but the mathematical treatment is complicated and beyond the scope of this chapter. The advantage of this method over the phosphorescence detection technique is the generally solvent-independent behavior of the calorimetric references (in contrast, Φ_Δ is often solvent dependent, which precludes the use of a given sensitizer as a phosphorescence standard in multiple solvents).

Chemical acceptor methods.

The general principle of these methods is to quench singlet oxygen, at least partially, with an acceptor, and monitor either the loss of this acceptor or the appearance of products. The most popular monitoring techniques include absorption (time-resolved or steady-state), steady-state fluorescence, ESR when the product is paramagnetic, and chromatography. The quenchers may be physical or chemical. β-Carotene is a prime example of the former for time-resolved studies where singlet oxygen is monitored through the absorption of its triplet state. Other popular acceptors are diphenylisobenzofuran, DPBF, anthracene derivatives, rubrene, and 2,3-dimethyl-2-butene. The chemical acceptor methods have been used to determine primarily quenching rate constants and Φ_Δ values but also as qualitative analysis tools to detect the presence of singlet oxygen in a given system. It cannot be overemphasized that chemical methods are *indirect* and similar products could be formed in reactions other than those involving singlet oxygen. Chemical acceptor methods are the techniques of choice to determine the reactive component of the total quenching rate constant. For the following reaction scheme in which singlet oxygen either decays through solvent interactions, is physically quenched by an acceptor Q or reacts with it to form the product QO_2 ,



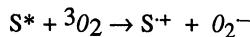
The following expression can be written for the quantum yield of formation of products under CW irradiation:

$$\frac{1}{\Phi_{\text{QO}_2}} = \frac{1}{\Phi_\Delta} \times \left(1 + \frac{k_p}{k_r} + \frac{k_d}{k_r} \times \frac{1}{[Q]} \right)$$

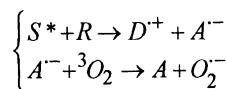
For pure chemical acceptors (*i.e.*, $k_r \gg k_p$) the measurement of initial $\Phi(QO_2)$ for a series of acceptor concentrations affords the ratio k_d/k_r , known in the literature as the β value. When physical quenching occurs at a comparable rate it is necessary to compare the $\Phi(QO_2)$ values for two different acceptors in order to be able to separate the physical and chemical components.

ACTIVATION BY ELECTRON TRANSFER

The processes leading to active oxygen forms by electron transfer can be summarized as follows: (1) Direct electron transfer from the excited sensitizer to oxygen:

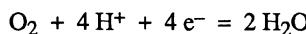


or (2) reaction of the excited sensitizer with an electron acceptor or an electron donor to produce a radical ion pair. In each case the radical anion can further react with ground-state oxygen to produce the superoxide anion:



The Electrochemistry of Oxygen

Oxygen is reduced to water in a multistep reaction involving the exchange of 4 electrons:



The standard reduction potential is 1.23 V at unit proton activity, or 0.87 V at pH 7. Oxygen is first reduced to the superoxide radical anion in a one-electron process. The reduction potential associated to this step is -0.33 V in water (pH 7) and -0.57 V in aprotic media due to loss of stabilization by solvation. As a consequence oxygen is a rather poor one-electron oxidizing agent, which limits its reactivity to relatively strong reducing species. This contributes to the already-mentioned low reactivity of ground-state oxygen. Since photoexcitation of an electron donor enhances its donating ability it is a straightforward conclusion that oxygen reduction to superoxide will be facilitated by photosensitization. The second step is the reduction of the superoxide to hydrogen peroxide, which in water at pH 7 has a potential of 0.94 V. Hence superoxide can act both as an electron donor or as electron acceptor and we will see later the implications of this duality.

Hydrogen peroxide is further reduced to the hydroxyl radical (0.46 V) and this species is finally reduced to water ($E = 2.18$ V). Thus, the hydroxyl radical is one of the most powerful oxidants known and, again, this will be reflected in its reactivity. A summary of

Table 4. Elementary processes in the reduction of O_2 to H_2O . Reduction potentials are calculated for pH = 7 assuming unit activity for all oxygen forms.

Reduction reaction	E / volt	Acid-base reaction	pK_a
$O_2 + e^- = O_2^-$	-0.33	$HO_2^- = O_2^- + H^+$	4.88
$O_2^- + e^- + 2 H^+ = H_2O_2$	0.94	$H_2O_2 = HO_2^- + H^+$	11.7
$H_2O_2 + e^- + H^+ = HO^- + H_2O$	0.46	$HO^- = O^- + H^+$	11.9
$HO^- + e^- + H^+ = H_2O$	2.18		

these half-reactions is given in Table 4. Since the hydrogen ion is involved in all reactions either directly or through the acid-base equilibria also shown in Table 4, the potential for the different reduction processes depends on the medium pH. This dependence is illustrated in Figure 9.

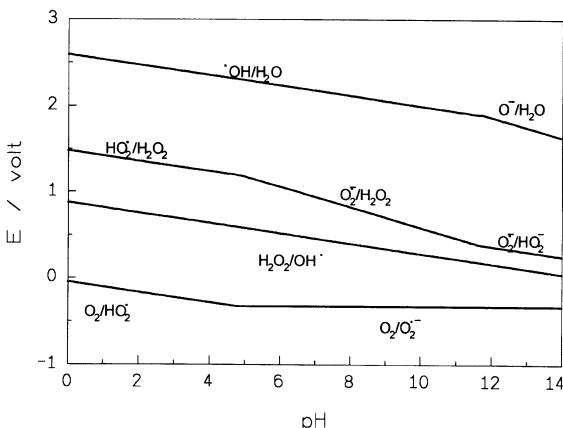
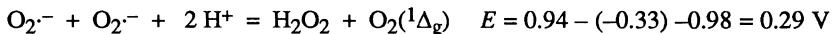
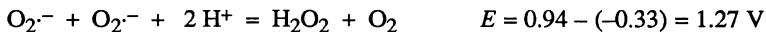


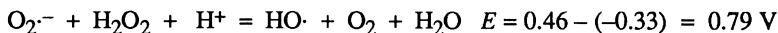
Figure 9. Diagram potential-pH for the various reduced oxygen species.

This diagram serves as a quick aid to ascertain the thermodynamic feasibility of any reaction involving these species. Because of their importance (*vide infra*) let us cite the following:

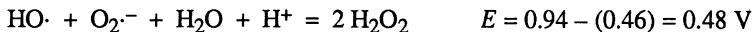
(1) the superoxide dismutation reaction, producing hydrogen peroxide and perhaps singlet oxygen (*vide infra*):



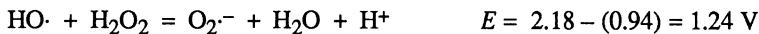
(2) the so-called Haber-Weiss reaction, producing the hydroxyl radical:



(3) the reaction between the hydroxyl radical and superoxide:



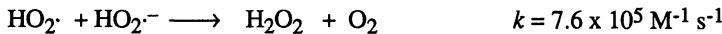
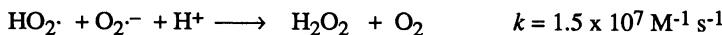
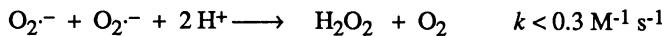
(4) the formation of superoxide by reaction of the hydroxyl radical and hydrogen peroxide:



Clearly, thermodynamics permit the interconversion of all species. This greatly difficults the task of identification of the particular species responsible for a biological effect. The picture becomes even more complicated when we try to predict the reaction occurring with, say, superoxide and a couple Ox/Red with potential $-0.33 < E < 0.94$. The diagram shows that superoxide may either oxidize Red or reduce Ox. The concentration of each species involved will determine the reaction pathway both from the thermodynamic point of view (the driving force for a reaction depends on the reaction quotient in addition to the standard electrode potentials discussed so far) and the kinetics point of view. The following sections discuss the main reactivity of each of these oxygen species.

THE SUPEROXIDE RADICAL

The most important reaction of the superoxide radical is dismutation. Since superoxide is in equilibrium with its conjugated acid the following equations can be written:



Thus, superoxide will be very stable in aprotic solvents. In water, the dismutation rate is strongly pH dependent (*cf.* Figure 10) and is maximum at pH = 4.88. At physiological pH the rate constant is about $1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. Deuteration of the solvent decreases the rate constant by a factor of 3.5 at pH = 5, effectively increasing the superoxide half-life.

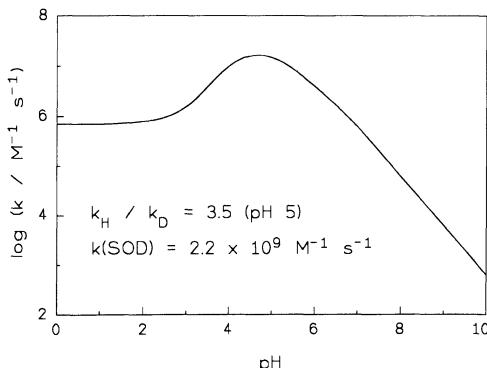
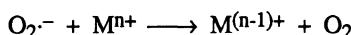


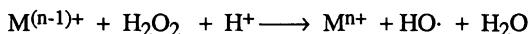
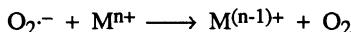
Figure 10. pH dependence of the rate constant for the superoxide dismutation reaction.

Since dismutation is second order in superoxide, the half-life of this species is strongly concentration dependent. Thus low concentrations of the species are very persistent and then amenable to diffuse a long distance. It is thermodynamically feasible that the dismutation reaction produces singlet oxygen. However, all evidence points to a very low, if any, singlet oxygen production yield. In addition, superoxide quenches singlet oxygen at a close-to-diffusional rate. Thus, any singlet oxygen formed by this reaction would be very efficiently quenched. The dismutation reaction is catalyzed by copper and iron complexes. The rate constant for the copper superoxide dismutase-catalyzed reaction increases to $2.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (a catalytic efficiency of 2×10^4). The mechanism seems to involve a cyclic oxidation-reduction of the metal:

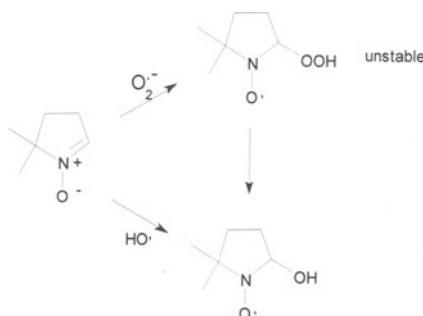


These two reactions illustrate another type of superoxide reactivity: its capability to reduce metal cations. It can also reduce several quinones, although the reverse reaction proceeds also efficiently. Superoxide is also capable to oxidize different substrates, but the rate constants are usually small. In particular, it reacts with the ascorbate anion with a rate constant of $2.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. A final reaction which warrants some discussion is the so-called Haber-Weiss reaction that was cited above. Its importance stems from the formation of the extremely reactive hydroxyl radical as a reaction product. However, the rate constant for

this reaction is very low ($k = 0.13 \text{ M}^{-1} \text{ s}^{-1}$) which, coupled with the fact that superoxide reacts with the hydroxyl radical with a rate constant $k = 1.01 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$, makes it a very unlikely way to produce any biologically significant amount of hydroxyl radicals. It has been suggested that the Haber-Weiss reaction occurs catalyzed, *i.e.*, the role of superoxide is to reduce a metal ion which subsequently reacts with hydrogen peroxide in a Fenton-like reaction:



It is also worth mentioning that superoxide *does not* react with DNA and lipids. Finally, it should be noted that the perhydroxyl radical, $\text{HO}_2\cdot$, is more reactive than its conjugated base and can overcompensate for its low concentration, two orders of magnitude lower than that of superoxide at physiological pH owing to the pK_a value of 4.88.



Scheme 3. Spin-adducts formation between DMPO and superoxide or the hydroxyl radical.

Detection of O_2^-

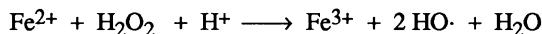
Superoxide can be monitored by absorption spectroscopy since its absorption spectrum shows a maximum at 245 nm ($\epsilon = 1100 \text{ M}^{-1} \text{ cm}^{-1}$ in water and $\epsilon = 2686 \text{ M}^{-1} \text{ cm}^{-1}$ in organic solvents). In most cases, however, it is detected indirectly using a chemical acceptor method. One method is to trap it with 5,5-dimethyl pyrroline-1-oxide (DMPO) to form the nitroxide which can then be monitored by ESR. This method must be used with caution since the spin adduct is unstable and changes with time to give the same product as the $\text{HO}\cdot$ radical. Another common method is the reaction with acceptors such as the nitroblue tetrazolium cation, NBT, which is reduced by superoxide leading to the formation of diformazan. The latter absorbs at 560 nm and can be monitored spectrophotometrically. A similar method is the reduction of tetrannitromethane to nitroform anion, which absorbs at 350 nm, the reaction rate constant approaching the diffusion-control value. As in the case of DMPO, the hydroxyl radical also reduces tetrannitromethane. A good indication of the involvement of superoxide in a process is provided by the inhibitory effect of superoxide dismutase.

THE HYDROXYL RADICAL

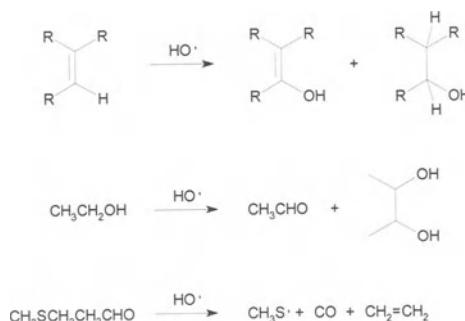
Hydroxyl radicals can be formed by direct photolysis of hydrogen peroxide,



or by reaction of hydrogen peroxide with iron(II) ions, the so-called Fenton reaction:



The hydroxyl radical is a very reactive species, as the thermodynamic data already suggested. Three main types of reaction are well established: (1) addition to double bonds and aromatic rings, eventually yielding the hydroxylated derivatives, (2) hydrogen abstraction from suitable hydrogen donors such as alcohols, and (3) electron transfer both from inorganic as well as organic compounds. The bimolecular rate constants for these elementary processes approach the diffusional-control value ($10^8 - 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) which implies that $\text{HO}\cdot$ reacts at the site where it is formed.



Scheme 4. Main reaction between the hydroxyl radical and organic compounds.

The hydroxyl radical is detected essentially by chemical acceptor methods. Common reactions include the oxidation of methional to form ethylene, the bleaching of *para*-nitroso-dimethyl aniline, which can be followed spectrophotometrically at 440 nm, and the above-cited formation of a spin adduct with DMPO, which is monitored by ESR spectroscopy.



Involvement of $\text{HO}\cdot$ in a process is usually ascertained from the inhibitory effect of $\text{HO}\cdot$ scavengers such as ethanol, mannitol, 2-propanol or *tert*-butanol.

CONCLUDING REMARKS

The interaction of light with a variety of chromophores may lead to the formation of reactive forms of oxygen, thus overcoming the relative inertness of the ground-state oxygen molecule. The energy-transfer pathway results in the formation of singlet oxygen, while electron transfer produces the superoxide radical anion. Singlet molecular oxygen reacts with electron-rich organic compounds such as amines, sulfides, phenols, alkenes and aromatics. The reaction rate constants usually fall in the $10^7 - 10^8 \text{ M}^{-1} \text{ s}^{-1}$ range. Superoxide is a much less reactive species, whose main reaction in biological media seems to be dismutation to produce hydrogen peroxide. It can also contribute to the formation of the most reactive oxygen derivative, the hydroxyl radical, either directly by means of a catalyzed Haber-Weiss reaction or indirectly through the Fenton reaction between hydrogen peroxide and iron(II). The hydroxyl radical then reacts very rapidly ($k = 10^8 - 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) with almost any substrate abstracting a hydrogen atom or an electron, or adding to double bonds.

ACKNOWLEDGMENTS

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BIOLOGICAL ANTIOXIDANT AND DEFENCE/REPAIR SYSTEMS

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INTRODUCTION

Molecular oxygen is the major biological oxidant, yet its direct reaction with most organic compounds is spin forbidden. Molecular oxygen is a biradical that has two unpaired electrons in parallel spins (triplet state) and prefers to accept extra electrons singly. Most biomolecules are covalently bonded non-radicals, and the two electrons forming a covalent bond have opposite spins and occupy the same molecular orbital (singlet state). Hence the reaction of oxygen with biomolecules is spin restricted. Therefore, dioxygen must be activated in order to be able to react with these compounds. The most important mechanism for dioxygen activation in biology involves complexation and/or reduction by transition metals like Fe^{2+} and Cu^+ . The ability of iron and copper to participate in one-electron redox reactions is used by many living systems to facilitate the metabolism of molecular oxygen. Iron and copper are common at the active sites of oxygenases, oxidases, and electron transport proteins¹.

Although, because of its high reactivity, activated oxygen remains bound to the enzymes as much as possible, cells normally contain $10^{-11}\text{-}10^{-12} \text{ M O}_2\cdot$ and $10^{-7}\text{-}10^{-9} \text{ M H}_2\text{O}_2$. If the reactive oxygen species (ROS) are released they are very dangerous and can even be fatal for the cell because they can damage several cell constituents. There is evidence that ROS are involved in a number of diseases e.g. emphysema, cancer, arthritis, cataract, and adult respiratory distress syndrome².

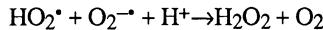
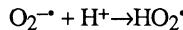
To protect themselves against the noxious effects of oxygen a cell contains an elegant system of antioxidants. An antioxidant can be defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate"³. The term "oxidizable substrate" includes almost everything found in living cells, including proteins, lipids, carbohydrates and nucleic acids.

Within cells the most important antioxidant systems are enzymes such as superoxide dismutases, catalases, and glutathione peroxidases which remove or control the level of ROS (preventive antioxidants). In extracellular fluids little of these enzymes are present although ROS are produced in such compartments e.g. during inflammation, enzyme reactions and substrate autoxidations. It is likely that the major antioxidant protection in extracellular fluids is afforded by preventing metal complexes interacting with ROS⁴. A second line of defence in all biological systems may depend on radical scavenging by low molecular weight species such as ascorbate (vitamin C), glutathione, glucose, urate, albumin, carnosine, and bilirubin⁵. The hydrophobic interior of membranes requires special lipophilic antioxidants such as α -tocopherol (vitamin E), reduced coenzyme Q₁₀ and β -carotene which act as chain-breaking antioxidants⁶.

PREVENTIVE ANTIOXIDANTS

Superoxide Dismutase

One-electron reduction of oxygen produces the superoxide anion radical, $O_2^{-\bullet}$. Superoxide is formed in almost all aerobic cells. A major source is the leakage of electrons onto O_2 from the cellular electron transport chains of mitochondria and endoplasmatic reticulum³. It is also well established that $O_2^{-\bullet}$ is produced during the respiratory burst of phagocytic cells⁷. Superoxide is not very stable in aqueous solutions. It undergoes dismutation which can be written as:

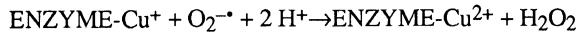
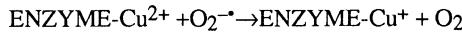


The overall reaction is strongly pH dependent. The pK of the first reaction is 4.8. The overall rate of dismutation at physiological pH is about $2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. Despite this high rate constant of dismutation and the moderate reactivity of $O_2^{-\bullet}$ all aerobic cells contain superoxide dismutase (SOD). This enzyme accelerates the dismutation reaction by about four orders of magnitude, thereby making the reaction diffusion controlled⁸.

SOD, previously referred to as hemocuprein or erythrocuprein, was first thought to be a storage protein for copper. In 1969 McCord and Fridovich discovered the ability of this protein to catalyze superoxide dismutation⁹.

At least three separate forms of SOD have been characterized. One is a dimer (MW= 32 kD) and contains copper and zinc and is present in the cytoplasm of eukaryotic cells. In mitochondria and in prokaryotic cells a Mn-containing enzyme is found; this enzyme is built up from four identical sub-units each containing one atom of Mn. A third form contains iron and is associated with the cytoplasm of E. coli¹⁰.

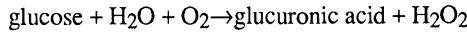
The mechanism of the Cu-containing enzyme is known:



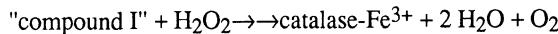
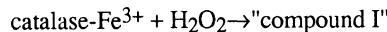
The Zn-ion is not involved in the catalytic cycle but appears to stabilize the enzyme.

Catalase

One electron reduction of $O_2^{-\bullet}$ produces the peroxide-ion O_2^{2-} . Because the pK_a of H_2O_2 is very high, the peroxide-ion is readily protonated at physiological pH. Hydrogen peroxide is an intermediate in electron transfer systems. It can also be formed by dismutation of $O_2^{-\bullet}$ and by some enzymes belonging to the group of the oxidases like glucose oxidase³:



H_2O_2 can be converted to H_2O and O_2 by catalase. Catalase (MW= 240 kD), which is present in the cytoplasm, in mitochondria and other organelles, consists of four subunits each containing a haemgroup (Fe^{3+} -protoporphyrin) bound to the active side, and generally one molecule of NADPH to stabilize the enzyme¹⁶. Like in SOD one-electron transfers take place by forming enzyme intermediates called "compound I, II, and III". The most important reactions are:



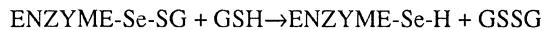
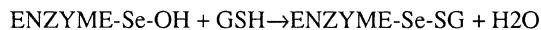
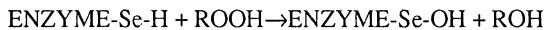
Glutathione Peroxidase

Besides catalase also glutathione peroxidase (GPX), a selenium-dependent enzyme, is able to convert H₂O₂ into harmless compounds via oxidation of glutathione¹⁹:



GPX does not only catalyze the conversion of H₂O₂ but also the conversion of organic peroxides like lipidhydroperoxides (LOOH). The antioxidant activity of GPX is tightly coupled to intracellular glutathione, glutathione reductase, and NADPH. Once oxidized, glutathione can be reduced by glutathione reductase with NADPH as the reducing agent. GPX is effective at low concentrations of H₂O₂, and under conditions of low oxidant stress, this enzyme system appears to play a more significant role than catalase in the protection of cells against H₂O₂-mediated injury²⁰.

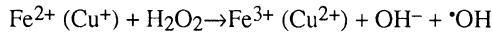
There are three different kinds of GPX's²⁰. The most important one is the Se-containing GPX. The proposed mechanism of this enzyme is:



Besides this enzyme a cell also contains a non-selenium containing GPX, a sulphur-transferase enzyme involved in the conjugation of GSH with "foreign compounds". Both enzymes are cytosolic and are incapable of acting on peroxidized lipids in organized membrane structures. Therefore a cell also contains a Se-GPX specific for phospholipid hydroperoxides in membranes (PH-GPX)²⁰.

Metal-ion "Deactivators" (Chelators)

Hydrogen peroxide is able to take up an electron by forming a hydroxyl ion (OH⁻) and a hydroxyl radical (·OH). The electron is donated in most cases by the transition metals iron or copper:

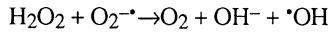


This reaction is called the Fenton reaction.

If H₂O₂ is present in the cell there normally is also O₂·⁻ formation. Superoxide is able to react with Fe³⁺:



If we add both reactions we get the Haber-Weiss reaction, with iron (or copper) as a catalyst:



Hydroxyl radical is fearsomely reactive: it reacts at the site where it is generated with almost all molecules in living cells, with rate constants of 10⁹-10¹⁰ M⁻¹s⁻¹²¹. Thus almost everything in a cell is an ·OH scavenger: no specific molecule has evolved for this role.

Therefore prevention of damage caused by ·OH in vivo will depend on binding of the transition metals needed for ·OH formation⁵. Most iron is chelated in protoporphyrin IX giving haem, the prosthetic group of proteins such as haemoglobin (70% of the 3-5 g present in the human body is bound to haemoglobin), catalase, peroxidase and cytochrome C. The remaining iron is stored in tissues inside molecules of ferritin (MW= 460 kD). The large internal cavity of ferritin can hold as many as 4500 ferric ions. Iron is transported in the

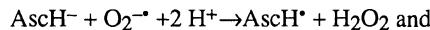
plasma by transferrin, a 77 kD protein that binds two ferric ions very tightly at pH 7. Normally only 20-30% of the transferrin is loaded with iron. Copper is bound to caeruloplasmin, a 134 kD protein that binds six or seven copper ions per molecule. Caeruloplasmin can also function as a ferro-oxidase and promote oxidation of Fe^{2+} to Fe^{3+} without the release of oxygen radical intermediates²². Iron bound to transferrin²³ and copper bound to caeruloplasmin²² can no longer participate in the $\cdot\text{OH}$ formation. However ferritin iron can be mobilized from the protein by $\text{O}_2^{-\bullet}$ and the released iron can then participate in $\cdot\text{OH}$ generation². Whether haem-bound iron can catalyse the Fenton reaction, is still unclear.

Copper also binds tightly to albumin, present in high concentrations in plasma (50-60 mg/ml). This bound copper may still participate in Fenton reactions and thereby the protein is damaged²⁴. However, albumin is much less significant as a target of damage than are membranes of erythrocytes or vascular endothelial cells, so that the binding of copper to albumin may represent a protective mechanism against $\cdot\text{OH}$ ⁵. The antioxidant role of urate (300 μM in plasma) is largely related to its ability to bind iron and copper ions²⁵. Melanins are also able to bind metals. Saturation of neuromelanin (found in brain) may be related to Parkinson's disease.

SCAVENGERS

Several compounds present in the cells and in the extracellular fluids can act as scavengers; they react with high rate constants with ROS. Perhaps the best known is ascorbate. It reacts with superoxide and hydroxyl radicals. Another well known compound is uric acid. Besides binding of transition metals, urate can also react with ROS by forming allantoin. This compound is detected in patients suffering from rheumatoid arthritis²⁶. Uric acid is an endproduct of purine catabolism. This is an example of the use of end products of catabolism as scavenger of ROS. Other examples are biliverdin and bilirubin, products of haem degradation.

Vitamin C (ascorbate, $\text{AsCH}^{\cdot-}$) reacts with $\text{O}_2^{-\bullet}$ with a rate constant of approximately $3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at pH 7.4¹¹. Many animal tissues (e.g. eye lens, lung, brain)¹² and the chloroplasts of green plants contain ascorbate at millimolar concentrations¹³, making scavenging of $\text{O}_2^{-\bullet}$ in vivo feasible. Ascorbate reacts with $\text{O}_2^{-\bullet}$ via the following reactions:



The formed ascorbate radicals are not very reactive¹⁴ and can be converted to ascorbate and dehydroascorbate (Asc):



while dehydroascorbate can be regenerated to ascorbate by glutathione peroxidase¹⁵:



Several keto-acids, such as glyoxylate, pyruvate and 2-oxoglutarate react with H_2O_2 . Rate-constants for the reactions have not been determined but are likely to be low, since millimolar concentrations of keto-acid have to be added to achieve high rates of H_2O_2 removal^{17,18} and it is not clear if such concentrations are achieved in vivo.

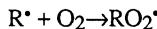
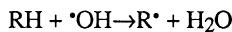
The reactivity of molecular oxygen can be increased by moving one of the unpaired electrons to the orbital containing the other unpaired electron, thus alleviating the spin restriction. Singlet oxygen (${}^1\text{O}_2$) has no unpaired electrons and is therefore not a radical, but it is known to be a powerful oxidizing agent. ${}^1\text{O}_2$ can be produced on or in the skin as a result of photosensitization reactions triggered by certain drugs²⁷, cosmetics²⁸, plant

toxins²⁹ or by the porphyrins that accumulate in some forms of porphyria³⁰. Singlet oxygen may also be generated in both the lens and the retina of the eye and contribute to the development of cataract. Tryptophan and its product kynurenine act as photosensitizers in this case³¹.

Ascorbate (present in high concentrations in the eye lens) is not only a chemical scavenger of $^1\text{O}_2$, but also quenches this molecule very effectively in aqueous solution³². Similarly, α -tocopherol can do the same within membranes³. Also carotenoids can both quench and scavenge $^1\text{O}_2$: this role is of greatest importance in protecting illuminated chloroplasts against photodestruction¹², but whether it is of relevance to normal mammalian metabolism is uncertain. Carnosine (β -alanyl-L-histidine), present in high concentrations in muscle tissue (40 mM), also acts as a scavenger of $^1\text{O}_2$ in vivo³³.

CHAIN-BREAKING ANTIOXIDANTS

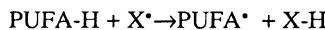
A very important group of scavengers are those reacting with peroxy radicals (ROO $^\bullet$). These scavengers are called chain-breaking antioxidants. Attack of $^\bullet\text{OH}$ upon biological molecules can proceed by addition, hydrogen abstraction or electron transfer reactions³. In all cases a radical is formed from the biomolecule attacked. In many cases, carbon-centred radicals are produced, that can react with O_2 to give peroxy radicals, e.g.



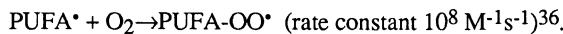
Formation of peroxy radicals is the major chain-propagation step in lipid peroxidation, but they can also be formed in non-lipid systems.

Scavengers able to remove peroxy radicals might be effective in the aqueous phase (e.g. dealing with radicals from DNA, thiols, uric acid etc) or in the hydrophobic (membranes) phase. Glutathione (GSH) reacts rapidly (rate constant $10^8 \text{ M}^{-1}\text{s}^{-1}$) with radicals resulting from the attack of $^\bullet\text{OH}$ upon DNA³⁴. Ascorbic acid also reacts very quickly (rate constants $10^8 \text{ M}^{-1}\text{s}^{-1}$)³⁵ with both peroxy and sulphur-containing radicals.

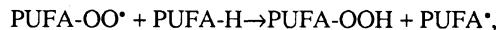
The scavengers of peroxy radicals that operate in the hydrophobic interior of biological membranes are the chain-breaking antioxidant inhibitors of lipid peroxidation. By far the most important of these in vivo is α -tocopherol (vitamin E, TOH)⁶. Polyunsaturated fatty-acids (PUFA-H) can react with radicals under hydrogen abstraction:



Propagation of this chain reaction is both thermodynamically and kinetically favoured because PUFA $^\bullet$ reacts rapidly with O_2 :



The peroxy radical formed is able to propagate the chain reaction:



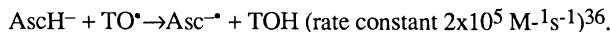
thereby bringing about the oxidation of other unsaturated lipids.

Because of the low rate constant for the propagation step ($10 \text{ M}^{-1}\text{s}^{-1}$)³⁶ vitamin E is able to outcompete the propagation reactions, forming PUFA-OOH:



When comparing the rates for the competing reactions for PUFA-OO[•] (with PUFA-H or with TOH), if TOH is to react with 90% of the PUFA-OO[•] produced, then the ratio [TOH]:PUFA-H] must be 1:1000. Indeed, the experimentally observed level of TOH in membranes is in the order of 1:1000³⁷.

Each tocopherol molecule can donate two electrons as a chain-breaking antioxidant, and then it is consumed. To be an effective antioxidant, each oxidized tocopherol must be recycled. At present, enzyme systems capable of reducing oxidized tocopherol have not been described. The current idea is that ascorbate recycles tocopherol via TO[•], producing the ascorbate radical¹⁵:



Asc[•] can be removed by dismutation, yielding AscH⁻ and dehydroascorbate (Asc). Both Asc and Asc[•] can be reduced by enzymes that use NADH or NADPH as reducing agents such as glutathione peroxidases; thus ascorbate is recycled¹⁵. A possible problem with this model is that the water soluble ascorbate and tocopherol being lipid soluble are in different phases. However, it has been demonstrated that in a membrane, the phenolic OH group of tocopherol is present at the membrane-water interphase and the phytol tail of tocopherol lies parallel to the phospholipid fatty acyl chains, so that the TO[•] can react with the AscH in the water phase⁵.

Other, less important, lipid soluble chain-breaking antioxidants are carotenoids³⁸ and reduced coenzyme Q₁₀ (CoQH₂). There is very little information about the mechanism of the antioxidant activity of the carotenoids. The most important antioxidant activity of CoQH₂ is probably in the mitochondrial membrane³⁹.

REPAIR

In spite of the abundance of preventive systems and the presence of antioxidants, oxidative damage to proteins, nucleic acids and lipids occurs. The cells contain effective systems in order to remove the damaged compounds, repair the molecules and synthesize new compounds.

The DNA excision repair system is an important example. It consists of many enzymes such as DNA glycosylase, endonuclease, exonuclease, polymerase, and ligase. Ligase and endonuclease activity are regulated by poly(ADP-ribose)polymerase. This activity is enhanced by DNA strand breaks⁴⁰.

It is well known that oxidatively damaged proteins have an altered conformation. This makes them more susceptible to proteases for breakdown⁴¹. The result of amino acids are used for de novo synthesis.

An important enzyme in removing damaged fatty acids is phospholipase A₂. A peroxidized fatty acid then can be processed by phospholipid hydroperoxide glutathione peroxidase and a new fatty acid is inserted by fatty acyl-coenzyme A³⁶. Another possibility is insertion of a new phospholipid with the aid of phospholipid exchange proteins.

Among the responses of cells to ROS is activation of specific genes, including the early genes c-fos, c-jun, and c-myc⁴². These genes are also involved in growth promotion and it is not at all clear what the significance is. It has been speculated that induction of a response similar to the growth response has an important role in replacing damaged cellular constituents with newly synthesized counterparts. Only when the amount of DNA damage exceeds the capacity of the repair system is cell division blocked via the activation of a yet unknown pathway⁴³.

Besides these genes, also stress genes are activated leading to the formation of stress proteins. Activation of these genes require signal transduction processes. In this newly and rapidly developing area of research it has been shown that src tyrosine kinase and map kinase are involved in the response to UV-C⁴³, and that in HpD-mediated photodynamic treatment of T24 human bladder transitional carcinoma cells a rescue signal is mediated by elevation of intracellular Ca²⁺⁴⁴, prostaglandin E₂⁴⁵ and cyclic AMP⁴⁶.

STRESS PROTEINS

The increased expression and accumulation of the stress proteins appears to represent a universal cellular defence mechanism and provides the cell with an added degree of protection. The stress proteins include (1) the heat shock or stress proteins (HSPs), which are induced by heat and other forms of stress; (2) the glucose regulated proteins (GRP78, and GRP94), share sequence homology with HSPs. They are not normally heat-inducible, but are produced by glucose deprivation or calcium ionophores. Cell fractionation shows that they are abundant endoplasmic reticulum-associated proteins, and perform within the endoplasmic reticulum the same functions as HSPs⁴⁷; (3) ubiquitin, a 76 amino acid polypeptide which plays a role in targeting of proteins for degradation; (4) metallothioneins, which are induced by various stressful conditions like hyperoxia, heat, ionizing radiation and exposure to xenobiotics. It is widely believed that the proteins play a role in metal homeostasis and probably also in scavenging of H₂O₂ and oxygen radicals⁴⁸; and (5) oxidation-specific stress proteins, such as haem oxygenase.^{49,50,51,52}

Studies over the past few years have revealed a role for some of the stress proteins as being intimately involved in protein maturation.⁵³ It also became clear that HSPs are not solely stress proteins, but that they play important functions in unstressed cells as well. The functions of HSPs in unstressed cells appear to relate to "chaperoning". Molecular chaperones (Hsp70s, Hsp60s, Hsp90s) bind transiently and noncovalently to nascent polypeptides and unfolded or unassembled proteins, aiding in protein biogenesis in two general ways: they block nonproductive protein-protein interactions, and they mediate the folding of proteins to their native state by sequestering folding intermediates, allowing the concerted folding by domains and assembly of oligomers.^{54,55,56}

There are, besides heat, many other inducers of the heat shock response, including oxidative injury.^{57,58} Hydrogen peroxide induces a heat shock response in bacteria, and several types of oxidative injuries (UV radiation, sodium arsenite, cadmium) induce stress proteins in human cells. HSPs are also induced in animal models for ischemia and reperfusion injury. During reperfusion, oxygen free radicals are generated from molecular oxygen via the metabolism, by xanthine oxidase, of xanthine/hypoxanthine accumulated as a consequence of ATP depletion secondary to ischemia. Synthesis of HSPs in these animal models suggest that oxygen free radicals also have the ability to induce HSPs *in vivo*.

The induction of HSPs by heat and oxidative stress suggests that they might be part of a common pathway. The possibility that the heat shock induces HSPs (as well as SOD) via generation of oxygen free radicals and lipid peroxidation is supported by several observations: (1) heat shock has been shown to increase lipid peroxidation as evidenced by the rise in 2-thiobarbituric acid reactive material; (2) modulation of the level of lipid peroxides by the use of free radical scavengers prevents lipid peroxidation as well as induction of HSPs by heat; and (3) both heat shock and oxidative stress also lead to accumulation of adenylated nucleotides or "alarmones".^{57,58} This hypothesis, however, remains controversial. Polla et al⁵⁷ found no generation of superoxide during exposure of human neutrophils to higher temperatures (37°C-45°C); rather heat shock was found to inhibit superoxide generation by neutrophils. Thus, the possibility that induction of HSPs and induction of oxidation-specific stress proteins are regulated by separate pathways has to be considered. In particular, a possible common mechanism for the induction of stress proteins by heat or oxidative injury does not account for the specific synthesis of haem oxygenase under certain conditions of oxidative injury. Indeed, haem oxygenase (the 32 kD oxidation-specific stress protein) has been shown to be induced in human cells specifically by oxidant stresses such as H₂O₂, UVA, menadione, PDT^{59,60,61,62}, and agents which are known to interact with or modify cellular reduced glutathione levels, but not by heat. An increase in haem oxygenase will increase cellular capacity to generate both biliverdin, and bilirubin, both potential antioxidants and reduce the amount of Hb-bound iron which under certain conditions can be involved in the formation of reactive oxygen species.

Potential mechanisms for protection from oxygen free radicals by HSPs include prevention from protein degradation, membrane lipid peroxidation or calcium intrusion from the extracellular milieu, and maintenance of ATP levels, induction of classical scavengers such as superoxide dismutase (SOD) or glutathione (which itself plays a role in induction of HSPs). And the inhibition of any of the multiple steps involved in oxidative injury-mediated cell death, such as uncoupling of oxidative phosphorylation, decrease in calcium-ATPase,

activation of phospholipase A₂, or maintenance of a normal cellular structure is prevented by HSPs.^{57,58}

In addition to these development at the biochemical level, investigators are being to explore the role of the stress response and stress proteins in various aspects of disease. In this regard, the potential role of the stress proteins as sensitive markers of cell injury, and their possible connection with the immune response and autoimmune diseases are under investigation. Finally, the idea that their expression may be exploited as a new way by which to monitor the status of our environment is also being explored.⁶³

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PHOTOSENSITIZERS

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INTRODUCTION

Light can interact with biological systems and thereby cause both beneficial and undesirable effects. Examples of the first are processes like vision, photosynthesis, photomorphogenesis and photomovement, that are essential for life on our planet. In all these examples light is absorbed by highly specialized pigments. The interaction can also cause damage to biological systems; this can be undesirable, e.g. when it leads to mutagenesis or carcinogenesis, but it is also used in protection of green plants against herbivores and disease organisms and in several therapies. Damage can be brought about in principle by two different pathways. The first possibility is that biological molecules like proteins and nucleic acids absorb light themselves and as a direct result are chemically changed. In the second pathway light is absorbed by certain molecules, so-called photosensitizers (Sen), which can transfer the absorbed light energy to other molecules (substrates or targets (T)). The definition of a photosensitized process thus is a process in which the targets are chemically altered in the process, but the originally photoexcited chromophore is not affected¹. However also the sensitizer molecules can act as a target and undergo a permanent modification ("bleaching"). The resulting photoproducts are sometimes photosensitizers themselves. Perhaps the best known example is the formation of kynurenine from tryptophan. This is of clinical importance in cataract formation, where crosslinking between the crystallines in the lens has been shown to occur.

PHYSICAL BACKGROUND

A prerequisite for a dye to be a sensitizer is that the molecules can undergo intersystem crossing from the initially excited singlet state (lifetime in the order of nsec) to the triplet excited state (lifetime μ sec-msec). Because of the longer lifetime of the triplet excited state compared to the singlet excited state, the sensitizer in the triplet state can then either transfer electrons to an acceptor or accept electrons itself from a suitable substrate (type I reactions), or transfer its energy to other molecules like ground-state oxygen (type II). In an energy transfer pathway the reactive singlet oxygen is formed, that can oxidize most biological molecules.

In homogeneous solutions, the probability and the efficiency of each process depends on various parameters. First of all, the redox potentials determine whether a reaction can take place or not². Secondly, kinetic considerations are important. The oxygen and target concentrations, the rate constants of the reaction of sensitizer triplet with target molecules and with oxygen, and the concentration of the excited triplet state, dependent amongst others on

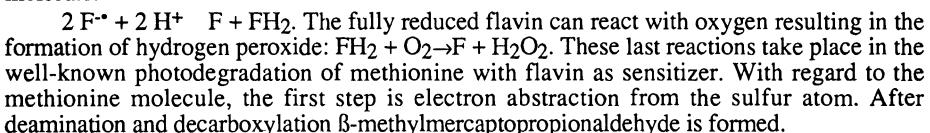
the yield of the excited triplet state and the rate of triplet decay, determine the reaction rates ($v_I = k_I[\text{Sen}(T_1)][T]$ and $v_{II} = k_{II}[\text{Sen}(T_1)][\text{O}_2]$). Thus electron transfer processes are more likely at high target concentrations and low oxygen tension and when the oxygen concentration is high, energy transfer reactions are favored. In this context it is clear that the efficiencies of the two processes can be lowered by various deactivation processes at subsequent stages of the process. Further, binding of the photosensitizer to the target is important; for an electron transfer reaction it is clear that this is a prerequisite. In general both processes are electrophilic reactions, i.e. an oxidative attack on electron-rich sites present on target molecules. These targets include the amino acids tyrosine, tryptophan, methionine, cysteine and histidine, the nucleic acid base guanine and the unsaturated sites in fatty acids and cholesterol. The presence of relative electron rich sites is supported by a pH above the pK_a . Thus the oxidation rate of tyrosine is higher above pH 9 and of histidine above pH 6.6.

In heterogeneous environments, for example in biological systems like cells and tissues, structural and functional parameters are also involved in determining the overall efficiency of the photosensitization process, because these parameters regulate uptake, localization and binding/interaction of the sensitizer molecules with the target molecules. The first step in photosensitization of a cell is binding of the sensitizer to the plasma membrane or permeation through the plasma membrane. In principle permeation can occur by diffusion, by an active transport system or by endocytosis or phagocytosis. Most important here is the way in which the sensitizer is presented to the cell, whether it is present in solution or bound to (lipo)proteins or even delivered with special systems like antibodies or liposomes. Thereafter the sensitizer is distributed throughout the cells. The final localization depends on the type of the cell, on the delivery system, on the physico-chemical properties of the sensitizer molecules, of the environment and of the target and also on the duration of the exposure and on the possibility that the sensitizer molecules are subject to metabolic changes. The final concentration of a sensitizer inside a cell is also determined by efflux from the cell.

Physico-chemical properties like the hydrophobicity of the sensitizers, the targets and the environment, also influence the overall efficiency of a photodynamic process. These properties determine e.g. directly the final localization of the sensitizers. They have to be present at or near the very site of photosensitization, because in electron transfer reactions an interaction between the target and the sensitizing molecule is necessary and in energy transfer reactions the generated singlet oxygen has a definite lifetime. Another example are dyes that form aggregates in a hydrophilic environment. They are not very good sensitizers in the aggregated form as there is efficient energy transfer between the aggregated molecules. However bound to e.g. membranes such dyes monomerize and can very often efficiently induce photodynamic damage.

In electron transfer reactions, semi-reduced anionic sensitizers are often formed, together with positively charged target radicals. These target radicals are very reactive. In lipids peroxides can be generated in this way, giving rise to lipid peroxidation.

The semi-reduced sensitizer can react with oxygen, resulting in the formation of the superoxide anion radical. Either by spontaneous or by catalyzed dismutation, hydrogen peroxide is formed. Certain semi-reduced sensitizers, especially the flavines, dismutate spontaneously with the formation of a ground state flavin and a fully reduced flavin molecule:



The above considerations make it obvious that, apart from some general features, it is impossible to say whether a certain dye is a good and efficient photosensitizer, because for one reaction it might be a good one, whereas in another situation a compound with different properties is needed. Common properties are, of course, the ability to efficiently absorb photons (a large extinction coefficient), a high triplet quantum yield and a long triplet lifetime, which markedly increases the probability of interacting with other molecules. A good electron transfer sensitizer can, in the triplet state, easily donate or accept electrons while a good energy transfer sensitizer gives a high singlet oxygen yield.

PHOTOSENSITIZERS IN BIOLOGICAL SYSTEMS

Many dyes present in biological systems can act as photosensitizers. First of all many compounds involved in normal metabolism are potential photosensitizers. The most well known examples are probably chlorophyll, iron-free porphyrins and flavins like riboflavin and flavine mononucleotide (FMN). Without wanting to be exhaustive, the list of endogenous sensitizers includes bilirubin, the already mentioned amino acid tryptophan and its photoproduct N-formyl kynurenone, NAD(P)H, pyridoxine, quinones (present in respiratory chains), all-trans retinal and many proteins containing these compounds. Two compounds deserve to be mentioned separately: lipofuscin, the so-called age pigment and melanin. The former can generate superoxide anion radicals when illuminated and melanin can generate all kinds of reactive oxygen species. Probably because of the photosensitizing ability, these compounds are shielded from environmental light or only present as intermediates in biochemical pathways. And of course many defense systems against reactive oxygen species exist in cells and tissues. When an enzyme deficiency occurs in a pathway, problems may arise, as in the case of inborn errors of metabolism. One of the best known examples is erythropoietic protoporphyrin with a defect in the enzyme ferrochelatase. The altered enzyme in the patients is less stable than the normal enzyme. This results in a higher protoporphyrin concentration in the patient's serum than normal, resulting in the photosensitivity of the skin of these patients.

Riboflavin is present amongst others in milk, beer and growth media for cell culture and these substances therefore should be kept in the dark.

There are also many kinds of naturally-occurring exogenous photosensitizers³. Examples of sensitizers produced by microorganisms are gilvocarcins by Streptomyces bacteria and cercosporin by Cercospora fungi. Many plants also make photosensitizers, most likely in order to protect the surface against viruses, fungal and bacterial spores, yeasts, small arthropods and their eggs and probably also against insects. Among the many different photosensitizers synthesized by plants are acetophenones, (furano)coumarins and other furanochromes, pterocarpans, (iso)quinolines, thiophenes, benzophenanthrenes, lignans, sesquiterpenes, hypericins and curcuminins. Among the many plant families that produce photosensitizers are the Apiaceae (carrot, celery, parsnip), the Araliaceae (ivy, ginseng), the Asteraceae (sunflower), the Hyperiacae (St.John's wort), the Liliaceae, the Moraceae (fig), the Orchidaceae, the Rubiaceae (coffee), the Rutaceae (citrus), and the Solanaceae (potato). Contact of the skin with parsnip plants (*Pastinaca sativa*) or with *Heracleum sphondylium* or extracts of various parts of these plants followed by exposure to sunlight results in bullous lesions and pigmentation. The last phenomenon was already known in ancient India and Egypt, where vitiligo patients were treated with plant extracts and light. In all of these cases the plant species used contained furocoumarin type photosensitizers. Pheophorbide, a breakdown product from chlorophyll, can cause photosensitivity both in animals and in humans. It has been observed in cattle and in rats fed on a diet of dried corn. Photodermatitis occurs occasionally in humans taking Chlorella tablets or eating certain Japanese foods like salted Takana and pickled greens. Bacteria present in the gut of grazing animals convert chlorophyll to phylloerythrin, which is normally excreted via the bile. With a liver dysfunction however, it gets to the skin and the animals become photosensitive. In eastern countries curcumin is used as a spice, in medicine and as a coloring agent. It is a yellow-orange compound derived from the root of *Curcuma longa* (Zingiberaceae family). It is also a potent antibacterial⁴ and it is very likely that adding curcumin to rice as a spice also prevents the rice from contamination when preserved for later consumption.

Animals and humans also produce photosensitizers in normal metabolism and in certain types of hereditary porphyrias, as discussed above, while β -carbolines appear in the urine of rats and humans after the ingestion of alcohol; the function of this process is unknown. The brown color of chicken egg shells is caused by porphyrins; they fluoresce brightly under dark light. Pheophytins have been shown to be present in the excreta of silkworms. This is used as a traditional Chinese medicine called Càn Shā against rheumatic and abdominal pains⁵. Photosensitivity of humans can be induced by exogenous sensitizers as well. Some examples have already been given of sensitizers present in food and food products. Another example is erythrosine, used as a red dye in some dairy products, in jam and in soft drinks. Some people show photosensitivity after preparing margaritas, a drink made primarily of tequila and lime juice. Further, the possibility of contact photodermatitis after contact with plants has also been discussed above. Finally it should be mentioned that some perfumes

contain bergamot oil, in which the sensitizer 5-methoxypsoralen is present.

Many drugs used in medicine are known to induce photodermatoses, a condition in which the skin reacts to light exposure after using these drugs. A distinction has to be made between phototoxic and photoallergic reactions. In phototoxicity the skin reaction is the direct consequence of damage caused by the photodynamic action of the drug, whereas in photoallergy the damage is caused by a secondary activation of the immune system. A photoallergic reaction is in general delayed, not observed immediately after administering the drug. In particular, halogenated compounds give rise to photoallergic reactions. Examples of drugs that can give rise to photodynamic reactions are antibacterials and antifungals like tetracyclines, sulfonamides and hexachlorophene, tranquillizers such as chlorpromazine and the phenothiazines, the diuretic chlorthiazide, anti-inflammatory drugs like benoxaprofen, piroxicam and chemotherapeutics like doxorubicin and adriamycin.

Photosensitizers have had applications for centuries, although unknown by the users. In the 20th century they were rediscovered, especially for use in medicine, as will be discussed below for therapeutic use in neonatal jaundice, PUVA, extracorporeal photochemotherapy, and photodynamic therapy of cancer, and for the sterilization of blood. Other applications of photosensitizers, including their use as herbicides and pesticides, and for the photodynamic purification of waste water will also be examined.

PHOTOTHERAPY OF NEONATAL JAUNDICE

One of the best known applications of photodynamic action is the phototherapy of neonates with rhesus antagonism. In these infants an enhanced breakdown of heme takes place, leading to neonatal jaundice. The concentration of non-glucuronic acid-conjugated bilirubin in the serum can become very high and exceed the capacity of serum albumin to bind it. This non-bound bilirubin can cause damage to the central nervous system. In order to avoid this, phototherapy with 450 nm-light is performed. Bilirubin acts as sensitizer and is also the target. In electron transfer reactions the conformation of bilirubin is changed in such a way that it becomes more hydrophilic and can be excreted. In the less important energy transfer reactions, self-destruction of bilirubin takes place, giving breakdown products that again can be more easily excreted^{6,7}.

PUVA

PUVA is a form of photochemotherapy involving the use of psoralen (furocoumarins) as sensitizers plus UVA radiation. On illumination, proraleins can form cross-links with the pyrimidine residues of nucleic acids by an electron transfer reaction; both mono- and diadducts can be produced, depending on the psoralen structure. Some psoralens sensitize damage to cell membranes and mitochondria by a singlet oxygen mechanism.

Psoralens can be used as sensitizers for the treatment of vitiligo and psoriasis in patients. It is also used in so-called extracorporeal photochemotherapy, a modality used to treat a wide range of diseases including cutaneous T-cell lymphoma, systemic sclerosis and (other) autoimmune disorders^{8,9}. PUVA treatment induces immunological effects, possibly the specific suppression of pathogenic T-cell clones^{10,11}. Recently it has been suggested that pre-illuminated psoralen is effective in the treatment of these diseases¹². The use of aminomethyltrimethyl-psoralen in the photosterilization of blood products is under investigation (vide infra).

PUVA therapy causes hyperpigmentation of human skin; for this reason, psoralen-containing oil of bergamot is sometimes used as a component of suntan preparations. It is not known whether the UV protection provided by the increased melanin production outweighs the possible mutagenic effects of PUVA treatment¹³.

A special issue of the J. Photochem. Photobiol., B: Biol. (vol.14, issue 1-2) has been devoted to psoralen photosensitization including a review and the latest developments in this field.

PHOTODYNAMIC THERAPY OF CANCER

Perhaps the best known application of photodynamic sensitizers in therapy today is their use for treatment of cancer (PDT). Porphyrins and porphyrin-like compounds appear to have the most suitable properties for PDT. In whatever mode they are given, i.v., i.p. or interstitially directly into the tumor, thereafter they appear to be localized in tumors at a (somewhat) higher concentration than in the surrounding normal tissue. This fact, together with the capability of treating a well defined area with laser light, of course including the tumor, permits a localized treatment modality. It should be stressed that PDT is not the easy treatment that it appears to be at first sight. Several choices have to be made: the sensitizer (and together with it the mode of delivery), the amount of it and the kind of injection, the time between administering and light treatment, the power density of the light at the tumor surface and the duration of the light treatment. At present some of the choices are relatively simple. Only Photofrin, an empirically developed complex mixture of monomers, oligomers and aggregates with hematoporphyrin as the starting material, is allowed as the sensitizer for clinical trials. It has been approved for normal clinical use so far only in Canada and only for prophylaxis of recurrent papillary bladder tumors. In most cases it is not only difficult to treat just the tumor, but there are also reasons to include a very small area around the tumor in the laser light field. One of the causes of tumor necrosis with Photofrin as sensitizer is as the induction of vascular stasis¹⁴. Thus it is useful to illuminate the small area around the border of the tumor. The other contribution to necrosis is direct tumor cell damage and death. The mechanisms of both processes are still largely unknown. In cells nearly all cellular functions can be compromised and all constituents damaged¹⁵. Recently a description was given of the processes involved in the photodynamically induced death of murine L929 fibroblasts. Inhibition of poly(ADPribosylation) together with minor DNA damage makes it impossible to repair this damage. Together with a Ca²⁺ influx, which activates endonuclease activity, this ultimately results in fragmentation of the DNA¹⁶.

The most obvious late effect after PDT has been performed, is the continuing cutaneous photosensitivity. This is probably caused by photosensitizer molecules still present in the circulation. If this is so, it can be diminished by rapid clearance of the sensitizer by excretion or breakdown or by quenching the excited states or the generated species. The second aspect that has to be considered is wound healing or the reaction of normal tissue to PDT. This depends on the tissue that has been treated¹⁷. Also there is an infiltration of PDT-treated tissue with lymphocytes; this, together with plasma cells and histiocytes and high levels of cytokines in the urine of the treated patients suggests an immune response^{18,19}. Several authors have shown that PDT can cause immunosuppression²⁰⁻²². Finally there is the possibility that PDT can induce mutagenesis and carcinogenesis. This is not judged to be of much importance, although with aluminum phthalocyanine as sensitizer, mutagenicity was detected at the *tk* locus of murine L5178Y lymphoma cell strains LY-S1 and LY-R16, which are heterozygous with respect to this locus, but no mutagenicity was found in strain LY-R83, which is hemizygous at this locus²³.

There are many theoretical considerations that lead to the conclusion that Photofrin is not the most suitable sensitizer for PDT. This led to development of a multitude of new photosensitizers and/or delivery systems, including the synthesis *in situ* of protoporphyrin after administration of δ -aminolevulinic acid, the use of (immuno)liposomes, LDL and antibodies as carriers. All of these approaches can lead to efficient PDT, but the underlying mechanisms of the induction of tumor necrosis are different from each other. At the moment it is not clear at all what the best choice would be and it could well be that different sensitizers will be needed for different tumors, for example in one type of tumor, shutdown of the vasculature could be much more important than for others. Even for direct (tumor) cell killing, different sensitizers can be used to target various (sub)cellular organelles. Cationic dyes, like rhodamine 123 and the kryptocyanines are accumulated in mitochondria^{24,25}. Methylene blue and, at low concentrations, acridine orange preferentially localize in lysosomes²⁶, while hydrophobic compounds like the non-sulfonated phthalocyanines are found in membranes²⁷. Psoralens and some cationic porphyrins can intercalate into DNA²⁸. Also, as mentioned before, dyes can be covalently coupled to antibodies and other ligands²⁹⁻³² and so be targeted to specific receptors, either at the membrane or inside cells. It has been shown that relocation of photosensitizers can occur during illumination, inducing damage leading to cell death elsewhere in the cell^{26,33}.

Most of the developed new sensitizers absorb at wavelengths of 700 nm or higher. Penetration of light in tissue depends on the wavelength and is higher at higher wavelengths, because normal cell constituents have a low light absorption at these high wavelengths and light scattering is relatively inefficient³⁴. Melanin absorption is also low, so that possibly melanin-containing neoplasms can also be treated with PDT. An additional and important advantage is that portable and relatively inexpensive diode lasers, emitting at these higher wavelengths, are being developed.

Sensitizers with absorption at the highest wavelength reported so far are bacteriochlorin *a*, naphthalocyanine derivatives and cadmium texaphyrin. They all absorb at wavelengths around 770 nm. The use of bacteriochlorin *a* has extensively been studied by Schuitmaker et al.³⁵⁻³⁸. Texaphyrin is only mentioned as a possibility, without any data^{39,40}. The photophysical properties of silicon naphthalocyanine derivative have been investigated by Rodgers et al.^{41,42}. These sensitizers are currently under investigation in animal and cell studies [Biolo, Rodgers and Jori, personal communication]. Water soluble sulfonated naphthalocyanines are not efficient sensitizers in NHIK 3025 cells⁴³.

For treatment planning, knowledge of the concentration of the photosensitizer in the tumor is necessary, together with the light dose that has to be used. The former must be determined in a non-invasive way, possibly by fluorescence measurements, although aggregated sensitizers do not give much fluorescence. Light dosimetry has not been developed well enough for PDT, although this is an area of the utmost importance to the successful management of cancer treatment. Ideally it should also be possible to assess the induced tumor damage during PDT, so that even at that time a decision could be made about the duration of the illumination. Maybe MRI could be used for this purpose.

For more reading, recent reviews can be consulted⁴⁴⁻⁴⁶.

Photosensitizers are also applied for early detection of cancer, not because of their photosensitizing ability, but because many of them are fluorescent. Again this is only possible because some of these dyes are present in the tumors in a higher amount than in the surrounding tissue and the fluorescence from the tumor is higher than from the surrounding⁴⁷.

PHOTODYNAMIC STERILIZATION OF BLOOD AND BLOOD PRODUCTS

Transfusions with non-cellular blood components, prepared from large pools of plasma are extremely safe compared to other clinical interventions. This is the result of a combination of donor screening for transmittable diseases and procedures like treatment with heat or detergents, that remove or inactivate remaining viruses such as human immunodeficiency virus-1, hepatitis B or C virus. In the case of transfusion with cellular blood components or individual plasma transfusions, the safety is solely based on donor selection. In the case of immunocompromised recipients, blood components can be depleted of white cells, thus removing viruses harbored in them.

It is obvious that approaches such as heating, solvent and detergent treatment are not applicable for cellular blood components. Experiments with photodynamic treatment suggest that methods can be developed to limit cellular damage, while the inactivation of lipid-enveloped viruses can still be achieved, even when present inside white cells. In the latter case, the white cells themselves are destroyed; the implications of this have to be explored.

Several different sensitizers have been used in the above described approach, including merocyanine 540, aminomethyltrimethylpsoralen and different phthalocyanines. Merocyanine binds more efficiently to enveloped viruses and virus containing cells than to red cells, but has several disadvantages like readily inducing an inactivation of platelets and absorbing at wavelengths where the hemoglobin absorption is still appreciable. Aminomethyltrimethyl-psoralen binds well to the nucleic acids of the viruses and can then inactivate them upon light exposure. Photodynamic treatment with this sensitizer causes relatively little damage to platelets. It has to be determined however, whether and to what extent phototreatment with this sensitizer is mutagenic and carcinogenic. Recently the phthalocyanines have also been investigated for this purpose. Some compounds of this class of sensitizers could be well suited for the sterilization of red cell concentrates⁴⁸. In Germany methylene blue is routinely used as a sensitizer for the sterilization of blood plasma.

The common prerequisite for sensitizers for sterilization of blood and blood products is that they must be able to efficiently kill viruses. The further requirements for the different blood products are so divergent that it will probably turn out that different sensitizers will be necessary for the various applications. For red cell concentrates, for example, a sensitizer is needed that absorbs outside the absorbance bands of hemoglobin and that will not bind to the red cells or not to the target in the cells responsible for the lethal damage. For plasma the sensitizer should not bind to the plasma proteins. The photodynamic approach seems to be very promising for the purification of blood and blood products, but many more studies have to be performed, including investigating the possible side effects and the technical feasibility on a large scale⁴⁹⁻⁵⁸.

PHOTODYNAMIC SENSITIZERS AS HERBICIDES AND PESTICIDES

The principle of using photosensitizers as herbicides and pesticides is to force plants and insects to accumulate amounts of photosensitizing compounds that are high enough to kill them when they are exposed to the sun. Ideally such compounds should be non-toxic themselves and not be taken up by other organisms in such high amounts that they are killed. Also they should not pollute the environment. Porphyrins and porphyrin-like structures seem to be a class of compounds with promising and useful properties, although a number of other possible compounds have been described in the literature as well⁵⁹. Some plant secondary metabolites have been recognized as insecticides and are frequently extremely phototoxic to unadapted insects, as discussed above. This area of research has been reviewed recently^{60,61}, as has the recent work on photoherbicides⁶².

PHOTODYNAMIC CLEANING OF WASTE WATER

It has been shown that bacterial cell counts and the concentrations of organic contaminants decrease in surface waters exposed to sunlight. Humic substances, complex mixtures of macromolecular phenols from decaying vegetation, act as photosensitizers. All of the known reactive oxygen species are generated, killing bacteria and destroying organic pollutants. For the cleaning of waste water, inorganic sensitizers like titanium dioxide are under investigation. Again all kinds of species are formed that react readily with most organic molecules, with the exception, perhaps, of some totally halogenated compounds^{63,64}. Titanium dioxide is also phototoxic to cells in culture⁶⁵.

PHOTODYNAMIC SENSITIZERS AS TOOLS IN BASIC RESEARCH

Photodynamic sensitizers are used in biochemistry to study the structure of nucleic acids and the structure-activity relations of enzymes. They can specifically bind to DNA or specifically destroy certain amino acids in enzyme proteins, thereby revealing whether a certain amino acid is necessary for activity^{28,66-68}. This approach, compared to treatment with other oxidative moieties, has shown that even a very limited protein oxidation has a pronounced influence on the conformation of proteins. With glyceraldehyde-3-phosphate dehydrogenase such a (pre-)treatment leads to potentiation of thermal inactivation^{69,70}.

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PHOTOADDITION BY FUROCOUMARINS

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INTRODUCTION

In photobiology, the photoreactions involving the formation of C_4 -cyclobutane type adducts in DNA, such as the formation of pyrimidine dimers (Setlow and Carrier, 1966) by short wave UV radiation and the photoaddition of furocoumarins to pyrimidines in DNA (Musajo and Rodighiero, 1972 ; Hearst et al., 1984), have been of particular importance because of the relatively high chemical stability of these lesions during biochemical analysis. Pyrimidine dimers have been important model lesions (see for review Kim and Sancar, 1993 ; Sancar and Tang, 1993 ; Hoeijmakers, 1993a, b), not only for elucidating major DNA repair mechanisms, but also for investigating the mechanisms of photoinduced mutagenesis and carcinogenesis. Likewise, photoadditions induced by furocoumarins have been useful for elucidating the structure and function of chromatin (Cimino et al., 1985) as well as for studying the repair and genotoxic consequences of well defined bulky photolesions in DNA (see for review Averbeck, 1989). These studies are especially interesting because of the wide use of furocoumarins in photomedicine (see for review Averbeck, 1989). The so-called psoralen plus UVA, PUVA-therapy, has been found very beneficial in the treatment of psoriasis (Parrish et al., 1974, 1982), the T-cell lymphoma mycosis fungoides (Gilchrest et al., 1976) and other skin diseases, such as atopic dermatitis (Jeckler, 1992), lichen planus (Ortonne et al., 1978), graft versus host disease (Hymes et al., 1985 ; Kapoor et al., 1992), pruritus of human immunodeficiency virus infection and acquired immunodeficiency syndrome (Gorin et al., 1989). Furthermore, the recently developed photopheresis therapy, i.e. extracorporeal irradiation of 8-methoxysoralen containing blood, has been shown useful in the treatment of cutaneous T-cell lymphoma and other T-cell mediated diseases, such as pemphigus vulgaris, lupus erythematosus, psoriatic arthropathy, myasthenia gravis and chronic lymphocytic leukemia and AIDS related complex (Edelson et al., 1987, 1991) and has been tested in an animal model (Van Iperen and Beijersbergen van Henegouwen, 1993). To what extent furocoumarin-plus-UVA induced photoadditions to DNA are involved in the photochemotherapeutic effects is still a matter of debate.

Many naturally occurring and newly synthesized furocoumarins are known (see for review Musajo and Rodighiero, 1972 ; Pathak et al., 1974 ; Scott et al., 1976 ; Song and Tapley, 1979 ; Ben-Hur and Song, 1984 ; Rodighiero et al., 1988 ; Dall'Acqua, 1989 ; Averbeck, 1989). Among these, the bifunctional furocoumarins, 8-methoxysoralen (8-MOP), 5-methoxysoralen (5-MOP) and 4,5',8-trimethylpsoralen (TMP), are currently in wide spread use in photochemotherapy (Parrish et al., 1982). In spite of the synthesis of many new furocoumarins of the monofunctional type, such as methylangelicins (Guiotto et al., 1984 ; Dall'Acqua et al., 1985) and pyridopsoralens (Moron et al., 1983 ; Dubertret et

al., 1985), showing very promising photochemotherapeutic activities and less side effects, these compounds have not yet found their commercial development. Fundamental studies with new linear (psoralens) and angular (angelicins) derivatives have indicated that there is no simple correlation between their photosensitizing (erythematogenic), pigmentogenic and photocarcinogenic properties (Rodighiero et al., 1988 ; Averbeck, 1989). The present review focuses on the formation of photoadducts of furocoumarins with DNA and other important biomolecules, their detection and interaction, as well as the repair and biological consequences of psoralen photoaddition in terms of genotoxicity and the induction of genes in eukaryotic cells.

FORMATION OF PSORALEN PHOTOADDUCTS

Due to the absorption properties of photoreactive furocoumarins in the far and near UV, they are easily brought to the singlet or triplet excited state after absorption of photons in that range of radiation energy and wavelengths. Thus, it is not surprising that upon UVA irradiation (320 to 400 nm) psoralen derivatives can undergo photoreactions with other molecules involving radical formation and electron transfer reactions (so called type I reactions), energy transfer reactions from the triplet excited state to molecular oxygen (so called type II reactions) generating singlet oxygen (Poppe and Grossweiner, 1975) as well as energy transfer to pyrimidine bases (Musajo and Rodighiero, 1972) or to other organic molecules (so called type III reactions) carrying an unsaturated double bond, for example unsaturated fatty acids (Caffieri et al., 1987, 1989a, b ; Specht et al., 1988a, b, 1989). While type I and type II reactions may involve or are dependent on the presence of oxygen, respectively, the reaction type III is independent of oxygen and results in the formation of covalent C₄-cyclobutane addition products between the furocoumarin and an acceptor molecule. In this way, also the photodimerization of psoralen (Caffieri and Dall'Acqua, 1987), methylangelicin (Caffieri et al., 1987) and 3-carbethoxypsoralen (3-CPs) (Moysan et al., 1988) can occur.

The photoaddition reaction of furocoumarins with pyrimidines in DNA and RNA has been worked out in some detail (see for review Dall'Acqua et al., 1979a, b ; Hearst et al., 1984 ; Cimino et al., 1985). It involves as a first step complexation of the furocoumarin molecule to DNA in the dark followed in the presence of UVA radiation by the induction of 3,4-pyrone side and 4',5'-furan side monoadducts of the C₄-cyclobutane type involving the 3,4 or the 4',5' double bond of the furocoumarin and the 5,6 double bond of the pyrimidine base. Schematically the photoaddition reaction of 8-MOP, a typical bifunctional furocoumarin, with the pyrimidine base thymine is shown in Fig. 1a. The photoreaction gives rise to the induction of fluorescent 4',5'-monoadducts and non fluorescent 3,4-monoadducts. Because of the limited absorption of 3,4-pyrone side adducts in the UVA region, only 4',5'-furan side adducts can absorb a second photon to form interstrand cross-links between two thymines from adjacent base pairs (Dall'Acqua et al., 1979a, b ; Kanne et al., 1982a ; Hearst et al., 1984 ; Tessman et al., 1985 ; Cimino et al., 1985). The yield of monoadducts is generally higher than that of interstrand cross-links. Most monoadducts induced are of the cis-syn type (see for review Averbeck, 1989). Because of the strong wavelength dependence of interstrand cross-links formation by bifunctional furocoumarins carrying two reactive double bonds, irradiations at specific wavelengths can be used as a tool to compare the effects of very reduced or increased levels of interstrand cross-links (Averbeck et al., 1987). Furthermore, re-irradiation protocols in which after a first exposure (at 365 nm or longer wavelengths) unbound furocoumarin molecules are washed out before a second exposure to 365 nm radiation, can be applied to obtain extremely high yields of interstrand cross-links in DNA (Tessman et al., 1985). The C₄-photocycloaddition of furocoumarins to pyrimidines in DNA appears to involve formation of kinks in B-form DNA (Peckler et al., 1982 ; Tomic et al., 1987). The structural modification may be a recognition signal for repair enzymes (Averbeck, 1989).

The C₄-cyclobutane photoaddition of furocoumarins to pyrimidine bases appears to be somewhat similar to the formation of C₄-cyclobutane thymine dimers by 254 nm short wave UV radiation (UVC). Indeed, the formation of thymine dimers by far UV is due to the reaction of a thymine molecule in one of its excited state with a neighbouring thymine in its ground state (Greenstock et al., 1967). It can be achieved also by triplet-triplet energy transfer

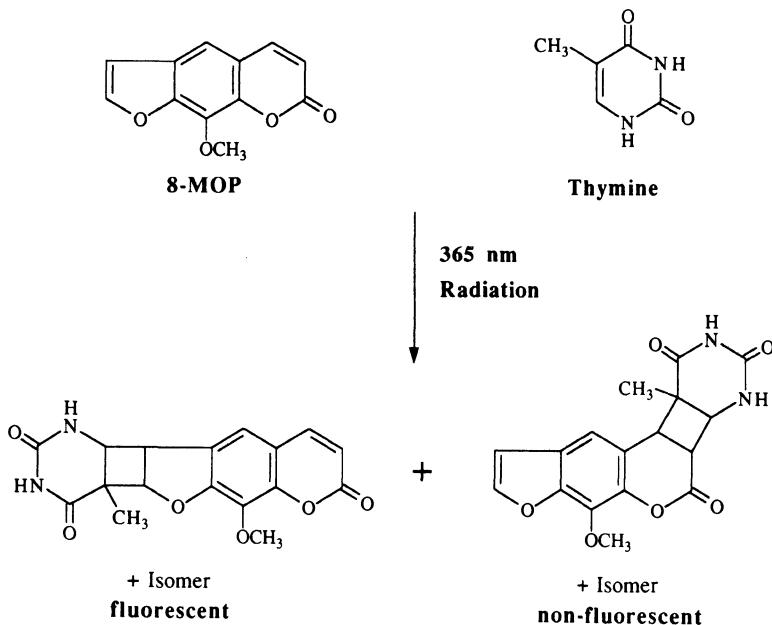


Figure 1a : Scheme of the formation of covalent C_4 -cyclobutane photoadditions of 8-MOP to thymine in the presence of 365 nm (UVA) radiation (according to Pathak et al., 1977).

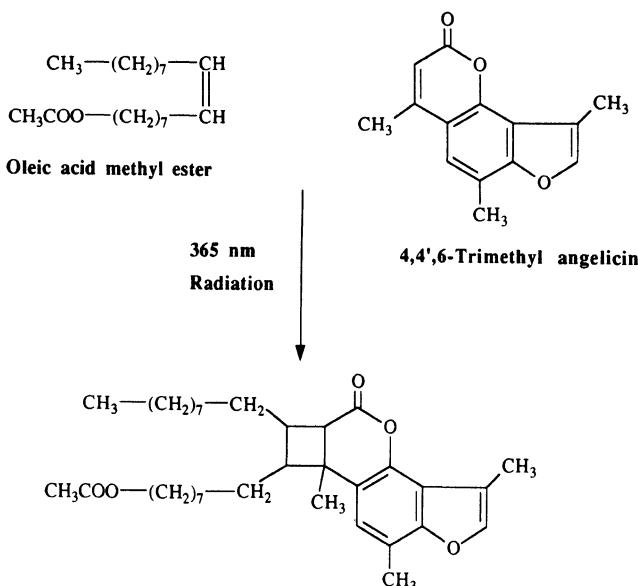


Figure. 1b : Schematic drawing of the formation of covalent C_4 -cyclobutane photoaddition products between 4,6,4'-trimethylangelicin and a fatty acid (oleic acid methyl ester) in the presence of 365 nm (UVA) radiation (according to Caffieri et al., 1989a).

from certain photosensitizers (for example acetone) to thymine (Charlier and Hélène, 1967 ; Von Wilucki et al., 1967 ; Greenstock and Johns, 1968 ; Lamola, 1968). Interestingly, it was shown recently (Costalat et al., 1990) that with the pyridopsoralens, 7-methylpyrido (3,4-c) psoralen (MePyPs) and pyrido (3,4-c) psoralen, the triplet excited state T1 energy is close to that of thymine (3.22 eV), i.e. 0.1 to 0.2 eV lower than that of thymine. The T1 energy values are much lower for 8-MOP and 5-MOP (0.4-0.5 eV). Therefore, the pyridopsoralens are able to generate the formation of C₄-cyclobutane thymine dimers by triplet-triplet energy transfer mechanism (Costalat et al., 1990 ; Moysan et al., 1991), concomitantly to the formation of C₄-cycloadditions to thymines (Moysan et al., 1993). There is some circumstantial evidence that the concomitant induction of thymine dimers and C₄-furan side cycloadditions in DNA by MePyPs may explain the unusual high photobiological activity of this monofunctional furocoumarin for the induction of antiproliferative effects in eukaryotic cells and in the treatment of psoriatic plaques (see for review Averbeck et al., 1992).

With regard to the mechanisms of the formation of C₄-cyclobutane adducts, it is interesting to note that covalent photobinding of furocoumarins to unsaturated fatty acids has been observed (Kittler and Löber, 1983 ; Caffieri et al., 1987, 1988, 1989a, b ; Specht et al., 1988a, b, 1989) at relatively low doses (see Midden, 1988), involving saturation of the 3,4 or 4',5' double bond of the furocoumarin and the 12,13 double bond of the fatty acid (Caffieri et al., 1987, 1988, 1989a, b ; Midden, 1988) (see also Fig. 1b). A biological role of these lesions can be expected (Dall'Acqua and Caffieri, 1988 ; Dall'Acqua and Martelli, 1991). As pointed out before (Specht et al., 1988b), such reactions interfere with cell proliferation by inhibiting indirectly the activation of protein kinase C (Nishizuka, 1986) and thus may be of importance for photochemotherapeutic responses to PUVA treatments. Furthermore, Beijersbergen van Henegouwen et al. (1989) and Schoonderwoerd et al. (1991) reported that, in rat epidermis, 3/5 of 8-MOP is covalently bound to lipids whereas 1/5 of 8-MOP is bound to nucleic acids and 1/5 of 8-MOP to proteins.

Furthermore, furocoumarins have been shown to differ strikingly in their capacities to generate singlet oxygen by triplet energy transfer to molecular oxygen (Potapenko, 1991). From this, the induction of damage to proteins and thus to membranes can be expected. Indeed, both are thought to be involved in hemolysis of red blood cells (Potapenko, 1991) and in the inactivation of certain enzymes (see for review Midden, 1988). In addition, photooxidation of furocoumarins may lead to covalent binding to proteins (Yoshikawa et al., 1979) and to cross-linking of membrane proteins (Hornicek et al., 1985 ; Malinin et al., 1986). Furthermore, the induction of DNA-protein cross-links by furocoumarins (Bordin et al., 1993) and the interaction with viral capsids (Hudson et al., 1993) have been reported. Also, the relaxation of supercoiled plasmid DNA by treatments with furocoumarins and UVA has been observed (Bordin et al., 1992 ; Chen and Kagan, 1993 ; Epe et al., 1993 ; Oroskar et al., 1993). However, the mechanisms involved are not yet fully elucidated.

As shown with the monofunctional furocoumarin 3-carbethoxypsoralen oxygen dependent reactions may increase cell inactivation without affecting the mechanisms of mutation induction (Averbeck, 1988a, b). Moreover, oxidative damage photoinduced in DNA by 3-CPs appears to be easily recognized and repaired by DNA repair enzymes (Sage et al., 1989), and not oxidative damage but 3-CPs-DNA photoadditions are primarily responsible for the induction of lethal effects in *Escherichia coli* (Boiteux et al., 1993).

The fact that furocoumarins form defined 4',5'-furan side and 3,4-pyrone side adducts to pyrimidine in DNA, a certain sequence specificity could be expected. Indeed, careful studies with mono- and bifunctional furocoumarins using exonucleases demonstrated a DNA sequence specificity of furocoumarin photoadditions depending on the sequence context (see for review Sage, 1993a). It was shown that furocoumarins preferentially photoreact with T in 5'-TpA (forming monoadducts and interstrand cross-links) rather than with T in 5'-ApT (forming only monoadducts). In T_n runs with n = 3, photobinding is maximum, TAT/ATA sites are very reactive and altering (A-T)_n sequences are true hot spots for photobinding. Furthermore, 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT), 4,5',8-trimethylpsoralen (TMP) and 8-methoxypsoralen (8-MOP) have been shown to form photoadditions to C in an AC or CA context (Boyer et al., 1988). Interestingly, with methylangelicin the photobinding to C residues was increased but there was a diminished preference for 5'-TpA versus 5'-ApT sites (Miolo et al., 1989a, b). In the case of pyrido (3,4-c) psoralen, in addition to photoadducts in TA sites, pyrimidine dimers were formed only at TT sites and preferentially

in T runs followed by TA, which are strong sites for PyPs-DNA photoaddition (Sage, 1993a). This makes it likely that the induction of TT dimers occurs very close to that of PyPs-DNA adducts, thus increasing the potential for DNA synthesis inhibition and the overall antiproliferative effectiveness in comparison to other psoralens (Averbeck et al., 1992).

Taking advantage of the characteristic absorption and fluorescence properties of furocoumarins, complexation of furocoumarin molecules, i.e. the (non covalent) interaction in the dark with DNA, an essential preparatory step for photoaddition, can be demonstrated by the red shift in the absorption maxima of psoralens in the presence of DNA and corresponding changes in fluorescence following equilibrium analysis (Dall'Acqua et al., 1978). The formation of 4',5'-furan side monoadducts can be most easily detected by the bright blue fluorescence of these photoadducts which is severely quenched when 4',5'-furan side adducts photoreact further and are converted to interstrand DNA cross-links (Dall'Acqua et al., 1979a, b ; Moreno et al., 1982). A rather complicated photochemical behaviour of the 4',5'-furan side psoralen-DNA monoadducts can be observed upon UVA radiation (Tessman et al., 1985). The 4',5'-furan side 8-MOP-thymidine monoadduct inside the DNA helix can be converted into a DNA interstrand cross-link which may be photosplit into a pyrone-side monoadduct. Photosplitting of interstrand cross-links has been used as an indication for their presence in DNA sequencing gels (Boyer et al., 1988). Unfortunately, 3,4-pyrone side adducts are only weakly fluorescent and are difficult to detect (Musajo and Rodighiero, 1972).

The most global technique is the detection of psoralen photobinding by using radioactively (mostly ^3H or ^{14}C) labelled psoralens (Tokura et al., 1991). After the photoreaction with isolated and purified DNA, the number of total photoadducts can be directly deduced from radioactivity measurements taking into account quenching factors and the specific activity of the radiolabelled psoralen. However, this technique is unable to distinguish between different types of photoadducts. In recent years, polyclonal (Zarebska et al., 1984 ; Pathak et al., 1986) and monoclonal antibodies (Santella et al., 1985) have become available for the detection of psoralen-DNA photoadducts using anti-DNA-psoralen polyclonal or monoclonal immune sera as immunofluorescent probes. The advantages of this technique is their relatively high sensitivity to detect psoralen-DNA adducts down to a few femtomoles (Santella et al., 1985) by competitive ELISA assays. In this way, in human lymphocytes treated extracorporeally with 8-MOP and UVA, even very low amounts of 0.7 adduct/ 10^6 bases could be detected (Santella et al., 1985). By the immunofluorescence technique, also 8-MOP photoadducts could be measured in mammalian skin after 8-MOP plus UVA treatment (Pathak et al., 1986 ; Yang et al., 1989). Similarly, the induction of thymidine dimers, as well as 6-4 pyrimidine pyrimidone photoproducts can be efficiently detected *in situ* by monoclonal specific antibodies (Mori et al., 1991 ; Roza et al., 1991 ; Potten et al., 1993) using fluorescent image analysis.

Different types of psoralen-DNA photoadducts can be determined by hydrolysis of aliquots of DNA treated *in vitro* or extracted from treated cells and analysis of the products at the level of nucleosides by reverse phase HPLC as described by Kanne et al. (1982b). DNA isolated from treated cells can be readily examined for the presence of psoralen-photoadducts after enzymatic hydrolysis by DNase 1, S₁ nuclease and acid phosphatase treatment (pH 4.6) and high performance liquid chromatography (Dardalhon et al., 1988 ; Moysan et al., 1988a, b, 1991, 1993). Using this method, the two *cis-syn* diastereoisomers of photoadducts formed between 3-carbethoxypsoralen (3-CPs) and thymidine and between methylpyrido (3,4-c) psoralen (MePyPs) and thymidine were determined in treated DNA *in vitro* and in DNA extracted from treated yeast and mammalian cells (Dardalhon et al., 1988 ; Moysan et al., 1988a, b, 1991, 1993 ; Dardalhon et al., 1993b). Using hydrolysis with formic acid at 180°C for 90 min of DNA treated with 254 nm UV radiation or MePyPs plus UVA radiation and subsequent HPLC analysis, the *cis-syn* cyclobutadithymine (pyrimidine dimers) could be detected as well (Moysan et al., 1991 ; Dardalhon et al., 1993b). With DNA sequencing methodology applied to defined DNA fragment (M13mp8 RF) after digestion with T4 DNA polymerase 3'-5' exonuclease and T4 endonuclease V, the sequence specificity of concomitantly MePyPs-plus-UVA induced MePyPs-DNA 4',5'-furan side monoadducts in DNA and of cyclobutadithymine was demonstrated. Adjacent thymine residues were found to be excellent targets for MePyPs-plus-UVA induced cyclobutadithymine in TTTTA and TTAAT sites which are also strong sites for MePyPs photoadditions (Moysan et al., 1991). HPLC analysis was also used to detect psoralen photoadditions in isolated DNA (Gasparro et al., 1993 ; Olack et al., 1993) and in human

platelets (Wagner et al., 1993). By HPLC analysis, it could be demonstrated that, in mammalian cells treated with 8-MOP in the presence of 419 nm radiation, approximately 10 times less interstrand cross-links are induced than in the presence of UVA (Gasparro et al., 1993). On the other hand, when using split dose protocols with 8-MOP and UVA, it was shown that the second exposure to UVA can convert 4',5'-furan side monoadducts into interstrand cross-links as well as into 3,4-pyrone side monoadducts (Tessman et al., 1985; Olack et al., 1993).

Chromatographic analysis using hydroxylapatite after alkali unwinding of DNA that was treated with bifunctional furocoumarins and UVA inducing DNA interstrand cross-links has been currently used in psoralen research to detect the presence of interstrand cross-links in DNA treated *in vitro* (Musajo et al., 1974; Dall'Acqua et al., 1979b) or DNA extracted from treated cells (Fujiwara et al., 1977; Poll et al., 1984; Dardalhon and Averbeck, 1988; Bankmann and Brendel, 1989). As classical means for the detection of psoralen induced interstrand cross-links, also velocity sedimentation in alkaline sucrose gradients, after denaturation/renaturation of DNA (Cole, 1970; Ben-Hur and Elkind, 1973), and neutral gradients (CsCl equilibrium sedimentation) (Kaye et al., 1980; Jachymczyk et al., 1981; Magana-Schwencke et al., 1982; Miller et al., 1982; Gruenert and Cleaver, 1985) have been used in pro- and eukaryotic cells. More recently, alkaline elution (Kohn et al., 1981) and alkaline step-elution as well as electron microscopy have been successfully employed for the quantification of psoralen induced interstrand cross-links in eukaryotic DNA (Cech and Pardue, 1976; Sogo et al., 1984; Averbeck et al., 1987, 1988; Papadopoulou et al., 1987; Rousset et al., 1990; Cundari et al., 1991a).

As 254 nm UV induced pyrimidine dimers can be detected by specific enzymatic induction of single strand breaks at dimer sites by the UV endonuclease from *Micrococcus luteus* (Ahmed and Setlow, 1979) or T4 endonuclease (Terleth et al., 1989; Meniel et al., 1993) and subsequent analysis of the cleaved single stranded DNA by denaturing (alkaline) agarose gel electrophoresis (Freeman et al., 1987; Terleth et al., 1989), psoralen photoadducts as well as other bulky DNA adducts are recognized and cleaved by the Uvr ABC excinuclease (Van Houten et al., 1986) and can be quantified by analysis in denaturing agarose gel electrophoresis (Gillardeaux et al., 1994).

The ^{32}P -post-labelling assay (Randerath et al., 1981; Gupta et al., 1982) is a very sensitive method for determining DNA adducts in DNA of cells and tissues after exposure to genotoxic agents. This method is not only useful for the detection of bulky base aromatic carcinogen-DNA adducts (Gupta et al., 1982) and oxidative DNA damage (see for review Cadet et al., 1992), but has recently been successfully adapted for the detection of psoralen-DNA adducts (Gillardeaux et al., 1994).

GENOTOXIC CONSEQUENCES OF PSORALEN PHOTOADDITIIONS

C_4 -cyclobutane photoadditions photoinduced by furocoumarins in DNA constitute bulky lesions that, when left unrepaired, block DNA polymerase progression and thus DNA synthesis and replication, as well as RNA synthesis (Ben-Hur and Song, 1984).

This is the basis of the so-called antiproliferative effect which appears to play an important role in the photochemotherapeutic effects of PUVA (Parrish et al., 1982). From early on (Cole, 1970), it has been expected that psoralen monoadducts are less effective than DNA interstrand cross-links in inhibiting DNA synthesis. Indeed, experiments comparing the effects of mono- and bifunctional furocoumarins on DNA synthesis have supported such a notion (Musajo et al., 1974; Nocentini, 1986). However, Baccichetti et al. (1976) found that the template activity inhibition of nucleic acid synthesis in Ehrlich ascites tumor cells was strongly affected by mono- and diadducts. However, 4,5'-dimethylangelicin and psoralen showed approximately the same photobinding rate and the same inhibition of DNA and RNA synthesis in Ehrlich ascites tumor cells. Knowing that 4,5'-dimethylangelicin photoinduced only monoadducts whereas psoralen photoinduced monoadducts and interstrand cross-links (20% of total adducts), this appears to indicate that inhibition of nucleic acid synthesis is independent of the type of photoadduct induced but only dependent on total photobinding activity (Rodighiero et al., 1988).

With regard to the inhibitory effect of monoadducts (induced by monofunctional furocoumarins) and mixtures of monoadducts and interstrand cross-links (induced by bifunctional furocoumarins) on the clonogenicity (colony forming ability) of yeast cells, it

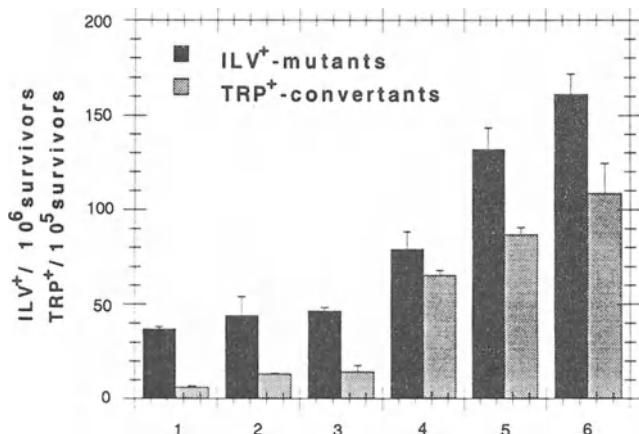


Figure. 2 : Induction of ILV⁺-revertants (mutations) and TRP⁺-gene convertants (mitotic intragenic recombination) in the diploid strain D7 of the yeast *Saccharomyces cerevisiae* by treatments with 8-MOP (5 µM) and single and double exposures to monochromatic radiation. 1 = Control treated with 8-MOP alone ; 2 = 8-MOP treated, washed out and exposed to 365 nm radiation (21 kJm^{-2}) ; 3 = 8-MOP plus 405 nm radiation (21 kJm^{-2}) ; 4 = 8-MOP plus 365 nm radiation (1.4 kJm^{-2}) ; 5 = first treatment with 8-MOP and 405 nm radiation (21 kJm^{-2}), followed by washing out and reexposure to 365 nm (1.4 kJm^{-2}) radiation ; 6 = first treatment with 8-MOP and 405 nm radiation, followed by washing out and reexposure to 365 nm radiation (21 kJm^{-2}) (based on results obtained by Cundari and Averbeck, 1988).

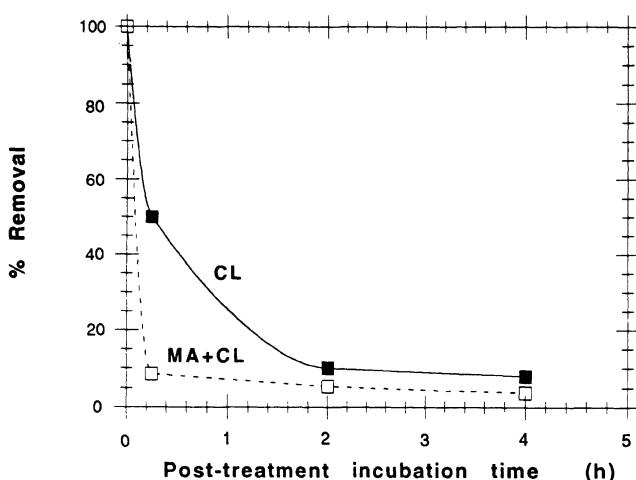


Figure. 3 : Kinetics of the removal of 8-MOP plus UVA induced total photoadducts (monoadducts plus interstrand cross-links) and interstrand cross-links in diploid yeast (D7) (*Saccharomyces cerevisiae*) as a function of post-treatment incubation time. Cells were treated with [³H]-8-MOP (5 µM) and 10 kJm^{-2} of UVA radiation and incubated in liquid complete growth medium (data redrawn from Cundari et al., 1991a).

was shown that, at the same level of total photoadducts induced the bifunctional compounds 8-MOP and 5-MOP were much more (~10x) effective than the monofunctional compounds MePyPs and 3-CPs (Averbeck, 1985). In other words, mixtures of monoadducts and interstrand cross-links affected more strongly cell survival than monoadducts alone suggesting that the presence of interstrand cross-links is more harmful. Furthermore, Bankmann and Brendel (1989) estimated that only 58 photoadducts induced by 8-MOP plus UVA, but 2500 monoadducts induced by 3-CPs plus UVA or 18000 254 nm UV induced thymine dimers are needed to decrease cell survival in yeast down to 37%. These results clearly indicate that the presence of interstrand cross-links confers higher genotoxicity than monoadducts alone.

Research work on the relationship between furocoumarin induced lesions and mutagenic and recombinogenic effects in the yeast *Saccharomyces cerevisiae* (Averbeck, 1985) demonstrated that, at equal levels of photoadducts, 4',5'-furan side monoadducts inducing compounds, such as MePyPs and 3-CPs, were clearly less efficient than compounds, such as 8-MOP and 5-MOP inducing a mixture of monoadducts and interstrand cross-links. 8-MOP and 5-MOP and also MePyPs and 3-CPs differed from each other in effectiveness. It was concluded that the ratio of monoadducts over interstrand cross-links may differ in 8-MOP and 5-MOP. Indeed it was shown by alkaline elution on cultured Chinese hamster V-79 cells that 5-MOP induced proportionally less interstrand cross-links than 8-MOP (Papadopoulou and Averbeck, 1985).

The difference in the responses elicited by treatments with MePyPs and 3-CPs plus UVA can be explained by differences in the repair of the cis-syn diastereoisomers of the 4',5'-furan side adducts to thymine (Dardalhon et al., 1988b, 1993a) and by the concomitant induction of thymine dimers by MePyPs (Moysan et al., 1991; Dardalhon et al., 1993b). Indeed, the apparent higher effectiveness of MePyPs induced photoadducts, in comparison to those induced by 3-CPs, may well be due to the concomitantly induced thymine dimers which, because of the close proximity with monoadducts, become refractory to repair activities such as photoreactivation (Averbeck et al., 1992).

The genotoxicity of monoadducts versus interstrand cross-links could be best evaluated for a given bifunctional furocoumarin, for example, by comparing, at equal photoadduct levels, the mutagenic and recombinogenic effectiveness of 8-MOP plus 365 nm radiation inducing monoadducts and interstrand cross-links to that of 8-MOP plus 405 nm radiation inducing mainly monoadducts and the genotoxic response at increased levels of interstrand cross-links induced by using the bifunctional furocoumarin 8-MOP plus a double exposure to UVA (365 nm radiation). By the latter procedure, the first exposure to UVA is followed, after washing out of unbound furocoumarin molecules, by a second exposure to UVA. It has been clearly shown in eukaryotic cells, such as the yeast *Saccharomyces cerevisiae*, that, in the presence of interstrand cross-links, the lethality, the induction of nuclear mutations and mitotic gene recombination are significantly increased over that observed in the presence of predominant monoadducts (Fig. 2). It is seen that 8-MOP alone or exposed to 365 nm radiation after washing out or 8-MOP plus 405 nm radiation have very little genetic effects. However, at the same total number of 8-MOP plus 365 nm radiation induced photoadducts, 8-MOP-DNA adducts, are apparently more photomutagenic and recombinogenic than those induced by 8-MOP plus 405 nm radiation. Furthermore, the genotoxic effects are clearly further increased when converting 8-MOP-monoadducts into interstrand cross-links by a reirradiation procedure. These effects have been well documented before (Cassier et al., 1984; Averbeck, 1988a, b; Averbeck et al., 1987, 1991; Cundari and Averbeck, 1988). The presence of increased amounts of interstrand cross-links in yeast cells was verified by alkaline step elution (Averbeck et al., 1987; Cundari and Averbeck, 1988). Ben-Hur and Elkind (1973) showed that increased TMP-DNA diadduct levels resulted in increased cell killing in Chinese hamster V-79 cells and Babudri et al. (1981) observed higher frequencies of thioguanine resistant mutants using the UVA re-irradiation procedure with 8-MOP.

Molecular studies on the distribution of photoadducts in relation to the mutational spectra observed (see for review Sage, 1993a, b) appear to be in line with the above findings. In the bacteria *Escherichia coli*, mutations induced by 8-MOP plus UVA in the *lacI* gene comprised base substitutions, frameshifts and deletions. Since in repair proficient cells 50% of frameshifts and 25% of base substitutions occurred at cross-linkable sites, it was concluded that 8-MOP-DNA diadducts play an important role in frameshift and base substitution mutagenesis due to a specific processing of interstrand cross-links (Yatagai and Glickman, 1986). Also, interstrand cross-links are suspected to be a source for deletion mutations (Yatagai and Glickman, 1986). In addition, the mutational spectrum obtained in the

lac promotor region and the *lacZ* gene regulatory region of M13mp10 after treatment with HMT plus UVA (Piette et al., 1985; Collet and Piette, 1993) showed mutational hot spots at cross-linkable sites. Using the shuttle vector pZ189 carrying the *supF* tRNA gene, Bredberg and Nachmanson (1987) showed an increased mutagenicity in primate cells due to the induction of 8-MOP-DNA interstrand cross-links. In a mouse lymphoma cell line (Piette, 1991) and in Chinese hamster ovary cells (Sage et al., 1993), respectively, HMT and 8-MOP induced photoadditions in the *HPRT* and *APRT* genes resulted predominantly in base substitutions located at cross-linkable sites. A model has been proposed to account for the results obtained at the *APRT* gene in rodents (Sage et al., 1993). If monoadducts were responsible for all mutations induced, 90% of the mutations would be due to the absence of repair of photoadducted thymine on the non-transcribed strand of the *APRT* gene. However, if, as stated above, the interstrand cross-links were the major premutagenic lesions, at all mutational hot spots, the cross-linked T would be first incised on the transcribed strand followed by a translesional synthesis past modified thymine bases persisting in the non-transcribed strand. Psoralen mutagenesis would then occur by a transient misalignment during translesional bypass (Strazewski, 1991). Such a mutagenic pathway can be expected to compete with the normally error free recombination repair (Sage, 1993b).

In contrast to the results obtained in rodents, after treatment with 4,5'-8-trimethylpsoralen (TMP) and UVA, large deletions were induced in the *HPRT* locus in human lymphoblasts (Papadopoulou et al., 1990). These findings may indicate differences in the processing of furocoumarin plus UVA induced interstrand cross-links in yeast and rodent cells versus human lymphoblasts. Furthermore, when determining the molecular spectra of mutations induced at the *HPRT* locus by TMP in a normal human lymphoblast cell line, mainly (84%) base substitutions were induced localised at sequences favorable for the induction of monoadducts. Interestingly, in the hypomutable Fanconi anemia lymphoblasts, the processing of TMP-DNA photoadducts led mainly (66%) to deletions and to only a few base substitutions (22%). Most mutations appeared to be targeted to thymine residues on the non-transcribed DNA strand (Papadopoulou et al., 1993). Moreover, 8-MOP induced mutagenesis may be part of a stress response (Boesen et al., 1992). Treatments of a mouse T-lymphoma cell line with 8-MOP plus UVA and fluctuation analysis of the progeny of treated cells showed an enhanced mutation rate persisting up to the 11th generation. 90% of all mutational events were untargeted suggesting that 8-MOP photoaddition induced a stress response involving untargeted mutagenesis (Boesen et al., 1992).

With regard to the mutational consequences of furocoumarin plus UVA induced monoadducts, only a few data obtained with angelicin and 4,4',6-trimethylangelicin in the *lacI* gene of *Escherichia coli* are available (see for review Sage, 1993b). Apparently, mutations induced by angelicins were highly targeted at hot spots of angelicin photoaddition.

REPAIR OF FUROCOUMARIN PLUS UVA INDUCED PHOTOADDUCTS IN EUKARYOTIC CELLS

A large body of data exists providing evidence for the repair of monoadducts and interstrand cross-links in eukaryotic cell systems (see for review Smith, 1988; Friedberg, 1988; Averbeck, 1989; Averbeck et al., 1990a, b). Models for the enzymatic processing of these photolesions were developed using the bacterium *Escherichia coli* (Van Houten et al., 1986; Yeung et al., 1987). Psoralen-induced furan-side monoadducts were shown to be processed *in vitro* by the Uvr ABC excision nuclease involving a double incision, one at the 5' eighth phosphodiester bond and one at the 3' fifth phosphodiester bond (Yeung et al., 1987) followed by the action of *Uvr D* protein DNA helicase II and polymerase I and ligase (Van Houten et al., 1986). Cross-link repair is more complex involving not only excision repair but also recombinational repair events (Van Houten et al., 1986). In *E. coli*, it needs the action of the Uvr ABC excinuclease, *recA* protein (recombination), helicase II (*Uvr D* protein) followed by excision repair involving Uvr ABC excinuclease, helicase II, polymerase I and ligase (Van Houten et al., 1986).

Apparently, during the repair of interstrand cross-links in *E. coli*, no double-strand breaks occur (Cole et al., 1976). As in bacteria, from the sensitivity towards psoralen photoaddition of specific mutants in eukaryotic cell systems, the repair pathways involved in the repair of psoralen induced photolesions could be determined. In the yeast *S. cerevisiae*, the excision repair pathway (deficient in mutants belonging to the *RAD3* complementation

group) and post-replication or mutagenic pathway (deficient in mutants belonging to the *RAD6* complementation group), known to operate on 254 nm UV induced lesions, are involved in the repair of psoralen induced monoadducts. The repair of cross-links appears to be dependent not only on the excision repair (*RAD3*), mutagenic repair (*RAD6*), but also on the pathways involved in double strand break and recombinational repair (deficient in mutants belonging to the *RAD50* complementation group), as well as on cross-link specific pathways (*PSO2* (*SNM1*) and *PSO4* mutants) (see for review Averbeck, 1989; Averbeck et al., 1991; Cassier et al., 1985).

Also, human cells derived from xeroderma pigmentosum and Fanconi anemia patients have been found sensitive to furocoumarin plus UVA treatment and defective in specific steps of psoralen adduct removal (Gruenert and Cleaver, 1985; Papadopoulou et al., 1987; Averbeck et al., 1988; Smith, 1988). Interestingly, Fanconi cells in culture were found to be partially deficient in the removal of TMP plus UVA induced 4',5' furan-side monoadducts and interstrand cross-links, depending on the complementation group (Averbeck et al., 1988). Generally, repair proficient normal human cells (fibroblasts) are quite efficient in the removal of furocoumarin-DNA adducts and interstrand cross-links (Nocentini, 1986; Vuksanovic and Cleaver, 1987; Averbeck et al., 1988; Smith, 1988), and Chinese hamster cells as well (Dardalhon and Averbeck, 1988a).

While the concomitantly induced oxygen-dependent damage had apparently no effect on the removal of 3-CPs induced specific diastereoisomers of 4',5' furan-side monoadducts in yeast and Chinese hamster cells (Averbeck et al., 1990a, b), one diastereoisomer of 4',5' furan-side monoadducts induced by MePyPs plus UVA was more effectively repaired than the other in yeast cells, possibly because of the presence of concomitantly induced pyrimidine dimers (Dardalhon et al., 1993a, b).

That the presence of one type of photolesion may hinder the repair of another has been observed in several cases. For example, the thymine dimers induced concomitantly to 4',5' furan-side monoadducts by MePyPs plus UVA are apparently not repaired by photoreactivation which effectively splits UV (254 nm) induced pyrimidine dimers (Averbeck et al., 1992). The proximity of monoadducts and pyrimidine dimers induced by MePyPs plus UVA may result in inhibition of repair and may explain the accumulation of single strand breaks in treated yeast cells during post-treatment incubation (Moustacchi et al., 1983; Magana-Schwencke and Moustacchi, 1985).

Another example for the interaction of photolesions affecting repair was reported by Papadopoulou et al. (1988). By alkaline elution analysis, it was shown that the incision of TMP-photoinduced interstrand cross-links in normal human fibroblasts is strongly affected by the amount of cross-linkable 4',5' furan-side monoadducts, whereas the total number of total photoadducts was of little importance. Large amounts of furan-side monoadducts could totally block the incision of TMP induced interstrand cross-links (Papadopoulou et al., 1988). However, incision of interstrand cross-links took place when converting some of the furan-side monoadducts into interstrand cross-links by re-irradiation with UVA. Furthermore, when measuring the removal of 8-MOP plus UVA induced interstrand cross-links in yeast cells by alkaline step elution, it was shown that the conversion of a number of cross-linkable furan-side monoadducts into interstrand cross-links generated a considerable increase in endonucleolytic cleavage of damaged DNA without changing the overall kinetics of interstrand cross-links removal (Cundari et al., 1991b). This appears to indicate that the presence of interstrand cross-links may act as a signal for endonucleolytic cleavage of DNA which may be related to the increased mutagenic and recombinogenic effects observed at increased levels of interstrand cross-links in yeast (Cassier et al., 1984; Cundari and Averbeck, 1988). The level of interstrand cross-links per total photoadducts in yeast was found to affect the removal of photoadducts induced by homopsoralen (pyranocoumarin) and 8-MOP (Averbeck et al., 1990a). These results clearly indicate that the processing of photoadducts induced by furocoumarins is not independent from the concomitant presence of different types of photolesions. Fig. 3 shows that, in diploid yeast, the mixture of total photoadducts (monoadducts plus interstrand cross-links) containing high amounts of monoadducts, is more efficiently removed than the interstrand cross-links induced by 8-MOP plus UVA (Cundari et al., 1991a), suggesting that in general psoralen plus UVA induced monoadducts are more efficiently removed in yeast.

Interestingly, there exists a difference in the contribution of the known repair systems in yeast to the repair of furocoumarin induced photoadducts in endogenous (genomic) DNA and exogenous (plasmid) DNA (Magana-Schwencke and Averbeck, 1991). For instance, the *RAD6* and *PSO2* gene products were found to be not involved in the repair of 8-MOP plus

UVA photoadducts in exogenous plasmid DNA level in endogenous DNA. The activity of these gene products appears to depend on chromatin structure. Psoralen photoinduced damage promoted also plasmid recombination in yeast in a *RAD1* and *RAD52* dependent manner (Saffran et al., 1992).

Experiments performed in yeast measuring the persistence of psoralen-photoinduced lesions showed that 8-MOP-DNA cross-linkable 4',5' monoadducts may persist in DNA for several cell generations and may be bypassed by the cellular replication machinery (Chenet et al., 1983). The bypass of 8-MOP induced monoadducts was found to be under the control of the *RAD6* gene product (Chenet et al., 1985). In line with this, the long persistence of monoadducts induced by 8-MOP plus UVA in human skin has been demonstrated *in vivo* (Gange et al., 1984; Ortel and Gange, 1990; Ortel et al., 1991). Human skin was photosensitized by application of dilute 8-MOP (0.003%) and a non erythematogenic radiation dose at wavelengths above 380 nm. Re-exposure to UVA converting monoadducts into interstrand cross-links elicited a phototoxic erythema up to 15 days after the initial exposure (Ortel et al., 1991), indicating a long persistence of cross-linkable monoadducts in human skin. The bypass efficiency of psoralen (HMT) monoadducts has been measured at the gene specific level in cultured mammalian cells (Vos, 1988) and was found to be very high in mammalian genes (Vos and Hanawalt, 1987; Wauthier et al., 1990) except in xeroderma pigmentosum variant cells (Misra and Vos, 1993). The removal of 8-MOP photoadducts in normal human keratinocytes was shown to depend on calcium concentration. This is an important finding in the light of recent findings on the dependence of ultraviolet light mutational hotspots on cellular calcium levels (Seetharam and Seidman, 1992). Addition of retinoids (etretinate, acitretin and 13-cis retinoic acid) suppressed 55 to 80% adducts removal but the suppression could be restored by adding the calcium ionophore A23287 (Tokura et al., 1991). Recently, it could be shown *in vitro* that a human cell free extract (from HeLa cells) is capable of eliciting unscheduled DNA repair synthesis that is associated with the removal of psoralen (HMT) photoadducts. In comparison to monoadducts, psoralen (HMT) induced interstrand cross-links induced a higher level of DNA synthesis associated with the removal of interstrand cross-links (Reardon et al., 1991).

GENE SPECIFIC REPAIR

In the last decade, the notion has been developed that DNA repair is heterogeneous at the level of the gene, i.e. repair takes place preferentially in actively transcribed genes (see for review Hanawalt, 1991). Zolan et al. (1984) showed first that psoralen photoadducts were less efficiently removed from repetitive non transcribed a-DNA sequences than from bulk link DNA in markers kidney cells. In the following, using renaturing agarose gel electrophoresis to quantify HMT plus UVA induced monoadducts and interstrand cross-links in the transcriptionally active *DHFR* gene in human cells and in ribosomal RNA genes in Chinese hamster ovary cells, Vos and Hanawalt (1987, 1989) and Wauthier et al. (1990) observed that, within 24 h, most of the interstrand cross-links but less than half of cross-linkable monoadducts were removed from both *DHFR* genes. However, interstrand cross-links were less efficiently removed in rodent and human cells from ribosomal genes sequences (Wauthier et al., 1990).

More recently, a new technique was developed to determine repair in specific sequences and bulk DNA (Islas et al., 1991). After alkaline denaturation, cross-linked DNA rapidly renatures in a CsCl gradient at pH 10.8 whereas un-cross-linked DNA remains single stranded. After equilibrium sedimentation in a CsCl gradient, the DNA in the various gradient fractions are slot-blotted and hybridized with ^{32}P -labeled probes for gene specific sequences. Autoradiograms are densitometrically quantitated and the frequency of interstrand cross-links determined in each sequence. The frequency of interstrand cross-links in bulk genomic DNA is measured at the same time. Interestingly, 90% of HMT-DNA interstrand cross-links in human fibroblasts carrying an amplified *DHFR* gene were repaired within 24 h in the active *DHFR* gene, but only 31% in bulk DNA and 0% in the transcriptionally inactive *fms* proto-oncogene. 63% of cross-linkable HMT-DNA monoadducts were repaired within 24 h in the active *DHFR* gene and only 39% in the bulk DNA (Islas et al., 1991). Since initially less interstrand cross-links were induced in *fms* than in bulk DNA and the *DHFR* gene, it appears that not only the induction of photoadducts but also the repair is dependent on the target DNA sequence and its transcriptional activity.

Knowing that preferential repair of UV (254 nm)-induced pyrimidine dimers in the active *MATα* compared with the inactive *HMLα* locus can be observed in the yeast *Saccharomyces cerevisiae* (Terleth et al., 1989), recently the repair of 3-CPs photo-induced monoadducts was determined in the same system using the *UVR ABC* assay (Meniel et al., 1993). Evidence was obtained for preferential repair of 3-CPs plus UVA induced monoadducts in the active *MATα* locus compared with the inactive *HMLα* locus in a *SIR⁺* strain. However, in comparison with the repair of UV (254 nm) induced pyrimidine dimers, the repair of 3-CPs-DNA monoadducts was less efficient than that of pyrimidine dimers (Meniel et al., 1993). This may indicate that, as in mammalian cells (Bohr, 1991), the preferential repair of active genes is most efficient for pyrimidine dimers, probably due to differences in the enzymatic recognition of the DNA damage induced.

Further studies are on the way to find out whether there is also preferential repair for 8-MOP plus UVA induced interstrand cross-links in the same genetic system (Meniel et al., in preparation) and to what extent this repair is dependent on the type of photoaddition induced.

INDUCTION OF GENES BY FUROCOUMARIN PHOTOOADDITION

It is well known that, in pro-and eukaryotic cell systems, certain genes are induced in response to environmental stress, such as oxidative stress (Farr and Kogoma, 1991) and the induction of DNA damage. For example, in bacteria, the OxyR and SoxRS regulons are induced in response to oxidative stress which encodes transcription factors associated with changes in the oxidative state of the cells (Demple and Arnabale-Cuevas, 1991). On the other hand, short-wave UV (254 nm) irradiation activates the SOS regulon involving a number of genetic and phenotypic modifications that increase the DNA repair capacity (Walker, 1985). In fact, DNA damaging agents can stimulate the expression of *UvrA*, *recA* and *UmuDC* genes in *Escherichia coli* thus assuming cell survival (see for review Devoret, 1992, 1993). Also, in eukaryotes, such as the yeast *Saccharomyces cerevisiae* (see for review Friedberg, 1988) and mammalian cells (see for review Sarasin and Hanawalt, 1987), DNA damage-inducible repair functions have been demonstrated. In *Saccharomyces cerevisiae*, several genes involved in excision repair, *RAD2* (Robinson et al., 1986; Siede et al., 1989; Siede and Friedberg, 1992), *RAD7*, *RAD23* (Jones et al., 1990; Madura and Prakash, 1990), in post-replication repair, *RAD6* and *RAD18* (Madura et al., 1990; Jones and Prakash, 1991), in recombinational repair, *RAD51* (Abousekhra et al., 1992) and *RAD54* (Cole et al., 1987, 1989), in ligation, *CDC9* (Peterson et al., 1985), in alkylation repair, *MAG1* (Chen et al., 1990), in photoreactivation, *PHR1* (Sebastian et al., 1990), in polymerisation, polymerase 1 (Johnston et al., 1987) and *RAD16* involved in the repair of dimers in transcriptionally active genes (Waters et al., 1993) have been found to be inducible. In addition, a number of unrelated non-DNA repair genes have been shown to be DNA-damage inducible. These include the *DIN1* gene encoding the large subunit of the ribonucleotide reductase enzyme *RNR3* and the *DNR48* gene involved in mutagenesis (Treger and McEntee, 1990; Yagle and McEntee, 1990), the *UBI1* gene involved in protein degradation (Treger et al., 1988), the thymidylate kinase gene *CDC8* and the *RNR2* gene encoding for the small subunit of the ribonucleotide reductase (Elledge and Davis, 1987). The latter gene *RNR2* was effectively induced by 254 nm UV radiation, 4-nitroquinoline 1-oxide (4NQO), methyl methanesulfonate (MMS) and agents blocking DNA replication (Elledge and Davis, 1989).

It is important to note that also protooncogenes like *c-fos* and *c-jun* encoding for nuclear transcription factors in mammalian cells have been found to be inducible by DNA damage (see for review Krämer et al., 1990; Ransone and Verma, 1990). Activation of these transcription factors appears to involve protein kinases (Krämer et al., 1990; Kerr et al., 1992). Activation is not only part of a signal transduction pathway (Kerr et al., 1992), but also linked to DNA repair processes (Xanthoudakis et al., 1992). The redox activation of *fos-jun* DNA binding activity is apparently mediated by an apurinic/apyrimidinic (AP) endonuclease which is involved in DNA repair (Xanthoudakis et al., 1992).

The induction of gene expression by nuclear DNA damage raises the question on the nature of the signal(s) involved. In *Escherichia coli* as well as in *Saccharomyces cerevisiae*, the amplitude of induction was found to depend on the type of treatment used (Oishi et al., 1981; Robinson et al., 1986). However, not much is known on the role of specific types of DNA lesions. On the other hand, a recent paper by Devary et al. (1993) has shown that

expression of the protein NF_kB may be even induced in unnucleated mammalian cells, thus in the absence of DNA damage.

Because of the specificity of the photoadducts induced by furocoumarins and UVA in DNA, the specific induction of a repair and a house keeping gene has been studied in some detail in the yeast *Saccharomyces cerevisiae* (Averbeck and Averbeck, 1994). For this, a yeast strain containing the damage inducible *RAD54* gene which belongs to the *RAD52* epistasis group involved in recombinational repair (Cole et al., 1987), as well as a yeast strain containing the damage inducible *RNR2* gene coding for the small subunit for ribonucleotide reductase which catalyses the first step in the pathway of deoxyribonucleotide production needed for DNA synthesis was used (Elledge et al., 1992). While the gene *RAD54* plays an important role in double-strand break repair, the ribonucleotide reductase gene *RNR2* plays a central role in the control of DNA synthesis in a number of organisms including *Escherichia coli*, *Saccharomyces cerevisiae* and mammalian (mouse) cells. The inducibility of both genes by various DNA damaging agents has been already demonstrated in the yeast *Saccharomyces cerevisiae* by measuring the induction of β -galactosidase in *RAD54-lacZ* and *RNR2-lacZ* fusion strains by a colorimetric assay (Cole et al., 1987 ; Elledge and Davis, 1987, 1989). However, neither the relationship to cell growth nor the relationship to cell survival or the type and level of DNA damage has been established.

In order to find out the possible importance of psoralen plus UVA induced DNA monoadducts and interstrand cross-links for the induction of these genes in *Saccharomyces cerevisiae*, the induction of β -galactosidase was determined in the *lacZ* fusion strains as a function of treatment dose, number of photoadducts induced and cell survival, i.e. colony forming ability. The colorimetric assay of β -galactosidase activity was based on the enzymatic cleavage of O-nitrophenyl- β -D-galactopyranoside (ONPG) and the spectroscopic detection of one of the products (nitrophenol) by its absorbance at 420 nm (Guarente, 1983 ; Rose and Botstein, 1983 ; Ruby et al., 1983). The optical densities obtained reflect the enzymatic activity by β -galactosidase induced by the DNA damaging treatment.

The haploid *RAD54-lacZ* fusion strain was provided by Dr. G.M. Cole (Cole et al., 1987). The haploid strain carrying the *RNR2-lacZ* fusion was constructed by Dr. J. Borges-Meira (Institut Curie, Section de Biologie, Paris, France) transforming the haploid yeast strain FF1852 (*MATA leu2-3 trp1-289 ura3-52 ade5 can1 RAD*) (Dr. F. Fabre, Institut Curie, Orsay, France) by the protoplast method (Magana-Schwencke and Averbeck, 1991) using the centromeric plasmid pMN403 with the *RNR2-lacZ* fusion (Dr. S.J. Elledge, Stanford University of Medicine, Stanford, California 94305, USA).

First, the survival, i.e. colony forming ability, of the two strains was determined after treatments with furocoumarins and UVA radiation using the methods previously described (Averbeck, 1985 ; Averbeck and Averbeck, 1994). Survival studies, i.e. measures of colony forming ability, after treatments with furocoumarins plus UVA performed with these yeast strains showed sensitivity patterns comparable to those previously observed with a diploid yeast strain (Averbeck, 1985). Figures 4a-c show data redrawn from Averbeck and Averbeck (1994). Fig. 4a shows a typical survival curve obtained after treatment with 8-MOP plus UVA for the *rad54-lacZ* fusion strain of the yeast *Saccharomyces cerevisiae*. After 8-MOP (5 μ M) plus UVA treatment, a growth delay is observed depending on the UVA dose (Fig. 4b). The induction of the *RAD54* gene is also dose-dependent ; the peaks of induction show a characteristic shift as a function of post-treatment incubation and a dose-dependent increase (Fig. 4c). Maximum induction is observed when growth is starting again after treatment (Fig. 4b).

Assuming that the number of photoadducts per 10^6 bp induced in DNA by the different psoralens was the same as measured before (Averbeck, 1985), the induction of *RAD54* gene in the *RAD54-lacZ* fusion strain was determined after treatments with 8-methoxysoralen (8-MOP), 5-methoxysoralen (5-MOP), 3-carbethoxysoralen (3-CPs) and 7-methylpyrido (3,4-c) psoralen (MePyPs) and different doses of UVA to obtain comparable levels of photoadditions. UVA radiation or the furocoumarins alone were unable to induce β -galactosidase activity in the range of doses and concentrations used. As shown in Table 1, after treatment with the furocoumarins at 5 μ M plus UVA radiation and 4 h of post-treatment incubation at the same number of DNA photoadducts induced per 10^6 bp, the β -galactosidase activity, expressed here as the optical density of the ONPG cleavage product found in the cell supernatants, was highest with the two bifunctional compounds 8-MOP and 5-MOP and lowest with the two monofunctional compounds 3-CPs and MePyPs.

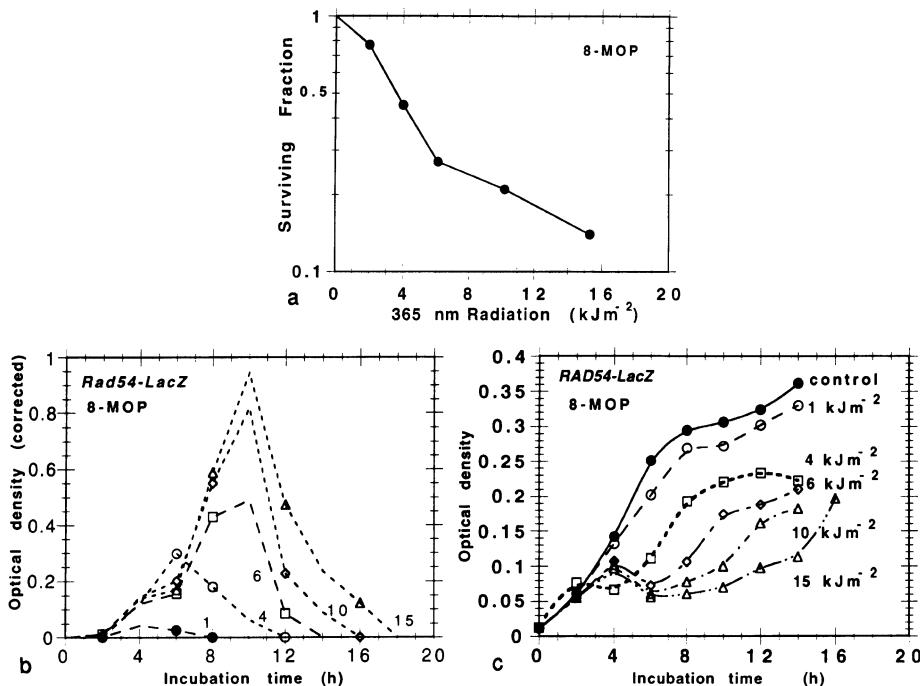


Figure. 4a : Typical survival curve obtained for the haploid strain *RAD54-lacZ* of the yeast *Saccharomyces cerevisiae* after treatment with 8-MOP (5 μM) and different doses of 365 nm (UVA) radiation.

4b : Cell growth of the haploid strain *RAD54-lacZ* of the yeast *Saccharomyces cerevisiae* following treatments with 8-MOP (5 μM) and different doses of 365 nm (UVA) radiation as a function of post-treatment incubation time in complete growth medium.

4c : Induction of the *RAD54* gene in the haploid strain *RAD54-lacZ* of the yeast *Saccharomyces cerevisiae* following treatments with 8-MOP (5 μM) and different doses ($\text{in } \text{kJ m}^{-2}$) of 365 nm (UVA) radiation as a function of post-treatment incubation time in complete growth medium.

Table 1. Induction of β -galactosidase in the *RAD54-lacZ* fusion strain after 4 h of post-treatment incubation following exposures to furocoumarins (5 μ M) and UVA radiation inducing 40 photoadducts/10⁶ bp

furocoumarins	optical density of the supernatants after cleavage of ONPG	Nb of photoadducts needed to induce a comparable β -galactosidase activity (in terms of optical density of cell supernatants at 420 nm)
8-MOP	0.39	4
5-MOP	0.27	8
MePyPs	0.12	34
3-CPs	0.02	100

Moreover, much more photoadducts are needed to be induced by the monofunctional furocoumarins than by the bifunctional furocoumarins to obtain a comparable induction of β -galactosidase activity. Both findings suggest that mixtures of monoadducts and interstrand cross-links induced by furocoumarins are much more effective in inducing *RAD54* gene expression than the monoadducts. As previously discussed (Averbeck, 1985; Averbeck et al., 1992), differences between the bifunctional compounds may reside in different ratios of monoadducts over interstrand cross-links induced and differences between the monofunctional compounds in the concomitant induction of other types of lesions photooxidative damage (3-CPs) and pyrimidine dimers (MePyPs).

When analysing the induction of the *RAD54* gene in relation to the growth delay (Fig. 4b) induced by 8-MOP and UVA, the peak of gene induction (Fig. 4c) lay in the lag period before the onset of cell proliferation. Also, when investigating the inducibility of the *RNR2* gene (in the *RNR2-lacZ* fusion strain) by furocoumarins and UVA radiation, the survival and growth inhibition responses were taken as a base line for comparisons between the different treatments. For the photosensitizing treatments, we used commercially available 8-MOP (Chinoin, Italy) and 5-MOP (Sarsyntex-Interchim, Montluçon, France). 3-CPs and MePyPs were kindly provided by Dr. E. Bisagni (Institut Curie, Orsay, France), and 4,4',6-trimethylangelicin (TMA) (Baccichetti et al., 1984) was a kind gift from Professor F. Dall'Acqua (University of Padua, Italy).

The induction of the *RNR2* gene was determined for doses leaving comparable survival levels, in the ranges of 9.5-11.5%, 22-27.5%, 47-53% and 70-79% survival (Averbeck and Averbeck, 1994).

Interestingly, as in the *RAD54-lacZ* fusion strain, the induction of β -galactosidase in the *RNR2-lacZ* fusion strain resulted in a slight shift in the maxima of induction at low treatment doses and high survival after 6-8 h of post-treatment incubation to maxima at higher treatment doses and low survival after 10-12 h of post-treatment incubation. Typical induction kinetics were obtained for treatments with 8-MOP, 5-MOP, TMA, 3-CPs, MePyPs plus UVA and 254 nm UV radiation. Fig. 5 shows the induction of β -galactosidase activity (corresponding to the induction of the *RNR2* gene in the *RNR2-lacZ* fusion strain of *Saccharomyces cerevisiae*) at the different doses and survival levels obtained after post-treatment incubation of 8 h and 10 h. At high survival levels (around 70%), the amplitudes of induction were quite different and depended on the type of photosensitizing treatment used whereas, at low survival levels (around 10%), the induction kinetics were comparable for the different treatments. Quite strikingly, the induction of the *RNR2* gene by 254 nm UV radiation and TMA plus UVA appears to be even higher than that obtained by treatments with 8-MOP plus UVA. In this respect, it is important to note that also the activation of HIV virus in a human cell line was found to be more important with TMA plus UVA than with 8-MOP plus UVA (Zmudска et al., 1993). However, in addition, the HIV-1 promoter can be also activated by oxidative stress mediated by singlet oxygen (Legrand-Poels et al., 1993).

Since at low doses and high survival levels (70-79%) (Fig. 5), the two monofunctional compounds 3-CPs and MePyPs, inducing mainly 4',5'-furan side monoadducts in DNA, and 5-MOP plus UVA, inducing a higher ratio of monoadducts over interstrand cross-links than 8-MOP plus UVA, show a reduced capacity for *RNR2* gene induction, it appears that furocoumarin induced furan side monoadducts in DNA act as less potent gene inducing

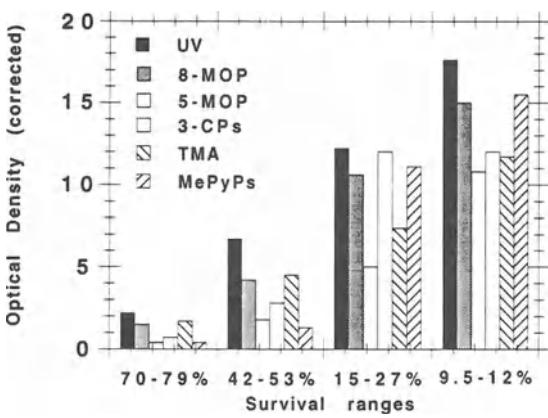


Figure. 5 : Induction of the *RNR2* gene in the haploid *RNR2-lacZ* fusion strain of the yeast *Saccharomyces cerevisiae* after treatments with 254 nm UV and 8-MOP, 5-MOP, 3-CPs, TMA and MePyPs plus UVA radiation at doses leaving survival levels of 70-79%, 42-53%, 22-27% and 9.5-12%.

Induction was determined at peak levels of induction, i.e. after 8 h and 10 h of post-treatment incubation for the two lower (42-53% and 70-79% survival) and the two higher (9.5-12% and 15-27% survival) dose ranges, respectively (see for details, Averbeck and Averbeck, 1994).

signals than mixtures of monoadducts and interstrand cross-links induced by 8-MOP plus UVA, as well as 254 nm UV induced and TMA plus UVA lesions.

Knowing that TMA photoinduces furan side as well as pyrone side monoadducts, photoadditions to membrane components such as fatty acids and was shown to activate protein kinase C signal transduction pathway (Dall'Acqua and Martelli, 1991), it seems possible that, in the case of TMA, the induction signal does not depend so much on the induction of damage to nuclear DNA, but does involve other pathways such as the activation of transcription factors via the protein tyrosine kinase and protein kinase C pathways. In this respect, also 254 nm UV radiation appears to be very effective for gene induction combining DNA damage and other not yet well defined non-DNA damage as inducing signals.

The apparent dose dependence of the gene induction may suggest that not only the type of photolesion induced, but also their local density may determine the induction of the genes. Indeed, higher levels of 3-CPs photoadducts (monoadducts) than 8-MOP photoadducts (monoadducts plus interstrand cross-links) were necessary to induce the *RAD54* gene and, only at higher dosage, 3-CPs plus UVA reaches comparable induction levels of the *RNR2* gene as 8-MOP plus UVA (Table 1).

In conclusion, photoadditions induced by furocoumarins have been shown to act as effective inducers of genes in an eukaryotic cell system, the yeast *Saccharomyces cerevisiae*. At low photoadduct levels, mixtures of interstrand cross-links and monoadducts appear to be more effective inducers than monoadducts. However, at high photoadduct levels, mono- and bifunctional agents show comparable effectiveness for gene induction possibly by interference with cellular signal transduction pathways that are independent of DNA damage.

On the other hand, recent studies on the kinetics of the occurrence of DNA double-strand breaks as repair intermediates in the repair of furocoumarin induced interstrand cross-links, high levels of monoadducts (Averbeck and Dardalhon, 1990 ; Averbeck et al., 1990b, 1992, 1994) and even after UV irradiation (Averbeck et al., 1992 ; Kiefer and Feige, 1993) would suggest that also the induction of double-strand breaks as repair intermediate may not only play a significant role in the induction of genetic damage, i.e. mutations and recombination, but also may provide a signal for gene induction (Averbeck and Averbeck, 1994). In this connection, it is interesting to note that the induction of the *RNR2* gene was very low with ionising (gamma) radiation and practically absent after induction of oxidative damage by photodynamic treatments with benzo(a)pyrene or 1,6-dioxapyrene plus UVA radiation (Averbeck and Averbeck, 1994), known to induce single strand breaks in DNA and a relatively low frequency of mutations (Averbeck et al., 1993).

Thus, as shown above, photoadditions by psoralens constitute photolesions that are specifically effective in the induction of certain genes in eukaryotic cells. The inducing signal may be related to the specific processing of photoadducts leading to the occurrence of double-strand breaks as repair intermediates.

IMMUNOLOGICAL CONSEQUENCES OF PSORALEN PHOTOOADDITION

As shown above, the photoaddition of psoralen can not only give rise to cell defined genetic responses, but also to more subtle cellular responses (Averbeck et al., 1990b ; Averbeck and Averbeck, 1994), such as the induction of genes and activation of the protein kinase C signal transduction pathway (Dall'Acqua and Martelli, 1991). Furthermore, the re-activation of Herpes simplex virus (Coppey et al., 1978 ; James and Coohill, 1979) and activation of SV40 in mammalian cells (Moore et al., 1983) have been reported. In addition, the *in vivo* activation of human immunodeficiency virus type 1 long terminal repeat by UV type A (UV-A) light plus psoralen and UV-B light in the skin of transgenic mice was reported (Morrey et al., 1991). More recently, the human immunodeficiency virus promoter has been shown to be activated by furocoumarins in a human cell line (HeLa) (Zmudzka et al., 1993). Interestingly, at equal concentration, 4,4',6-trimethylangelicin (TMA) was more effective than 8-MOP or 5-MOP and 4,5'-dimethylangelicin (DMA), and both, psoralen photoinduced monoadducts and interstrand cross-links, were able to activate the HIV promoter. The response appeared to be correlated with the photosensitizing potentials and the DNA photobinding constants of the furocoumarins (Gasparro, 1988 ; Rodighiero et al., 1988).

Considering that UV (280-320 nm) radiation results in local and systemic immuno-suppression (Kripke, 1984) and is known to induce pyrimidine dimers and pyrimidine pyrimidone (6-4) photoproducts (Mitchell, 1988), it has not been very surprising

that also treatments inducing bulky adducts in DNA, such as treatments with psoralen and UVA, are able to interfere with normal immunological responses (Horio and Okamoto, 1983 ; Kripke et al., 1983). Altered immune responses are likely to have some bearing on the development of tumors in humans (Stern, 1989 ; Kripke, 1990 ; Young, 1990). *In vitro* studies on epidermal cells showed that, after UV irradiation (Kim et al., 1990), as well as after treatment with psoralens and UVA (Aubin et al., 1991c), immuno-suppressive factors are released from keratinocytes which may include growth factors stimulating the growth of tumor cells (Aubin et al., 1991c), as well as clastogenic factors (Alaoui Youssefi et al., 1994). Studies undertaken with mono- and bifunctional furocoumarins suggest that immuno-suppression can be elicited monoadducts, as well as interstrand cross-links inducing agents (Alcalay et al., 1989 ; Aubin et al., 1991a, b). From this, it can be expected that immuno-suppressive responses may involve not only DNA damage, but also damage to lipids, proteins and membrane receptor sites (Laskin et al., 1985) leading to the activation of genes, transcription factors and signal transduction pathways. To what extent defined psoralen photoadducts are involved in this needs further research work.

CONCLUSION AND PERSPECTIVES

Defined photoadditions to cellular DNA induced by furocoumarins have directed several lines of research work including mechanistic studies on photoinduced genotoxicity and the basis of photochemotherapy. In recent years, the spectrum of furocoumarins application as specific tools for investigating the mechanisms of cellular responses has been widened considerably. Research lines concerning cellular transduction pathways including the activation of genes and modification of immunological responses as well as the activation and inactivation of viruses, such as the human immunodeficiency virus (HIV) are of general importance. In this connection, recent advances in the development of extracorporeal photopheresis techniques for the treatment of certain autoimmune diseases (see for review, Edelson, 1991) and the inactivation of virus particles in human platelets (Wagner et al., 1993) by furocoumarins and UVA are especially promising. In this respect, the use of psoralen photoaddition as diagnostic (Cimino et al., 1990 ; Isaacs et al., 1990) and possible new therapeutic tools (Giovannangeli et al., 1992 ; Sastry et al., 1992 ; Grigoriev et al., 1993) has to be mentioned. Thus, it appears that it is the advanced understanding of furocoumarin photoaddition and furocoumarins plus UVA induced photoreactions with living matter which is promoting further more detailed studies, that are of great relevance for human health.

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PHOTOPIGMENTATION

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INTRODUCTION

The energy levels present in ambient illumination from various sources, particularly from solar irradiation, are sufficiently high to pose a potential hazard to living systems. In the case of man the surface structures exposed to this potential hazard are the eye, which by virtue of its special transparent structure permits a wide range of wavelengths to impinge on sensitive elements such as the lens and the retina, and the skin. Ocular effects of light are dealt with in Chapter 8.

Human evolution has resulted in a primate species which is relatively hairless with the consequence that the skin is relatively exposed and thus susceptible to actinic damage. The damaging effects of light on tissue are fully discussed in other sections of the present volume and consist essentially of direct effects in which light-activated tissue components react with neighbouring molecules to form adducts, including dimerization where the reactants are closely apposed as in the case of the formation of pyrimidine dimers in DNA, or oxygen-dependent effects which may involve singlet oxygen as an intermediate or the formation of peroxides and other reactive (partially reduced) oxygen species (ROS). As a result of such reactions many secondary effects may ensue, such as the induction of new gene products or the enhancement of certain enzymatic activities. Moreover, the products generated by such secondary phenomena may diffuse to surrounding tissues and provoke an amplification of the response which may even have systemic implications. Examples of this are severe inflammatory reactions in sunburn, immune suppression by the release of cytokines from light-damaged epidermis, and of course the possibility of mutations leading to skin cancer. According to figures released by the Government in the U.K. (*Ultraviolet Radiation and Skin Cancer*, Dept. Health PC/CMO (93)6) nearly 30,000 people in England now develop skin cancer each year, a figure which has risen by a third over the last decade. In 1992 there were 1065 deaths from Malignant Melanoma (43% higher than in 1980) and 457 deaths from other types of skin cancer (27% higher than in 1980). These trends are believed to be due principally to the extent to which people with relatively unpigmented skin expose themselves to sunlight. People with darkly pigmented skin are minimally at risk of developing skin cancer and this constitutes the most convincing argument in favour of a photoprotective role of melanin in the skin.

PHOTOPROTECTION

In general terms the natural photoprotection of the skin is due to (1) limitation of light penetration i.e. various forms of light shielding; (2) scavenging of light-induced reactive species; (3) repair of molecular damage caused by light; (4) removal of irreversibly damaged cells.

There is currently much interest in the induction of damage-limiting and repair systems especially in relation to DNA damage (e.g. induction of P53 and excision repair enzymes) and also the possible importance of stress proteins (e.g. ubiquitin) in accelerating the degradation of proteins damaged by light-induced oxidation. Pigmentation does not appear to be closely involved in these light-reactive processes although it is conceivable that the induction of tyrosinase, and tyrosinase-related proteins (see below) could be encompassed in a wider definition of stress-related protein synthesis. However, melanin is certainly implicated in light shielding, scavenging processes and (possibly) in the removal of damaged cells by a process which does not involve apoptosis.

MELANIN

Melanins are indolic polymers of uncertain structure. They seem to have arisen early in evolution and are widely distributed in nature. It has been argued that they are products of orthoquinones which possess strong reactivity towards nucleophiles forming covalent adducts with, for example, thiols and amino agroups. Thus, the primary purpose of the generation of orthoquinones may have been to furnish a protein-tanning agent (see Riley, 1992). Such a reactant may have many important potential functions (Table 1). In vertebrates, melanins are present in many organs that are not normally exposed to light (e.g. the gut, the meninges) suggesting that there are properties subserving functions that have not yet been identified. In man there is pigment present in the inner ear which appears to modify the rate of recovery of acoustic acuity following a loud sound which suggests a possible energy conduction or dispersive role (Barrenas and Lindgren, 1990). In fish and amphibia melanin is one of the pigments present in dermal melanophores and is involved in camouflage and display. (Cott, 1940; Fox & Vevers, 1960, Hadley & Quevedo, 1966).

In humans, melanin is present in hair and conspicuously in the epidermis except of the palms and soles. It has been proposed that this epidermal distribution of melanin in the hairless primate is evolutionarily related to the need to excrete dietarily-derived transition metals by desquamation through an organ of large surface area (Riley, 1992). There is a strong possibility that epidermal pigmentation is able to act as a detoxification pathway, in particular for metals. The skin is the largest organ of the body and the transfer of melanin to cells that are finally desquamated (see below) presents a large potential excretory pathway. It is known that melanin acts as a strong ligand for cations (Felix *et al.* 1978) and this provides a means of chelating and excreting toxic metals and other potentially harmful cationic compounds. The forensic value of estimating lead and arsenic in hair has long been recognized and may be related to the degree of pigmentation. The notion of melanin as an excretory pathway for metals, first advanced by Horcicko *et al.* (1973), is also consistent with the proposed littoral evolution of humans. The theory of littoral evolution of man accounts for several unusual features characteristic of aquatic mammals, such as subcutaneous fat deposits and loss of body hair (see Morgan 1985). The potential of learning manual skills at the seaside seems much more plausible than the hunter-gatherer model which is so frequently depicted as the earliest stage in human evolution. The most plentiful source of food would have been molluscs and these are known to concentrate potentially toxic metals, such as copper (Dodd 1973), hence a mechanism for increasing the bodily excretion of heavy metals, such as would be provided by increased epidermal melanization, may have been of crucial evolutionary importance. I have previously (Riley 1992) drawn attention to the possible connection between increased melanization and iron overload in haemochromatosis. However, there can be little doubt that epidermal melanin serves an important function as a photoprotective pigment.

THE SYNTHESIS AND DISTRIBUTION OF EPIDERMAL MELANIN

The melanin of the mammalian epidermis and epidermal appendages (including hair) is derived from specialized dendritic cells. These cells, the melanocytes, are embryologically derived from the neural crest and migrate during early embryonic development to populate the epidermis. Here they synthesize melanin which is transferred to the epidermal keratocytes (Riley, 1975; Prota, 1992). The transference occurs by a process of phagocytosis of which there are several possible outcomes (Riley 1978). It has previously been suggested (Slater and Riley 1966) that the free radical properties of melanin necessitates

Table 1. Evolutionary Significance of o-Quinones (from Riley, 1992)

Antibiotic	
	Microorganisms (bacteria, fungi)
Plants	
Insects	
Cephalopods	
Tanning	
	Spores and seeds
	Sclerotization of insect cuticle
Precursor of melanin	

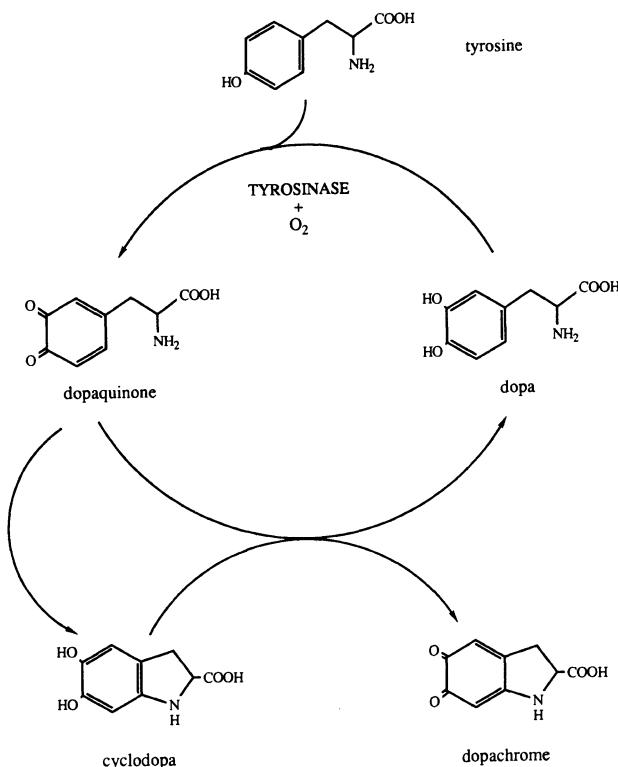


Figure 1. Phase 1 Melanogenesis

Schematic outline of Phase 1 Melanogenesis showing the dopa/ dopaquinone cycle which is driven by the tyrosinase-catalysed oxidation of dopa to dopaquinone. Tyrosine enters the oxidative mechanism by tyrosinase-catalysed conversion to dopaquinone. Dopamine undergoes a cyclization reaction to form cyclodopa which then is converted to dopachrome by a redox reaction with dopaquinone (Chedekel *et al.*, 1984). Dopamine in the process is converted to dopa which can be reoxidised by tyrosinase.

encapsulation of the pigment in order to protect the cells responsible for its synthesis and indeed the reactive nature of the melanogenic intermediates (Hochstein and Cohen 1963) is a further reason for the process of melanogenesis to be conducted in a membrane-bound sequestered organelle. There seem to be three possible topological outcomes of the process of pigment donation depending on the actual process involved. The simplest process would be release of the individual melanin granules to the exterior of the synthesizing cells and their ingestion by the phagocytosing epithelium. An isolated melanin granule coated with a single layer of membrane would be phagocytosed to form a phagosome in which the melanin is surrounded by two layers of membrane. Since the melanin is topographically outside the membrane, the apposed membrane layers have the same polarity and, assuming that there is no impediment to the fusion of these membranes, direct continuity would be established between the melanin and the cytosol of the recipient cell. This may be the case under circumstances such as those in the experiment of Johnson *et al.* (1972) in which melanin was phagocytosed by macrophages. However, it is doubtful whether melanin transfer occurs by this means in normal epithelium. A possible variant of this melanin transfer would be in the case of compound melanosomes (the product of autophagy) which would have four layers of membrane. It can be shown to be topographically equivalent to the case of isolated melanin granules although this process is probably rare. The generally accepted mechanism of melanin transfer from melanocytes to keratocytes is by phagocytosis of a portion of the melanocyte dendritic cytoplasm containing melanin granules. The subsequent fusion of the phagosome membrane with the enclosed melanocyte-derived plasma membrane results in the release of membrane-coated melanin granules. The possibility that by fusion with lysosomes this would be analogous to the uptake of hydrophilic photosensitizers has previously been suggested (Riley 1978). The factors controlling the ingestion of melanocyte dendrites and membrane fusion and hence the state of granule aggregation in the epithelial cells are not known but they appear to be under genetic control (Szabo *et al.*, 1988). The melanocyte population density in the basal layer of the epidermis is essentially similar for persons of quite divergent levels of pigmentation (Szabo, 1967). Szabo has shown that there is a characteristic density of melanocytes for each body region. The regional differences are quite small in the fetus but become accentuated in the adult. Possibly the differences are more apparent than real and may be caused by differences in melanogenic activity and especially in the degree of undulation of the basal layer of the epidermis since the melanocyte density was estimated by the number of cells per unit area of separated sheets of epidermis. By this criterion there are in man about 2,000 melanocytes per square millimetre of epidermis from the head and genitalia and about 1,000 melanocytes per square millimetre in the rest of the body. Melanocytes are also present in mucous membranes but their enumeration is less well-defined. There are no sexual or racial differences in melanocyte population density in the skin. Racial differences in epidermal pigmentation arise from differences in rates of melanogenesis (synthesis of melanin), the type of melanin produced (*vide infra*), and possibly effects of granule size and the degree of granule aggregation in the epidermis.

MELANOSOMES

In melanocytes (and in retinal pigment epithelium, RPE) melanin is synthesized in specialised cytoplasmic organelles known as melanosomes. It is these structures that become progressively pigmented and finally are observed as dark structureless granules (melanin granules) which are either retained in the generating cells (as in RPE and so-called dermal "melanophores") or transferred to surface structures such as skin, hair or feathers by the pigment donation process mentioned above. Melanosomes are formed in melanocytes from at least two vesicles derived from the Golgi (Mishima, 1992). The vesicles carrying the oxidative enzyme of the melanogenic pathway (tyrosinase) are derived from the first lamellae of the Golgi and are known as coated vesicles. It is probable that the other tyrosinase-related proteins, TRP 2 (which is now recognized as the melanogenic intermediate converting enzyme dopachrome tautomerase) and TRP 1 (a protein coded by the "brown" locus in mice possessing an amino-acid sequence similar to tyrosinase but with currently unknown function), are also routed through the coated vesicular traffic. Another vesicle, resembling a secretory vesicle or lysosome is derived from the Golgi and contains an albumin-like protein which is thought to be involved in the formation of an internal protein lamina. There appear to be two basic internal structural arrangements of pre-melanosomes: a granular matrix or a lamellar matrix respectively resulting in spherical or ellipsoid granules. Both can be

generated in the same cell (Jimbow & Takeuchi, 1979). It is possible that zinc is required for the organization of the internal structure of pre-melanosomes (Borovansky, personal communication). In normal melanogenesis there is fusion of the coated vesicles with premelanosomes to give rise to melanosomes proper which are designated in stages I-IV according to their degree of melanization as observed by electron microscopy. Melanin is formed by the oxidation of L-tyrosine and is deposited on the protein stroma. Fully melanised granules (stage IV melanosomes) are devoid of enzymatic activity indicating that the protein-coating process also involves the inactivation of enzymes present in the melanosome.

It has been shown (Mishima *et al.*, 1979) that the coated vesicles possess tyrosinase activity and some melanin precursors are found in these organelles although melanin does not form until fusion occurs with the other vesicular elements. There may be complex control mechanisms that limit the reactivity of melanogenic intermediates in the vesicular compartments (Riley, 1993). These include O-methylating enzymes which can convert any prematurely-formed melanogens into excretable compounds (Smit *et al.*, 1990). Reactions with glutathione have also been proposed as de-toxifying pathways.

MELANOGENESIS

Once the vesicular fusions have taken place the conditions exist in the melanosome for pigment formation to occur. The process of melanogenesis is conveniently divided into three phases. The first phase consists of the (enzymatic) oxidation of L-tyrosine to the corresponding ortho-quinone known as dopaquinone. The enzyme responsible for this oxidation is called tyrosinase. In the absence of tyrosinase no pigment is formed. Albinism is due to a molecular defect in the enzyme with the result that the oxidation is greatly reduced or absent. Tyrosinases are abundant in nature. The human enzyme is a protein of about 60-70 Kd MW with two copper-binding sites. The enzyme requires the copper ions to be in the reduced form in order to bind molecular oxygen which is arranged at the active site in a peroxy conformation (Lerch, 1981; Solomon & Lowery, 1993). The reaction with tyrosine results in a hydroxylation in the *ortho* position followed by a dehydrogenation yielding the quinone as the reaction product. As shown many years ago by Evans & Raper (1937) dopa (3,4-dihydroxyphenylalanine) is generated as a secondary product in the reaction sequence by dismutation of dopaquinone to give dopa and dopachrome (Fig. 1). Dopa is readily oxidised by tyrosinase to dopaquinone by a reaction which donates electrons to the tyrosinase copper atoms and thus "activates" any tyrosinase which is unable to bind oxygen. This process is thought to account for the unusual kinetics of tyrosinase oxidation which exhibits a "lag" or induction period (Naish-Byfield & Riley, 1992). The product of the first phase reaction is relatively stable. Dopachrome is able to exist in the quinone-imine form as a reddish pigment and undergoes slow conversion to 5,6-dihydroxyindole (DHI) with spontaneous release of CO₂. DHI is able to autoxidise rapidly in air to give a black insoluble pigment. However, there is now ample evidence that this is not the major route of biological melanogenesis and it has been shown that there exists an enzyme (dopachrome tautomerase) which catalyses the conversion of dopachrome to DHICA (5,6-dihydroxyindole-2-carboxylic acid) (Aroca *et al.*, 1990). This pathway is the basis of phase 2 melanogenesis followed by (probably indirect) oxidation of DHICA to give the corresponding quinone (Fig. 2). The indirect oxidation is thought to require a redox exchange with dopaquinone (Chedekel *et al.*, 1984), an oxidation reaction vicariously catalysed by tyrosinase (Riley, 1993). The quinone that is formed, indole-2-carboxylic acid-5,6-quinone (ICAQ), is able to exist in 3 isomeric forms: either as the quinone, the quinone-imine or the quinone-methide. There is evidence from pulse radiolysis data (Lambert *et al.*, 1991) to implicate the quinone-methide in the polymerization reactions that lead to dimers or oligomers (melanochrome) and finally the melanin polymer. Phase 3 consists of the polymerization reactions and is currently under investigation. Basically there are two mechanisms currently proposed for the polymerization step, either self-condensation as postulated by Bu'Lock & Harley-Mason (1951), or radical-radical interaction (see Riley 1988). A series of studies have been conducted using pulse radiolysis to examine the reactions of azide-induced one electron oxidation products of DHI and DHICA and the more stable model compound N-methyl-5-dihydroxiindole (Lambert *et al.* 1989; Lambert *et al.*, 1990; Al-Kazwini *et al.*, 1990) which indicate that the likely mechanism for the second order decay of the radical derived from

dihydroxyindole is by disproportionation giving rise to 5,6-indole quinone and, if this is so, the lack of variation in the rate of decay of the quinone with the dose excludes a process of melanochrome formation by self-condensation. Prota and co-workers (Corradini *et al.* 1986; d'Ischia and Prota 1987) have isolated in low yield several types of indole dimer after oxidation of dihydroxyindoles and have suggested that these are formed by coupling of the corresponding phenoxyl radicals. Structures of possible unstable dimers which might isomerise unimolecularly have been suggested by Land (1988). Various arguments based on kinetic data (see Bensasson *et al.*, 1993) seem to exclude significant polymerization by condensation of semi-quinones or by a reaction of semi-quinone with DHI, or a reaction of 5,6-indole quinone or its tautomers with DHI. The pulse radiolysis data suggest that polymerization may occur by a reductive addition of a tri-hydroxyindole to the 5,6-quinone, or to its isomeric quinone imine, or possibly more reactive quinone methide. The overall scheme for phase 3 melanogenesis, or at least its initiation as suggested by Lambert *et al.*, (1989), is shown in Figure 3. The nature of the intermediates and the reaction mechanisms leading to the final melanin polymer are discussed by Prota (1992). Clearly any theory of the polymerization mechanism will need to embrace the reaction with the protein matrix of the melanosome and it may be that the nature of the matrix proteins will hold the clue to the polymerization process *in vivo*.

TYPES OF MELANIN

In describing the melanogenic pathway we have so far concentrated only on the so-called eumelanogenesis: the formation of the dark brown or black pigment characteristic of highly pigmented races. At the other end of the colour spectrum are the phaeomelanins (see Thomson, 1974) which are defined by their sulphur content. These pigments are red or yellowish and result from interaction of dopaquinone with cysteine to give the corresponding cysteinyl adduct. This pathway in its classical form leads to benzothiazine pigments characteristic of red chicken feathers (Minale *et al.*, 1969). There is evidence that in most cases of melanogenesis there is some admixture of phaeomelanins in the polymer - a process known as "intermeshing". For purposes of classification "pure" eumelanin may be regarded as predominantly a polymer of indole-2-carboxylic acid quinone (ICAQ). "Hypo-indolic" melanin contains other intermediates in the oxidation pathway. Pure phaeomelanin by such a definition would consist of a polymer of cysteinyl dopaquinone. At present it is considered that the melanin polymer is composed of an assortment of intermediate oxidation products of tyrosine incorporating more or less cysteine.

Several studies have been made of the possible crossbinding between the monomers in melanin. It is probably enough to state that at present the structural details are not known. Some physical evidence has been adduced that there is 3-4 Å spaced stacking of "sheets" of the polymer (Thathachari, 1976), and it is known that some melanins (generally synthetic melanin produced by the auto-oxidation of dopa) have properties of an amorphous semiconductor (Proctor *et al.*, 1974). Melanin has unusual light absorbing properties by virtue of its high degree of conjugation and it is this property that is perhaps the most significant with regard to skin photoprotection (Kollias *et al.*, 1991).

SKIN PHOTOPROTECTION

Light Shielding

The distribution of melanin in the form of granules in the epidermis permits it to function as a light shielding pigment both by absorption of photons and by a light-scattering effect. Light scattering is most significant in the blue part of the spectrum (Rayleigh scattering) and is a function of the size and aggregation of the granules. However, as is evident from the pallor of albino skin which contains the granules in an unmelanized form, the major light shielding effect is the result of photon absorption by melanin. Melanin has efficient photon-phonon coupling which enables photonic energy to be readily dissipated as heat. The distribution of melanin in the keratocytes throughout the epidermal thickness limits light penetration to the generative cells in the basal layer and into the upper dermis. There can be little doubt that this is a major and significant function of epidermal melanin in man, although the evolutionary importance of this light barrier function has been disputed (Riley,

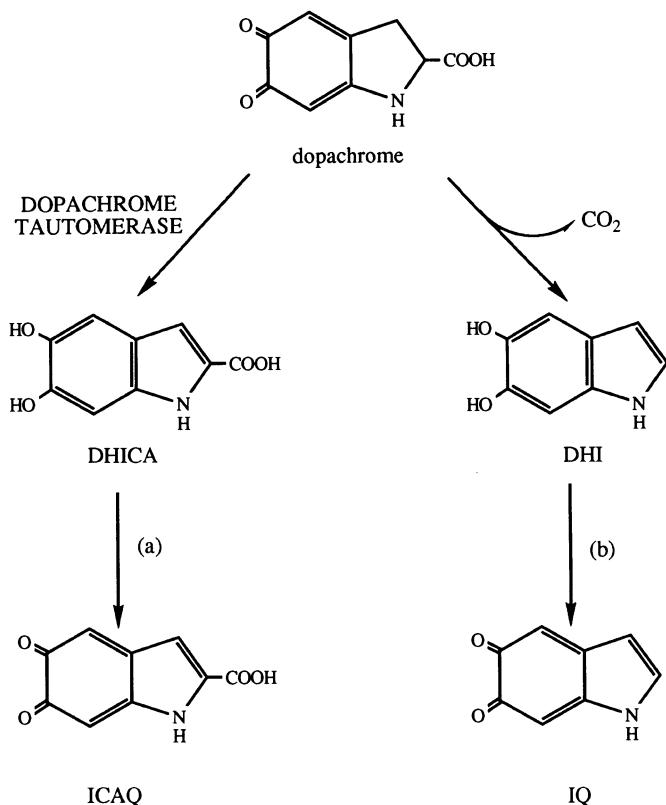


Figure 2. Phase 2 Melanogenesis

The schematic outline shows that dopachrome undergoes a rearrangement to form dihydroxyindole-2-carboxylic acid (DHICA) which is catalysed by the enzyme dopachrome tautomerase. (a) DHICA is oxidised, probably by redox exchange with dopaquinone, to form the corresponding quinone i.e. indole-2,carboxylic acid-5,6-quinone (ICAQ). The alternative pathway from dopachrome is by spontaneous decarboxylation to give rise to 5,6-dihydroxyindole (DHI) which (b) can undergo spontaneous autoxidation to give rise to indole quinone (IQ).

1992) on the basis that, whilst present-day epidemiological evidence makes it clear that skin pigmentation is protective against genetic damage giving rise to skin malignancies, in selective terms this effect is insignificant, as Haldane (1949) pointed out many years ago, since skin cancer is of relatively late onset and thus unlikely to have any significant impact of procreation. Other possible functions subserved by epidermal pigmentation might be an influence on body temperature regulation (Barnes, 1963) and could have evolved as a heat loss mechanism. In addition, there is the conventional explanation regarding the need for cutaneous synthesis of Vitamin D (Loomis 1967) which may have had some influence on the degree of epidermal pigmentation, particularly in races migrating to high latitudes where the incidence of solar radiation is less and where the incidence of childhood rickets may have had some significant effect on an evolutionary timescale.

Scavenging Functions

As previously mentioned, natural melanins are anionic polymers being largely composed of subunits with free carboxylic acid groups and have significant ion exchange properties (Sarna *et al.*, 1976). Many cationic dyes are known to bind strongly to melanin by ionic bonds and melanin is a powerful chelator of metals.

Metal chelation, in addition to the possible excretory function already alluded to, may be beneficial to melanized cells since it may help to diminish the availability of free transition metal ions in other cellular locations where they may act as single-electron reductants of peroxide e.g. in the generalized Fenton reaction which yields the hydroxyl radical:



Since HO^\cdot attack on sensitive molecules is a significant source of disruption of normal cellular processes the metal chelating property of melanins may be regarded as an important indirect cellular antioxidant. There is evidence that melanin is able to act as a reducing agent with respect to bound metals which would favour oxidising reactions of the type mentioned above occurring vicinal to the pigment (Pilas *et al.* 1988). This probably accounts for the effects of H_2O_2 on melanin pigmentation (e.g. in peroxide blondes). Although the details of the mechanisms have not been fully delineated it is likely that, in the presence of reduced transition metals bound to melanin, reaction with H_2O_2 will generate hydroxyl radicals in sufficient density to attack and fragment the polymer with consequent loss of the extended conjugation and hence a diminution of the light absorbent properties. Such peroxide "bleaching" of melanin may be an example of a protective property of melanin as an alternative and dispensable target for oxidative attack.

Another aspect of the scavenging role of melanin is the ability of the polymer to act as a free radical sink. The system of conjugation between (essentially) catecholic and orthoquinone subunits permits ready delocalisation of electrons so that the polymer can exist in an equilibrium state (Sealy *et al.* 1980) between the oxidised and reduced form with a pH-sensitive steady-state level of semiquinone radicals:



(reduced) (semiquinone) (oxidised)

It has long been recognized that melanin has free radical properties (Mason *et al.*, 1960; Longuet-Higgins, 1960; Commoner *et al.*, 1964) and this may enable the polymer to trap extraneous radicals by radical-radical interactions:



or alternatively by acting as a facile electron donor or acceptor, e.g.:



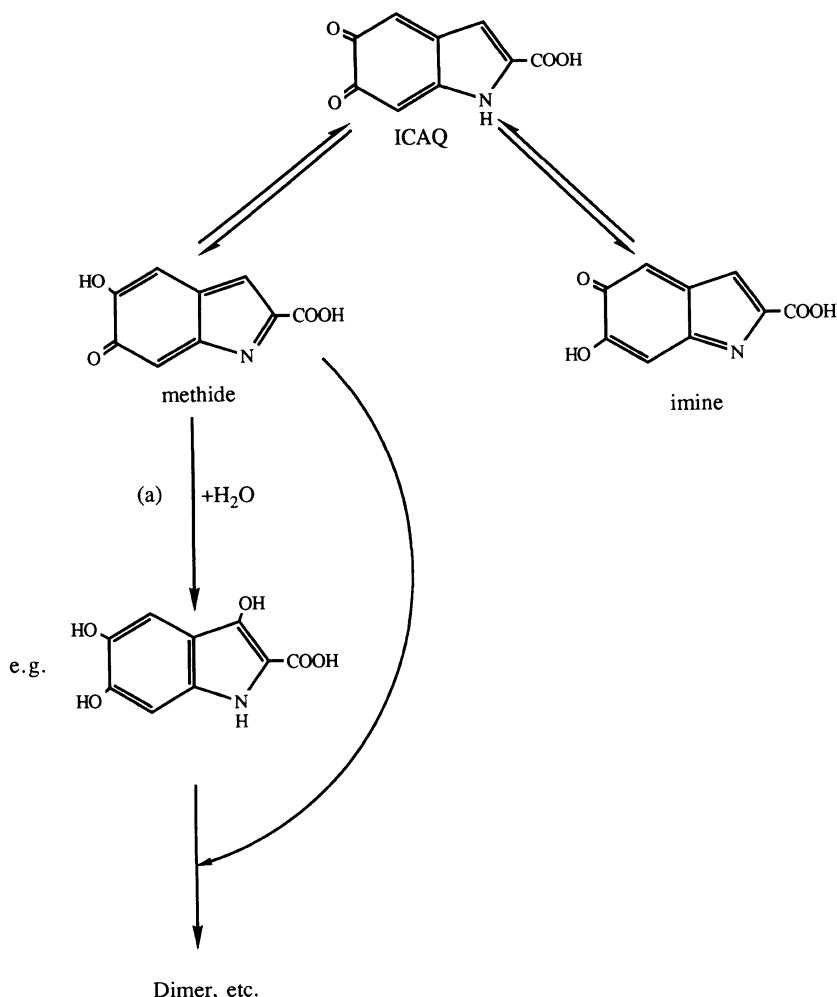


Figure 3. Phase 3 Melanogenesis

The scheme shows the possible initiation of polymerization as proposed by Lambert *et al.*, (1989). Indole-2-carboxylic acid-5,6-quinone (ICAQ) may exist in three isomeric forms of which the imine is regarded as the most stable and the methide the most reactive. The methide (a) may undergo facile nucleophilic addition, for example with water, to give rise to a tri-hydroxy compound which (b) can condense with a further molecule of methide to form a dimer and this may initiate the process of polymerization to form melanin.

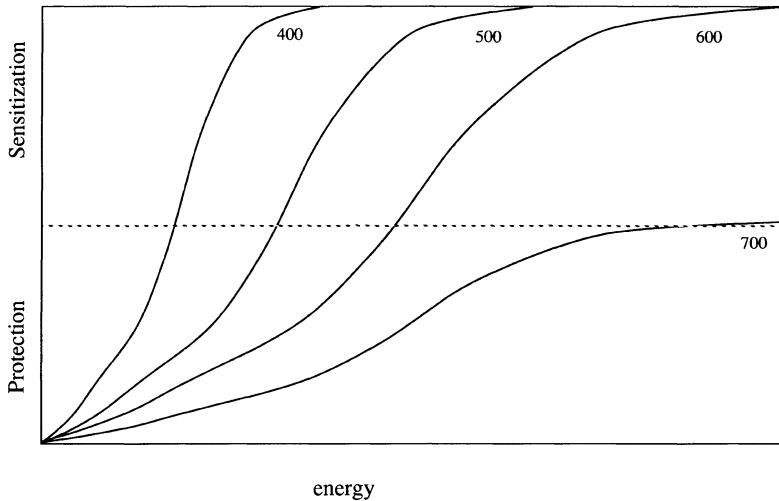


Figure 4. Cross-over Effect of Melanin: Conversion from Photoprotective to Photosensitizing Role

The Figure (based on Riley (1973)) suggests that the extent of energy absorption by melanin may convert the pigment from a protective pigment in which the absorbed energy is dissipated as heat to a photosensitizing pigment when sufficient energy is present in the incident radiation to generate high radical concentrations and hence raised intracellular concentrations of reactive oxygen species. The effect would be expected to be a function of the wavelength of the incident radiation indicated for the purposes of illustration as 400, 500, 600 and 700 nm.

The ability of the semiquinone radicals, which give rise to the ESR signal of melanin, to react with extraneous materials naturally depends on the accessibility of the semiquinone radical. For example, Blois *et al.* (1964) have argued that the ESR signal is due to unpaired electrons in semiquinone radicals embedded within the polymer and inaccessible to chemical change. However, the semiconductor properties of melanins make it likely that delocalization of these electrons throughout the length of the polymer will enable the redox equilibrium postulated by Sealy *et al.*, (1980) to exist with accessible sites at the surface of the polymer. Sarna *et al.*, (1980) have shown that the free radical population in melanin suspensions is a function of the pH of the medium and Felix *et al.* (1978) have shown that the ESR signal of melanins is enhanced by diamagnetic ions. Both of these phenomena imply effects on accessible sites, although the nature of the interaction is not clear. In the case of metal ion interactions this may be due to binding to non-radical sites with effects on the pKa or other interactions (see Sarna *et al.*, 1976).

Of course, the potential ability of melanin radicals to generate secondary reactive species needs to be recognized and, as mentioned below, may account for possible photosensitizing effects.

Removal of Damaged Cells

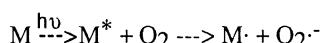
One of the most biologically evident aspects of epidermal pigmentation in man is the inverse correlation between the degree of melanization and the incidence of cancers of the skin, in particular basal cell carcinoma, squamous cell carcinoma and melanoma. It has been proposed that this is the result of melanin acting as a means of initiating a destructive mechanism in cells that have been irradiated with light of sufficient energy to cause damage to the genome of sufficient severity to result in deleterious mutations (Riley 1973). There is considerable evidence that melanin is able under certain circumstances to act as a photosensitizer (Hill & Hill, 1987). The process may be regarded as a threshold phenomenon with melanin acting as a protective pigment at low energy levels but becoming sensitizing at high energy levels. Clearly the type of response will be dependent on the wavelength (see Fig. 4) since the quantum efficiency of energy capture will be a function of the absorbance of melanin which increases with the frequency of the incident radiation.

One of the characteristic features of melanin (see Enochs *et al.*, 1993) which has long been recognized (Pathak & Stratton, 1968; Norins, 1962) is the large increase in the ESR signal, and therefore presumably in the proportion of semi-quinone radicals present in the polymer, that is generated by illumination. This implies a photooxidation with electron loss to suitable surrounding acceptor molecules. The effect appears to be oxygen-dependent (Sarna *et al.*, 1984) and therefore one of the most important electron acceptors is oxygen giving rise to the formation of superoxide and hydrogen peroxide (Felix *et al.*, 1978b). The essence of the proposal outlined in Figure 4 is therefore that under circumstances of relatively low energy absorbance, the amounts of reactive oxygen species generated are insufficient to damage cells, but under conditions of high energy absorption sufficient ROS are generated to place the cells in a condition of oxidative stress resulting in oxidative damage such as lipid peroxidation, etc. that may be fatal to the melanin-containing cells. Moreover, peroxide-induced degradation of melanin (*vide supra*) may release transition metals into the cytoplasm of oxidatively stressed cells, thus amplifying the potential damage.

PHOTOPIGMENTATION

In relation to melanin there are two principal pigmentary responses of illuminated epidermis: (1) Immediate Pigment Darkening; and (2) Increased melanin synthesis.

Immediate Pigment Darkening (IPD). On exposure to UVR there is a rapid tanning response in skin. This phenomenon is probably more spectacular *in vivo* than *in vitro* and may be in part due to alterations in upper dermal blood flow as part of an erythematous response. Nevertheless, as mentioned above, it has been shown that irradiation of melanin produces an oxygen-dependent increase in absorbance and a rise in the free radical content. It is probable, therefore, that the process is described by:



with internal adjustment of the redox balance of the polymer towards the more oxidized (quinonoid) form. The increase in the proportion of carbonyl functions in the pigment results in a stronger absorbance especially at longer wavelengths (a phenomenon described as bathochromicity). Presumably, in the cellular environment the redox status will determine the absorption characteristics of melanin and this can be rapidly altered by any oxidative stimulus including light.

Melanin Synthesis

About 10 days after exposure to light with a significant proportion of UVA in the spectrum, there is evidence of an increase in epidermal melanization. This is due to the synthesis of new pigment by the melanocytes and the transfer of melanin granules to the epidermal keratocytes. The most significant elements in this photopigmentary response are those responsible for the induction or up-regulation of melanogenesis. This involves expression of the tyrosinase gene and also the related genes involved in the synthetic processes leading to the generation of melanin granules previously described.

At the present time the controls of these processes are not known. In skin the new synthesis of melanin is maximally stimulated in the spectral region 290-320 nm. Although there is an effect due to exposure to UVA, the response involves stimulation of tyrosinase activity in basal melanocytes which can be observed by an increase in dopa-positive cells in skin split preparations (see Pathak, 1985). There is no evidence for an increase in melanocyte mitosis, although Rosdahl (1979) reported that repeated UVB irradiation increases the melanocyte population not only in exposed skin but also in unexposed areas suggesting the release of a melanocyte mitogen in the course of epidermal cell damage. It seems probable, in view of the relationship between the erythematous response to UV irradiation and long-term pigment synthesis, that the stimulation of melanocyte activity is the result of the release of inflammatory mediators from damaged epidermis, including prostaglandins and cytokines. It has been shown however, by Friedmann and Gilcrest, (1987) that UVB applied directly to melanocytes can stimulate melanogenesis and Bologna et al., (1989) have raised the possibility that UV-induced melanogenesis is mediated through stimulation of MSH receptors, possibly by increasing the availability of the hormone. Seechurn & Thody, (1990) however have shown that α -MSH is not involved in UV-induced melanogenesis *in vivo*. Nordlund *et al.* (1986) have shown that prostaglandin E2 and arachidonate increase proliferation of epidermal melanocytes although MSH does not. The mechanisms involved in signal transduction are also not clear and there are conflicting reports about the effect of other potential mediators such as Vitamin D3. Suffice it to say that at the present time the mechanisms that determine the reaction of melanocytes to epidermal cell damage are unknown and much further work will be necessary to furnish the understanding of the controlling mechanisms responsible for the melanogenic response of the skin to light.

CONCLUSIONS

It seems likely that in evolutionary terms melanogenesis is a by-product of a mechanism for generating reactive orthoquinones. It is clear that the resulting light-absorbing pigment has been put to a number of important biological uses, some of which are still to be identified. It is argued that the epidermal pigmentation of man is the result of evolutionary pressures which are unrelated to photoprotection, but there is undeniably an important photoprotective role of melanin in preserving the structural and genetic integrity of the exposed human skin. This photoprotective role comprises light shielding, scavenging of metals and light-induced radicals, and possibly the initiation of irreversible cytotoxic damage in overexposed cells.

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SYSTEMIC EFFECTS FROM PHOTOTOXIC DRUGS

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INTRODUCTION

Xenobiotics extensively used in *e.g.* drugs, cosmetics, food and agriculture chemicals, can produce adverse biological effects. When light is an essential condition for its toxicity the xenobiotic is called phototoxic.

The reader is referred to a review article¹ for information about important points such as phototoxic *versus* photoallergic xenobiotics and phototoxicons *versus* phototherapeutics. Besides, attention is given in that review to the sequence of events which starts with the absorption of a photon of UV-radiation or visible light by the phototoxicon and which eventually leads to the adverse biological effects. Further, the primary photoreactions and secondary molecular processes which the xenobiotic can undergo, the molecular targets in a biological system and the ultimate phototoxic effects are dealt with. The foregoing is illustrated with a number of chemicals with completely different molecular structure.

In this chapter emphasis will be put on the possibility that phototoxic effects do not only concern the skin or eyes but can also affect inner organs: *systemic* effects. This will be illustrated with some *N*-oxidized compounds *e.g.* nitroarenes and imino-*N*-oxides.

N-OXIDIZED COMPOUNDS

N-oxidized compounds find extensive application as constituent of for instance drugs, cosmetics, food and agriculture chemicals. Although very useful and almost indispensable they can also produce adverse biological effects. These toxic (side-) effects include genotoxicity, for instance carcinogenicity, but also immune diseases such as allergy. Enzymatic activation of *N*-oxidized compounds as a possible cause of this, already received much attention. It is assumed that reactive intermediates or instable products formed during (minor) metabolic processes damage biomacromolecules of *e.g.* the genetic or immune system.

That biological effects can also result from reactive intermediates which are non-enzymatically formed in (sun)light exposed parts of the body will be shown with some representatives of two classes of compounds: nitroarenes and imino-*N*-oxides. Most of the examples dealt with are drugs, while some of them concern chemicals which find extensive application in pig and poultry feed. In each case the photochemistry will be discussed and special emphasis will be put on reactive intermediates formed on irradiation. The possibility of a connection between photoreactivity and toxic effects reported will be investigated. As

will be shown, light can be a contributing factor in the occurrence of toxic effects which concern inner parts of the body.

Nitroarenes

Nifedipine. This drug belongs to an important group of calcium antagonists of which the prototypes had already been synthesized in the sixties but of which new therapeutic possibilities were discovered in the early seventies. They are used in the treatment of angina pectoris and arterial hypertension.

Nifedipine (NIF) is extremely sensitive to ultraviolet radiation and to visible light up to 450 nm. The quantum yield for photodegradation is ~0.5²; statistically this means that of every two photons absorbed, one causes decomposition of a NIF molecule.

The *ortho*-nitrophenyl derivatives, NIF and nisoldipine appear to be far more photolabile than corresponding dihydropyridines with a *meta*-nitro group e.g. nicardipine, nitrendipine and nimodipine (Figure 1³). An explanation for this may be an intramolecular redoxreaction which simultaneously leads to reduction of the nitro group to nitroso and oxidation of the dihydropyridine ring to pyridine. This redoxreaction proceeds more smoothly if both groups involved, nitro and dihydropyridine, are at small distance from each other.

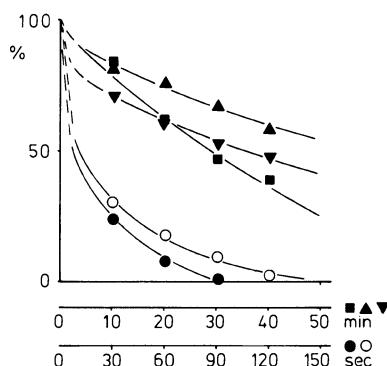


Figure 1. Photodecomposition of nifedipine [●], nisoldipine [○], nicardipine [■], nimodipine [▲] and nitrendipine [▼] in aqueous solution (10^{-2} mg mL⁻¹) as a result of exposure to UVA (70 W m⁻²; 345-410 nm; maximum, at 370 nm.)

By far the main product of the photodecomposition is a nitroso compound^{2,4} (Figure 2). De Vries and Beyersbergen van Henegouwen³ found that in water/methanol 50/50 the UVA-induced conversion, to this 4-nitrosophenyl derivative of NIF (NONIF), is even quantitative (more than 99%). The structure of NONIF was established by NMR and mass spectrometry. However, when NIF is irradiated in (rat)blood *in vitro* NONIF can not be detected. Instead of this, there is another compound which appears to be the only product by HPLC analysis. The structure of this photoproduct (NHNIF) was determined by NMR and mass spectrometry. That UVA exposure of NIF in blood produces NONIF as an intermediate was established as follows. In the presence of glutathione (GSH), of which the concentration in blood is ~ 10^{-3} M, UVA exposure of NIF in water/methanol 50/50 gives NHNIF as the only product. On the other hand, if GSH is added to a water/methanol 50/50 solution which contains NONIF as the only product, NHNIF is formed quantitatively within a few seconds.

As NIF is complexed with plasma proteins for ~95%, its photolability and high photoreactivity of its first and main photoproduct, NONIF, implies the possibility of irreversible photobinding to proteins. This has been found indeed by several investigators^{5,6,7}. Irreversible photobinding to protein may provide an explanation for photosensitivity associated with NIF which has been reported several times⁸.

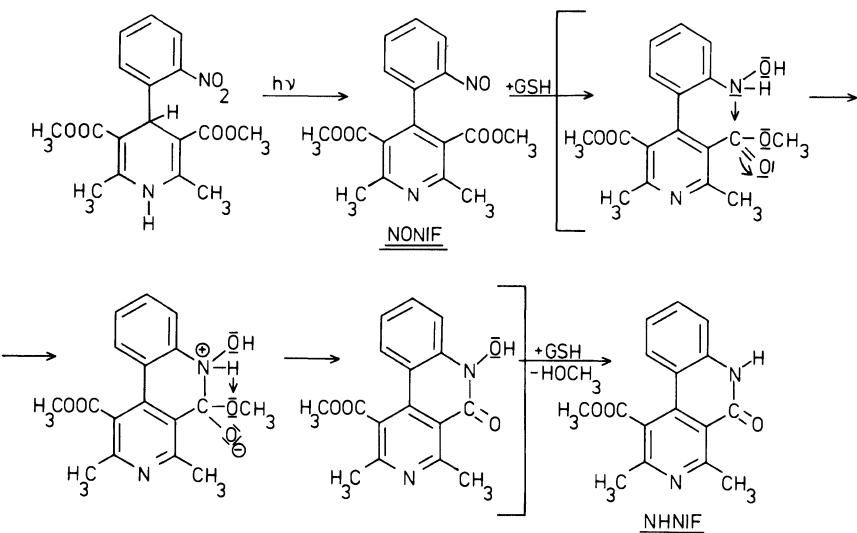


Figure 2. Light-induced formation of NHNIF from nifedipine in blood (in parentheses: supposed intermediates).

Preliminary results indicate that the photoreaction of NIF, with NHNIF as an endproduct, also takes place *in vivo*⁹. Besides, the clearing of NIF from the blood is faster in the UVA-exposed rat than in the animal kept in the dark⁹.

Chloramphenicol. Chloramphenicol (CAP) is an antibiotic effective against a wide range of life-threatening bacteria. It is administered systemically, *e.g.* in the treatment of central nervous system infections, but also topically, *e.g.* against deep ocular infections, where it is a first choice drug. CAP is a bone marrow toxicon affecting primarily the erythroid precursors. Although rare, anemia as a side effect of CAP can be fatal; this has also been observed after topical application¹⁰.

It is supposed that reactive intermediates damage the bone marrow cells. One of the routes, along which these reactive intermediates may occur, is metabolic reduction of the nitro group in the liver. In this regard, *p*-nitrosochloramphenicol (pNOCAP) has been extensively investigated; *in vitro* it proved to be a very potent bone marrow suppressing agent¹¹.

That reactive intermediates can also be formed as a result of exposure to sunlight was pointed out by De Vries et al.¹². They showed that CAP *in vitro* was photodecomposed for ~80% on exposure to sunlight for 45 min (UVA = 14 W m⁻²). The initial concentration of CAP (10 mg L⁻¹ in phosphate-buffered saline, PBS) was comparable with that found in blood after systemic administration or in the aqueous humor of the eye up to one hour after topical application. In addition to the 20% CAP remaining, 25% *p*-nitrobenzaldehyde (pNB), 36% *p*-nitrosobenzoic acid (pNOBA) and 15% *p*-nitrobenzoic acid (pNBA) were determined (Figure 3).

The rapid photodecomposition of CAP under conditions relevant to the *in vivo* situation, and the reactivity of some of the photoproducts necessitated further research¹³. The toxicity of the photoproducts towards bone marrow cells *in vitro* was determined. For CAP and pNB, the values found correspond quite well with those reported in literature¹⁴ (Table 1).

As can be seen pNB and pNOBA are far more potent bone marrow depressants than CAP itself with a similar value to pNOCAP (1.2).

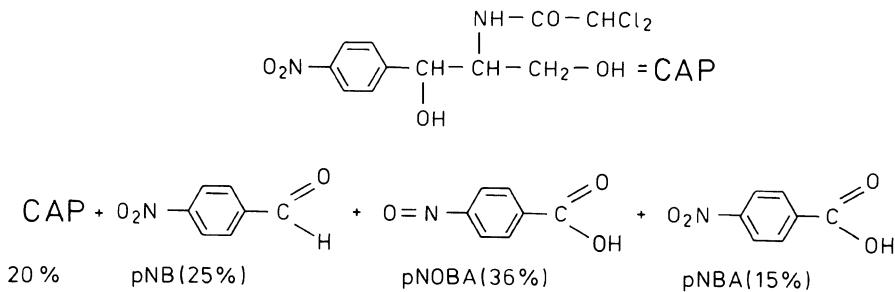


Figure 3. Composition (mol%) of a CAP solution (10 mg L^{-1} in PBS) after exposure to sunlight ($\text{UVA} = 14 \text{ W m}^{-2}$) for 45 min. CAP = chloramphenicol, pNB = *p*-nitrobenzaldehyde, pNOBA = *p*-nitrosobenzoic acid, pNBA = *p*-nitrobenzoic acid.

To act as a bone marrow toxicon, a reactive intermediate should be stable enough to be transported by the blood from its site of formation to the target tissue. Eyer et al.¹⁵ found that pNOCAP was rapidly eliminated from human blood (half life is less than 10 s). For this reason, they found it unlikely that pNOCAP, enzymatically formed in the liver, could be the cause of bone marrow depression by CAP. In this regard, the photoproducts pNB and pNOBA seem to be more serious candidates; their half life in human blood appeared to be 1.7 min and 3.7 min respectively. Thus, once formed photochemically in the skin they are stable enough to reach the bone marrow.

Table 1. *In vitro* bone marrow cell depressing activity (represented is % ${}^3\text{H}$ -thymidine incorporation; control without compound is put at 100%)

Abbreviations (also see Fig. 3): pHABA = *p*-hydroxylaminobenzoic acid, pABA = *p*-aminobenzoic acid, pNBOH = *p*-nitrobenzylalcohol; n.d., not determined; n.a., not available.

Compound	% ${}^3\text{H}$ Thymidine incorporated ¹³	% ${}^3\text{H}$ Thymidine lit. values
pNOCAP	n.d.	1.2 ¹¹
CAP	44	42.6 ¹⁴
pNBA	57	n.a.
pNB	2	1.7 ¹⁴
pNOBA	7	n.a.
pHABA	7	n.a.
pABA	73	n.a.
pNBOH	73	n.a.

In the presence of glutathione (GSH), endproducts of the photoreaction of CAP are *p*-nitrobenzoic acid (pNBA), *p*-nitrobenzyl alcohol (pNBOH) and *p*-aminobenzoic acid (pABA). pNB and pNOBA are intermediates only; dissolved in PBS containing glutathione, pNB appeared to be converted to pNBA and pNBOH, while pNOBA gave pABA¹³. It was proved, also by others¹⁶, that *p*-hydroxylaminobenzoic acid (pHABA) is an intermediate in the conversion of pNOBA to pABA. The *in vitro* bone marrow cell depressing activity of pHABA is comparable to that of pNOBA (Table 1).

UVA-exposure of CAP in rat blood produced pNBA, pNBOH and pABA. Incubation of possible intermediates in the dark gave the same results as described for the photoreaction in PBS with GSH, indicating the same reaction mechanism¹³.

With regard to the side effects of CAP, irreversible binding to plasma proteins and cell constituents in the blood is considered important. Irreversible binding is likely to occur simultaneously with the formation of the reactive intermediates mentioned, pNB, pNOBA

and pHABA. This was found indeed when ^3H -CAP in human blood was irradiated with UVA *in vitro*; irreversible binding was found to plasma proteins as well as to cell constituents¹³.

In various adverse effects from drugs, oxidative stress plays a key role. To investigate whether this may be relevant to the occurrence of the side effects of CAP, photoproducts were tested for their ability to generate oxidative stress¹³. This was done by the determination of the amount of methemoglobin (MetHb) formed during incubation of the photoproducts in blood (Table 2).

Table 2. Generation of methemoglobin (MetHb as % of total Hb) in rat blood by CAP and photoproducts (0.1 mg mL^{-1}). UVA: 60 W m^{-2} ; 345-410 nm; maximum, at 370 nm; 30 min. Abbreviations see Table 1.

	% MetHb
Untreated blood	0.3
Blood + UVA	1.1
CAP	0.2
CAP + UVA	4.3
pNBA	0.2
pNOBA	30.9
pHABA	16.1
pABA	0.2
pNBOH	0.3

Only the photoproducts pNOBA and pHABA, both without irradiation, appeared to be able to generate considerable oxidative stress. This indicates that the photoproducts pNOBA and pHABA play an important role in the occurrence of oxidative stress resulting from CAP + UVA.

To determine whether oxidative stress also occurs *in vivo*, Wistar rats, whose backs were shaved, were given intraperitoneally (i.p.) 100 mg CAP and exposed to UVA (45 W m^{-2} ; 345-410 nm; max. at 370 nm) for 4 hours. This resulted in 3.6 times the basic level of MetHb¹³.

Further evidence that CAP photodecomposes indeed *in vivo* was investigated as follows¹³. Each of six rats (200 g) of which the back was shaved, was given 30 mg ^3H -CAP (i.p.). Three of them were exposed to UVA (50 W m^{-2} ; 345 - 410 nm; max. at 370 nm) for 10h and three kept in light-poor environment. This was repeated the next day after which the rats were killed. After dialysis under non-equilibrium conditions, irreversible bound CAP was determined and expressed per mg protein (the latter is a measure for the amount of dorsal skin taken). Values found were 463 ± 13 and 51 ± 15 disintegrations per minute (dpm) per mg protein for the UVA-irradiated and non-exposed rats respectively. Irreversible binding found *in vivo* corresponds with data from *in vitro* experiments with blood and evidences that the photodecomposition proceeds via the formation of reactive intermediates derived from CAP such as pNB, pNOBA and pHABA.

The results from the *in vitro* and *in vivo* photochemical research described above, justify the conclusion that the possible relationship between sunlight exposure and anemia, as a systemic side effect of CAP, deserves further research.

Nitrofurantoin. The urinary tract disinfectant nitrofurantoin (NFT) is notorious for its high incidence of serious side effects. Of these side effects, 40% are thought to be allergic reactions especially concerning the lungs and the skin.

NFT is also known to cause a considerable number of hematologic reactions; no less than 20% of the fatalities by NFT are due to blood dyscrasias. Blood dyscrasias include methemoglobinemia, a clinical situation in which more than 1% of the blood hemoglobin has been oxidized to the ferric form.

Incomplete metabolic reduction of the nitro group of NFT in the liver resulting in reactive intermediates, has been proposed as the origin of most of the side effects of this drug. However, the exact mechanism involved is not known.

In this context and taking into consideration that NFT is photolabile on irradiation with UVA, the possible role of light in the activation of NFT was investigated.

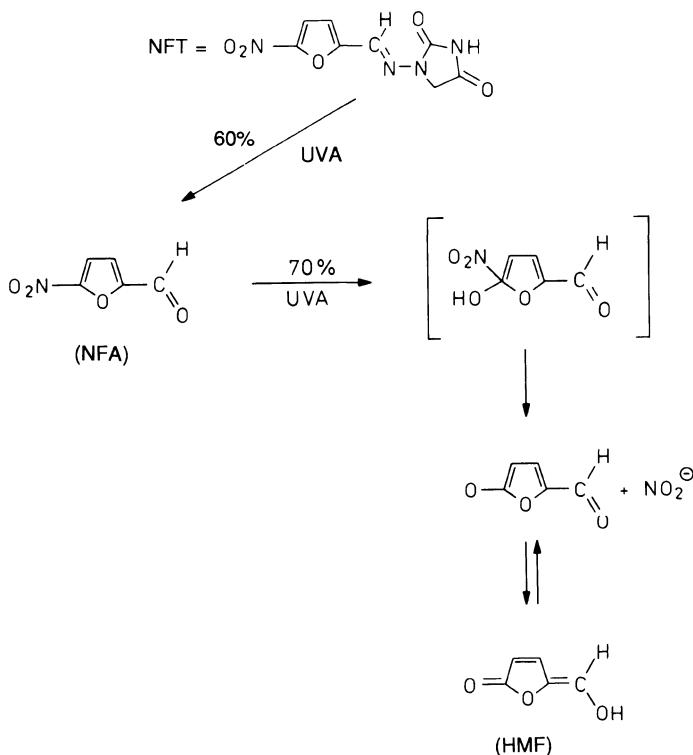


Figure 4. Photodecomposition of nitrofurantoin (NFT) into 5-nitrofurfural (NFA) and 5-hydroxymethylene-2(5H)-furanone (HMF). See text.

On UVA-exposure of an NFT solution in PBS (1 mM, pH = 7), 60% of the photodecomposed starting material appeared to be converted into 5-nitrofurfural (NFA). NFA is also photolabile. Under the same conditions, up to 70% of NFA loses nitrite with formation of 5-hydroxymethylene-2(5H)-furanone (HMF). Extensive research of product formation and reaction kinetics has revealed that HMF is a tautomer of 5-hydroxyfurfural and that the conversion of NFA into the latter aldehyde probably proceeds via nucleophilic substitution with a σ complex as an intermediate¹⁷ (Figure 4).

As nitrite is a photoproduct of NFA (40 mol%) and a well known inducer of methemoglobin (MetHb), it was investigated whether photodecomposition of NFT in human blood *in vitro* results in increased MetHb and nitrite levels. A considerable increase in both MetHb and nitrite concentration was only observed after simultaneous exposure to NFT and UVA. The first photoproduct of NFT, NFA, was found to play an important role in the formation of MetHb¹⁸.

To investigate the possible relationship between exposure to (sun)light and methemoglobinemia (as a side effect of NFT), *in vivo* experiments were performed with rats¹⁸. The experimental conditions and results are summarized in Table 3. NFA was given intraperitoneally (i.p.) instead of orally (p.o.) and in a lower dose per day, because it is a (photo)metabolite of NFT. Exposure to UVA alone (B) or administration of NFT or NFA without exposure to UVA (C and E respectively) gives a MetHb level which does not differ significantly from the normal value (A) (For more detailed values see¹⁸).

The rat can nullify an increased MetHb level very efficiently and is not the most suited species for this experiment. Nevertheless the increase of the level of MetHb is clear; it only occurs as a result of simultaneous exposure to NFT (or NFA) and UVA (Table 3, D and F respectively).

Together with the *in vitro* photochemical data mentioned above, these *in vivo* experimental results strongly points to a relation between (sun)light exposure and methemoglobinemia as a systemic side effect of NFT.

Table 3. MetHb level of rats after the treatment indicated (normal MetHb = 0.5%; also see text). ^a UVA exposure: 25 W m⁻²; 320 - 410 nm; maximum at 370 nm; for 12 h day⁻¹. ^bp.o., oral administration; i.p., intraperitoneally. ^c C and D (as well as E and F) differ significantly: p < 0.01; Student t-test¹⁸.

Experiment	Compound	Days	Dose (mg day ⁻¹)	UVA ^a	MetHb (%)	n
A	-	4	-	-	0.5	10
B	-	4	-	+	0.6	10
C ^c	NFT	4	12 (p.o.) ^b	-	0.5	8
D ^c	NFT	4	12 (p.o.)	+	1.0	12
E ^c	NFA	2	3 (i.p.) ^b	-	0.4	5
F ^c	NFA	2	3 (i.p.)	+	1.3	6

Allergic drug reactions are considered to arise from an immune response against adducts between the drug and biomacromolecules, *e.g.* plasma proteins. The formation of adducts may result from activation of the drug involved by biotransformation. However, because of the photolability of NFT, irreversible binding to biomacromolecules may be caused as well by UVA irradiation.

In vitro, efficient photobinding to human serum albumin was demonstrated for both NFT (up to 50 nmol mg⁻¹ protein) and its primary photoproduct NFA. However, NFA and HMF, the endproduct of the photodecomposition of NFT and NFA, already irreversibly bound to plasma proteins in the dark. Protein amino, and to a lesser extent thiol groups, proved to be targets for binding. Furthermore, a significant decrease in iso-electric point of albumin was observed¹⁹.

Whether adducts are also formed *in vivo* was investigated with rats²⁰. Eight rats (150g), whose backs were shaved, were given 80 mg kg⁻¹ ¹⁴C-NFT orally. Four were exposed to UVA (50 W m⁻²; 345 - 410 nm; maximum at 370 nm) for 10h day⁻¹ and four were kept in light-poor environment. This was repeated the next day, after which the rats were killed. Irreversible bound NFT was determined and expressed per milligram of protein as a measure for the amount of organ material taken. The results are represented in Figure 5.

More irreversible binding was found in the UVA exposed animals than in those kept in light-poor environment. This concerns the skin of the back (both epidermis and dermis), ears, plasma protein and spleen and, not given in Figure 5, the tail and eyes. When rats were kept at 32°C, instead of 22°C during NFT-UVA treatment, even more irreversible binding was observed (Figure 5; this is probably caused by an increased dermal blood flow.) Under these conditions, irreversibly bound radioactivity was even found in the lungs and, not represented, in kidneys and liver²⁰. (Other experiments showed that the photobinding increased with dose and light intensity. Furthermore, the same amount of irreversible binding was found in rats killed immediately after the last NFT/UVA treatment and 5 days after. The latter observation gave further evidence that photobinding was irreversible.)

The irreversible binding found in inner organs can be explained by systemic distribution of plasma proteins to which NFT has been photobound in the skin. In addition, reactive photoproducts of NFT, such as NFA and HMF, can be transported and eventually covalently bind to tissue biomacromolecules far away from their site of formation.

The immunogenic properties of photoadducts between NFT and plasma proteins have also been demonstrated²¹. Photoadducts produced *in vitro* with plasma proteins of rabbits, were injected into the same animals of which blood was taken. It appears that photoadducts can indeed induce formation of antibodies (titers ranging from 150 to 1500 were found; controls were less than 60).

The wide variety of dermatologic reactions appearing in NFT therapy are frequently of

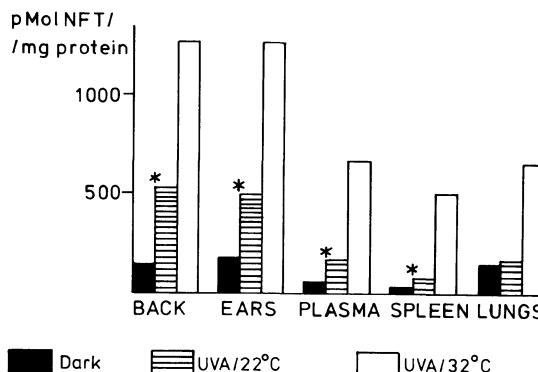


Figure 5. Irreversible photobinding of NFT to biomacromolecules in various organs. * means that UVA/22 °C and Dark differ significantly ($p < 0.001$; Student t-test). See also text.

the allergic type. This may be caused by covalent binding after incomplete enzymatic reduction of NFT in the liver. However, we found significantly more covalent binding in rats exposed to UVA than in those kept in the dark. Therefore the photoactivation of NFT in the skin should be considered as a route to (immunologically mediated) skin damage or rash.

Furthermore, the presence of irreversible bound NFT throughout the system (blood and organs, especially the spleen) indicates that light-induced adverse reactions are not restricted to light-exposed skin. They may also appear as systemic allergic reactions.

Imino-N-oxides

Chlordiazepoxide. Extensive research into the *in vitro* photochemistry of phototoxic chlordiazepoxide (CDZ), known under the trade name Librium, has shown that an oxaziridine is the first and main product. This reactive intermediate is also found as a result of UVA exposure of other imino-N-oxides such as the major metabolites of CDZ²² (Figure 6).

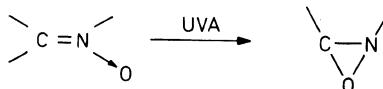


Figure 6. Photoisomerization of an imino-N-oxide into an oxaziridine.

In vitro, imino-N-oxides irreversibly bind to plasma proteins on UVA exposure. This appeared to proceed via an oxaziridine as an intermediate²³. Analogs of CDZ without an oxygen attached to the nitrogen are photostable and photobinding to proteins does not occur²³.

Based on these *in vitro* results it was expected that the N-oxide group in CDZ and its metabolites was responsible for the phototoxicity. This was confirmed with a microbiological test system; survival of *Salmonella typhimurium* TA100 was plotted against concentration of the compound investigated. Compounds lacking an oxygen to the nitrogen of the C=N group were not phototoxic. Furthermore, phototoxicity curves of CDZ and some other imino-N-oxides appeared to correspond quite well with curves for the toxicity (without UVA exposure) of their oxaziridines^{22,24}.

UVA-induced effects were also extensively investigated *in vivo* with rats^{25,26,27}. Some typical results are summarized here (see also Figure 7). As can be seen, UVA has a considerable influence on CDZ metabolism. The percentage of CDZ and N-oxymetabolites present after irradiation is only half of that found without UVA irradiation. In contrast to

that, the percentage of metabolites with no oxygen attached to nitrogen, including reduced CDZ (R.CDZ), is about 5 times higher in UVA-exposed rats.

An explanation can be proposed based on the reaction of oxaziridines, formed by photoisomerization in the UVA-exposed skin, with compounds containing SH groups. In the presence of glutathione (GSH), which is abundantly present in the body, CDZ (on UVA irradiation) and its oxaziridine (in the absence of light) react spontaneously with GSH to give R.CDZ as the ultimate product²⁷. These reactions with GSH occurring in the UVA-exposed rats will be the cause of a lower percentage of *N*-oxides (*e.g.* CDZ) and a higher percentage of reduced metabolites (*e.g.* R.CDZ).

Another observation with regard to metabolism of CDZ was, that the percentage of glucuroconjugated metabolites was two times lower with UVA exposed rats²⁵.

As was expected from *in vitro* photochemical data, irreversible binding to organ material was also found with rats simultaneously exposed to CDZ and UVA. In a typical experiment²⁵, four rats (200g) were given one single dose only of ¹⁴C-CDZ (25 mg kg⁻¹, i.p.). On 18 consecutive days (on which no CDZ was administered), two were exposed to UVA (14 W m⁻²; 320 - 380 nm; maximum, 350 nm) for 10 h day⁻¹ and the two others were kept in light-poor environment. Thereafter the rats were killed and organ material was submitted to dialysis for 26 days under non-equilibrium conditions. Irreversibly bound radioactivity was only found in UVA-exposed rats (*e.g.* skin of the back, 2440 ± 30; skin of the abdomen, 910 ± 20; ears, 1940 ± 80 and liver, 1030 ± 90 (units, counts per minute (c.p.m.) x 10⁻² mg⁻¹ tissue)).

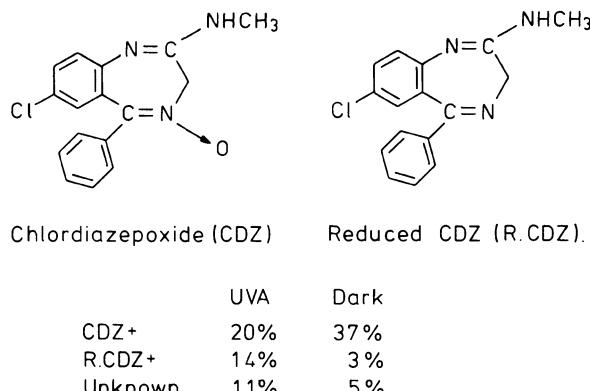


Figure 7. Urinary excretion (~45% of dose) of CDZ metabolites with rats either UVA-exposed or kept in light-poor environment. CDZ+ are imino-*N*-oxides, including CDZ; R.CDZ+ are metabolites lacking the oxygen attached to nitrogen, including R.CDZ (For more experimental details see ref. 25).

The considerable change in metabolism and the irreversible binding to biomacromolecules in the liver show that the phototoxicity of CDZ is not restricted to the UVA-exposed area but can involve inner organs as well.

Further confirmation of the responsibility of the *N*-oxide function in the molecule for the phototoxic effects was obtained by comparing CDZ with R.CDZ *in vivo*. These investigations with R.CDZ were performed under the same conditions as with CDZ. (For instance, with an administered dose of R.CDZ 1.5 times that of CDZ, both compounds had the same UVA absorption and bioavailability in the skin as a function of time.) The UVA exposure of rats had no influence on the metabolism of R.CDZ and no irreversible binding to biomacromolecules was observed²⁶. Similar differences has been found by these authors between diazepam-*N*-oxide and diazepam²⁷. In contrast with diazepam-*N*-oxide, diazepam, commercialized as *e.g.* Valium, appeared to be non-phototoxic.

Olaquindox. Olaquindox (OLAQ), commercialized as BAYO-N-OX, belongs to a group of quindoxin (QUIN) derived compounds. (Unlike OLAQ, Figure 8, QUIN does not have side chains.) OLAQ finds extensive application in cattle breeding, pig husbandry and poultry farming, as a feed additive with antimicrobial and growth-promoting effects. QUIN has also been used as such, but has been removed from the market; it proved to cause persistent photocontact dermatitis in man. In a number of cases, light sensitivity continued for more than 4 years²⁸.

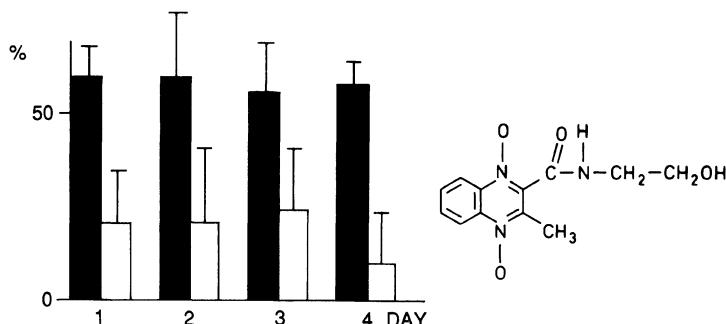


Figure 8. Daily urinary excretion (mol% of dose) of unconverted olaquindox by rats exposed to UVA (□) or kept in light-poor environment (■). □ differs significantly from ■ ($p < 0.001$; Student t-test; see text).

More recently OLAQ has also been reported to cause photoallergy in man²⁹. Symptoms of OLAQ induced photoallergy resemble those of QUIN. Furthermore, severe phototoxicity has been observed with pigs who got OLAQ.

QUIN-derived compounds belong to the group of imino-*N*-oxides and appear to form an oxaziridine on irradiation *in vitro* (see also Figure 6). Furthermore, they are highly photoreactive with proteins, indicating photoallergic properties³⁰.

Rats exposed to the combination of OLAQ and UVA, and not to either OLAQ or UVA alone, suffered from severe erythema of the skin, edema of the feet and necrosis of the ears³¹. The same experiments were performed with QUIN and some analogs of OLAQ, carbadox (CARB) and cyadox (CYAD) which are also extensively used as a feed additive with antimicrobial and growth-promoting effects. The results show that CARB is the most phototoxic followed by QUIN and OLAQ. CYAD, although very reactive *in vitro*³⁰, was not taken up from the intestine into the bloodstream like the other compounds and thus could not be reached by the UVA. Because of this, a photochemical reaction could not take place, which provides an explanation for the fact that this compound was not phototoxic after oral administration.

Besides skin phototoxicity, a profound influence on metabolism was found. Eight rats (140g), whose backs were shaved, were given OLAQ (60 mg kg⁻¹, orally). Four were exposed to UVA (60 W m⁻²; 345 - 420 nm; maximum 370 nm) for 12 h day⁻¹ and the other four were kept in light-poor environment. Urine was collected every 24 h. The procedure was repeated on each of four consecutive days (intervals, 24 h).

With rats kept in light-poor environment, about 60% of the daily dose of OLAQ is excreted. However, with UVA irradiation, the unconverted OLAQ found in urine is only about 20% of the dose (see also Figure 8).

Without UVA-exposure reduction of the parent imino-*N*-oxide is only a minor metabolic route. This situation changes drastically as a result of UVA irradiation; instead of about 2% desoxyolaquindox-4-monoxide (desoxyOLAQ-4-M), about 30% of this compound is found (see also Figure 9).

The decrease in imino-*N*-oxide in favor of a reduced metabolite has already been mentioned above for chlordiazepoxide (Figure 7) and diazepam-*N*-oxide.

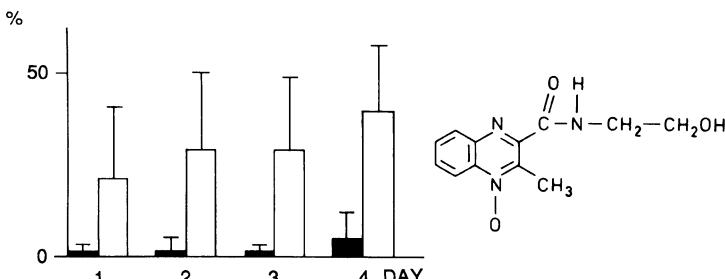


Figure 9. Daily urinary excretion (mol. % of dose of olaquindox) of the metabolite desoxyolaquindox-4-monoxide with rats exposed to UVA (□) and kept in light poor environment (■). □ differs significantly from ■ ($p < 0.001$; Student t-test; see text).

Together with the *in vitro* photochemical data (occurrence of an oxaziridine and its photoreactivity with proteins), the formation of desoxyOLAQ-4-M *in vivo* is an important indication that the photoisomerization of OLAQ into a reactive oxaziridine is responsible for the phototoxic and photoallergic effects observed.

CONCLUSIONS

The research with some nitroarenes and imino-*N*-oxides demonstrates that:

- 1) Phototoxic effects can involve inner organs as well. More attention should be paid in drug research to this possibility of light-induced systemic effects.
- 2) Knowledge of the part of the molecular structure responsible for phototoxicity, can provide the opportunity to alter the structure of the drug in such a way that the adverse photobiological effects diminish while the desired ones are conserved (*e.g.* research on chlordiazepoxide and derivatives). This aim can be reached more efficiently if data from *in vitro* and *in vivo* investigations are combined.

SUMMARY

In this review emphasis is put on the possibility that phototoxic effects do not only concern the skin or eyes but can also affect inner organs: *systemic* effects. This is illustrated with some *N*-oxidized compounds, *e.g.* nitroarenes and imino-*N*-oxides. Most examples dealt with are drugs, *e.g.* nifedipine, chloramphenicol, nitrofurantoin, and chlordiazepoxide.

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PHOTOTOXICITY OF THE EYE

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INTRODUCTION

The biological function of the human eye is to provide individuals with visual information about their physical surroundings. This is achieved through light reception, which consists of a series of complex reactions that involves photophysical and photochemical transformations of the visual pigment rhodopsin, biochemical processes that modify the ion permeability of the photoreceptor cell membrane, generation of the membrane potential and processing of the visual signal at the level of neural retina (Dratz, 1981). Before reaching the photoreceptor cells in the outer retina, light has to travel through several ocular media - the cornea, lens, vitreous and neural retina. Thus, all main eye segments are exposed to substantial fluxes of light whose intensity and spectral content is, to a significant extent, determined by the light-filtering properties of the underlying ocular tissues (Lerman, 1987).

Although the anterior eye tissues filter out the most damaging components of solar radiation that impinges upon the eye, the retina and retinal pigment epithelium (RPE) may be subjected to intense illumination from focal light that includes the relatively energetic photons from the blue part of the solar spectrum. As a result, photic retinopathy may develop. This photodamaging reaction affects the most critical, for acute vision, part of the retina — the *fovea* region (Young, 1988).

Even though the primary photoprocesses that ultimately lead to degeneration of the outer retina, have not been identified, it is postulated that retinal phototoxicity may contribute to the development of age-related maculopathy, which is the predominant cause of blindness in people over the age of 60 in many developed countries.

Phototoxic phenomena in all major segments of the human eye are briefly reviewed in this article, and the role of environmental and endogenous factors that can modify the ocular tissue susceptibility to light is discussed, and a hypothesis about a possible role of RPE melanin and lipofuscin in retinal phototoxicity is presented.

OPTICAL PROPERTIES AND MAIN ANATOMICAL FEATURES OF THE ANTERIOR EYE SEGMENTS

The amount and spectral content of the light that reaches photoreceptor cells in the retina, mainly depend on the optical properties of the anterior eye tissues - the cornea, the lens and the iris (Fig. 1). The cornea and the lens are the major optical system in the eye, and their optical properties are determined by their refractive indices, their radii of curvature and their chemical composition. Both ocular tissues are completely avascular and this, in part, explains their remarkable transparency to visible light^t. Light scatter is almost absent in these

tissues, and this results from a high degree of spatial order of the structural elements of the corneal stroma and the lens-fiber cells. Thus, while the geometric arrangement of the collagen fibrils in the corneal stroma, can be credited not only for high tensile strength of this relatively thin membrane, it also plays a significant role in corneal transparency (Benedek, 1971). An important feature of the corneal transparency is extreme sensitivity to its state of hydration. The proper state of the corneal hydration is maintained by the metabolic activity of the corneal epithelium and endothelium (Lerman, 1987).

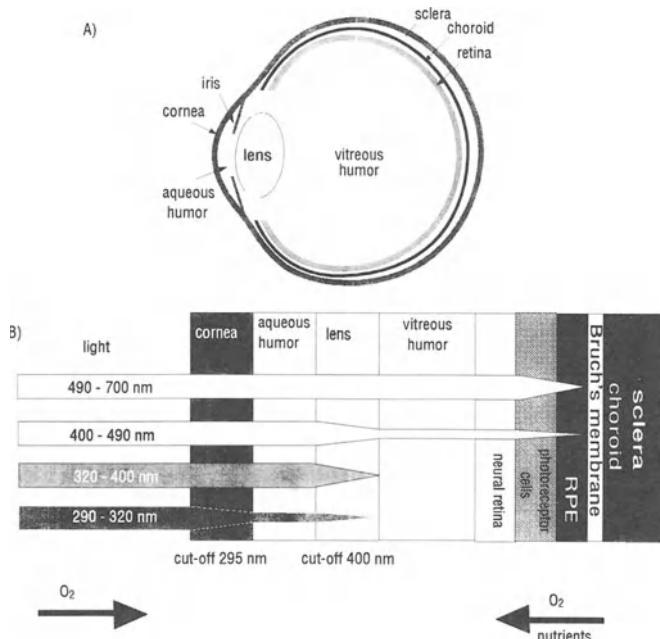


Figure 1. Schematic outline of main anatomical features (A) and optical properties (B) of the adult human eye.

The cornea contains mostly water (75%), proteins (20%), polysaccharides (1%), lipids, and nucleic acids (Lerman, 1987). Over 99% of the chemical composition of the cornea is composed of molecules that absorb light only between 200-295 nm. The major protein of the cornea is collagen which has a relatively low content of aromatic amino acids. Effectively, a cornea acts as a cut-off filter for the UV and very little light, if any, with the wavelength below 295 nm is transmitted through this optical medium (Boettner and Wolter, 1962). It is important to emphasize that optical properties of the cornea change with age and, as a result, the percent transmission of UV and visible light significantly decreases in the human cornea of older individuals.

The light intensity at the human lens is actively controlled by the iris. It is estimated that the "mechanical" iris aperture of the eye that is responsible for the dilation and constriction of the pupil, can account for up to 10^2 of the intensity range of sensitivity adaptation of the eye (Dratz, 1981). Blocking of light by the iris is mainly due to the presence of melanin in the stroma and in epithelial cells of the iris. Melanin pigment in the iris is formed in two distinctively different cell types - the melanocytes of the stroma and the neuroepithelial cells (Prota, 1992). Interestingly, while melanin in the iris stroma of all normal caucasian newborns does not appear until some weeks after birth, neuroepithelial melanin can be observed in human embryos at early stages of their development. No melanin is present in the iris of individuals with the most severe cases of ocular albinism (Kinnear *et al.*, 1985). Thus, in tyrosinase-negative oculocutaneous albinism, iris translucency produces the "pink-eyed" appearance. Such albinos experience significant photophobia. It is held that the

excessive amount of light that enters through the non-pigmented iris, causes glare and can contribute to degradation of visual acuity.

The human crystalline lens is a unique, biconvex organ that represents a pure cell line and preserves the complete population of the cells from fetal life to death (Lerman, 1987). Cell mitosis occurs only in a part of the epithelial layer which is attached to an outer collagenous capsule. Lens growth takes place as the mature cells migrate to the lens bow, where they denucleate and become strap-like fiber cells extending forward to the anterior and posterior poles (Dillon, 1991). Consequently, the major portion of the lens is composed of long thin fibers that have a hexagonal cross section and form closely packed, onion-like layers.

Water and protein are the primary chemical constituents of the lens (Lerman, 1987). The degree of hydration, and perhaps the action of the mucopolysaccharides as cement substance between the lens fibers, may play a role in lenticular transparency. The metabolic activity of the lens is mainly directed toward maintaining normal hydration (60 to 65%) and protein synthesis. There are three main structural proteins in the human lens called α -, β - and γ -crystallins (Lindley *et al.*, 1985). These proteins vary in structure and aggregative abilities. They are also unique in being organ specific and not species specific, immunologically. With aging, the structural proteins in the human lens undergo changes that are manifested on both the macromolecular and molecular levels (Lerman, 1987).

The young lens is almost transparent to visible radiation (400-700 nm) (Boettner and Wolter, 1962). Beside the minute amount of cytochromes in the lens epithelial cells, only the α - β -glucoside of 3-hydroxykynurenine (3-HKG) absorbs light above 320 nm in the lens of the infant (Bando *et al.*, 1981). Protein-bound tryptophan, on the other hand, absorbs only 5% of the photons that are transmitted by the cornea (using the spectral output of the sun) (Barker *et al.*, 1991). Even though the concentration of 3-HKG decreases with age, the total filtering capacity of the human lens increases due to generalized yellowing of the lens proteins (Lerman, 1987). Thus, as the lens ages, it displays an increasing absorbance in the UVA and visible region, which is associated with increased pigmentation of the lens core (nucleus). It is also accompanied by an increased lenticular fluorescence. This visible fluorescence of the ocular lens (emission maxima at 420-440 nm and 500-520 nm) has been shown to be present in the soluble and specially insoluble high molecular weight fractions of the human lens (Lerman and Borkman, 1976). Aggregation and the formation of large water-insoluble proteins can lead to light scattering and opacification. An advanced stage of such age-related changes are called by a generic name "senile cataract" (Spector *et al.*, 1975; Dilley and Pirie, 1974).

The vitreous is the largest body of the human eye. Its main function is to exert pressure that helps to hold in place the retina which is not permanently attached to the choroid. The normal vitreous is a gel-like material composed of water, collagenous protein and long-chain carbohydrates (hyaluronic acid) (Lerman, 1987). These constituents do not have any significant chromophores that absorb above 240 nm, although some aromatic aminoacids are also present. Since the vitreous contains some tryptophan residues, it is potentially able to absorb UV-radiation longer than 295 nm. Indeed, exposure of the vitreous to UV radiation up to 320 nm, resulted in the denaturation of the collagen network (Balazs *et al.*, 1959). However, it must be stressed that in normal, phakic eyes this radiation is already fully blocked by the lens. With aging the vitreous shrinks, and this leads to a reduced pressure exerted on the retina. As a result, probability of the retinal detachment increases in older age.

THE EFFECT OF AGING ON STRUCTURAL INTEGRITY AND FUNCTIONAL PERFORMANCE OF THE POSTERIOR POLE OF THE HUMAN EYE

Out of all eye segments, the most important functionally and most complex anatomically is the retina. Its chief biological function is transducing the ocular light image and processing the neural signals before sending them toward the brain. Transduction of the visual signals begins in a highly specialized part of the retina - the photoreceptor cells. In humans, there are two types of photoreceptor cells: the rods and cones (Dratz, 1981). The highest surface density of the photoreceptor cells is in the central 1.5 mm region of the retina which is called the fovea. It contains almost exclusively the cones and is responsible for high visual acuity and for color vision. Rods, on the other hand, are responsible for high

sensitivity detection of light. However, signals that are transmitted by rods to the brain are perceived as colorless images (Dratz, 1981).

With all its complexity, the retina is a remarkably compact organ - its thickness is between 100 to 500 μm . Interestingly, the photoreceptor cell layer is located in the most posterior portion of the retina. Thus, light in the human eye has to travel through the entire retina before reaching the rods and cones. Fortunately, the composition of the neural retina minimizes undesirable light absorption and scatter. In the *macula lutea*, located in the central region of the retina (5.5 mm in diameter), there is a selective absorption of blue light due to the presence of zeaxanthin and lutein (Bone *et al.*, 1985). The biological role of these macular pigments is still uncertain. It has been proposed that the selective filtering of light in that wavelength region may improve the contrast of the retinal image (short wavelength light being more scattered by the atmosphere and the ocular media) and may limit chromatic aberration of the lens (Reading and Weale, 1974). Antioxidant and photoprotective role of the macular pigments (*vide infra*) has also been considered (Kirschfeld, 1982).

Under normal, bright lighting conditions, only a small fraction of the radiation that reaches the retina is absorbed by the visual pigment. The rest travels to the overlying retinal pigment epithelium (RPE) cells and the choroid where it is absorbed by the melanin. Even though a significant individual variation in the amount of the RPE melanin has been reported, no systematic racial differences exist. Pigmentation of the choroid, on the other hand, strongly depends on genetic factors and, generally, is consistent with the cutaneous pigmentation (reviewed by Sarna, 1992). The biological role of the RPE and choroid melanin remains highly speculative. The remarkable ability of melanin to absorb photons from the entire spectral range of radiation that can reach the retina, may suggest an important photoprotective function of this pigment (Handelman and Dratz, 1986). Alternatively, absorption of light by the RPE (and choroid) melanin may contribute to visual acuity by minimizing spurious signals (Taylor, 1978). Such spurious signals could arise in the nonpigmented fundus due to light reflection back from the sclera to the photoreceptor cells.

The primary photophysical event in visual signal transduction is absorption of light by rhodopsin, followed by an efficient and extremely rapid photoisomerization reaction. Within hundreds of femtoseconds after the rhodopsin has been excited by visible light, its chromophore (11-cis isomer of vitamin A aldehyde) undergoes isomerization from the 11-cis-retinal to all-trans-retinal configuration (Schoenlein *et al.*, 1991). Because the rate of the photoreaction is so high, it can easily compete with any processes that could potentially utilize the energy of the electronically excited rhodopsin molecule.

With aging, the human retina undergoes significant structural and physiologic changes that have deleterious effects on visual acuity (Weale, 1975), color perception (Ohta and Kato, 1975) and dark-adaptation sensitivity (McFarland *et al.*, 1960). Even though all segments of the human eye are affected by senescence, it is believed that age-related changes of the retina are by far the most important factor in senile degradation of vision. A monotonic decrease in the photoreceptor cell density with age has been described in a recent study of human retinas from donors spanning almost 8 decades of life (Gao and Hollyfield, 1992). Since the ratio of photoreceptors to RPE cells showed no significant change with age, parallel loss of these closely apposed cells has been suggested. Although the relative vulnerability of rods and cones to loss during aging remains controversial (Liem *et al.*, 1991), it now is documented that both photoreceptor cell types decay with aging. In addition, senile degeneration of the photoreceptor cells is accompanied by the loss of neurons such as cells in the ganglion cell layer (Gao and Hollyfield, 1992).

Dramatic age-related changes can also be observed in the RPE and Bruch's membrane (Marshall, 1987). As a result of aging, the RPE cells exhibit significantly modified biochemical indicators (Katz and Robinson, 1984; Boulton, 1991) and show changes in its optical properties (Feeney-Burns, 1980). The latter is mainly due to the loss of melanin, appearance of complex melanin granules (e.g. melanolysosomes) and formation of lipofuscin (Feeney, 1978). In addition, the distinct polarity of location of the RPE melanosomes, which in young eyes are predominantly present in the apical processes that envelop the photoreceptor outer segments, is lost in senescence (Feeney, 1978).

Although the molecular origin of the RPE lipofuscin is not fully understood, it is believed that these amorphous pigment granules arise from incomplete molecular degradation of the rod and cone outer segments that are constantly shed, transported to the RPE via phagosomes and degraded by the action of RPE lysosomes (Young, 1982). Interestingly, the highest rate of lipofuscin accumulation in the human RPE occurs during the first two decades of life (Wing *et al.*, 1978). Between the second and sixth decade of life the RPE lipofuscin

content remains relatively constant, and only at the age of 60 and above, a second rise in the lipofuscin accumulation becomes apparent. By age 80, lipofuscin granules can occupy twenty or more percent of the RPE cell volume (Feeney-Burns *et al.*, 1984). It is very likely that this alone could contribute to disruption of normal biological functions of these important cells that provide key metabolic support for the underlying photoreceptor cells, and are involved in an extensive phagocytosis and molecular renewal processes (Dorey *et al.*, 1989; Boulton and Marshall, 1986).

Bruch's membrane is an avascular membrane, composed of collagen and elastin, immediately adjacent to the RPE. It separates the RPE from the choroid, and it also enables transfer of all of the nutrition of photoreceptor cells from choroid and facilitates an outflow of water from retina to choroid (Marshall, 1987). Calcification, progressive accumulation of lipids and appearance of highly refractile bodies, usually called drusen, are indicators of the aging Bruch's membrane (Bird, 1992). These changes contribute to decline in metabolic transfer capacity of the organ.

ENDOGENOUS AND EXOGENOUS FACTORS AFFECTING THE SUSCEPTIBILITY OF THE EYE TO PHOTOOXIDATIVE DAMAGE

Most of the human eye tissues are inherently at risk of photooxidative damage. This is due to their exposure, over the lifetime, to substantial fluxes of light, to high concentration of oxygen and to the presence of endogenous and exogenous molecules with potential photosensitizing abilities.

Even though the levels of artificial illumination that humans are exposed to may vary substantially, it is estimated that the present indoor and outdoor ambient illumination, derived from such sources as the ubiquitous fluorescent or mercury-vapor street light, are between 20 and 50 fc (3.2 to 8.1×10^{-5} W/cm²) (Lerman, 1987). These apparently low levels are at least partially offset by the fact that the blue and UVA component of most artificial light sources account for a quarter to a third or more of their spectral irradiance within the 320 to 700 nm band. Furthermore, artificial light extends blue-light-UVA exposure beyond that available from daylight, to the full 16 to 18 hours of the working day. Of course, outdoor daylight levels are significantly higher; they typically exceed the levels of artificial illumination by a factor of 30 to 1000 (Simons, 1993). The amount of visible and, in particular, UV light that reaches the eye can vary enormously. In addition to predetermined, well-defined changes that are related to season, time of day and latitude, sun light exposure is reduced by heavy clouds, natural landscape, trees and man-made structures that obstruct part or all of the horizon, and can be enhanced by reflection by the ground with the amount depending greatly on the type of surface.

There is a number of factors that protect the eye and shields it from excessive light exposure. Thus, the normal horizontal alignment of the eye and the orbit significantly reduces ocular exposure to whole-sky irradiation. Further anatomic protection is provided by the brows, the nose and the cheek (Sliney, 1983). The eyelids provide protection that is enhanced by squinting. It has been estimated, based on a study using mannequins, that the ratio of the ocular to ambient solar UVB dose is approximately 0.2 (Rosenthal *et al.*, 1985). The amount of light and its spectral content that reaches the human retina is, to a significant extend, controlled by the iris and by optical properties of the lens. Other important factors that can influence ocular light exposure in a given environment, include wearing a hat and the use of eyeglasses.

An important environmental factor that is involved in photooxidative damage to the eye is oxygen. Its tension gradient throughout the cat eye was measured using an oxygen-sensitive microelectrode (Weiter, 1987). The cornea is well oxygenated because its surface is in constant contact with air. From the cornea to the lens there is a gradient with decreasing concentration of oxygen with possible range of 72-24 mm Hg. The lens epithelium contains moderately high oxygen concentration reflecting this tissue metabolic activity. The vitreous is, in general, poorly oxygenated since no vascularization is present in this eye segment. The retina, on the other hand, exhibits a nonuniform gradient of oxygen tension with the highest concentration of oxygen in the outer retina (above 70 mm Hg). It has been found that the photoreceptor-retinal pigment epithelial complex accounts for two thirds of total retinal oxygen consumption. So, the photoreceptor-RPE complex is exposed to a very high oxygen concentration that is supplied by the choroid capillary network. Interestingly, the retinal

oxygen tension increases with illumination, as shown in the intact cat retina which is likely to be representative of mammalian retinas with similar vascular supplies (Linsenmeier, 1986). Changes in oxygen tension were maximal at the level of the outer nuclear layer which had the lowest oxygen concentration in the entire cat's retina. It is understood that these changes result from light-induced decrease in oxygen consumption (Kimble *et al.*, 1980).

Among the endogenous molecules of the anterior human eye segments which have photosensitizing ability, N-formylkynurenine (N-FK) is the most prominent (Walrant and Santus, 1974). Although this well known UVA photosensitizer is not normally present in the lens of young individuals, its appearance in the lens of older people is a result of photooxidation of the protein tryptophan residues (Creed, 1984).

The outer retina and the RPE contain a number of chromophores that strongly absorb visible light and potentially are able to participate in photosensitized oxidation reactions. Mitochondrial enzymes, such as cytochrome C oxidase, and products of photopigment bleaching (all-trans retinal and all-trans retinol) have been considered as potential photosensitizers (Young, 1988). The role of the RPE melanin in photooxidative reactions that may take place in the RPE-retina complex remains controversial. It has been established that melanin can act as a powerful antioxidant in model systems (Sarna and Swartz, 1993). On the other hand, the ability of melanin to photogenerate superoxide anion (Felix *et al.*, 1978), hydrogen peroxide (Korytowski *et al.*, 1985) and hydroxyl radicals (Korytowski *et al.*, 1987) may suggest possible involvement of this pigment in retinal phototoxicity (Handelman and Dratz, 1986).

There are relatively few exogenous photosensitizing molecules that can locate intraocularly. In this respect, psoralens (e.g. 8-MOP) and fenothiazines are most noticeable exceptions (Lerman, 1986). This may be due to the screening effect of the blood-aqueous and the blood-retina barriers which usually are very tight and significantly limit the transfer of unwanted molecules from the blood to the ocular tissues (Potts, 1991).

There are a number of biological and anatomical mechanisms that protect the eye tissues against photooxidative damage. The efficient repair process that takes place in the corneal epithelium, is an example of biological photoprotection of the eye. Thus, in spite of the fact that cornea is exposed to the most damaging light that is transmitted to the Earth surface, this anterior eye tissue is relatively impervious to photodamage. This is mainly due to the wound healing capability of the epithelium (Dillon, 1991). The metabolic activity of the ocular lens is substantially lower than that of the cornea, consequently the lens repair mechanisms are not very efficient (Lerman, 1987). Hence, any photodamage that may appear in the lens is likely to accumulate with age.

To minimize the effects of photooxidative damage, the photoreceptor cells developed a unique and remarkably efficient system of cellular repair. On a daily basis a certain fraction of the photoreceptor outer segments is shed and phagocytosed by the RPE (Young, 1971). The need for the rapid outer segment turnover (approximately 10-15 days) is probably related to the high degree of photooxidative damage they endure. It is important to realize that the rod and cone outer segments consist of a dense stock of about 2000 layers of phospholipids that contains a very high concentration of polyunsaturated fatty acid, including the most unsaturated fatty acid - the decosohexaenoic acid (22:6) (Gordon and Bazan, 1990; Bazan *et al.* 1984). Thus, the system is exceptionally susceptible to lipid peroxidation which is further aggravated by the high oxygen tension and exposure to light. The mechanism of biological renewal of the photoreceptor outer segments is so efficient that the cells can function normally for years, despite photooxidative insult they are constantly subjected to.

Biochemical mechanisms of the protection of the eye tissues against photooxidative damage include the action of antioxidant enzymes and low-molecular-weight antioxidants. Thus, the cornea, lens, retina and RPE contain glutathione, ascorbic acid and vitamin E which are powerful quenchers of photooxidative reactions (Handelman and Dratz, 1986). It must be stressed that the levels of low-molecular-weight antioxidants that are normally present in various ocular tissues decrease with age (Lerman, 1987). It has also been shown that dietary supplements of ascorbate efficiently protects the ocular lens (Spector and Garner, 1981) of experimental animals against photodamage from UV and have photoprotective effects on the retina and RPE (Organisciak *et al.*, 1992; Li *et al.*, 1985). The antioxidant function of the macular pigments has also been considered (Kirschfeld, 1982). These two dihydroxy carotenoids, zeaxanthin and lutein, can be found throughout the retina but their highest concentration is in the foveal region. It has been established that they are mainly located in the photoreceptor axon layer and inner plexiform layer (Handelman *et al.*, 1988). Among all carotenoids, zeaxanthin is one of the most efficient quenchers of singlet molecular

oxygen (di Mascio, 1989). Therefore, it is tempting to speculate that the biological function of this pigment is to protect the critical area of the human retina against oxidative damage that might arise from photosensitized reactions. Interestingly, there appears to be no systematic racial differences in the density of the human molecular pigment (Bone and Sparrock, 1971).

All eye tissues contain, in various amounts, powerful antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase (Armstrong, 1981; Bhuyan and Bhuyan, 1978; Atalla *et al.*, 1987; Atalla *et al.*, 1988; Rao *et al.*, 1985). The enzymes are found in high levels in the retina but only in small amounts in the lens (mainly in the cortex) (Lerman, 1987). The activities of both catalase and SOD decrease with age, and catalase is inactivated by exposure to UV (Zigman, 1985).

PHOTODAMAGING REACTIONS TO THE CORNEA AND LENS

Clinically, corneal photodamage from UV has long been appreciated (Lerman, 1987). The typical example is "snow blindness", experienced by polar explorers. This condition, which is also called Labrador Keratopathy and is due to chronic exposure to UV light (mainly to light scattered from the snow), is found in about 15% of the Eskimo population (Dillon, 1991). Studies using artificial lighting indicate that the maximum efficiency for experimental photokeratitis and photokeratoconjunctivitis peaks sharply at 288 to 290 nm, with a smaller peak at 254 nm (Bachem, 1956; Scherashov, 1970). This action spectrum can be ascribed to absorption by tyrosine and tryptophan and by the nucleotides in the cornea. Thus, UV-induced damage to the cornea may result from photomodifications of these corneal epithelium constituents. In another study, it has been shown that the most active wave band for corneal damage in both monkeys and rabbits is 270 nm (Pitts, 1970). The action spectrum for the human cornea is generally similar to that of subhuman primates, although the human cornea is more sensitive to UV than is cornea of either monkeys or rabbits (Lerman, 1987).

Experiments on animals clearly suggest that UV may cause cataract. Thus, cortical and posterior subcapsular clouding and opacities have been induced by exposures to UV between 293 and 315 nm (Pitts *et al.*, 1976). Action spectra of the lens photodamage have been obtained for species whose lenses do not have the absorptive properties of the human lens. These spectra show maximum damage at about 300-305 nm (Parrish *et al.*, 1978). Permanent lens opacities could be induced *in vivo* in laboratory animals with either acute high-dose or chronic low-dose exposure to UV. Interestingly, no nuclear opacities and brunescent changes have been ever induced in these experiments. This may be due to the fact that the experimental animals used have lenses with significantly different properties than those of the humans. Epidemiologic and clinical observations also suggest a link between sun-light exposure and cataracts. In general, cataracts are more common in tropical areas than in regions with more moderate climate (Taylor, 1989). In one of such a study, involving 64,307 Australian aborigines, a strong positive correlation between the intensity of UVB radiation in the zone of residence and the presence of senile cataract has been found (Hollows and Moran, 1981). Since many of such studies were "ecological" in that the exposure of individuals to sunlight or UV were not ascertained, and none of them have taken into account the possibility that the UV could preferentially affect selected parts of the lens, Taylor has examined selfselected samples of watermen who worked on Chesapeake Bay (Taylor, 1988). The personal ocular exposure index for UVB was constructed based on interview data and laboratory and field measurements. The ocular to ambient exposure ratio, defined as the average fraction of ambient radiation that reached the eyes of a subject not wearing spectacles or sunglasses, had been measured. The ocular exposure was corrected for the use of eyeglasses according to laboratory measurements of UV attenuation by typical eyewear. The data clearly indicate an association between individual exposure to UVB (but not to UVA!) and cortical cataract. No association was seen with nuclear opacities. In the same study of watermen, two corneal disorders - pterygium and climatic droplet keratopathy - were significantly associated with a broad band of UV exposure (both UVB and two bands of UVA) (Taylor *et al.*, 1989).

It has been suggested that the UV radiation could affect the lens and cause cataract by at least four different mechanisms (Taylor, 1989): 1) photooxidation of free or protein-bound tryptophan; 2) photosensitized oxidation reactions involving activated oxygen species; 3) disruption of the membrane-cation transport system; and 4) damage to nucleic acids in lens epithelial cells.

Among the mammalian lens crystallins, the tryptophan residues of α -crystallin undergo a more rapid photooxidation when exposed to 300 nm irradiation *in vitro* (Andley, 1987). H_2O_2 and O_2^{*-} , the tryptophan photoproducts, have been found in the solution of the irradiated α -crystallin (Andley, 1984). It has been proposed that the reaction mechanisms may involve photosensitized formation of O_2^{*-} , with NFK being the intrinsic photosensitizer generated by photooxidation of the protein tryptophan residues (Creed, 1984). However, the photoinduced changes by NFK were found to be less severe than those observed in the presence of extrinsic photosensitizers and visible light. For instance, methylene blue-sensitized changes in α -crystallin showed a dramatic decrease in the near-UV CD spectrum, a loss of SH-group content and an exposure of cysteine residues to more hydrophilic sites (Mandal *et al.*, 1986). Evidence for the involvement of electron transfer and singlet oxygen in riboflavin photosensitized oxidation of α -crystallin was also reported (Andley and Clark, 1987).

Since γ -crystallins contain high percentage of cysteine and methionine, it is postulated that the photoinduced aggregation of these lens proteins might be due to the formation of intermolecular disulfide bonds (Blundell *et al.*, 1981). Alternatively, modification of the surface charge of the protein, caused by photooxidation, would result in disruption of the protein structure, and lead to aggregation and insolubilization (Lindley *et al.*, 1985). An increase in the yellow color of the lens nucleus is observed as the lens ages (Lerman, 1980). This age-related pigmentation of the lens may play a role in the development of so-called nuclear sclerosis. In about 10% of the human population, this process progresses at an accelerated rate, resulting in the development of the brunescent (nuclear) cataract (Lerman, 1987) which is an extreme example of age-related photochemical changes of the lens. This type of discoloration could actually be beneficial since it gives the lens an additional filtering ability, particularly for UV and short-wavelength visible radiation, providing the accumulating age pigment does not increase the susceptibility of the lens proteins to photochemical destruction.

UV radiation or visible illumination in the presence of extrinsic photosensitizers have been demonstrated to affect the activity of key lens enzymes. Thus, exposure of rat lens to 360 nm radiation caused an inactivation of the membrane-bound Na^+/K^+ ATPase (Zigman, 1987). However, the UV-effects could be ameliorated by ascorbate (Zigman, 1987). Also, when whole rat lenses were exposed to riboflavin-photosensitized oxidation reaction, the carrier-mediated transport function in these lenses was decreased (Varma and Mooney, 1986).

Very high concentrations of hydrogen peroxide have been reported in the aqueous humor (Garner and Spector, 1980). Even though the exact mechanism of H_2O_2 formation is not known, it is postulated to be due to interactions of ascorbate with UVB (Spector and Garner, 1982). It is possible that, under certain conditions, elevated levels of H_2O_2 could contribute to the lens toxicity and cataractogenesis.

8-Methoxysoralen (8-MOP) can be found in a variety of ocular tissues within a couple of hours after the experimental animal or patient is given a single dose. Of course, a concurrent exposure of the eye to UVA may result in photobinding of 8-MOP to DNA and lens proteins (Lerman, 1986). Dose-related ocular damage, consisting of dense central corneal opacification in 8-MOP (and 5-MOP) treated animals exposed to UVA, was observed (Lerman *et al.*, 1977; Lerman *et al.*, 1981; Lerman *et al.*, 1984). Cataract formation in human cataracts as well as in experimental animals, has now been documented in Psoralen-UVA (PUVA) therapy (Lerman *et al.*, 1976; Lerman *et al.*, 1982). It has been established that this drug can generate specific photoproducts in human lens, which is associated with the formation of PUVA cataracts in experimental animals.

PHOTIC RETINOPATHY AND POSSIBLE ROLE OF LIGHT IN AGE-RELATED MACULAR DEGENERATION

Solar retinopathy is probably one of the most common photodamaging reactions in the human eye following sun gazing (Gladstone and Tasman, 1978; Henkes, 1977; MacFaull, 1969). It has been described in numerous cases as a result of viewing the solar eclipse without the proper eye protection (Sadun *et al.*, 1984; Penner and McNair, 1966; Flynn, 1960). This type of retinal lesion is localized in the center of the macula, and is primarily situated in the outer layers of the retina (Sykes *et al.*, 1981; Parver *et al.*, 1983). It damages

photoreceptor cells and the retinal pigment epithelium (van Norren and Schellekens, 1990; Marshall *et al.*, 1972; Adams *et al.*, 1972; Tso and Woodford, 1983). Based on a laboratory study, it can be predicted that a lesion of clinical proportions can be produced in less than a minute, when the eye is fixated directly on the sun, and the sun is near zenith on a cloudless day (Sadun *et al.*, 1984). It has been observed that light from an indirect ophthalmoscope can cause irreversible retinal damage after only 15 minutes exposure in the monkey (Parver *et al.*, 1983). Interestingly, high concentrations of the inspired oxygen appeared to potentiate retinal lesions in rhesus monkeys, produced on light exposure from the operating microscope (Jaffe *et al.*, 1988). The retina is much more sensitive to experimental damage from UV or blue light than it is from longer wavelengths of visible light (Ham *et al.*, 1980), even though the lens in adult phakic eyes filters out most of the UV. A significant loss in short-wave cone sensitivity, due to exposure to UV, was found in the eyes of patients who had undergone bilateral cataract extraction and implantation of intraocular lens that transmitted UV (Werner *et al.*, 1989). The results of this study suggest that 5 years of exposure to ambient UV produces loss in sensitivity that is equivalent to over 30 years of normal aging of the short-wave cones.

It is important to emphasize that in all experiments and observations discussed here, the fluence rates of the damaging radiation were well below the levels required to damage the retina in a thermal way. For example, it has been estimated that the focal energy flux, delivered to the retina and choroid by the indirect ophthalmoscope, is approximately 0.1 to 0.2 W/cm² which approximates the amount of irradiance received at the retina surface from the sun (Lerman, 1987). It is estimated that in photochemical damage to the retina, its temperature seldom raises by more than 1° to 2°C (Lawwill *et al.*, 1980; Weiter, 1987). On the other hand, thermal damage, or photocoagulation, results when enough light is absorbed by the tissue (usually the RPE) to raise the temperature in the retina by 10° to 20°C (Birngruber and Gabel, 1983).

The nonthermal type of photodamage - actinic in nature - was first recognized by Noell *et al.* (1966), who reported that irreversible retinal damage occurred in normal laboratory rats exposed continuously to illuminated environment. The wavelength that produced maximum damage corresponded to the peak absorption of rat rhodopsin, and the damage depended on the animal's body temperature (Noell, 1980). It is called class 1 photic retinopathy. It appears to be mediated by the visual pigments and is mostly observed in nocturnal animals whose retinas are extremely sensitive to low intensity visible light. White light threshold damage to the rat's retina was found in the range 1 to 10 J/cm² (van Norren and Schellekens, 1990).

At higher light levels, photic retinopathy of different origin may develop. So-called class 2 photodamage to the retina was originally observed in monkeys after brief intense exposure to visible light (Ham *et al.*, 1978). The threshold damage for white light was about 300 J/cm², and the retinal damage action spectrum increased towards the UV (Ham *et al.*, 1980). Class 2 photic retinopathy seems to be additive, and is enhanced by increased oxygen concentration (Ruffolo *et al.*, 1983). It has been suggested that the initial site of damage, induced by blue and UV light, is the RPE (Mainster, 1987). Indeed, experiments on the Long Evans rats showed that while retinal damage by green light was characterized by significant derangement of the rod inner and outer segments (with no apparent changes in the RPE); in the UVA-exposed retinas there was minimal disorganization of the outer segments and severe swelling and distortion of rod inner segment and RPE mitochondria (Rapp *et al.*, 1990).

The predominant cause of blindness in people over the age of 60, in many developed countries, is age-related maculopathy (ARM) (Young, 1987). Although many factors that may contribute to the development of the disease, have been analyzed (Van der Schaft, 1992; Vinding *et al.*, 1992), there is presently no established etiology that could serve as a basis for preventive medicine. The etiology of ARM is most likely multifactorial (Marshall, 1987). However, one environmental factor that is supported by a growing body of evidence is solar radiation (Young, 1988). It is remarkable that the retinal damage produced by bright light is most severe precisely in the location that deteriorates most rapidly in ARM.

A recent population-based survey of 838 fishermen from Chesapeake Bay indicates strong association between the patient's exposure to blue (400 to 500 nm) or the whole visible light (400 to 700 nm) over the preceding 20 years and advanced ARM (Taylor *et al.*, 1992). Similar conclusion, about the association of ARM with exposure to sunlight, has been reached in a smaller, most recent population-based study of ARM in people from

Beaver Dam, Wisconsin (Cruickshanks *et al.*, 1993). On the other hand, no association has been found between individual ocular exposure to UVB and UVA and symptoms of ARM (West *et al.*, 1989). The fact that individual ocular exposure to UV appears to have little effect on increasing risk of ARM, can be explained if both the damaging efficiency of light and the transmission curve of the ocular media of normal phakic adults are taken into account. Since in a normal adult eye, the cornea and the lens filter out most of the light below 400 nm (Boettner and Wolter, 1962), the resultant action spectrum of the *in vivo* retinal damage is a product of the effective transmittance of the ocular media and the action spectrum of photodamage of the unprotected retina. Of course, the situation in aphakic eyes is different and their retinas show much stronger susceptibility to UV (Werner *et al.*, 1989).

MOLECULAR MECHANISM OF RETINAL PHOTOTOXICITY; ROLE OF THE RPE MELANIN AND LIPOFUSCIN

In spite of substantial research efforts over the last two decades, neither the principle reactions nor the key chromophores that are responsible for the phototoxic phenomena in the mammalian retina, have been identified (Young, 1988; Mainster, 1987; Dillon, 1991). Nevertheless, several theories have been proposed to explain the mechanism(s) for the light effect on the retina. Hence, in one such theory, photodynamic action on the photoreceptor cells is postulated, in which generated free radicals could lead to peroxidation of the lipid portion of the photoreceptor outer segments (Wiegand *et al.*, 1983; Shvedova *et al.*, 1983; De La Paz and Anderson, 1992). A second theory considers the possible effects of light on specific metabolic pathways in the retina. An example is the observation that the oxygen consumption of the retina is suppressed by a steady exposure to light (Kibble *et al.*, 1980). A third possibility is the formation of a toxic photoproduct (result of the photopigment bleaching) which under normal lighting conditions is rapidly eliminated, but with chronic bleaching it accumulates as a toxic agent. It has been proposed that the accumulation of opsin (product of the rhodopsin bleaching) may result in destruction of the photoreceptor disc membranes due to unstable nature of this protein moiety (Lawwill, 1982). Alternatively, the damaging species, arising from the rhodopsin bleaching, might be retinol, which is known for its membranolytic properties (Schroder and Black, 1980). Even though this reduction product of all-trans-retinal is, in its free form, quite unstable, a recent study on the distribution of vitamin A compounds in bovine eyes indicates that after bleaching of rhodopsin, a substantial amount of all-trans-retinol, along with minor amounts of 11-cis-retinol, accumulates in the RPE (Bongiorno *et al.*, 1991).

From photochemical, mechanistic view point, perhaps the first theory is the most attractive one. It is also quite challenging for it requires unambiguous identification of the molecular nature of the photosensitizer(s) and the key photochemical reactions that are involved in generation of the ultimate phototoxic products. Two, most often considered, molecules that may play the role of endogenous photosensitizers, responsible for photodynamic damage to the retina, are flavins and porphyrins (Young, 1988). An acute ocular toxicity, localized predominantly in the retina of pigmented rabbit eyes, has been observed after intravenous administration of clinically relevant doses of hematoporphyrin derivative and visible light illumination (Gomer *et al.*, 1984). Since *in vitro* photoactivation of protoporphyrin IX, which is a precursor molecule of hemoglobin, leads to the generation of superoxide anion and singlet oxygen (Gottsch *et al.*, 1990), hematogenous photosensitization has been proposed as a mechanism of retinal phototoxicity. It was further speculated that hematogenous photosensitization could play a role in ARM. An *in situ* action spectrum of photodamage to the RPE, in enucleated bovine eyes, was shown to encompass the absorption spectrum of the oxidized and reduced forms of cytochrome C oxidase (Pautler *et al.*, 1990). Therefore, it was concluded that cytochrome C oxidase might be a prominent photosensitive locus and that absorption of blue light by hemoproteins may contribute to the observed photodamage.

If the original site of class 2 photodamage to the retina is located in the RPE, then melanin and lipofuscin must also be considered as potential photosensitizers. These two unusual RPE pigments are amorphous, polymeric materials, that have heterogeneous chemical composition, appear *in situ* in particle form, and are not soluble in most solvents (Prota, 1992). Very little is known about possible similarities of their biophysical properties and, in particular, the photochemical reactivity. However, their molecular origin and their biogenesis are very different (Feeney-Burns, 1980). While the RPE melanin can clearly be

seen in human embryos at the 7-week stage, lipofuscin, as the age pigment, accumulates in the human RPE during the entire life (Weiter *et al.*, 1986). Even though melanogenesis may involve free radical reactions and stochastic processes (Prota, 1988), key transformations of the melanin substrate molecules are controlled by specific enzymes (Aroca *et al.*, 1990). In addition, the entire process of the melanin biosynthesis is confined to within specialized, membrane-bound organelles, the melanosomes (Riley, 1994). RPE lipofuscin granules, on the other hand, are believed to originate from incomplete lysosomal digestion of the photoreceptor outer segments (Feeney, 1978). Free radical processes and lipid peroxidation reactions might be involved in lipofuscinogenesis (Katz *et al.*, 1984), and the role of vitamin A derivatives has been postulated (Eldred, 1989).

Melanins in general are efficient quenchers of electronically excited states such as the triplet excited states of positively-charged photosensitizing dye molecules (Bielec *et al.*, 1986) and the singlet molecular oxygen (Sarna *et al.*, 1985). In model systems it has also been shown that melanin can act as a free radical scavenger (Sarna *et al.*, 1986), and is able to exert substantial antioxidant activity. These properties of melanin can be explained by its ability to reduce and oxidize many compounds (Sarna and Swartz, 1993) and to bind, hence sequester, certain photosensitizing dye molecules and transition metal ions (Sarna, 1992). If the results, from such model-system studies, could be extrapolated to the *in situ* - *in vivo* situation, then the RPE melanin might be viewed as a unique electron- and ion-exchange polymeric resin that efficiently protects the RPE-retina complex against oxidative damage, induced by reactive free radicals, photosensitizing dye molecules and redox-active metal ions. It can be argued that the ability of melanin to bind many potentially cytotoxic species is most relevant to the postulated photoprotective role of this RPE pigment. This is because such a binding significantly reduces the effective radius of oxidizing reactions that might be induced by the bound-to-melanin species. In practice, the formation and decay of reactive species will become strongly site-specific and mostly confined to within the RPE melanin granule.

On the other hand, the particle nature of the RPE melanin makes its free-radical scavenging abilities of less significance in cellular protection against oxidative damage. It is important to realize that any reactive, short-lived species that are generated randomly in the RPE cell, would as readily be deactivated by other cellular constituents that might be present in the proximity. Melanin will scavenge reactive free radicals and quench electronically excited molecules only if they are formed within the melanin granule or in its nearest proximity. Such conditions may occur if copper and iron ions or photosensitizing molecules, such as porphyrin derivatives, are complexed by the RPE melanin (Korytowski and Sarna, 1990). However, substantial accumulation of potentially cytotoxic species in the melanin granule carries a certain risk for the RPE cell. Thus, if melanin suddenly releases the accumulated species, the cell may be subjected to an aggravated oxidative damage.

The ability of melanin to bind multivalent transition metal ions is determined by several factors. Among environmental factors, the pH, presence of competitive metal-ion chelators and ionic strength of the aqueous media are most relevant (Sarna *et al.*, 1976). Oxidation state and structural integrity of the melanin polymer are important intrinsic factors that should be considered (Sarna *et al.*, 1980). Even though the mechanisms of cellular homeostasis normally prevent the cells from any dramatic changes in the pH and content of important electrolytes, the RPE melanin could, *in vivo*, be exposed to an acidic environment. This may happen when the melanin fuses with RPE lysosomes, forming melanolysosomes which are numerous in RPE's of older individuals (Feeney-Burns *et al.*, 1990). Since the lysosomal interior is generally considered to be quite acidic (Bainton, 1981), melanin complexes with metal ions are likely to be less stable in such an environment. Thus, depending on saturation of the metal-ion binding sites in the RPE melanin subjected to a lysosomal "digestion", some release of the accumulated metal ions (or other positively charged species) becomes highly probable.

Structural modifications of the melanin, due to degradative oxidation reactions, could also induce the release of accumulated cations. So, it can be postulated that not only does the aging RPE melanin lose its antioxidant capability, but also it may become a prooxidant. Indeed, using synthetic polymers as models for natural melanins, it has been shown that their antioxidant action is mainly due to efficient sequestration of iron ions (Korytowski *et al.*, 1993). Bleaching of such melanins, induced by prolong exposure to hydrogen peroxide, results in a significant decrease of their antioxidant activity, determined by their ability to inhibit lipid peroxidation (Zareba *et al.*, 1993). In this respect an intriguing question arises: whether or not the RPE melanin undergoes any *in vivo* photobleaching. Is the age-related

formation of the so-called RPE melanolipofuscin granules (Feeney-Burns *et al.*, 1984) related to such photooxidation of melanin?

Both questions remain unanswered at this time. However, results of a recent study indicate that the RPE melanosomes can be bleached *in situ* (in human and bovine eyecups) by illumination with intense visible light (Różanowska *et al.*, 1993). This process is accompanied by the formation of H₂O₂ and is wavelength-dependent. Blue light, as expected, causes faster bleaching of the RPE melanosomes than the green-red light does. If results of this preliminary study are to be extrapolated to the *in vivo* situation, then one may expect that the RPE melanin will gradually "bleach" with age (with the accumulated light dose). Of course, progress of such a "photoaging" of the RPE melanin should depend on many constitutional and facultative factors, of which the effective light dose and the efficiency of the cellular systems to prevent oxidative stress reactions, are perhaps most important.

In contrast to melanin, the RPE lipofuscin exhibits remarkably brilliant golden-yellow fluorescence upon excitation with near-UV light. Using chloroform-methanol extracts of whole human RPE and of isolated lipofuscin granules, Eldred and Katz (1988) were able to separate chromatographically four groups of fluorophores that exhibited different spectroscopic properties. Distinct changes in the fluorescence properties of the lipofuscin granules with age, have been described by Boulton *et al.* (1990). Most recently Eldred and Lasky, based on a very elegant analytical study, identified the molecular nature of one of the major lipofuscin fluorophores (1993). It is an amphoteric quaternary amine that arises from a Schiff's base reaction of retinaldehyde and ethanolamine.

The ability of lipofuscin to fluoresce and its molecular origin may suggest some photochemical reactivity of this RPE pigment. Indeed, it has recently been found that the RPE lipofuscin might be a free radical generator (Boulton *et al.*, 1993b). Purified lipofuscin granules, isolated from human RPE's, consume oxygen when illuminated with monochromatic light (Bielec *et al.*, 1993). The efficiency of the oxygen photoconsumption markedly increases with decreasing wavelength of the illuminating light. Even though superoxide anion, hydrogen peroxide and singlet oxygen appear to be only minor products of aerobic lipofuscin photoexcitation (Bielec *et al.*, 1993), their *in situ* formation may have deleterious effects on the RPE-retina complex. Interestingly, substantial photoconsumption of oxygen has been observed in suspension of isolated human RPE's (Boulton *et al.*, 1993a). Since the action spectra of oxygen consumption for both systems, the purified lipofuscin granules and isolated RPE cells, are similar, and the yield of oxygen photoconsumption correlates with the amount of lipofuscin in the RPE from humans of different age, it can be concluded that the *in vitro* photoreactivity of the RPE cells is mainly determined by their lipofuscin content. It is tempting to speculate that the RPE lipofuscin, being derived from photoreceptor outer segments in response to photooxidative processes may, under some circumstances, contribute to retinal phototoxicity by generation of oxygen reactive species.

Clearly, significantly more studies, particularly on photophysical and photochemical properties of intact lipofuscin granules as well as their organic-solvent extracts are needed to determine unambiguously whether or not lipofuscin plays any substantial role in the retinal phototoxicity and the etiology of ARM.

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PHOTOACTIVATION OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INVOLVES COMPLEX SIGNALING PATHWAYS

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INTRODUCTION

The mechanisms of toxicity by ultraviolet light (UV) or by photosensitizing agents have been explored for a long time as an approach to the development of antiviral agents to be used in many applications such as for the treatment of Herpes simplex viruses¹ or in suitable inactivation procedures to guarantee virus safety of purified plasma protein preparations like albumin or clotting factors^{2,3}. Since most of the laboratory tests are based on the detection of viral antibodies rather than of the virus itself, they fail to identify recently infected donors who have not yet seroconverted, donors infected by a mutant virus, or donors who have reverted to a seronegative state⁴. There is also concern about the transmission of viruses for which screening procedures have not yet been developed or whose potential for causing disease has not yet been recognized. Furthermore, the prevalence of certain viruses, like the human cytomegalovirus (HCMV), in the donor population may be so high that the pool of seronegative donors is too small to meet the growing demand for virus-free blood products. This situation has led to an important renewed interest in blood product sterilization procedures like treatments with mixtures of organic solvent and detergent, heat treatment in the lyophilized state or those involving light such as UV- or visible-light associated with photosensitizers⁵. Among all these technical approaches, the photoinactivation procedures are very attractive because they seem ideal to manufacture virus-safe fresh frozen plasma and for producing virus-safe plasma protein preparations because the phototreatment can decontaminate single plasma or cell unit in their containers, i.e. plastic bags.

One of the major drawback in the photoinactivation procedures is the possibility to reactivate viruses from latently infected cells or to activate virus replication from a quiescent provirus integrated in the host cell genome⁶. This last point is especially important in the case of the human immunodeficiency virus type 1 (HIV-1) which is present in T-lymphocytes or in monocytes in an integrated and quiescent state. This review will focus on the different mechanisms underlying UV- and photosensitized- activation of cells harboring copies of the HIV-1 provirus and on the consequences of this activation on HIV-1 replication.

HIV-1 INFECTIOUS CYCLE

The natural history of HIV disease is best described as a continuous process in which immune dysfunction and the loss of CD4⁺ helper T-cells begins at the time of infection and progressively increases^{7,8,9} leading finally to the wasting syndrome, opportunistic infections and malignancies that constitute clinically defined AIDS (the acquired immunodeficiency

syndrome) 10,11. Three consecutive stages can be viewed : a first acute stage corresponding to primary infection with HIV-1 (CDC stage I), a chronic stage representing a period of clinical latency (CDC stage II and III) and a crisis stage where the profound immunodeficiency is manifested by the development of opportunistic infections and other pathologic conditions (CDC stage IV) 10.

The symptoms of the primary infection consist of fever, headache, lymphadenopathy, myalgia 12,13, and in such patients have been found transiently high levels of infectious virus in the plasma with a significant percentage of infected peripheral blood lymphocytes (PBL) (up to 1 % of CD4+ T-cells) 14. The number of CD4+ falls acutely but it is not known whether it is due to their absolute loss or simply to their redistribution to extravascular sites. One month after the infection, the number of infected CD4+ found in the peripheral blood declines and the appearance of an anti-viral humoral and cellular immune response correlates with the diminished level of HIV-1 replication 15.

When the symptoms of the primary infection disappear with the evidence of an anti-viral immune response, HIV-1 infected patients enter in a chronic, clinically asymptomatic state often called the latent period. This stage is estimated to be 8-11 years before entering the last stage 16,17. However, AIDS can also occur in 2 to 4 months 18,19, whereas some infected individuals may remain asymptomatic for more than 13 years 20. Although clinically latent, recent studies suggest that the virus continues to replicate at a low level throughout this period but only low levels of plasma viremia are found with relatively few infected CD4+ T-cells (1 per 10,000 or 100,000) 21,22.

The development of clinically significant immunodeficiency is presaged by evidence of increasing virus burden, a significant level of plasma viremia and elevated levels of HIV-1 DNA and RNA in the blood 23. Increasing virus replication has also been correlated with accelerated depletion of CD4+. As CD4+ counts fall, the percentage of HIV-1 infected lymphocyte increases, and in patients with AIDS, the percentage of HIV-1 infected CD4+ may exceed one in 40 24. However, only a fraction of HIV-1 infected cells present in the blood express measurable levels of virus gene products at any one moment in time 25. In patients with AIDS, it is estimated that the number of infected T-cells containing HIV-1 DNA exceeds those producing HIV-1 RNA by a factor of 10 26.

One important point which should be mentioned is the dependence of the first step of the HIV-1 replication cycle on the state of the T-lymphocyte. Incomplete reverse transcription may occur in unstimulated cells leading to an unstable partially reverse transcribed HIV-1 DNA intermediate 27. This molecule is composed of partial minus strand DNA sequences including one Long Terminal Repeat (LTR) but no *gag* sequences. Recent data suggest that the efficiency of the completion of viral DNA production from these partial transcripts is very poor. Only 5 % of this intermediate can be rescued to produce virus 15 h after infection 28. Thus, this intermediate may contribute to inefficient infection of resting T-cells but is unlikely to provide a state of true virus latency. A second report shows that unstimulated lymphocytes in cell culture fully transcribe HIV-1 but the proviral DNA does not integrate in the host genome 29. Only after cellular stimulation is the proviral DNA able to integrate and productively express virion progeny. Unintegrated viral DNA is shown in PBL of certain HIV-1 infected individuals and stimulation of these cells with mitogens in cell culture leads to viral integration 30. Similarly, HIV-1 proviruses integrated into the genome of cells of the monocyte-macrophage lineage, which are permissive for proviral synthesis and integration even in the absence of cellular proliferation, might also be capable of maintaining a latent state. Subsequent activation of these cells by antigens, cytokines, or other stimuli might then result in reactivation of a productive HIV-1 infection (Fig. 1).

It has been proposed that unintegrated linear HIV-1 species may function as a reservoir of latent HIV-1 infection in resting T-cells and this condition has been called pre-integration latency 31 (Fig.1). When the viral DNA successfully integrates into the genome, it can be transcribed in activated cells or untranscribed in resting cells. In this case, the stage is called post-integration latency 31 (Fig.1). In activated cells, the transcriptional machinery aided by virus factors determines the rate of virus transcription and the extent of productive infection. It has been shown recently 32, using Polymerase Chain Reaction (PCR), that in asymptomatic patients there are many infected cells in the lymph node cells, more than in the blood and that these lymph node cells are more likely to become virus-productive than those

in the blood. Furthermore, in combining PCR and *in situ* hybridization with autoradiography, Embretson *et al*³³ demonstrate that a high fraction of cells, around a quarter in germinal centres of lymph nodes, are infected and that these cells tend not to be expressing viral RNA. In some sense, these cells can be called latently infected cells and most of them are in the post-integration latency stage. It is now clear that there are many cells harbouring virus in an HIV-infected individual, even early after infection, and that the infection takes many different forms including one in which the virus is completely latent. One implication of that, is for a preventive vaccine to be effective against HIV-1, it must block the virus from ever establishing residence in the host, particularly the seeding of lymphoid organs.

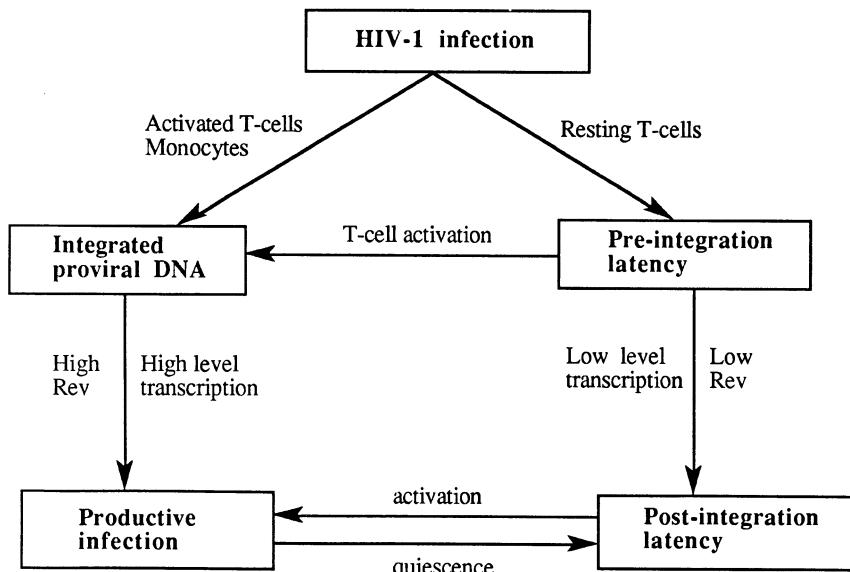


Figure 1. Regulation of HIV-1 latency showing that both activated T lymphocytes and non-dividing monocytes are able to support the synthesis and integration of HIV-1. In contrast, resting T-cells are non-permissive for this initial step of the replicative cycle.

The reactivation of HIV-1 from latently infected cells is a major step in the infectious cycle of the virus, and the characterization of all the events capable to trigger the reactivation is worth to know in details. Now numerous papers have demonstrated the key role of several cellular factors and of HIV-1 LTR in the passage between a latently infected cell and a virus producing cell. In many respects, the HIV-1 LTR functions like the promoter of a T-cell activation gene³⁴ (Fig. 2). Transcriptional induction driven by the LTR is critically dependent upon the presence of two juxtaposed κB enhancer elements that specifically engage members of the NF-κB / Rel family transcription factors³⁵. The binding of NF-κB to its enhancer element is an inducible event in most cell types but the genes encoding the p50 and p65 subunits are constitutively expressed. These two factors, p50 and p65, are sequestered as a complex in the cytoplasm by an inhibitory protein termed I-κB³⁶. Cellular activation by cytokines like IL-2 or TNF-α or by antigen recognition in turn promotes disassembly of this cytoplasmic complex permitting the rapid translocation of the NF-κB p50/p65 complex to the nucleus where it acts. The cytoplasmic-nucleus shuttling characterizes all members of the Rel family and is linked to their diverse biological activities^{37,38}. In terms of their individual functions, the p65 subunit appears to play a major role in transcriptional activation, while the p50 subunit serves mainly to increase DNA binding affinity of the complex³⁹.

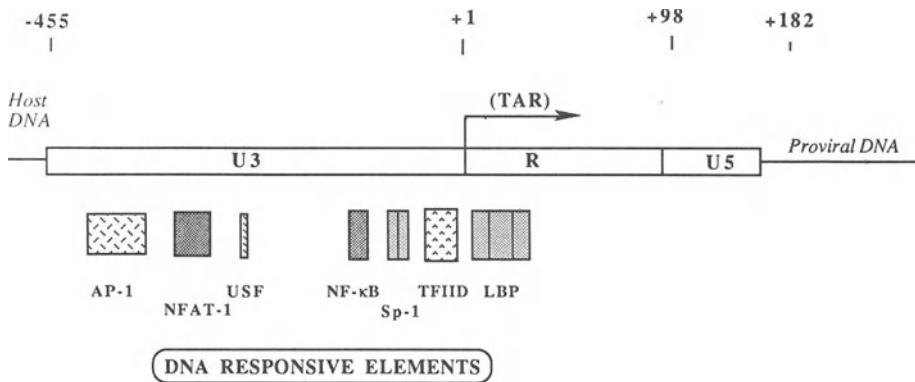


Figure 2. DNA responsive elements within the 5'Long Terminal Repeat (LTR) of an integrated HIV-1 provirus. LTR's are divided into three subsections, termed U3, R and U5. The border between U3 and R marks the site of transcription initiation (arrow). The size of each HIV-1 LTR subsection is given in base pairs. Also indicated are the locations of the single binding sites for NFAT-1, USF, AP-1 and TFIID, the two adjacent binding sites for NF- κ B and the three adjacent target sequences for the cellular factors Sp1 and LBP. The transactivation response (TAR) element maps to positions 1-59, but is recognized as an RNA sequence.

Additional DNA-binding proteins have been identified which bind to the downstream LTR region like the LBP, UBP-1 and CTF/NFI factors or to regions upstream the transcription initiation site like Sp1, USF, NFAT-1 and AP-1 sites⁴⁰. Therefore, it is important to consider that various stimuli or cofactors favoring the induction of one or several of these regulatory proteins can promote the switching towards the severe stage of the disease by disrupting the latent stage of HIV-1.

UV ACTIVATES THE EXPRESSION OF HIV-1 GENES.

Awareness that UV light can activate HIV-1 dates back to work done in 1988 by Valerie *et al*⁴¹ at a time when there was a strong focus in the AIDS research community on identifying the potential reactivating agents of the latent HIV. These authors chose to look at UV radiation, which is known to have DNA-damaging effects, having in mind that anything that could alter the DNA might have the potential to trigger HIV-1 activation. Because at that time a good system for assaying the activity of the HIV genome was needed, these authors constructed a hybrid gene by fusing the HIV-1 LTR to a reporter gene known as CAT (for chloramphenicol acetyltransferase) that encodes a bacterial protein which is readily detectable in cell extracts when the transcription of this gene is induced. In that case, the transcription of the CAT gene is completely dependent on the regulation elements found in the HIV-1 LTR. After introducing a plasmid containing this construct in HeLa cells to obtain a cell line which has stably integrated the LTR-CAT cartridge, these cells have been exposed to UV around 260 nm (UVC). The idea was that any increase in gene expression in response to UV radiations would be reflected in increased CAT activity in the cells. Indeed, 48 h after UV irradiation, a 50 to 150 fold increase of CAT activity was measurable in the cell extracts, comparable to the levels achieved with other factors that had previously been shown to activate HIV gene expression.

This original finding has been subsequently confirmed by showing that not only UVC but also UVB activates latent HIV in chronically infected cultured monocytes⁴². By monitoring HIV-1 reverse transcriptase (RT) activity in cell supernatants, these authors have shown that similar RT levels can be seen after exposure of these cells to UVB, although somewhat higher doses are required compared with UVC.

A step further in the understanding of the mechanism underlying HIV-1 reactivation by UV has been accomplished by Stein *et al*^{43,44} by using chimeric constructs carrying all or parts of the HIV-1 LTR linked to the CAT gene to transfet into HeLa- or T-cells and to

study their response to UV irradiation. These experiments have demonstrated clearly that the LTR region encompassing the *cis*-acting NF- κ B elements are required to confer the UV responsiveness. The examination, by gel retardation assay, whether the NF- κ B binding to its cognate sequence into the LTR is influenced by irradiating cells with UV confirmed that this cellular factor plays a crucial role in HIV-1 activation by UV. Indeed, whole cell extracts from non-irradiated cells exhibit a low level of binding activity detectable by this assay. This binding is specific for the HIV-1 LTR since only competition with the authentic sequence obliterates the complex formation. Extracts from UV-irradiated cells contain more NF- κ B binding activity which increases about 5-fold by 60 min and continues to rise to more than 10-fold by 8h after irradiation. Thus, UV-induced expression of HIV-1 is regulated at the transcriptional level and is based on an activation of NF- κ B and its binding to the major enhancer element contained in the LTR. Since this cellular factor is normally localized in the cytoplasm in an inactive form, the UV-induced signal transduction pathway must pass through the cytoplasm where NF- κ B is activated before migrating into the nucleus and turning on HIV-1 gene expression by binding to its responsive elements situated in the LTR.

The demonstration that the initial event in UV activation of HIV-1 is DNA damage has been obtained by determining the action spectrum of UV-induced gene expression⁴⁴. In fact, the primary target of HIV-1 reactivation could be anyone of a large number of molecules carrying chromophores that absorb UV, e.g., nucleobase compounds, tyrosine-containing substances, steroids and pyrrole rings. These chromophores are characterized by their UV absorption spectra, and to determine which of these transmit the relevant stimulus to NF- κ B, these authors⁴⁴ have measured the action spectrum of HIV-1 reactivation to obtain clues about the chemical nature of the primary target. The action spectra for the HIV-1 activation peaks at 265 to 275 nm and falls off steeply at longer wavelengths. Half maximum induction per quantum is seen with 280 to 290 nm. At 303 nm, the efficiency is down below 1/60 of the peak value, and no gene activation is found with irradiation of 345 and 360 nm at doses up 2,000 J/m². The spectrum obtained between 240 and 303 nm is superimposable on the cell-killing spectrum carried out with the same UV lamp and the spectrum of pyrimidine dimer formation. Thus, the most likely interpretation of the HIV-1 induction spectrum is that UV radiation is absorbed by DNA or RNA, whereas proteins and other compounds with conjugated double bonds such as those found in membrane constituents are ruled out.

A strong support for cyclobutane pyrimidine dimers involvement as primary event in HIV-1 reactivation mediated by UV has been brought recently by Yarosh *et al*⁴⁵. Two experiments have demonstrated that DNA lesions such as pyrimidine dimers can initiate the signaling pathway : (i) UV-induction is greater in DNA repair-deficient cells and (ii) T4 endonuclease V abrogates viral gene activation. This last point has been investigated by studying UV activation of latent HIV-LTR after the delivery of exogenous DNA repair enzymes. Liposomes encapsulating T4 endonuclease V, which specifically removes cyclobutane pyrimidine dimers from DNA, have been added to UV-irradiated cells. The liposomes produce an inhibition of the UV-induced CAT gene expression in proportion to the liposome dose showing that pyrimidine dimers and not 6-4 pyrimidine-pyrimidone photoproducts constitute the initial event in the activation process. Thus, the signal which leads to HIV-1 gene activation is initiated in the nucleus by DNA damage, migrates to the cytoplasm where it activates NF- κ B by releasing its inhibitory subunit I- κ B and then goes back to the nucleus where the activated NF- κ B binds to its DNA responsive elements situated in the LTR part of the HIV-1 provirus. However, it has also been shown by Devary *et al*^{46,47} that the mammalian UV response is triggered by the activation of tyrosine kinases situated at the plasma membrane level, and importantly, cells enucleated by cytochalasin B treatment are still fully responsive to UV both in NF- κ B induction and in activation of another key signaling event. Thus, HIV-1 activation by UVC could involve two distinct pathways one being initiated by lesions in DNA such as pyrimidine dimers, the other one by other types of cellular damage likely to consist of membrane or protein damage (Fig. 3).

Interestingly, this signal can also be spread from UV-irradiated cells to unirradiated one by co-cultivation^{43,45}, implicating the release of soluble factors. Exposure to UV irradiation is in fact an environmental factor for the general human population but most of the HIV-1 bearing cells are normally not exposed to UV irradiation. The description of a mechanism involving an extracellular factor which can indirectly affect those HIV-1 bearing cells is very important in the understanding of the UV effects.

Although numerous experiments have been performed on cell cultures transfected with reporter plasmid and then UV-irradiated, more recent work describe that UV irradiation also

induces HIV gene activation in a living animal. For these experiments, Cavard *et al*⁴⁸ have used genetically altered mice which they created by introducing into the animals a gene construct made by the linking of the HIV-1 LTR to the *E. coli* β -galactosidase gene (*lac Z* gene). These mice spontaneously express the transgene in the epidermis and the lens of both adults and embryos. UV-irradiation causes a significant increase of β -galactosidase activity in the skin. The same model has been experimented by Frucht *et al*⁴⁹ who have used the CAT gene as a transgene and exposed the animals to 254 nm UVC or to 312 nm UVB radiations. At optimal exposure times, a 20 fold increase in HIV LTR-CAT directed expression is observed in ear specimens. Kinetic data reveal that the transgene expression begins 120 min after exposure and reaches a maximum after 3 days. One approach based on the HIV-1 LTR driving the *tat* gene has also been used to generate transgenic animals⁵⁰. The presence of both of these regulatory elements has approximated the requirements of intact HIV for viral gene expression in the infected cell allowing to analyze their interactions with various cofactors. The transgenic mice selectively express the LTR-*tat* gene in their skin, and this expression is localized in the epidermal portion of the skin. Exposure to a variety of different UV wavelengths markedly but transiently increases the expression of the *tat* gene. Very recently, Zider *et al*⁵¹ have shown by using mice epidermal extracts that NF- κ B sites of the HIV-1 LTR are one of the targets of UV induction confirming the data obtained in transient expression assays. Secondly, they have generated transgenic animals carrying the *lac Z* gene under the control of the LTR partially deleted for the NF- κ B sites. Unexpectedly, all the animals, even those bearing the deleted LTR, display a UV-inducible epidermal expression suggesting that, in mice, the UV induction might be mediated through other sites than the κ B sites and may also depend on changes of the chromatin state.

Among the other important responsive elements found in the HIV-1 LTR, there are AP-1 sites situated far upstream and downstream the two NF- κ B sites (Fig. 2). AP-1 is one of the first discovered examples of transcription factor that respond to external influences. Initially detected as an activity that stimulates SV40 early promoter activity *in vitro*⁵², it has been purified as a family of proteins that bind to the sequence TGAGTC recognized as phorbol ester responsive elements in the collagenase gene^{53,54,55}. The structure of several members of the family has been resolved, and the transcription factor consists of two subunits linked by coil-coil interactions⁵⁶. Each subunit carries separable domains for DNA contact, for dimerization and for transactivation^{57,58}. The different subunit members of this family contribute to the complexity of AP-1 by the large number of combinatorial associations to hetero- or homo-dimers, each of which having slightly different DNA sequence preference⁵⁹. The prototype AP-1 operating as the critical factor at the collagenase enhancer consists of the products of the two oncogene *c-Fos* and *c-Jun*⁶⁰. AP-1, in most cells, controls genes that are required for cell growth and its activity is enhanced by agents that promote cell proliferation^{44,61} but also by UV⁴³. In fact, UV causes an increase in transcription of genes that code for transcription factors themselves like *c-Fos*⁶², and *c-Jun*⁶³. In HeLa cells, the transcription of the *c-Jun* gene is activated strongly (200 fold induction) and rapidly (less than 1 hour) by UV in comparison to other genes but also in comparison with other stimulating treatments like phorbol esters (a 50 fold induction peaked 2 hours after induction). The promoter of *c-Jun* carries two enhancers resembling to the consensus sequence of the AP-1 binding sites: the *Jun 1* UV response element and the *Jun 2* URE. These two elements act independently in the UV expression of *c-Jun* but they do not seem to be required for *c-Jun* induction by other agents.

It is now clearly established that UV induces the transcription of a number of cellular genes as well as promoter constructs carrying single or multiple sites for a specific transcription factor. For some of these genes, transcription occurs in the absence of protein synthesis, indicating that transcription factors are direct targets of an induced signal transduction cascade and that even long-term induction of these promoters is achieved by immediate changes of pre-existing transcription factors by post-translational modification. The transcription factor AP-1 is such a direct target and the post-translational modification has been confirmed by two types of experiments : (i) potent inhibitors of protein synthesis, such as anisomycin or cycloheximide, do not inhibit the activation process and (ii) chimeric Jun-Gal4 proteins composed of the transactivation domain of Jun and the DNA binding domain of the yeast transcription factor Gal4 are able to stimulate transcription of reporter plasmids containing two Gal4 binding sites⁶⁴. Since neither preexisting nor newly

synthesized AP-1 can work on the reporter plasmid or dimerize with the exogenously expressed Jun-Gal4 chimeric proteins, these data prove that Jun is activated by post-translational modification. The molecular characterization of these post-translational modification has been achieved by using Jun protein from [32 P] orthophosphate labeled HeLa cells resolved either in one-dimensional PAGE or for changes in phosphorylation of specific amino-acid residues by two dimensional tryptic phosphopeptide analysis⁶⁴. In Jun from unstimulated cells, mono-, di-, and triple phosphorylation of a peptide situated in the DNA binding domain (Thr231, Ser243, Ser249) can be detected together with one phosphorylated peptide (Ser73) located in the transactivation domain. UV causes a reduction in Ser249 phosphorylation which has been proposed to cause an increase in DNA binding but also an hyperphosphorylation of Ser73 and a phosphorylation of Ser63. In order to prove that the UV-induced modifications in the transactivation domain are relevant for the activation by UV, wild type Jun-Gal4 fusion protein has been compared with an identical fusion protein of the double mutant in Ser63 and Ser73. Transfection of this double mutant in cells is barely UV-inducible while the wild type constructs respond to UV.

MAP-2 kinase is involved in the signal transduction pathway leading to Jun

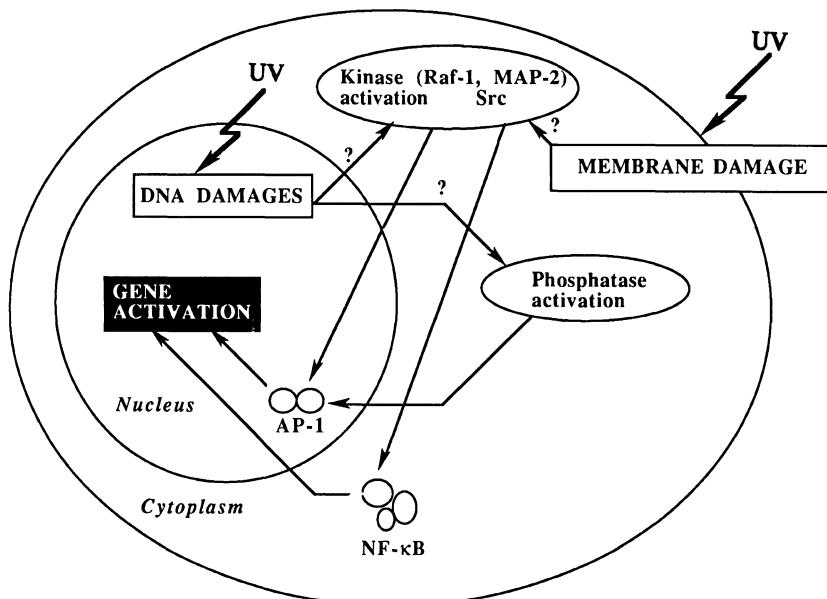


Figure 3. Schematic representation of the UV-induced signaling pathway with a potential signal transmission from the nucleus, where DNA damages are induced, to cytoplasm where both kinases and phosphatases are activated. Another pathway would involve membrane damages leading also to kinase activation. These enzymes in turn activate regulatory factors in the nucleus or in the cytoplasm which turn on or off genes.

phosphorylation⁶⁴. Indeed, in response to UV, MAP-2 kinase seems to be transiently modified (a slow migrating MAP-2 kinase band can be observed in *Western blot* analysis) and this modification occurs within 10 min before to disappear rapidly. However, a modification of MAP-2 kinase itself is also required for its activation and is due to phosphorylation at Ser/Thr and Tyr residues⁶⁵. This suggests that either, in addition to a protein Ser/Thr kinase, at least one Tyr kinase or a protein kinase with a dual specificity would be activated upon UV irradiation. Raf-1 kinase could be one of these because it has been shown to affect MAP-2 kinase activity⁶⁶.

In conclusion of this part of the paper, the peculiarities of the UV response have led to the detection of an information flow from the nucleus (with pyrimidine dimers as primary

events) to the cytoplasm (post-translational modification of transcription factors) back to the nucleus (gene activation by activated transcription factors) (Fig. 3). In addition to this pathway, another one initiated at the membrane or cytoplasmic level can also play a very important role in transcription factor activation. This exchange is very important in general linking cellular reactions to the state of the genome.

REACTIVE OXYGEN SPECIES CAN TRIGGER HIV-1 REACTIVATION

A common denominator shared by some agents capable to activate HIV-1 replication is their ability to cause stress responses in cells (see above). Because latently infected T lymphocytes can be the target of hydrogen peroxide and oxygen radicals commonly produced by the mononuclear and polymorphonuclear leukocytes⁶⁷, we have decided to investigate whether reactive oxygen species like hydrogen peroxide and singlet oxygen can induce the expression and replication of HIV-1 in human cell lines. Furthermore, these two activated oxygen species are known to be produced during photosensitization reactions⁶⁸. These photoreactions involve a photosensitizer which is a molecule absorbing light undergoing excitation directly to a singlet state. The singlet state has a lifetime in the order of nanoseconds, too short to allow reaction with a substrate in most cases. The singlet state is often converted to a triplet state having a much longer lifetime to transfer its energy to oxygen by different mechanisms capable to generate several oxygen reactive species like superoxide anion, hydrogen peroxide, hydroxyl radicals or singlet oxygen⁶⁹. Thus, it seemed worth to know in more details whether or not these reactive oxygen species could be involved in the mechanism of photoinduced HIV-1 reactivation.

Hydrogen peroxide as part of a general mechanism of HIV-1 reactivation

To determine the eventual role of H₂O₂ in HIV-1 reactivation, we have used two cell lines supporting a latent HIV-1 infection : the promonocytic U1 and the lymphocytic ACH-2 cell lines. These cell lines have been used as model systems to explore HIV-1 post-integration latency in cell culture^{70,71}. In the base line unstimulated state, these cells express the multiply-spliced HIV-RNA and some singly-spliced HIV-1 RNA but an extremely low level of the full-length unspliced RNA⁷². Upon stimulation, this pattern undergoes a switch to the synthesis of unspliced transcripts with a concomitant upregulation of total viral RNA transcription⁷². Thus, these cells express a specific RNA pattern analogous to the early stage and appear to be blocked in progressing to the late stage of productive infection unless they are stimulated.

A similar situation is recorded in numerous asymptomatic patients, in which by *in vitro* reverse transcription coupled to PCR, it has been shown that the ratios between multiply-spliced to unspliced HIV-1 RNA are dramatically higher in these patients than in patients with AIDS⁷³. Over 70 % of peripheral blood mononuclear cells (PBMC) samples from asymptomatic individuals reveal a viral RNA pattern with blocked early-stage latency whereas over 80 % of PMBC samples from patients with AIDS exhibit a productive pattern. It can be concluded that the asymptomatic HIV-1 infection in human could be characterized by a majority of HIV-1-infected PBMCs which have HIV-1-specific RNA expression patterns consistent with a blocked early-stage latency.

U1 and ACH-2 cell lines have been treated with increasing concentrations of H₂O₂ to characterize their susceptibility to these stress conditions⁷⁴. Fig. 4 shows the lethal effect induced by H₂O₂ on U1 cells. These cells are rather sensitive to H₂O₂ and small increases in concentration lead to a proportional decrease in viability. The viability begins to significantly drop at a H₂O₂ concentration higher than 200 µM and leads to a very low surviving fraction above 2 mM. The lymphocytic cell line ACH-2 appears to be more sensitive to H₂O₂ than U1; a significant lethal effect is already observed at a very low H₂O₂ concentration such as 50 µM.

To estimate the effect of H₂O₂ on HIV-1 reactivation in these latently infected cells two different techniques have been used : (i) the measurement of a reverse transcriptase (RT) activity in cell supernatants and (ii) the detection of intracellular viral antigen. An increasing RT activity can be determined in supernatant fluid after H₂O₂ exposure and this phenomenon turned out to be transient with a maximum arising between 48 and 72 h. In addition, a dose-

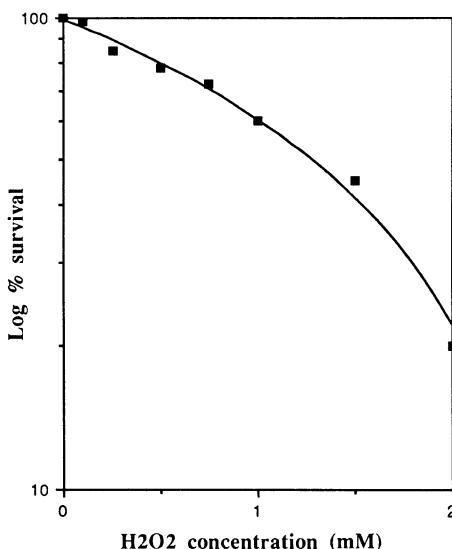


Figure 4. Lethal effect induced by H₂O₂ on the U1 promonocytic cell line. U1 cells were exposed to varying concentrations of H₂O₂ before being replaced in culture for 24 h and counted by trypan blue exclusion. The Log₁₀ of the percent of survival is plotted vs the H₂O₂ concentration in mM

response curve is obtained (Fig. 5). An increase of H₂O₂ concentration up to 1 mM in the case of U1 cells produces a proportional stimulation of the RT activity in the supernatant and above this concentration, the degree of stimulation decreases probably due to an excessive lethal effect of H₂O₂.

With the ACH-2 cells, the optimal concentration of H₂O₂ which leads to the higher HIV-1 reactivation is much lower than observed with U1 cells, and corresponds to a range of H₂O₂ concentrations observed in physiological conditions. An increase of intracellular HIV-1 proteins synthesis has been evidenced by an immunofluorescence (IF) assay ⁷⁴. Treatment of U1 cells with H₂O₂ between 0.5 and 1 mM leads to an increase in the frequencies of IF positive cells with an optimum 48 h after the oxidative stress.

In an attempt to define the molecular steps involved in the HIV-1 reactivation, we have used as cellular target a cell line carrying an integrated DNA cartridge containing an indicator gene under the control of the HIV-1 LTR. These cells are subjected to increasing concentrations of H₂O₂ and the reporter gene product activity is determined in cells extracts. The expression of the reporter gene under the LTR control is increased after exposure to H₂O₂ and as for the U1 and ACH-2 cells, a dose-response curve can be observed. Maximal stimulation of the gene expression ranged from 3 to 4 fold when the H₂O₂ concentration approaches 500 μM. These results clearly demonstrate that the first event in the HIV-1 reactivation following an oxidative stress is the LTR transactivation by transcription factors.

The nature of the transcription factor induced in these pro-oxidant conditions has been first elucidated by Schreck *et al.* ⁷⁵ who have shown that the treatment of T-cells with H₂O₂ activates NF-κB, this activation is specific and can be blocked by an anti-oxidant compound such as N-acetyl-L-cysteine ^{76,77,78}. We have undertaken similar experiments on ACH-2 and U1 cells to try to correlate the HIV-1 reactivation with the NF-κB and/or AP-1 activation. These two cell lines have been subjected to a oxidation reaction with H₂O₂ and then used either to monitor NF-κB and AP-1 DNA binding activity by gel retardation assay and virus reactivation by measuring RT in the supernatants. The results of NF-κB experiments are shown on Fig. 6. A very strong NF-κB activity can be demonstrated in the nuclear extracts of ACH-2 cells treated by increasing H₂O₂ concentrations; this induction is comparable to that observed with phorbol ester. However, it should be pointed out that the stimulation of the NF-κB DNA binding activity cannot be perfectly correlated with the increase in RT observed in cell supernatants demonstrating that the HIV-1 reaction process is not completely

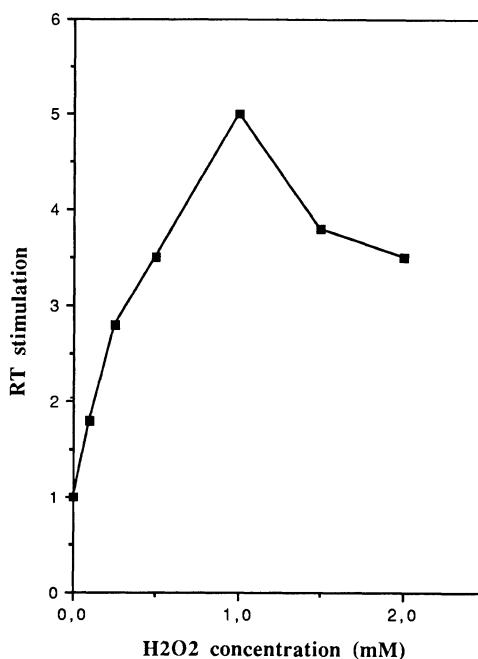


Figure 5. Effect of various H₂O₂ concentrations on virus production by U1 promonocytic cells expressed in term of RT activity stimulation. RT assays were performed 72 h after the oxidative stress on 1 ml of supernatant fluid.

dependent on the activation of one transcription factor such as NF-κB. Similar results are observed with the promonocytic cell line U1 but these cells are much more resistant to H₂O₂ than ACH-2 cells.

In the case of AP-1, a slight increase in AP-1 DNA binding activity can be observed at short time after the stress (60 min) (Fig. 6) but there is no correlation with the RT determination demonstrating that the activation of this transcription factor in the HIV-1 reactivation cannot be fully attributed to H₂O₂. Thus, there is a clear distinct responsiveness of NF-κB and AP-1 to H₂O₂ in T-cells. There are differences with respect to the strength of the response, kinetics as well as susceptibility to the levels of antioxidants. Indeed, Meyer *et al*⁷⁹ have observed that antioxidants such as pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) enhance DNA binding and transactivation of AP-1 in response to phorbol ester activation, whereas H₂O₂ is suppressing phorbol ester activation of the factor.

These experiments demonstrated the central role played by NF-κB in T-cells, but it still remains to be determined which is the pathway that triggers the release of the inhibitory subunit I-κB from its complex with p65 and p50 in the cytoplasm allowing the translocation of NF-κB to the nucleus and DNA binding on the HIV-1 LTR. Recently, it has been shown that the *in vivo* mechanism of NF-κB activation operates through the phosphorylation⁸⁰ and the subsequent loss of the I-κB subunit by a rapid proteolysis due to an inducible chymotrypsin like protease⁸¹. Because antioxidants like PDTC and NAC can decrease the level of NF-κB activation upon stimulation with phorbol esters, it has been postulated⁷⁵ that reactive oxygen species like H₂O₂ are second messengers involved in a general pathway leading to gene activation. Their role in AP-1 activation is still unclear.

Singlet oxygen generated intracellularly can reactivate HIV-1.

Because an oxidative stress mediated by H₂O₂ can promote HIV-1 reactivation through a mechanism involving NF-κB, we want to know (i) whether or not other reactive oxygen

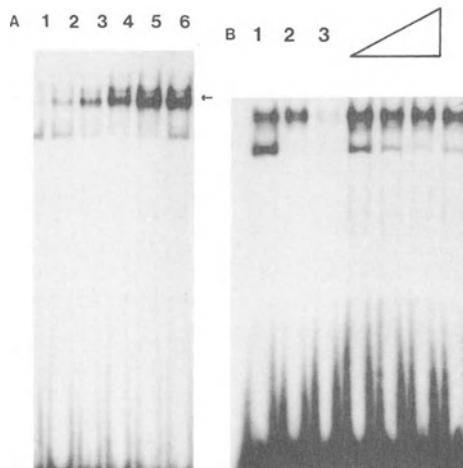


Figure 6. Induction of NF-κB (A) and AP-1 (B) DNA binding activity to their DNA responsive elements contained in the HIV-1 LTR after treatment of ACH-2 cells with increasing H₂O₂ concentrations. A Nuclear cells extracts were prepared 120 min after the stress, then mixed with a [³²P]-labeled oligonucleotide encompassing the NF-κB region of the HIV-1 LTR and resolved by native PAGE : (1) untreated ACH-2 cells, (2) to (6), ACH-2 cells treated, respectively, with 50, 100, 200, 250 and 500 μM H₂O₂. B same as in A except that nuclear extracts were taken 60 min after the stress and bound to the AP-1 responsive elements, (1): control cells, (2) control cells induced with PMA, (3) same as in (2) except that competition was done with a cold probe, the other samples correspond to cells treated, respectively, with 50, 100, 250 and 500 μM H₂O₂.

species can promote a similar effect, especially ¹O₂ which is produced by activated eosinophils ⁸² and which reacts far more specifically than other radical species ⁸³ and (ii) whether or not the generation of this species at different locations inside the cell can also initiate HIV-1 reactivation. This second point is very important. Indeed, the penetration of H₂O₂ in the cytoplasm is probably the central event in the post-translational activation of NF-κB by a mechanism leading to the release of the inhibitory subunit and the migration of the p65/p50 complex to the nucleus ⁷⁵. Thus, it is very important to know whether such a mechanism of activation can be triggered by an oxidative stress initiated outside the cytoplasm like in the nucleus or in the extracellular medium. Answering to this question would permit to characterize eventually other signalling pathways capable to promote LTR transactivation.

To study the effect of ¹O₂ on HIV-1 reactivation, we have used the same cellular model as described above (U1 and ACH-2 cell lines) and the generation of ¹O₂ by photosensitization. This reaction involves a molecule absorbing light and transferring the absorbed energy to molecular oxygen which in turn is activated and generates ¹O₂ ⁶⁹. This reactive oxygen species is a powerful oxidant capable to react with almost all biomolecules like cholesterol and phospholipids ⁸², aromatic amino-acids of proteins ⁸⁵ and with nucleic acids ^{86,87}. One interesting point concerning photosensitization is the possibility to initiate the reaction at various places inside the cell depending on the localisation of the photosensitizer.

In our work, we have assayed three photosensitizers : (i) Rose Bengal bound (RB) to beads which generates ¹O₂ outside the cells because the beads block the dye uptake, (ii) free RB which is water soluble, penetrates in the cytoplasm and in the nucleus and (iii) proflavine (Pf) which intercalates inside the DNA, produces ¹O₂ and a type I mechanism ⁸⁸ generating predominantly 8-oxo-dG in DNA without any other alteration in other cellular compartments.

Both U1 and ACH-2 cells lines have been reacted with $^1\text{O}_2$ produced in the three conditions described above and the susceptibility of these two cell lines to $^1\text{O}_2$ generated in these conditions has been investigated by measuring cell survival. No lethal effect can be detected in the absence of light demonstrating that these photosensitizers by themselves have no effect on the viability of these cells. However, when visible light is used to irradiate the cells complexed with the photosensitizers, cell survival starts to decrease proportionnally with the irradiation time. In all cases, ACH-2 cells turn out to be more sensitive than U1 cells to the treatment and an especially important cytotoxic effect is observed when the photosensitization reaction is mediated by RB bound to beads generating $^1\text{O}_2$ from the extracellular medium. When the photosensitization reaction is mediated by free photosensitizers like RB or Pf, survival is dependent on the photosensitizer concentration. Fig. 7 shows an example of U1 cell survival after photosensitization during increasing period of time in the presence of Pf.

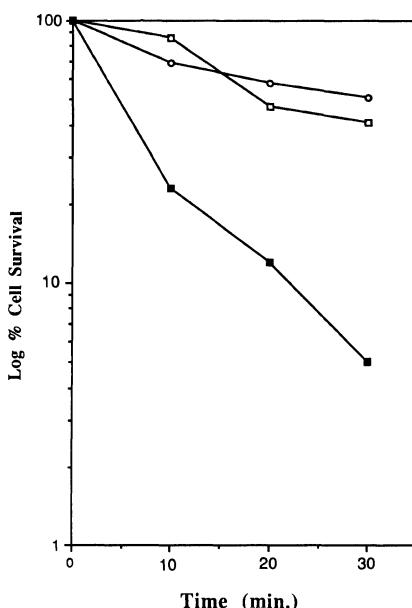


Figure 7. Survival of the U1 promonocytic cell line following an oxidative stress induced by photosensitization in the presence of various concentrations of proflavine (Pf) : - - - 1 μM , - - 0.5 μM and - O- 0.25 μM . The percentage of survival is plotted vs the irradiation time in minutes (min).

HIV-1 reactivation from both U1 and ACH-2 cell lines photosensitized in these three conditions is determined by measuring RT activity in the cell supernatants 24 and 48 h after the oxidative stress. RT activities measured 24 h after the photosensitization reactions are always very low whatever the irradiation condition chosen. However, 48 h after photosensitization, significant RT activities can be detected in the cell supernatants taken from cells photosensitized with free RB and with Pf. In the case of RB bound to beads, no RT activity can be detected although cell viability is strongly affected by this kind of treatment. Fig. 8 shows the reactivation of HIV-1 from U1 and ACH-2 cells treated with free RB and increasing irradiation times. Two important features can be pointed out from RT measurements : (i) RT activities exhibit an optimal value as a function of the irradiation time and, (ii) at comparable survival levels, RT activities are somewhat higher for ACH-2 than for U1 cells.

From the experiments described above, it clearly appears that HIV-1 can be activated by photosensitization treatments only when the photosensitizer can be uptaken by the cells. These results are highly relevant for the blood transfusion centres which use

photosensitization to clean-up blood products. Indeed, to avoid photoinduced HIV-1 reactivation from contaminating lymphocytes which could eventually remain in plasma, it would be safer to use a photosensitizer remaining outside cells or eventually, binding to membranes. In such experimental conditions an important cytotoxic effect should be observed without viral photoactivation.

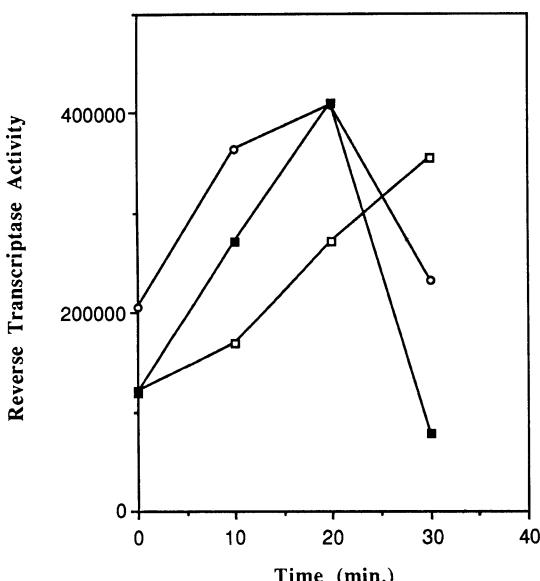


Figure 8. Induction of HIV-1 reactivation by photosensitization of U1 and ACH-2 cells by free rose bengal (RB). Supernatant were taken 48 h after the photoreaction. -○- ACH2 treated with 0.25 μ M RB, -■- U1 cells treated with 0.25 μ M RB and -□- U1 cells treated with 0.4 μ M RB.

In order to better understand the mechanisms underlying this photosensitized reactivation, both NF- κ B and AP-1 DNA binding activities are followed in both ACH-2 and U1 cell lines treated either with RB or Pf. Thus, cell lines have been photosensitized in similar conditions than above in order to determine whether or not there is a correlation between the activation of these transcription factors and HIV-1 reactivation. Nuclear salt extracts have been prepared either 120 min after the photoreaction for NF- κ B determination or 60 min for AP-1. Gel retardation assay shows an important NF- κ B DNA binding activity in ACH-2 cells after photosensitization with both PF (Fig. 9) and RB ; a lower enhancement of this binding activity being also observed with U1 cell extracts. However, only slight AP-1 DNA binding activities can be observed in these cell extracts which does not correlate at all with the RT measurements. These data demonstrate, like in the case of H_2O_2 , that NF- κ B plays a crucial role in HIV-1 reactivation. Generation of reactive oxygen species in the nucleus by a photosensitization reaction mediated by Pf demonstrates that oxidative DNA damage could also be involved as a primary event in a signaling pathway going from the nucleus to the cytoplasm to activate NF- κ B and then going back the nucleus to bind to several promoters with among them the HIV-1 LTR.

In conclusion of this part of the paper, it is obvious that an oxidative stress mediated by 1O_2 can reactivate HIV-1 remaining latent in both monocytes and T-cells. However, important differences can be detected with regard to the site of 1O_2 generation. Indeed, when 1O_2 is produced outside the cells, it produces a strong cytotoxic effect probably due to oxidation of the cellular membranes but no HIV-1 reactivation can be seen. These results demonstrate that an intracellular prooxidant state has to be reached in order to get reactivation. This situation can be obtained when 1O_2 is produced either in the cytoplasm or in the cell

nucleus. In the last case, it has been shown that Pf photoreaction induces only DNA damages without any side effects outside the nucleus. Thus, it can be concluded from these experiments that DNA lesions can be the primary event of a signaling pathways which could either (i) directly modify the chromatin structure, promoting its unwinding and then allowing the activation of integrated HIV genes as proposed by Valerie and Rosenberg ⁸⁹ and Verdin *et al* ⁹⁰ or (ii) send secondary messengers to the cytoplasm which could induce the post-translational modification of transcriptional factors through the activation of kinases, phosphatases or proteases.

- 0 5 10 15



Figure 9. The effects of photosensitization mediated by Pf on the NF-κB activity in ACH-2 cells. Nuclear extracts were prepared and incubated with ³²P-labeled oligonucleotide followed by analysis by native polyacrylamide gel electrophoresis. - control cells; 0, 5, 10 and 15 are ACH-2 cells photosensitized by Pf during respectively 0, 5, 10 and 15 min.

Finally, we will conclude this review by mentionning that UV light has also been shown to be an important factor in reactivating several other viruses like the Herpes simplex virus type 1 and type 2 ⁹¹. These recurrent infections result from the reactivation of latent virus remaining in the sensory ganglia with transport of the virus to the periphery where its replication produces characteristic herpetic lesions ⁹². Several animal models have been developed to understand the mechanism by which UV initiates this reactivation ^{93,94,95}; and it turns out that a virally encoded protein called ICP0 plays an important role in triggering photoinduced HSV-1 reactivation ⁹⁶. Many points still remain unclear in this phenomena, specially, how does UV light lead to ICP0 gene activation. Furthermore, inhibitors of prostaglandin synthesis, such as indomethacin can strongly decrease the level of UV-induced recurrent herpes simplex virus disease in guinea-pigs probably by preventing local immunosuppression or by directly inhibiting reactivation of HSV from latently infected neurons ⁹⁷. Thus, the mechanism responsible for the reactivation could then involve prostaglandins, since exposures to UV are known to increase local levels of prostaglandins ^{98,99} which could produce local immunosuppression ^{100, 101} allowing the reactivated HSV to replicate. In the case of HSV reactivation by UV, it is evident that the signaling pathway which initiates reactivation and virus replication in neurons is closely controlled by the immune system.

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GENERAL ASPECTS OF UV-IRRADIATION ON THE IMMUNE SYSTEM

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INTRODUCTION

In the last decade it has become evident that exposure of skin to ultraviolet light has an effect on the immune system. In this chapter the immune system and the effects of UV-light on the immune system will be reviewed briefly.

The essential function of the immune system is defence against neoplastic cells and infectious agents such as parasites, viruses, fungi, and bacteria. For this reason, ultraviolet light may have deleterious effects on the resistance against neoplastic cells and infectious agents.

THE IMMUNE SYSTEM

Antigens

An antigen is by definition a non-self protein or (poly)-saccharide which can induce an immune reaction. Antigens are characterized by the fact that they have a foreign (non-self) tissue structure. In some cases it is possible that own (self) tissue becomes a non-self protein and hence is recognised by the immune system as an antigen. In such a situation an immune response directed against own tissue antigens will create an autoimmune-disease.

HLA (MHC) Molecules

HLA (human major histocompatibility complex, MHC) molecules are present on the surface of cells in most tissues. These molecules will determine whether or not the tissue will be recognized as self or non-self.

The molecules are divided into HLA class I molecules and HLA class II molecules. There is a large polymorphism of these molecules. The molecules are inherited in a codominant way.

The Immune Response

In general the immune system, which serves to identify and to remove the antigen, is divided into two parts. A non-specific part that is characterized as a physical/chemical barrier found in for example the skin and mucosal tissues of the respiratory and gastro-intestinal tracts. In addition to the cells of the physical/chemical barrier that includes epithelial and mucus cells, also natural killer cells, macrophages and polymorphonuclear cells play an important role in the aspecific immune response.

The specific part of the immune system can be divided into a humoral and a cellular immune system. In the humoral immune system B-lymphocytes and in the cellular immune system T-lymphocytes play a predominant role.

The humoral Immune System. B cells, originating from the bone marrow, mature into plasma cells and produce immunoglobulins that can bind antigens via the combining sites of their variable regions. Each matured B cell (plasma cell) can produce antibodies with one particular specificity. Dependent upon the type of antigen, help of T cells (Th cells) is necessary. Diversity in antibody specificity is created by somatic recombination of genes encoding for different regions of the immunoglobulin-molecules. All the immunoglobulins have a molecular structure in which the specificity is ascertained. Additionally, immunoglobulins have a common structure which binds to plasma-membranes. In this way immunoglobulins are able to have a very specific binding site to certain antigens. On the other hand they have a possibility to activate certain cell types by binding to the plasma-membrane. In general antibody-binding serves to identify the antigen. Antigen-antibody complexes are cleared with the help of complement and phagocytic cells. In contrast, immunoglobulins may also directly neutralize the action of some bacterial toxins.

Immunoglobulins can be subdivided into several classes, i.e.: Immunoglobulin A, M, E, G and D (IgA, IgM, IgE, IgG and IgD). The function of IgA is predominantly restricted to the mucosal membranes. It plays a role in the defence-system of these membranes against foreign antigens. IgE is present on mast-cells and basophils. When these molecules are cross-linked by an antigen, the mast-cells will release histamine and other vaso-active substances. These substances have a profound effect on various systems. IgE plays a role in allergic immediate type reactions and during parasitic infections. IgM and IgG play a role in the defense system against antigens in the bloodstream and in all tissues. The binding of these immunoglobulins to antigens facilitates the destruction of these antigens by the immune system.

Cellular Immune System. Although T cells play a predominant role in cellular immunity also polymorphonuclear cells, macrophages and natural killer cells can play a role in the effector phase of the cellular immune response. However, these latter three cell types act non-specifically in contrast to the very specific action of T cells. The specificity of these non-specific cells is often brought about by T cells that recognize foreign structures and attract or stimulate non-specific cells like macrophages to it. The early development of the T cell system occurs during early youth in the thymus.

It is known that when an antigen complex is presented to a T cell receptor in a proper way, these cells are activated. Every T cell selects its antigen receptor by rearranging the available receptor genes. The antigen complex is "embedded" in a HLA (MHC) molecule. The specificity of the T cell response is regulated by the fact that certain sites (epitopes) of the antigen which is embedded in the HLA molecule are presented to certain binding sites (agretopes) of the T cell receptor. In this way a specificity, which is restricted by HLA molecules, is obtained. T cells recognize antigens mostly in the context of specialized "self" molecules, encoded by the major histocompatibility complex (HLA(MHC)-restriction). In general, all T cells are MHC-restricted and are especially important in the immune response against cells with an altered surface phenotype, e.g. cells changed by neoplastic transformation, intracellular pathogens and chemicals. In addition, T cells can only be stimulated in the presence of certain surface adhesion molecules, costimulatory signals (figure 1). Recently it has been shown that binding of the surface molecule B7 to CD28 present on the human T cell is important for the induction of the T cell response¹.

The T cell population can be divided into various subtypes of T cells. These cell types can be discriminated by the fact that they have different surface molecules and that these cells have a different response to certain cytokines and/or have a different function.

In general the majority of mature T cells can be subdivided into CD4⁺ and CD8⁺ lymphocytes. The CD8⁺ T cells have predominantly a cytotoxic effect. These cytotoxic T cells are stimulated by helper T cells (CD4⁺). The cytotoxicity of these cells is directed against tissues or cells which have a foreign (non-self) antigen on their surface (mostly virus infected cells or neoplastic cells). In this condition the antigen is presented to the cytotoxic T cell in combination with a HLA class I molecule which is present on all nucleated cells. This will also determine the specificity of the cytotoxic T cell response.

The CD4⁺ T cells are called T-helper cells. These cells respond to antigens only in the context of HLA class II molecules. In 1986, the existence of at least two CD4⁺ T helper cells was discovered in mice, designated Th1 and Th2 cells². These Th subsets can not be differentiated on the basis of a cell surface marker. They produce, however, defined patterns of cytokines that lead to strikingly different functions.^{3,4} Roughly speaking, Th2 cells are more efficient B cell helpers, especially in the production of IgE, whereas Th1 cells mediate delayed type hypersensitivity like immune responses. In addition, they crossregulate by producing mutually inhibitory cytokines. These two types of Th cell subsets were originally identified in the mouse. Recently, it has become clear that they also exist in humans⁵. The stimulation of the Th1 and Th2 cells has been investigated intensively.

Apart the existence of T cells with a helper or cytotoxic function, also T cells which can suppress the immune response exist. These cells are called T suppressor cells. It has been postulated that Th2 cells may be able to induce the proliferation of the suppressor T cells which are mostly CD8⁺ T cells.

Antigen Presenting Cells

Antigen presenting cells are cells which are able to present antigen to lymphocytes. These cells can be roughly divided into dendritic cells and non-dendritic cells. The dendritic cells are able to present antigens to naive T cells and to induce a proliferation of these naive T cells. Therefore dendritic cells have a very strong antigen presenting capacity.

Another type of antigen presenting cells are macrophages, which have a large phagocytic activity and can also present antigens to the T cell system. It is believed that these antigen presenting cells can only activate T cells which have already been primed. Finally B cells can also present antigens to lymphoid cells. It is suggested that B cells especially present antigens to Th2 cells and not Th1 cells.

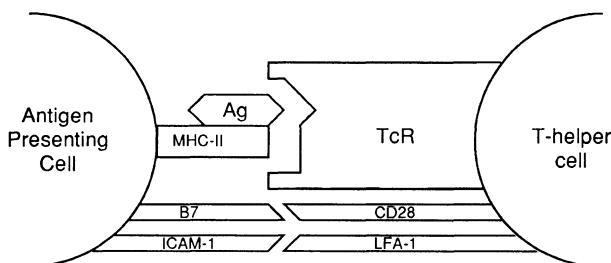


Figure 1. Antigen recognition by T cells. T cells recognize antigen in association with self-MHC molecules and get activated in the presence of costimulatory signals (e.g. B7-CD28 and ICAM1-LFA1 binding).

Cytokines

Cytokines are intercellular regulatory proteins that mediate a multiple of immunologic as well as nonimmunologic functions. Insufficient production of cytokines, such as interleukin-1 or interleukin-2, or the inability to respond adequately to these as well as to other immunomodulatory cytokines, oftentimes results in a state of immunosuppression. Conversely, exorbitant cytokine production may cause severe shock, autoimmune disease, or immunopathology associated with a given disease.

Many different cytokines have been isolated. The cytokine network consists of a complicated system in which also complement factors and factors important for the clotting system can be included.

THE SKIN IMMUNE SYSTEM

Several lines of evidence support the fact that in the skin the immune system is compartmentalized in the so-called skin immune system (SIS, figure 2). This is reviewed by Bos et al.⁶ Streilein⁷ extensively studied the SIS in rodents and called the SIS in these species SALT, i.e. skin associated lymphoid tissue, because of the presence of lymphoid cells in the epidermis. The term SALT is analogous to terminology used for other local lymphoid tissues such as BALT (bronchus associated lymphoid tissue) and MALT (mucosal associated lymphoid tissue) reviewed by Sminia et al.⁸

The terms skin immune system and skin associated lymphoid tissue suggest that within the skin the cellular elements which are immunological active represent a specific compartment. It has been shown that within the skin and the regional lymph nodes all prerequisites are present to induce a specific T cell response. Within the epidermis the most important cell in this respect is the Langerhans cell. This cell has a dendritic shape and is able to present antigens. The cell contains the enzyme ATP-ase and expresses HLA class II and B7 molecules. One of the characteristics of the Langerhans cell, in contrast to other dendritic antigen presenting cells, is the presence of Birbeck granules within the cytoplasm. The function of this Birbeck granule is still debated.⁹⁻¹¹

It has been shown that after stimulation of Langerhans cells these cells migrate from the epidermis through the basement membrane and dermis to the regional lymph nodes.¹² The adhesion molecules needed for this migration can be detected on their membranes. Stimulated Langerhans cells are able to present antigens very efficiently. Schuler¹³ has shown that cultured Langerhans cells have an increased antigen presenting capacity.

Apart from playing a role in the induction of the T cell response, the Langerhans cell also contains IgE receptors. For this reason this cell may be important in IgE mediated immune responses such as atopic dermatitis.^{14,15}

In murine epidermis, another type of dendritic cell is present which is called Thy1 DEC-cell (dendritic epidermal cell with the pan T cell marker). This cell has been shown to be an immature T cell. The finding that DEC-cells do not exhibit diversity in the T cell receptor suggests that they may perform functions that are quite different from normal T cells.¹⁶ According to Havran et al.¹⁷ DEC-cells can specifically recognize self antigens produced by skin-derived keratinocytes. It is suggested that the cells may function in identifying and perhaps even destroying those keratinocytes that have been damaged by various forms of stress.

A great majority of the epidermal cells are keratinocytes (approximately 97%). It has been shown that these cells are able to produce various inflammatory mediators such as prostaglandin E2 (PGE2) and cytokines such as tumor necrosis factor alpha (TNF α), interleukin 1 (IL-1), and interleukin 10 (IL-10). Co-culture models of keratinocytes and fibroblasts have demonstrated that the cytokine production of keratinocytes induces the production of other cytokines by dermal fibroblasts and endothelial cells. By this mechanism the keratinocytes play a regulating role in the immune system and environment of the skin.

EFFECTS OF UV-LIGHT ON THE IMMUNE SYSTEM

Animal Studies

Tumor Models. Already in the late seventies and early eighties it was demonstrated that UV light can have an effect on the immune system leading to immunosuppression. These studies were in majority restricted to animal studies. Kripke et al.¹⁸⁻²¹ demonstrated that exposure of murine skin to UV-light renders these mice less able to reject antigenic carcinoma tissues. If UV induced skin tumours were transplanted to syngeneic mice these tumours were rejected. However, if the recipient mice were pre-exposed to UV light the tumours were accepted. This was one of the first indications that UV can inhibit the immune system locally

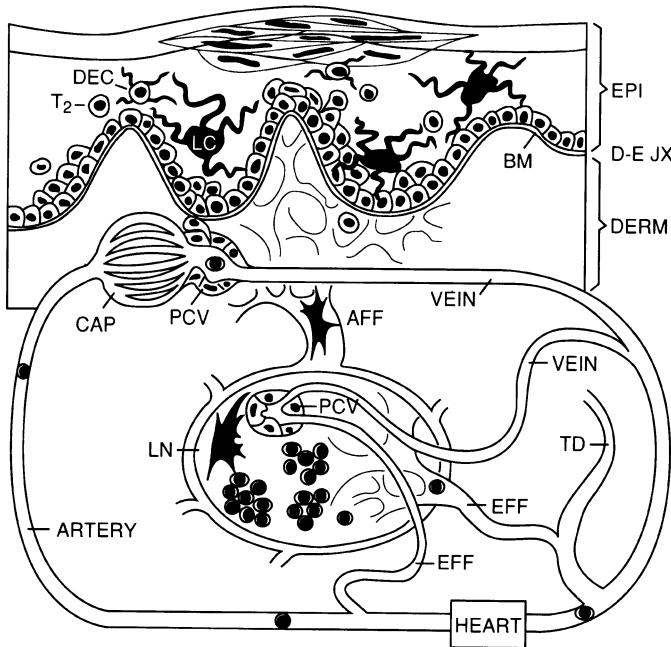


Figure 2. Skin Associated Lymphoid Tissues (SALT) (Reprinted with permission,⁷ CRC Press, Boca Raton). Abbreviations: EPI: epidermis; PVC: post capillary venule; D-E JX: Dermal epidermal junction; LC: Langerhans cell; DERM: dermis; CAP: capillary; TD: thoracic duct; LN: lymph node; AFF: afferent lymphatic; ART: artery; EFF: efferent lymphatic; BM: basement membrane; T₂: TCR-2 cells (α/β chains comprise T cell receptor); DEC: dendritic epidermal cell (γ/δ chains comprise T cell receptor).

as well as systemically. Later it was demonstrated that lymphocytes were responsible for this phenomenon.²² If lymphocytes from UV-irradiated mice were injected into syngeneic mice, transplanted non-melanoma skin cancers in these recipient mice were not rejected. This UV induced tolerance or immune suppression was specific for the UV induced carcinomas. The outgrowth of UV induced squamous cell carcinomas transplanted into athymic mice suggests that UV provokes immune deficiencies in athymic hairless mice that can also be observed in non-UV-exposed athymic mice.²³ Thus, changes in thymus dependent immunity may play a role in altered susceptibility to UV-induced carcinogenesis.

The effect of UV-B on the formation of melanomas suggests that also immunosuppression may be important. When syngeneic melanoma cells are injected into UVB irradiated ear skin, the melanomas appear earlier and with a higher incidence on the UVB irradiated skin than on a normal non-irradiated skin.²⁴⁻²⁶ This effect of UVB appears to be immunologically mediated. It may be related to the finding that immunity to a melanoma challenge is abrogated when the challenge dose of melanoma cells is injected into UVB exposed skin. Recently a correlation between exposure to UVB and the incidence of uveal melanomas has been found.²⁷

The immunological resistance against other types of "antigenic" tumours such as those induced by chemicals is also affected. Exposure of mice to UVB radiation results in enhanced and accelerated growth and metastasis of benzo[a]pyrene induced tumors.^{28,29}

Infection Models. The effect of UVB on the resistance against infectious diseases described so far is mainly restricted to skin-associated infectious diseases. *Herpes simplex* virus has been widely used as an experimental model of virus infection in mice. The immune response against this virus is suppressed by suberythermal doses of UVB.^{30,31} In addition, it is demonstrated using animal models that the immune response and pathogenesis induced by the parasite *Leishmania* can be affected by UVB exposure.³² In a yeast infection mouse

model (*Candida albicans*) it is demonstrated that the cellular immune responses against this fungal pathogen is inhibited by UVB exposure.^{33,34}

Recently it is demonstrated that the resistance against non-skin-associated infectious disease can also be affected by UVB exposure. The resistance against *Trichinella spiralis* in rats is inhibited by UVB exposure.³⁵ A widely used systemic bacterial infection model is *Mycobacterium bovis* causing tuberculosis. Mycobacteria still give major problems in tropical and subtropical countries in man and animal and it may be hypothesized that this is influenced in part by the high level of UVB radiation in these areas. Exposure to UVB, before and during infection, reduces the DTH against mycobacteria bovis in mice. Also the size of the lymph nodes is reduced. The total number of bacteria is increased in the spleen and lymph nodes after UVB exposure. An increase in the number of bacteria at unirradiated sites indicates a systemic effect of UVB.³⁶ Recently it was demonstrated that also the phagocytosis of these bacteria is affected by UVB.³⁷

In addition to these tumor and infection animal models effects of UVB on the immune system are extensively studied using several functional tests for the immune system such as contact hypersensitivity (delayed type hypersensitivity), mixed lymphocyte responsiveness, antigen presenting capacity tests, immunoglobulin titers, etc. Experiments of Toews et al.³⁸ showed that when skin areas of mice were irradiated with UV-light prior to sensitisation with an contact-allergen (DNPFB), the mice became tolerant to this specific allergen. The challenge with DNPFB after 14 days (which in normal conditions would evoke an allergic contact hypersensitivity reaction, skin swelling) did not cause any reaction in the UV-irradiated animals. This unresponsiveness was also found on non-irradiated skin areas indicating that UV can induce systemic immunosuppression. The immunosuppression in this model was also antigenspecific.

It has been postulated that the induction of immune tolerance in mice is regulated by genetic factors which are localized in the genes which are ruling the TNF α production. It was demonstrated that the induction of immunosuppression is dependent upon the mouse strain used.³⁹

Cellular Events (Possible Mechanisms of UVB Induced Immunosuppression)

Langerhans Cells. It is suggested that Langerhans cells play a crucial role in the induction of immunosuppression by UV light. A number of UV-induced changes of Langerhans cells is described such as: a decrease in number of Langerhans cells,^{38,40-43} a change in their morphology^{40,42,44} and a change in their antigen-presenting function.⁴⁵⁻⁴⁷

It is demonstrated that the UVB induced impairment of contact hypersensitivity responses is correlated with a decreased number of Langerhans cells in murine skin.³⁸ However, this form of UVB induced immunosuppression does not occur in all strains of mice, despite the fact Langerhans cell depletion occurs in all strains of mice. This indicates that a loss of Langerhans cells does not necessarily correlate with "local" immunosuppression.³⁹ Narrow-band UV exposure of mice showed that a decrease in number and change in Langerhans cell morphology is not always causally involved in the systemic suppression of the immune system.⁴² It is suggested that in the mouse the DEC T cell is involved in UVB induced immunosuppression instead of Langerhans cells.³⁹ In humans the UVB induced depletion of Langerhans cells is followed by infiltration of other HLA-DR expressing cells in the epidermis.^{48,49} These bone-marrow derived macrophage like cells (CD1A negative, DR positive) that restore the Langerhans cell function, appear in the epidermis following LC depletion due to UVB or UVC irradiation but not to UVA irradiation.⁵⁰ It is suggested that these infiltrating cells can activate a different population of CD4 positive cells, which may activate suppressor pathways.^{50,51}

Another hypothesis is that the function of Langerhans cells is altered. The inhibition of the Langerhans cell function by UV-light occurs already at a very low dosage of UVB. This means that exposure of skin to a low amount of UV-light has an effect on the antigen presenting capacity of the skin. The consequence is that the Langerhans cell cannot present the antigen in a proper way and the induction of T cell proliferation is blocked. It is hypothesized that UVB converts the antigen-presenting function of Langerhans cells for T helper cell activation from one that is immunogenic to one that is tolerogenic (TH2 instead of TH1 activation).^{45,47}

The exact mechanism by which the Langerhans cell function is affected by UV is not yet known. Early experiments showed that UV-irradiation decreases the presence of HLA class II molecules on Langerhans cells. However, recent experiments have demonstrated that when antigen presentation is inhibited by a relatively low dose of UV-irradiation, the HLA class II molecules are still present. Moreover, immuno-electronmicroscopical studies of the Langerhans cells in the skin have shown that the class II antigens are still present in the same cytoplasmic organelles after irradiation with UV-light.⁵²

T Cells. A direct effect of UVB on lymphocytes is discussable. However, Bruls et al.⁵³ demonstrated that 10% of 310 nm UVB light is able to penetrate the epidermis and reach the upper bloodvessels in the dermis. Whether circulating lymphocytes are present long enough in the dermis to be exposed to sufficient amounts of UV irradiation in order to have a chance to be affected directly by UVB is not known.

Indirect effects of UV light on lymphocytes are extensively studied. It is demonstrated in animals and man that UVB exposure can inhibit the function of lymphocytes from the blood stream, spleen and lymph nodes. Simon and Bergstresser have shown in mice that UVB especially inhibits T cell responses in which Th1 cells play a crucial role. The hypothesis is that the antigen presenting activity of the Langerhans cells is perturbed or changed in such a way that only Th2 cells (IL-4 responding T cells) are proliferating, and IL-2 responding T cells (Th1) are inhibited.⁴⁵⁻⁴⁷

In addition to these effects, UVB may also affect the homing and distribution of lymphocytes. UVB exposure of mice during 6 days induces a change in lymphocyte recirculation and homing to regional lymph nodes. Migration to peripheral lymph nodes seemed to be increased. This effect persists for about 10 weeks.⁵⁴ Prolonged retention of lymphocytes in these lymph nodes results in hyperplasia of these lymph nodes. The number of high endothelial venules (HEV), which play a crucial role in lymphocyte-homing is increased in these peripheral lymph nodes. This correlates with an increased cell number per peripheral lymph node.^{54,55} Recently it is demonstrated that UV can also affect adhesion molecules which are also important for lymphocyte homing.⁵⁶⁻⁵⁸

Macrophages. Cooper et al.⁴⁹ have shown that exposure of human skin to a large amount of UV (2 à 3 MED) inhibits within 24 hours the immune response as expected. However, after 48 hours there was an enhanced immune activity in the skin. This enhanced immune activity was based on the fact that OKM5⁺ macrophage-like cells were present within the skin. These cells were able to present antigens and induce a T cell proliferation. These authors postulate that these macrophage-like cells migrate to the skin because of UV-irradiation and may be able to induce immune suppression via the activation of Th2 cells.^{45,47-51}

Jeevan et al.³⁷ demonstrated recently that UVB exposure can inhibit phagocytosis of *Mycobacteria (leprea and bovis)* by spleen cells. Thus, in addition to alteration in antigen presentation also phagocytosis may be affected by UVB. It is remarkable that these effects can be found in the spleen, i.e. a systemic effect.

Keratinocytes. Other cells which are influenced by UV-light are the keratinocytes. Exposure of skin to UV may induce various factors or cytokines which are produced by the keratinocytes.

Work of Kupper et al.⁵⁹ has shown that the IL-1 production of cultured keratinocytes is stimulated by the UV-light. However, Krutmann et al.⁶⁰ found that cultured human keratinocytes upon UVB radiation secrete a suppressor factor, which blocks IL-1 activity. Vermeer et al.⁶¹ have shown that UV-irradiation of the skin will result in a release of TNF α by keratinocytes. Both modulation of the IL-1 activity and the induction of TNF α can influence the immune system.

The work of Ullrich and Kripke on cultured skin cells have provided data that exposure of these cells to UV-light induces the release of various factors.⁶²⁻⁶⁴ When these factors, released in the medium of these cultures after UV-irradiation, were injected into mice, it was found that these factors can induce immunosuppression.^{37,62,64}

From these experiments it was concluded that TNF α may be responsible for the decrease in contact hypersensitivity reactions and that IL-10 may be responsible for the decrease in delayed type hypersensitivity.

Mast Cells. Mast cells are present in the skin (especially dermis) and can be influenced directly by UVB exposure because many of these cells are located just beneath the basement membrane of the epidermis. These cells are involved in several immune processes in the skin such as type I and IV hypersensitivity like reactions.^{65,66} The effect is dependent upon the dose of UV light. A low doses of UVB inhibits mast cell degranulation and for this reason UVB exposure may have positive effects on eczema and atopic dermatitis patients. In contrast, high doses of UVB can induce mast cell degranulation itself.⁶⁷ Because only a small part of UV light penetrates the epidermis, it is suggested that effects on mast cells are partly indirectly induced via mediators such as cytokines and prostaglandins.

Photoreceptors

There are several reasons to believe that UVB induced effects are not always due to direct interactions with epidermal cells. It is demonstrated that UV can interact with molecules in the skin, which after chemical reaction can interact with parts of the local and/or systemic immune system. These molecules are called photo-receptors.

As known for many years, UV-irradiation can lead to DNA-damage directly in epidermal cells. This DNA damage, which is maximal in the outermost cells of the epidermis, is crucial for the induction of skin tumours but may also change functions of immunocompetent cells in the skin. When this DNA-damage is repaired or prevented by the addition of repair-enzymes the induction of UV induced immunosuppression is inhibited.^{68,69} For this reason the authors suggest that DNA is an important photoreceptor for photo-immunosuppression.

Based on dose-relationship data of UV-irradiation and the inhibition of contact hypersensitivity in mice it has been postulated that urocanic acid is a photoreceptor for UVB and involved in the induction of immunosuppression.⁷⁰⁻⁷² Urocanic acid is present in the upper layers of the skin (stratum granulosum and stratum corneum) and is produced by the enzyme histidase from histidine. Urocanic acid, which is present in the trans isomer, is isomerized into a cis isomer by UVB-irradiation. Recent data have shown that trans urocanic acid can bind to a histamine-like receptor on fibroblasts and stimulate cAMP production. Isomerization of trans to cis urocanic acid will result in down regulation of cAMP by cis urocanic acid. Moreover, the injection of cis urocanic acid into mice induces immunosuppression.⁷³

Most recently Gibbs et al.⁷⁴ demonstrated that the role of cis-UCA in UV induced immunosuppression is less clear as was published earlier by Noonan and De Fabo.⁷⁰ The action spectrum of UCA photoisomerization and the action spectrum for immunosuppression are not exactly similar in mice.⁷⁴

At last the cascade of events which lead to immunosuppression can be depicted in figure 3.

CLINICAL RELEVANCE OF IMMUNOSUPPRESSION BY UV-LIGHT

Physiological Role

Immunosuppression is easily induced by UV-light. Even by a very low dosage of UV-exposure this appears to occur. In addition, UV-light can easily induce (neo)antigens which can cause an inflammatory reaction. The photo-immunosuppression can prevent this event (figure 4). Such a physiological role may also be seen in solar keratosis. In this condition there is an increased thickening of the epidermis and in the histological picture several abnormal keratinocytes surrounded by an inflammatory reaction is observed.⁷⁵ This inflammatory reaction could be caused by the antigenic property of the keratinocytes. Because of the immunosuppressive effects of UV light, the inflammation of solar keratosis patients is not severe. When photo-immunosuppression means that the antigenic keratinocytes can persist, a beneficial environment for the development of skin cancer may be present.

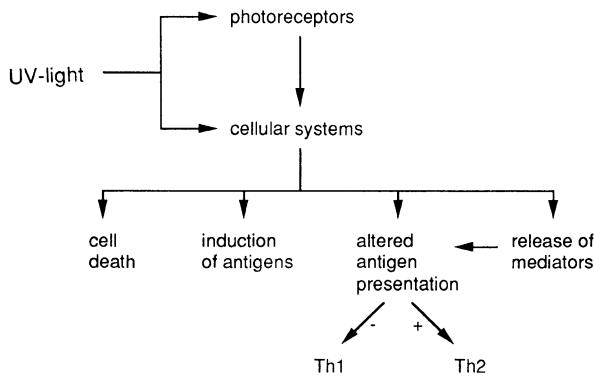


Figure 3. The cascade of events which can lead to UV-induced immunosuppression. UV-light can interact with photoreceptors (urocanic acid, DNA) and cellular systems (Langerhans cells, keratinocytes, macrophages), resulting in cell death, induction of antigens, altered antigen presentation and release of mediators (TNF α , IL-1, IL-6, IL-10, PGE $_2$). These cytokines can mediate the homing pattern of lymphocytes and the influx of OKM5 $^+$ cells in the epidermis. They can alter the antigen presenting function of Langerhans cells in such a way that the Langerhans cells preferentially activate Th2 cells and not Th1 cells.

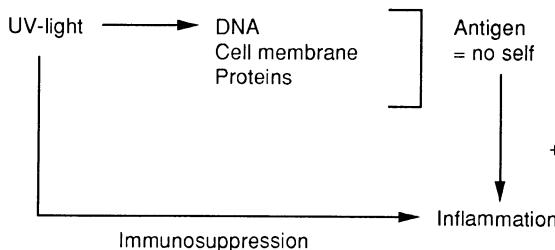


Figure 4. Physiological role of UV light. UV light can alter DNA, cell membranes and proteins which can lead to the induction of non self antigens and finally inflammation. The simultaneous UV-induced suppression of the immune system prevents the occurrence of a severe inflammation.

Risk Factor For Skin Cancer

It can be postulated that under certain circumstances the photo-immunosuppression can play an additional role in the development of skin cancer. This may especially be true in individuals who are already immunocompromised or in patients who have a genetic defect which causes an increased risk for the development of skin cancer.⁷⁶⁻⁷⁸

Individuals can suffer from a general immunocompromised condition due to the use of immunosuppressive drugs like corticosteroids, azathioprine, cyclosporine. Also an infection with human immunodeficiency virus (HIV) can cause an immunocompromised situation. The additional role of local photo-immunosuppression can render the sunexposed skin more susceptible for the development of skin cancer in these patients. The increased risk for the occurrence of skin cancer on sun exposed skin in transplant recipients can thus be explained. In our studies⁷⁹ we observed that not only skin cancers but also many wart-like lesions were predominantly present on sun exposed areas in renal transplant recipients (table 1). From these data we conclude that photo-immunosuppression can be relevant for the development of skin cancer and viral infection (human papilloma virus) of the skin for these patients.

Apart from exposure to sunlight also immunogenetic factors play a role in the susceptibility of renal-transplant patients for the development of skin cancer.⁷⁸ Patients with HLA-

A11 tissue antigens had a decreased risk for the development of skin cancer and patients with HLA-B27 an increased risk. The fact that immunogenetic factors also provide a risk factor, suggests that photo-immunosuppression also is regulated by immunogenetic factors. The investigations of Tseng et al.⁸⁰ and Yoshikawa et al.⁸¹ have provided evidence that the induction of photo-immunosuppression in mice and in men are ruled by genetic factors. In these studies it was shown that the induction of antigen specific (DNCB) tolerance by UV-light was nearly absent in healthy volunteers, but was present in the majority of patients with skin cancer.

Patients with a repair defect of thymine-dimers of DNA (Xeroderma Pigmentosum) have an increased risk for the development of skin cancer after UV-irradiation.⁷⁶ But in the Xeroderma Pigmentosum patients also the immunological function is disturbed. Moreover, it has been shown that UV-irradiation decreases the number of Langerhans cells in the epidermis more severe in these patients than in normal individuals. The photo-immunosuppression may have an additional role in the development of skin cancer in the Xeroderma Pigmentosum patients.

From these clinical data we can speculate that also in other patient groups who have an increased risk for development of skin cancer (i.e. Rotmund Thomson syndrome, Basal cell nevus syndrome, Epidermolyticus verruciformis) immuno-suppression may play an important role. Also in patients who have used co-carcinogens (arsenics) in early youth, the photo-immunosuppression can be important. Until now no good study has been performed to discriminate photo-immunosuppression as an additional risk factor in these patient groups.

It can be concluded that photo-immunosuppression is especially an additional risk factor in certain patient groups and may be of importance in a minority of the normal population.

Table 1. Mean number and localization of keratotic skin lesions in renal transplant recipients.⁸⁵

Localization and estimated percentage of body surface	surface area (%)	Mean number of lesions per 10% body surface
head and neck	9	5.7
forearms	6	19.2
backs of the hands	3	59.7
sun-exposed skin	18	19.2
non-sun-exposed skin	82	4.0

	100	

Viral infections

Apart from the development of skin cancer, photo-immunosuppression may play a role in the development of viral infections. From epidemiological and clinical findings it has been shown that exposure to sunlight may activate a herpes simplex infection. Both the activation of herpes virus and immuno-suppression may explain this phenomenon.^{82,83} The recent finding that the use of sunscreens can prevent the occurrence of herpes simplex infection demonstrate clearly that sunlight may provoke this infection.⁸⁴ Photo-immunosuppression may also play a role in human papilloma virus (HPV) infection.⁸⁵ In addition, it has been shown that HIV is activated by UV-light.⁸⁶

Parasitic, Bacterial, Yeast Infections

In animal studies it has been shown that the course of infections like leishmania,³² tuberculosis,³⁷ trichinellosis³⁵ and also candida infections^{33,34} are aggravated by the exposure to UVB. Also in humans the course of these infections may be aggravated by

exposure to UV-light. However, other socio/economic factors and factors of inoculation are most likely more important for the course of these diseases.

In animal models it has been shown that UV-exposure of the skin prior to inoculation of an antigen into the skin will perturb the immune response against this inoculated antigen. The interpretation may be that when a vaccination in humans is performed into skin which has just been exposed to sunlight, this vaccination might be less effective.

It can be concluded that photo-immunosuppression plays a role in herpes simplex virus infection and that its role in other virus infections is still hypothetical.

Prevention

When photo-immunosuppression is relevant (i.e. immunocompromised host), the prevention of photo-immunosuppression is important. The pigmentation of the skin does not seem to be protective against photo-immunosuppression.⁸⁷ The investigations with sunscreens are inconsistent. In several studies sunscreens did not prevent immunosuppression induced by fluorescent UV tubes: local and systemic suppression of contact hypersensitivity in mice⁸⁸ and suppression of the allo-activating capacity of human epidermal cells⁸⁹ were not prevented by sunscreens. Elmets et al.⁹⁰ found a photoprotective effect of sunscreens in cosmetics on Langerhans cell damage. Hersey et al.⁹¹ showed that sunscreens can prevent suppression of natural killer cell activity induced in human subjects by radiation of solarium lamps. In the study of Roberts et al.⁹² sunscreens were able to prevent suppression of allergic contact dermatitis in mice induced by a solar simulator. Therefore, the exact role of sunscreens with respect to the prevention of photo-immunosuppression is not yet established.

The Therapeutic Use

The therapeutic efficiency of UVB or psoralen + UVA treatment for psoriasis vulgaris, pityriasis lichenoides et varioliformis acuta, eczema and other inflammatory disorders of the skin, is partly based on the effect of UV-light on the immune system.

In the case of bloodtransfusions, donor thrombocytes can be irradiated with UVB prior to transfusion, to prevent an immune reaction of the host against the donor thrombocytes. This irradiation of blood cells by UVB is still in an experimental phase.

In conclusion it can be stated that photo-immunosuppression may have relevance in the development of skin cancer, especially in immunocompromised individuals. Further study has to reveal the exact nature of this relevance, especially for the development of melanoma and the occurrence of viral infections the photo-immunosuppression may be important. Moreover, photo-immunosuppression has also advantageous features, as it is being used as therapeutic regiments.

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PHOTODYNAMIC THERAPY OF CANCERS

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INTRODUCTION

Genomic stability ensures genetic continuity in proliferating cells and multicellular organisms. Cellular genomes are subjected to endogenous and environmentally induced structural alterations. Our environment contains a multitude of substances which are carcinogenic and which, in many cases, are thought to act via direct damage to DNA. Such damage can lead to mutations, gross chromosomal abnormalities, or gene amplification. It is becoming increasingly clear that DNA lesions at specific genomic sites can lead to corresponding local changes in nucleotide sequence and to the activation of protooncogenes which have been implicated in subsequent tumorigenesis^{4,6}. Resistance to malignant transformation thus depends in part upon a variety of enzymatic schemes for repairing lesions in DNA. Since a direct correlation between unrepaired DNA damage and carcinogenesis in humans was established, a number of other human hereditary diseases with predisposition to malignancy have been shown to be abnormal in their processing of damaged DNA.

Since most chemical carcinogens damage DNA, much research has focused on the mutagenicity of these agents. Evidence for a mutational basis of at least some types of neoplasia is provided by recent studies of transforming genes of the ras family. Activation of ras protooncogenes in human tumors is apparently due to point mutations at unique sites in the ras coding sequence. In these cases, an error-prone DNA replicative process at lesion sites might have played a direct role in the initiation of cancer after exposure to a chemical carcinogen.

Quite often carcinogenesis is a multistep process^{5,9,19} that occurs in association with abnormalities in the control of both cellular proliferation and differentiation¹⁷. It has been shown that UV irradiation, at dosages that initiate carcinogenesis, induces stable and heritable defects in stem cell differentiation; an initiating dose of carcinogen can induce specific defects in the ability of mouse epidermal cells to terminally differentiate. The fact that many metaplastic and dysplastic disease states that are associated with aberrant differentiation represent preneoplastic lesions also supports this conclusion. Metaplasia is the process in which a normal stem cell changes its pathway of differentiation so that one adult cell type is replaced by a different adult cell type, and dysplasia is the process by which normal cells develop variations in shape, proliferation, and differentiation.

Neoplasia can be considered as a stem cell disorder^{12,23,29} in which the normal growth restraints regulating self-renewal and production of terminal progeny are malfunctioning.

Regardless of the molecular basis of that abnormality, an early phenotypic change at the cellular level appears to be an imbalance in cell population dynamics possibly resulting from a shift towards an increased self-renewal capacity. This could occur in two ways: increased probability of self-renewal of stem cells or the differentiating progeny retain replicative capacity instead of becoming terminal.

Tumor progression probably starts when tumor cells diversify in their unique microenvironments, and variants with altered properties arise in the tumor cell population. To survive and proliferate in a competitive environment, such variants must have selective growth properties and other competitive advantages over other cells. In a tumor, these variant cells may rapidly become dominant leading to changes in tumor cellular composition.

It is thus becoming evident that cancer development in many tissues or organs is a stepwise process involving altered cell populations at the site of origin for yet another "new" or altered non cancerous cell population, and with each step, the process show an increased probability of evolving ultimately into a more malignant neoplastic population. The carcinogenic process in most cases appears to be one of cellular evolution based upon repeated selection of rare cells, more differentiated towards a cancer phenotype as the system evolves through the noncancerous steps. It must be emphasized that the different cell populations that constitute the long precancerous period show no evidence of cancer. Three steps in Cancer Development are generally identified although the mechanisms of the process remains poorly understood.

Initiation

This was formulated early in terms of the induction of an altered base, a miscoding lesion, and a mutation in the classical sense. Rearrangements of segments of DNA, the activation and modulation of gene expression by neighboring base sequences, and the increasing evidence for gene duplication could play also an important role.

Promotion

Promotion refers to the process whereby the initiated cells become expanded into "precancerous lesions" (nodules, papillomas, or polyps), one or more of which can act as a population or origin for the ultimate development of cancer. These focal proliferations are the immediate indices for promotion.

Progression

Progression, involves the stepwise process whereby the occasional expanded initiated cell, the nodule, the polyp, or the papilloma, evolve into a cancer, as well as the further steps the cancer undergoes as it becomes progressively more malignant. This sequence is the least understood of any segment of the carcinogenic process.

In GI the sequence known as the *adenoma-carcinoma hypothesis* is a multistep process that, in the colon, involves the transition from normal epithelium sequentially through a hyperproliferative state, adenomatous changes with increasing degrees of dysplasia, and finally frank malignancy with eventual metastasis and death. The prevalence of both adenomas and colon cancers increases with age, with the mean age of adenoma subjects exceeding that of cancer subjects by 5 to 10 years. In familial adenomatous polyposis patients, the affected individuals develop hundreds of colorectal adenomas that will progress to cancer in virtually 100 per cent of untreated cases. Removal of adenomas has been reported to reduce the incidence of subsequent colon and rectal cancers.

Pathologically, it is extremely rare to find a small focus of cancer in normal mucosa, whereas this is common in adenomas. The genetic abnormalities are identical to those in the cancer cells arising within that adenoma, although the cancer cells have additional mutations.

Mutations known to cause oncogenic activation of the K-ras gene have been found in a high percentage of early adenomas. Larger and more dysplastic adenomas frequently have abnormalities in the p53 gene located on chromosome 17p and the DCC (deleted in colon cancer) gene located on chromosome 18q.

Successive mutations will thus lead to tumor heterogeneity^{19,23}. Tumors are architecturally complex, differing regionally in vasculature, host infiltrates, connective tissue components, and other characteristics which can alter the phenotype of otherwise identical cells. Marked differences in the proliferation behavior of tumor cells within a single cancer

are quite common. Some cells, perhaps most, are reproductively dead; others are out of cycle; and still others are cycling but are, at a given time, at different stages in the process. Furthermore, many cellular phenotypes, such as antigen expression, membrane composition, response to chemotherapy, metastatic incidence are themselves functions of the cell cycle. These differences have to be distinguished from genetic differences.

During the development of tumors the survival of patients is affected by the metastatic process^{5,10}. Some cancer cells detach from the primary site and then either directly or via a first metastatic site anchor distantly from the initial cancer site. The process of metastasis consists of a series of sequential steps that must be completed by tumor cells if a metastasis is to develop. Metastases can have a clonal origin, and different metastases can originate from the proliferation of single cells. The outcome of metastasis depends on the interaction of metastatic cells with different organ environments. Organ-specific metastases have been demonstrated in a variety of experimental tumor systems. Thus, growth in the environment of specific organs can be selective and the environment per se influences this process.

Major steps in the formation of a metastasis are as follows :

- a) after the initial transforming event, either unicellular or multicellular, growth of neoplastic cells must be progressive;
- b) extensive vascularization must occur if a tumor mass is to exceed 2mm in diameter.
- c) local invasion of the host stroma by some tumor cells could occur by several mechanisms that are not mutually exclusive
- d) detachment and embolization of small tumor cell aggregates occurs next.
- e) tumor cells that survive the circulation must arrest in the capillary beds of organs.
- f) extravasation occurs next, probably by the same mechanisms that influence initial invasion.
- g) proliferation within the organ parenchyma completes the metastatic process.

Apparently kinetic factors are of critical importance for a comprehensive therapeutic approach of cancers. Tumor or metastases heterogeneity is a major barrier to the treatment. This heterogeneity is exhibited in a wide range of genetic, biochemical, immunological, and biological characteristics, such as cell surface receptors, enzymes, karyotypes, cell morphologies, growth properties, sensitivities to various therapeutic agents, and ability to invade and produce metastasis.

Finally one major difference between many solid tumors and surrounding normal tissue is the nutritional and metabolic environment^{29,33}. The functional vasculature of tumors is often inadequate to supply the nutritional needs of the expanding population of tumor cells, leading to deficiency of oxygen and many other nutrients. The production of lactic acid under anaerobic conditions and the hydrolysis of ATP in an energy-deficient environment contribute to the acidic microenvironment which has been found in many types of tumor. Regulation of pH may play an important role in maintaining the viability of tumor cells.

A tumor has two types of vessels²¹: those recruited from the preexisting network of the host vasculature; and those resulting from the angiogenic response to cancer cells. Since a tumor rarely invades the arteries and arterioles of the host vasculature, the smooth muscle cells, with their contractile and nervous apparatus surrounding these vessels, may respond to physical or chemical stimuli. Since the newly formed vessels lack smooth muscle cells, they may not respond to these stimuli. As a result, the overall response of tumors will depend on the ratio of host vessels to newly formed vessels. This ratio would vary from one location to another in the same tumor and from one tumor to another. Microcirculation plays an important role in the growth, metastasis, detection, and treatment of tumors. For example, angiogenesis and the resulting microcirculation are essential for supplying nutrients and removing waste products during tumor growth. Similarly, blood and lymph vessels provide the vehicle for cancer cells to metastasize to distant organs/tissues. In radiotherapy, the efficacy of treatment depends upon the local oxygen concentration which is governed by the local blood flow. In chemotherapy and immunotherapy, blood flow plays an important role in the delivery of appropriate pharmacological agents. Finally, in hyperthermia, the temperature distribution and interstitial microenvironment in tumors are influenced by the local perfusion rates. It is probable that all these factors will play a role of dramatic importance in the control of tumor growth by PDT.

Clinical Observation

Photodynamic therapy, (PDT) is a potentially useful treatment for cancers of small volume. This form of phototherapy is based upon a simple principle: HPD, a photodynamic agent retained in significant amounts by tumor tissues^{16,13}, is activated upon irradiation. The resulting photodynamic reaction gives rise to reactive intermediates, such as singlet oxygen³⁶, which destroy neoplastic cells while causing only slight damage to adjacent normal tissue. Furthermore, HPD-PDT has been shown to decrease tumor blood flow, probably by direct action on vessels, leading to partial anoxia and thus reducing the tumor growth rate^{31,3}. Successful PDT would ideally be based on a high HPD retention by all cancer cells followed by an intense reaction after light exposure leading to irreversible cancer cell lesions and death. However although numerous studies have been devoted to fundamental approaches, few teams all over the world are directly involved in research immediately applicable to improve clinical treatments Endoscopic hematoporphyrin derivative (HPD) (PDT) of small tumors used as primary treatment for gastrointestinal neoplasms have been carried out in our department for 10 years^{25,26} as well as by authors in other medical fields^{7,8}.

Previous studies involving PDT on cell cultures, demonstrated that PDT was able to induce 100% cell death but treatment parameters leading to such results were different according to cell types. L1210 murine leukemic cells were shown to be more sensitive to PDT than corresponding semi-syngeneic hemoprogenitors in primary cultures. This pattern was found whatever the irradiation parameters used, although the greatest difference in sensitivity was for fractionated laser dose and irradiation performed at 4°C. Although the sensitizer was identical, C6 murine glioma cells (adherent) required about 10 fold less sensitizer than L1210 (non adherent) to achieve the same PDT effects. Despite such an heterogeneity in cell response in 1983 we started clinical trials in our department.

Actually about 150 inoperable patients have been endoscopically treated by PDT for their small (early) gastrointestinal neoplasm (Tis, T1 N0, M0) in our department, among them 138 being analysed for this paper (Figure 1).

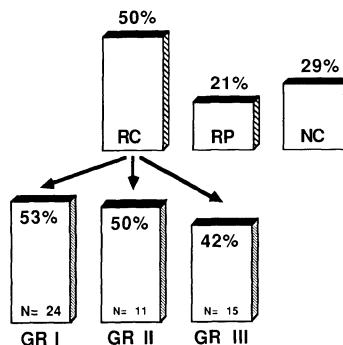


Figure 1. Clinical results obtained after HPD-PDT of 102 patients analysed for Tis/T1 N0 M0 cancers of the GI tract. Completely responding patients were analysed as a function of the initial histology of their tumor (lower part of the figure). The mean follow-up period was 25 months for all 102 patients, 34 for surviving patients and 36.2 for completely responding and surviving patients. The follow-up of completely responding patients with oesophageal squamous cell carcinoma was 30.2 months, 35.5 for adenocarcinomas of the upper GI tract and 42 months for rectal adenocarcinomas.

The sensitizer used was HPD, synthesized according to Lipson and Gregorie^{18,22}. Criteria for a complete response to PDT were an absence of malignancy on biopsies performed after at least 6 months as well as an absence of endoscopic evidence of persistent malignant tissues. Such severe criteria would explain the slightly worse results obtained by our team (50 % of complete responses) as compared to other studies involving similarly

enrolled cohorts of patients. In addition one third of our patients did not show any evidence of response to PDT. Patients were divided into 3 groups: oesophageal squamous cell carcinoma, gastric adenocarcinomas or rectal adenocarcinomas. In all cases, results of PDT were highly comparable. It thus indicates that a similar histological initial classification was not sufficient to accurately predict the result of PDT. Although patients were included according to the same entry criteria, results were unpredictable, some tumors being "resistant" and some other not. One patient was successfully treated for a rectal adenocarcinoma for 4 years (T1 N0 M0) until he developed an esophageal squamous cell carcinoma. This second cancer did not respond at all although it had been PDT treated twice. It appears from this case that at least in some patients resistance to PDT is not linked to specific patterns of a patient but rather to the biological features of a tumor.

Effect of cell type on tumour response to PDT

Different responses to PDT according to cell type have been observed by irradiation of cells under similar conditions^{13,15}. For some cell models it was possible to observe a selective destruction of cancer cells as compared to normal cells¹³. Even differences of photosensitivity between progressive and regressive colonic cancer cells cloned from the same murine chemically-induced colonic cancer were seen. This is of great importance as it is well known that cancers are polyclonal rather than monoclonal, particularly in GI. We could imagine that in PDT like for other treatments some populations of photosensitive cancer cells would be totally destroyed while other, resistant cells would survive. In addition, suppressing some cancer cell populations would also allow a more rapid progression of resistant cells by reducing mutual growth control of the previously coexisting clones. Towards this aim we chose to analyse cell DNA content or cell kinetics throughout the cell cycle with flow cytometry (FCM, according to ref 35) before and after HPD-PDT in 32 patients bearing Tis or T1 cancers of the GI tract and we compared results in near-diploid cancers with those obtained in normal corresponding tissue. During a period averaging 15.7 months complete local tumor destruction and negative histology (CR) were observed in 17 out of 33 cases. FCM DNA analysis was feasible in 32 patients. Aneuploidy was found in 15 out of 32 patients and was found to be a factor of bad prognosis as 5 out of 15 aneuploid patients were classified CR as compared to 12 out of 17 near-diploid patients. Changes in ploidy after PDT were found in 11 patients. It consisted in a reduction of the number of aneuploid peaks in 8 cases and the appearance of one aneuploid peak in 3 cases, suggesting that destruction of sensitive cell populations allowed the growth of initially non FCM detectable aneuploid clones. Results obtained in this series of patients confirmed previously published results but changes occurring in ploidy of PDT treated patients demonstrated that PDT acts directly on cancer cells in human beings and not only on the tumor vasculature. DNA analysis could be used routinely to improve HPD-PDT prognostic determination and to monitor the efficacy of cancer HPD-PDT in patients as observed for other cancer treatments particularly in non surgically removed malignancies. Aneuploidy appeared to be of bad prognosis for HPD-PDT and a criterion independent of Lamina Propria invasion or tumor differentiation so that conventional histology would be unable to replace DNA FCM analysis in prognosis determination. However response to PDT varies from one cell population to the other (Figure 2).

Understanding the mechanisms leading to resistance to HPD-PDT in some cell populations, particularly aneuploid cells, is of critical importance as treatment failures probably directly depend on such resistances.

Effect of adjuvant treatment on the efficiency of PDT

The efficiency of PDT can be also limited by the simultaneous use of symptomatic treatments. Thus, we failed to demonstrate a potentiation of HPD-PDT effects by incubating leukemic cells in vitro with the mitochondrial photosensitive stain rhodamine 123¹⁴. Actually, the intracellular HPD fluorescence decreased proportionally to rhodamine doses added. Moreover, exogenous drugs can limit the photodynamic reaction by quenching excited oxygen species. We paid a particular attention to non steroid antiinflammatory agents¹⁵ as it has been demonstrated that HPD-PDT could increase prostaglandin production,

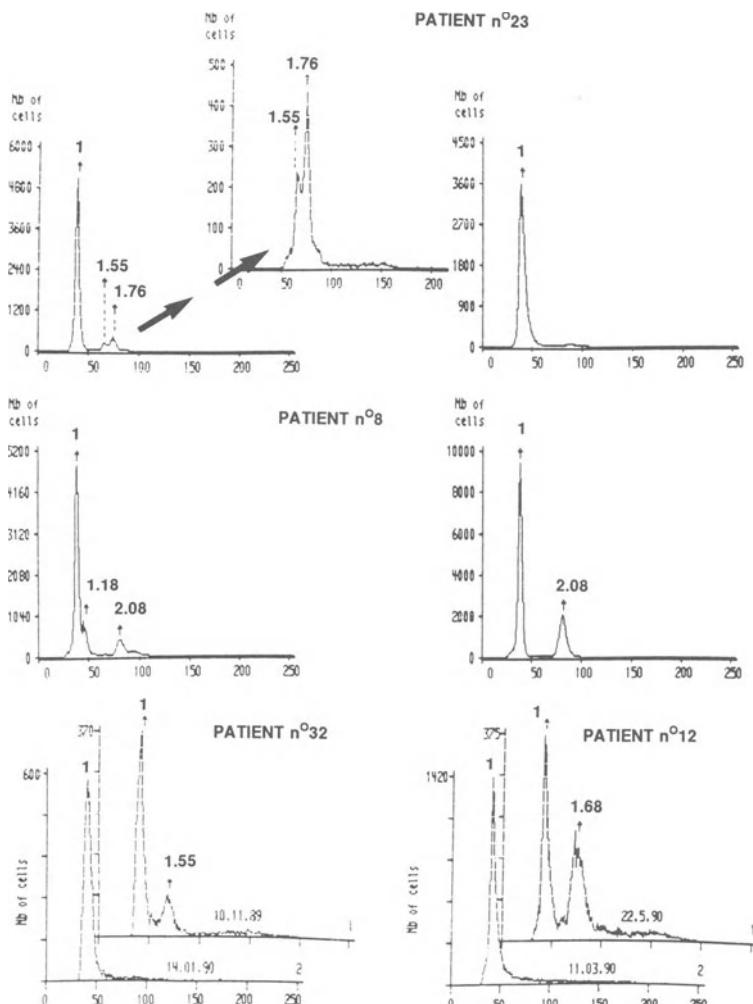


Figure: 2. Histograms of aneuploid tumors of the GI tract treated by HPD-PDT. At least 5 grip biopsies were taken and frozen. DNA was then propidium iodide-stained. In patient n° 23 in group 2, 2 aneuploid cell populations (DNA index 1.55, 1.76) were present before PDT. Only the near-diploid population was detectable after PDT. Patient n°8 in group 3 had 2 aneuploid populations (DNA index 1.18, 2.08) before PDT but only the 2.08 population was detectable after PDT. Patient n°32 in group 1 had an aneuploid cell population (DNA index 1.55) on the day of PDT (November 10, 1989) which disappeared after PDT. In patient n° 12 in group 3 an aneuploid cell population appeared after PDT (May 22, 1990) which was not detectable at the time of treatment (March the 11th 1990).

thereby increasing PDT effects^{11,20,27} (it is well known that antiinflammatory agents inhibit endogenous prostaglandin synthesis). Indomethacin was an inhibitor of PDT in vitro when added prior to cell irradiation but the level of the inhibition depended greatly on cell type. More recently, similar inhibition of PDT effects was demonstrated with aspirin³⁰. Finally we identified calcium antagonists as a powerful class of inhibitors of PDT effects (unpublished data). This action seems to be linked to the reduction of membrane peroxidations rather than to a reduction of calcium intake by the cell. Calcium antagonists like nifedipine or flunarizine are highly lipophilic and localize in the membrane lipid bilayer, hence they can inhibit ischemic induced peroxidations occurring after myocardial infarction^{34,24}. Such a decrease

of PDT effects was observed either in vitro or in vivo. Diuretics have been administered to reduce the side effects of HPD-PDT, such as skin photosensitization. It is probable that several drugs used during adjuvant or symptomatic treatments can modulate sensitizer uptake or repair of PDT effects. This is of great practical importance as patients selected for PDT are often treated for several diseases other than cancer and thus receive various drugs susceptible to interfere with PDT. Finally, as plasma membranes are the first targets reached by the sensitizer, cancer cell antigen or receptor expression could be modified either by the sensitizer alone or by light exposure.

Effect of photosensitizer concentration in tumor

Among the major prognostic criteria for HPD-PDT efficacy, tumour sensitizer concentration is evidently a prominent one. Knowing the sensitizer concentration would allow the determination of sensitizer uptake kinetics which probably varies for different tumors, and also between normal and cancer tissues in a given patient. Routine determination of this concentration would imply a simple and reliable device like a laser fiberoptic spectrofluorimeter (LFOSF). As an example of the potential applications of such a device, we studied in cooperation with Roy Pottier (RMC, Kingston, Canada) kinetics of endogenous protoporphyrin (PP) IX synthesis after d-amino levulinic acid injection in mice. Maximum PPIX (wavelength excitation: 488nm) was seen in tumors after 3 h (Figure 3) but totally disappeared when tumours were irradiated at 632 nm (100J/cm^2).

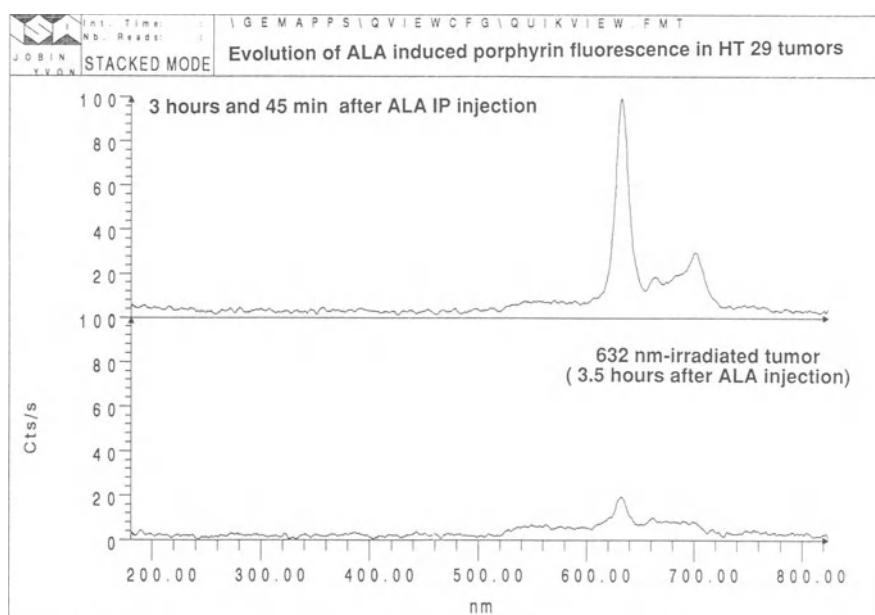


Figure 3. Protoporphyrin IX fluorescence in HT 29 tumors 3 hours after delta amino levulinic acid injection (25 mg/kg, IP) recorded in vivo in mice through a laser fiberoptic spectrofluorimeter (up) or followed by laser irradiation (lower part of the figure). Excitation wavelength was 488nm.

Simultaneous subcutaneous administration of the iron chelator desferrioxamine reduced significantly PP IX fluorescence in various tissues (Figure 4).

The main applications of LFOSF will be the determination of the optimum delay between sensitizer injection and tumour irradiation according to the specific sensitizer pharmacokinetics and the semiquantitative determination of the tissue concentration. Such determinations would help to discriminate between patients that are potential responders to

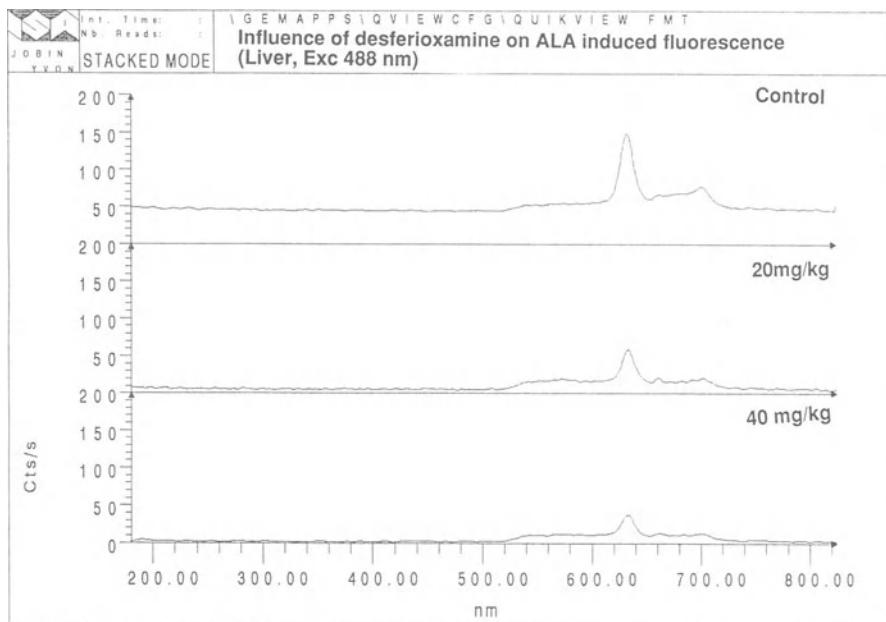


Figure 4. Changes in protoporphyrin IX fluorescence in liver 3 hours after delta amino levulinic acid injection (25 mg/kg, IP) and 1 hour after desferrioxamine administration (20 or 40 mg/kg subcutaneously) recorded *in vivo* in mice through a laser fiberoptic spectrofluorimeter as compared to liver treated with ALA alone (control).

PDT and those who have little chance to respond to PDT because of a too low sensitizer concentration. Kinetic data can be also used to modify clinical protocols for phase I/II trials in PDT. HPD-PDT gives more or less 50% of complete tumour response for 5mg/kg injected doses. However, increasing the injected dose (but by doing that, modifying also the tumor uptake kinetics and the ratio of concentration between the tumor and healthy adjacent tissues) would increase the therapeutic ratio, some resistances being over matched by increasing drug doses.

Finally, one can evaluate HPD-PDT effects, by analysing tissue damages on biopsies taken after PDT. We designed a miniaturised procedure to quantitate malondialdehyde (MDA, an ultimate product of membrane lipid peroxidation) occurring after PDT^{33,2} on a single biopsy (1mm³). MDA can easily be measured by the fluorometric thiobarbituric acid assay^{37,1}. To determine whether MDA content represents a reference for PDT intensity, tissue content in male, 7-week-old nude mice was studied on biopsy samples after PDT (HPD 5 mg/kg i.v. 24 hrs before irradiation at 632 nm) with or without I.P. injection of WR-2721 (an aminothiol radioprotector) 40 min before treatment. Among the already described procedures, the FeCl₃-method requiring only 15-min incubation in a 95°C water bath proved to be most effective. The best results were found for 60-min interval between treatment and biopsy freezing until processing. MDA concentration in HPD-PDT-treated samples was significantly higher than in HPD-tested controls ($p < .01$) and increased at laser irradiation doses ranging from 0 to 50 J/cm² (Figure 5).

Administration of WR-2721 intraperitoneally significantly reduced MDA concentration (from 70% to 38%). A maximal effect was obtained with 100 mg/kg for brain (-70 %) and 200 mg/kg for muscle (-47%). Higher doses gave no additional changes. The MDA assay is a simple tool for indirect evaluation of PDT, and WR-2721 can decrease MDA content in normal tissues, suggesting the possibility of good protection for normal tissues during PDT.

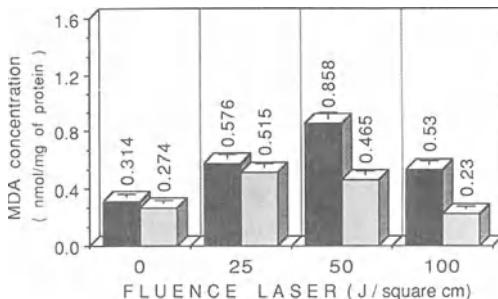


Figure. 5. Influence of laser fluence on MDA concentration in muscle after HPD-PDT (fluence ranging from 0 to 100 J/square cm), HPD-PDT-treated tissues (■), controls: no HPD, no laser.

CONCLUSIONS

To summarize, PDT appears to be an effective modality for cancer treatment, leading to neurosis of malignant cells in culture, in xenografts, in humans. The mechanism of PDT is not well defined as biochemical, biological, histological factors influence the prognosis. Despite such an evident effectiveness, few patients have yet been treated. Reasons for such an underdevelopment are numerous. The first point is that PDT is not just a new drug to be evaluated by comparison to existing ones in a given well established procedure for a well identified disease. It is a new concept associating one drug and one physical agent thus raising problems of biochemistry and pharmacology as well as problems of light dosimetry. Then, PDT is devoted to the treatment of the few small malignant lesions detected which are also the best potential results of surgeons. Evaluation is thus difficult involving slow-growing cohorts of patients and recruited by competition with already existing techniques. Finally, the overall market being small at least to-day, pharmaceutical companies remain anxious about the future.

Introducing a new technology is not simple. The easiest part of the work is the laboratory part as in our societies, strongly built and stable, modifying the equilibrium requires a lot of external energy. Only an extension of the market could represent the extra force able to introduce PDT in routine procedures. It means that besides coordinated clinical research, more powerful drugs will have to be designed. But the most important is that after a ten year period of research in this field we know that PDT works and will have a future among other applications for photobiology.

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