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Dyes and Pigments 58 (2003) 113–120
DYES and PIGMENTS
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Vitamin B2-sensitized photooxidation of structurally related dihydroxyflavonoids

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Received 18 October 2002; received in revised form 16 January 2003; accepted 28 February 2003

Abstract

A kinetic study of the processes involved in the vitamin B2 (riboflavin, Rf)-sensitized photooxidation of selected flavonoids (**F**) was carried out in methanolic solution. Under aerobic visible-light-irradiation conditions a complex picture of competitive interactions takes place: the singlet and triplet excited states of Rf are quenched by three **F**, namely 5,7-dihydroxy-3′,4′-dimethoxyflavanone (FNN); 5,7-dihydroxy-4′,6,8-trimethoxyflavone (FLV) and 2′,4′-dihydroxychalcone (CHL). Concomitantly, the species singlet molecular oxygen [$O_2(^1\Delta_g)$] and superoxide anion radical ($O_2^{\bullet-}$) are generated and interact with **F** and with Rf. CHN and FNN are photooxidised, probably by means of $O_2^{\bullet-}$, whereas FVN, the poorest quencher of excited triplet Rf, allows the generation of $O_2(^1\Delta_g)$, which oxidises the very flavonoid. The photodegradation of the vitamin is delayed due to an electron transfer process to ground state oxygen.

Keywords: Chalcone; Flavanone; Flavone; Riboflavin; Singlet molecular oxygen; Superoxide anion radical

1. Introduction

When natural irradiation penetrates external biological barriers and reaches an adequate light-absorbing agent, a series of unpredictable photorreactions may start. A daylight-absorbing pigment of particular interest is the naturally-occurring Vitamin B2, riboflavin (Rf), (Scheme 1), which is synthesised by green plants and participates in a variety of enzyme catalysed oxidation-reduction reactions [1]. Rf has been postulated as a possible sensitiser for the in vivo photooxidative degradation of numerous relevant substrates present in different types of living organisms [2,3].

A particular class of compounds present in higher plants are the flavonoids (**F**). Their antioxidative properties have received considerable attention [4–6]. The antioxidative process includes the scavenging or trapping of activated electrophilic species, oxygen radicals, singlet molecular oxygen [$O_2(^1\Delta_g)$], superoxide radical anion $O_2^{\bullet-}$ or hydroxyl radicals [7]. Nevertheless, the characteristics of the antioxidant properties of certain flavonoids have been the subject of contradictory interpretations, and even the promotion of oxidizing activity has been suggested [8]. Results from other authors and from our laboratory indicate

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0143-7208/03/$ - see front matter
doi:10.1016/S0143-7208(03)00049-4

that the effectiveness of flavones as $O_2(^1\Delta_g)$ quenchers mainly lies on structural effects, particularly on the number and position of the OH groups in the flavonoid skeleton, and more specifically on those located in the aromatic ring [7,9].

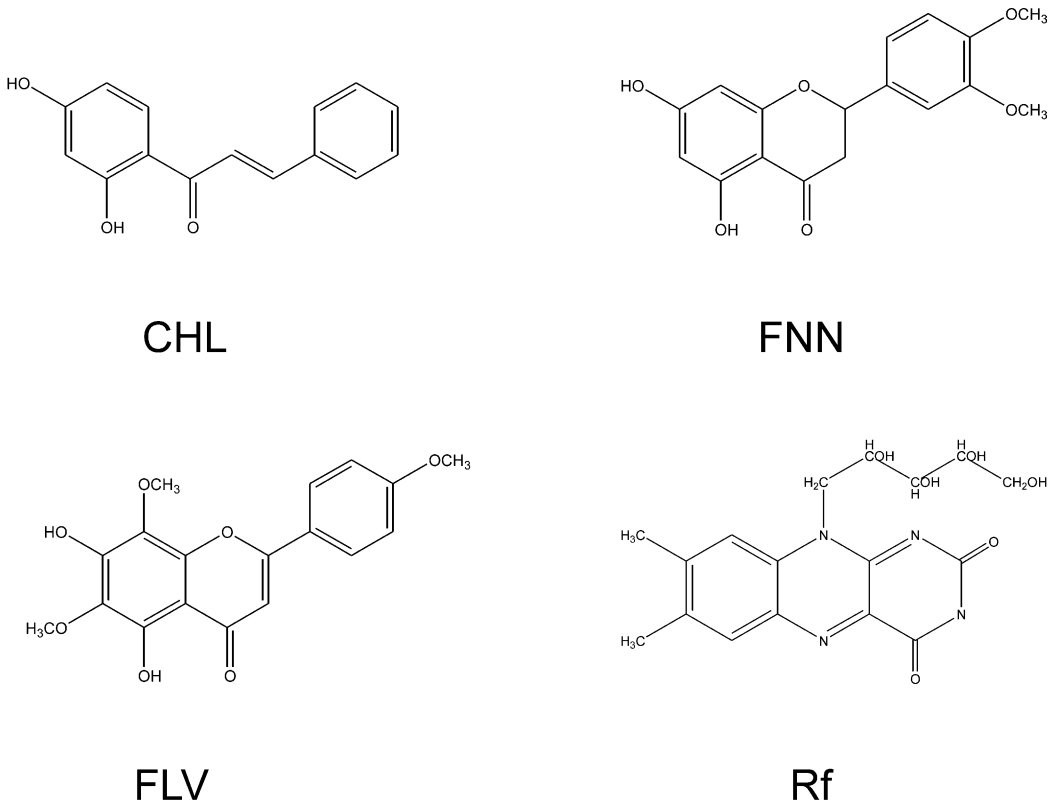
Since Rf and **F** can occupy common locations in complex biological structures in higher plants, kinetic information about the visible-light-photopromoted interactions between these compounds can help to understand the behaviour of Rf-generated oxidative species in general, the potential photorreactions on **F** derivatives in nature in particular, and the propensity of such processes to occur under given environmental conditions.

We chose for the present study three types of **F** (Scheme 1) structurally related, namely 5,7-dihydroxy-3′,4′-dimethoxyflavanone (FNN); 5,7-dihydroxy-4′,6,8-trimethoxyflavone (FLV) and 2′,4′-dihydroxychalcone (CHL).

2. Experimental

2.1. Chemicals

5,7-Dihydroxy-3′,4′-dimethoxyflavanone (FNN) was obtained from *Baccharis calliprinos* [10]; 5,7-dihydroxy-4′,6,8-trimethoxyflavone (FLV) (Nevadensin) was isolated from *Baccharis grisebachii* [11] and 2′,4′-dihydroxychalcone (CHL) was obtained from *Zuccagnia punctata cav*. [12]. All flavonoids were characterized employing UV, NMR, MS and IR spectroscopy. 9,10-Dimethylanthracene (DMA) and Rose Bengal (RB) were from Aldrich, Riboflavin (Rf), and superoxide dismutase (SOD) were purchased from Sigma Chem. Co (USA). The solvents employed were MeOH (HPLC quality, Sintorgan, Argentina) and MeOD (Sigma Chem Co., USA). Water was triply distilled.



Scheme 1. Chemical structures of 5,7-dihydroxy-3′,4′-dimetoxyflavanone (FNN); 5,7-dihydroxy-4′,6,8-trimetoxyflavone (FLV) and 2′,4′-dihydroxychalcone (CHL) and Vitamin B2 [riboflavin (Rf)].

2.2. Instrumentation and methods

For the the stationary Rf fluorescence experiments, an RF 5301-PC Shimadzu spectrofluorimeter was used, at 25±1 °C in air equilibrated solutions. Excitation and emission wavelengths were 446 and 515 nm, respectively. Ground state absorption spectra were registered in a Hewlett Packard 8452A diode array spectrophotometer.

Static aerobic photolysis of methanolic solutions containing **F** and *R*f were carried out in a PTI unit provided with a high pass monochromator and 150 W Xe lamp, irradiating with 440±10 nm, or in a home-made photolyser for non-monochromatic irradiation (150 W quartz-halogen lamp). In this case cut-off filters (420 nm) ensured that the light was only absorbed by the sensitiser.

The rates of the Rf-sensitised oxygen uptake of **F** and Rf in H_2O–MeOH 1:1 (V/V) were determined by evaluation of the initial slopes of the profiles of oxygen consumption versus irradiation time, employing a specific oxygen electrode (Orion 97-08).

Anaerobic and aerobic photodecomposition rates of Rf were determined by evaluation of the initial slopes of Rf consumption (decrease of absorbance at 446 nm) versus irradiation time.

The k_t values were obtained by time-resolved phosphorescence detection (TRPD), using a laser kinetic spectrophotometer already described [13], provided with a nanosecond Nd:YAG laser system (Spectron). The $O_2(^1\Delta_g)$-sensitizer RB, with a quantum yield of $O_2(^1\Delta_g)$ generation of 0.76 in MeOH [14], was employed at concentrations corresponding to absorbances at 532 nm in the range 0.4–0.5. $O_2(^1\Delta_g)$ lifetimes were evaluated in the absence (τ^0) and in the presence (τ) of **F**, and the ratio τ^0/τ was plotted as a function of the quencher concentration, according to a simple Stern–Volmer treatment, using the expression $\tau^0/\tau = 1 + k_t\tau^0[\mathbf{F}]$. The determination of k_t was made in MeOD due to the enlargement of the $O_2(^1\Delta_g)$ lifetime in this solvent.

The rate constants for the chemical reaction between FVN and $O_2(^1\Delta g)$ were determined by a comparative method, in which the k_r value for the sensitised photooxidation of a reference compound must be known [15]. The commercial dye Rose Bengal was employed as a photosensitizer and the reference was DMA, with a k_r value of 1.69×10^7 [16]. The DMA disappearance was monitored by spectrofluorimetry with 395 nm and 450 nm as excitation and emission wavelengths, respectively. The disappearance of **F** was followed by absorption spectroscopy in a Hewlett Packard 8452A diode array spectrophotometer, reading absorbances at 334 nm for FVN, at 337 nm for FNN and at 346 nm for CHL.

3. Results

Rf, is probably the most extensively studied biomolecule with respect to their complexation ability with other molecules of biological and environmental relevance, including hydroxyaromatic derivatives [17]. However, no ground state interactions between Rf and **F** could be detected in the present investigation, by means of absorption spectroscopy, under work conditions, ca. 0.05 mM Rf and 0.5–0.05 mM F.

The Rf-sensitised irradiation ($\lambda_{irr} > 420$ nm) of air-equilibrated methanolic solutions of **F** produces different changes in the whole absorption spectrum of the mixture, which reflect the addition of chemical changes in both the **F** and the very Rf. (Fig. 1). In the spectral region higher than 400 nm,

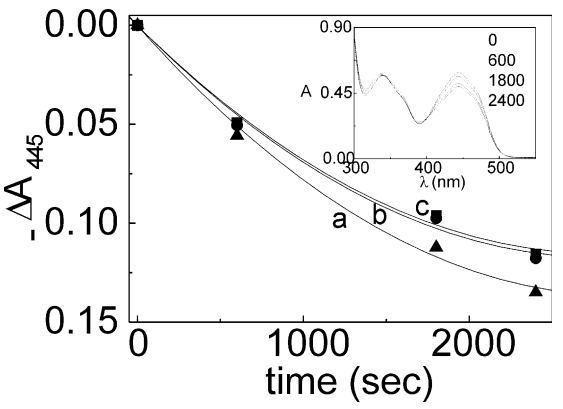


Fig. 1. Rates of Rf decomposition in the presence of (a) FVN; (b) FNN and (c) CHL. Inset: spectral evolution of the mixture Rf + FNN 0.070 mM upon photoirradiation at wavelength higher than 420 nm in MeOH. Numbers on the inset figure represent irradiation times (s).

the light absorption corresponds exclusively to Rf. The rates of Rf consumption (Δ Absorbance$_{446\ nm}$ vs irradiation time, Fig. 1) in the presence of **F** 0.075 mM, indicate comparatively low values for CHL and FNN, whereas the rate of Rf decomposition is faster, in relative terms, in the presence of FVN. The graphical representation corresponds to the mean value of 3 runs, which did not differ from each other in more than 5%. The relative mean rate values are shown in Table 1.

Parallel photoirradiation of diluted individual aqueous solutions of Rf (0.02 mM) + **F** (0.5–1 mM) in H_2O–MeOH 1:1 (v/v) gave rise to oxygen consumption (Fig. 2). The rates of oxygen uptake followed the order rate CHN $\cong$ rate FNN $>$ rate FLV. The rate of oxygen consumption was greatly reduced in the comparative aerobic irradiations of: (a) A solution of Rf (0.02 mM), but in the absence of **F**. In this case the rate of oxygen uptake was the smallest, approximately 0.06 ppm/min (in the experimental conditions of Fig. 2). (b) The mixture Rf + CHN or FNN + 14 μg/ml SOD. The enzime, which catalizes the dismutation of $O_2^{\bullet -}$, has been utilised [18–21] in similar concentrations to those employed in this work, as a quencher of $O_2^{\bullet -}$- mediated photooxidations.

From the experimental data, three observations are particularly relevant: (1) **F** and Rf are chemically affected by photolisys with visible light; (2) oxygenated species participate in the process and (3) **F** could interact with electronically excited states of Rf.

The overall photoprocess may involve different steps, and the elucidation of the reaction mechanism can contribute to the understanding of the relative importance of certain reaction environmental factors such as individualisation of the active excited states of the sensitiser, optimal sensitising conditions, oxygen requirement, and even local concentration necessary for the effective photoreaction of the involved targets.

At this point we decided to investigate the specific interactions of **F** with singlet and triplet excited states of the pigment, under aerobic and anaerobic conditions.

3.1. Quenching of excited singlet Rf by *F*

Rf presents an intense fluorescence emission band, centred at 515 nm, with a reported fluorescence quantum yield of 0.25 [3]. In the presence of >1 mM **F**, the quenching of the fluorescence from excited singlet Rf (^{1}Rf*) produces a decrease in

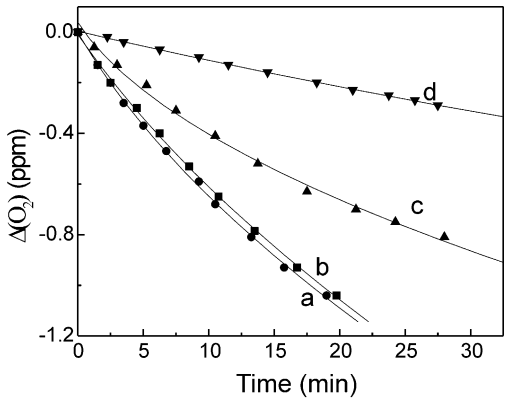


Fig. 2. Profiles of oxygen consumption as a function of irradiation time (irradiation wavelength higher than 420 nm) in MeOH–H_2O 1:1 (v/v) for the systems: (a) Rf (0.02 mM) + CHL (1 mM); (b) Rf (0.02 mM) + FNN (1 mM); (c) Rf (0.02 mM) + FVN (1 mM); (d) Rf (0.02 mM) + CHL (1 mM) + SOD 14 μg/ml.

Table 1
Rate constants for: the overall quenching of $O_2(^1\Delta_g)$ in MeOD (k_t); the reactive quenching of $O_2(^1\Delta_g)$ in MeOH (k_r); the quenching of excited singlet riboflavin (^1k_q); the quenching of excited triplet Rf in MeOH ($^3k_{qapp}$); and relative rates of Rf consumption upon aerobic photoirradiation in the presence of the flavonoids 0.075 mM (Rate$_{rel}$)

Comp.	k_t 10^{-6} (M^{-1} s^{-1}) ± 15%	k_r 10^{-6} (M^{-1} s^{-1}) ± 10%	^1k_q 10^{-9} (M^{-1} s^{-1}) ± 5%	$^3k_{qapp}$ 10^{-7} (M^{-1} s^{-1}) ± 10%	Rate$_{rel}$ ± 5%
FVN	1.1	0.30	19	3	1
FNN	$\leqslant$0.1	NR[a]	6.4	20	0.86
CHL	$\leqslant$0.1	NR[a]	18	23	0.81

[a] No reaction was observed.

the stationary emission intensity, but the shape of the emission spectrum does not change.

The Stern–Volmer constant K_{SV} was obtained from the graphical treatment of the data (Fig. 3), i.e. the fluorescence intensity in the presence (I) and absence (I_0) of **F**, through the Stern Volmer equation ($I_0/I = 1 + K_{SV}$ [**F**]). In a pure collisional quenching process, $K_{SV} = {}^1kq \times {}^1\tau_o$, where ${}^1kq$ is the dynamic rate constant for the quenching of ^{1}Rf* [process (3) in Scheme 2] and ${}^1\tau_o$ the lifetime for the ^{1}Rf* state [22]. Employing the value reported in the literature of 5.75 ns for ${}^1\tau_o$ [23] the ^{1}kq values were obtained, all they very close to the range values of the expected ones for a diffusional process of quenching in H_2O–MeOH [24] (Table 1).

3.2. Quenching of triplet Rf by F

It is known that anaerobic photodegradation of Rf under visible light irradiation predominantly proceeds through the triplet state [25], and the rate of the process can be estimated by absorption spectroscopy. Comparative irradiations of N_2-saturated methanolic solutions of Rf under identical experimental conditions, in the absence and in the presence of **F** showed that this rate decreased in the presence of **F** in the range 0.1–0.01 mM (Fig. 4, inset). At the said **F** concentrations, no fluorescence quenching of Rf occurs, and the experimental data strongly supports the idea that a long-lived triplet state, intermediate in the photolysis of Rf, can be quenched by relatively very low **F** concentrations. Hence, data were evaluated through a simple Stern–Volmer treatment: $V_0/V = 1 + {}^3k_{qapp} \times {}^3\tau_o \times$[**F**] (Fig. 4), where V and V_0 are the respective velocities of Rf photobleaching at 446 nm in the presence and absence of **F**, ${}^3k_{qapp}$ the apparent rate constant for the process of quenching of ^{3}Rf* (process (5), being ${}^3k_q = {}^3k_{qapp}$) and ${}^3\tau_o = 12$ μs, the ^{3}Rf* lifetime in MeOH [26]. Employing the available laser described in the experimental section, the rate constants ${}^3k_q$ could not be conventionally determined by laser flash photolysis due to the strong absorption of **F** at 355 nm, the excitation wavelength of Rf.

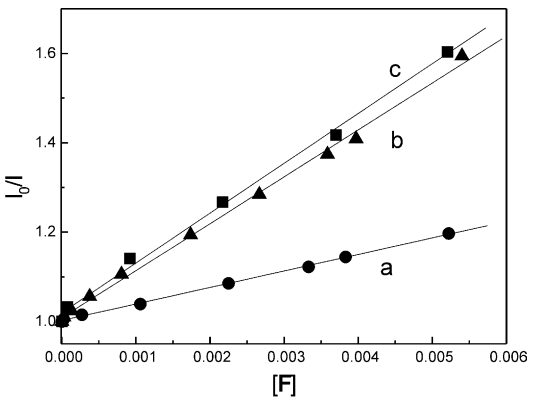
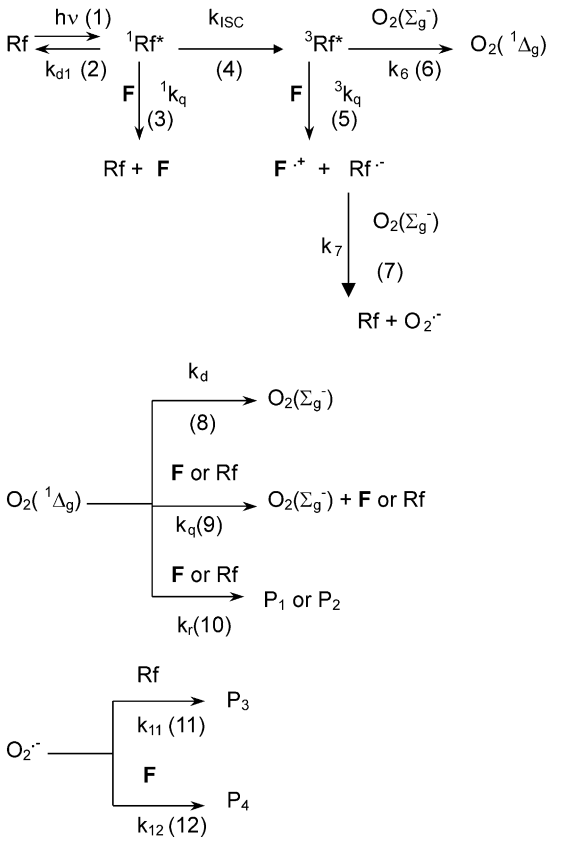


Fig. 3. Stern–Volmer plots for the quenching of Rf fluorescence in MeOH by (a) FVN; (b) CHL and (c) FNN.



Scheme 2. Possible reaction steps in the aerobic Rf-sensitised photoirradiation of the flavonoids FVN, FNN and CHL.

3.3. Quenching of $O_2(^1\Delta_g)$ by FVN

The interaction of **F** with $O_2(^1\Delta_g)$ was determined employing TRPD and RB as a sensitiser (Fig. 5). In parallel we performed runs of **F** consumption, monitored through the changes in the respective absorption spectra, as a function of irradiation time (Fig. 5). The rate constant values k_t and k_r obtained for FVN in MeOD and MeOH (Table 1) are in the same order of magnitude as reported values for other flavones [7,9]. Only a slight decrease in the $O_2(^1\Delta_g)$ lifetime (τ^0, see experimental section) was observed through the TRPD experiments in the presence of ca. 10 mM CHL and FNN, hence a rate constant value of $k_t \leqslant 1\times10^5$ M^{-1} s^{-1} was quoted for these **F**.

4. Discussion

The reaction mechanism will be discussed on the basis of the following Scheme 2.

Rf is the sensitizer, i.e. the species that absorb radiation typically in a wavelength range of the visible light, where **F** are transparent. The absorption of incident light promotes Rf to electronically excited singlet [reaction (1)] and triplet [reaction (4)] states. Both states can be quenched through reactions (3) and (5), (6) respectively. From the triplet state, an energy transfer reaction to the ground state-triplet molecular oxygen $O_2(^3\Sigma_g^-)$, dissolved in the medium, can take place, yielding the excited state oxygen species $O_2(^1\Delta_g)$ [reaction (6)]. This can decay either by collision with surrounding solvent molecules [reaction (8)] or by interaction with **F** and/or Rf through an exclusive physical [reaction (9)] or chemical [photooxidation, reaction (10)]. An overall rate constant for $O_2(^1\Delta_g)$ quenching (k_t) is defined as the sum of the rate constants for processes (9) and (10). By means of the electron transfer reaction (5), the respective semireduced and semioxidized Rf and **F** forms are produced. Reaction (7) represents the generation of the reactive species superoxide anion ($O_2^{\bullet-}$) which can react with **F** and/or with the pigment [reactions (12) and (11) respectively]. P_1–P_4 represent eventual photoproducts.

The experimental results, upon aerobic Rf-sensitised irradiation of **F**, denote the occurrence of chemical transformations with the participation of reactive oxygen species in the photopromoted process.

In principle both singlet ant triplet excited states of Rf should be directly or indirectly involved in

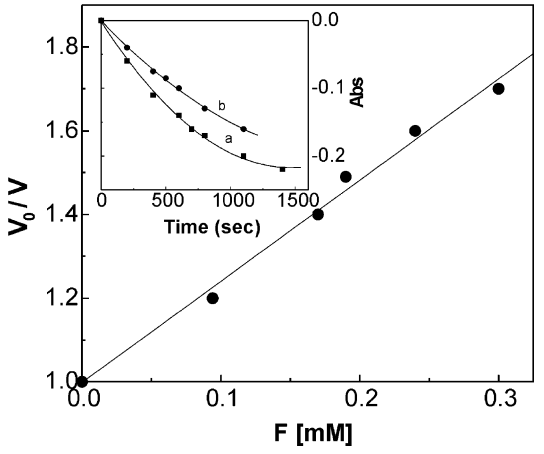


Fig. 4. Stern–Volmer plot for the inhibition of absorbance decrease at 446 nm of Rf (0.02mM) in MeOH by FNN upon photolysis with wavelength higher than 420 nm in deoxygenated solutions. Inset: profiles of absorbance decrease of Rf at 446 nm as a function of irradiation time for the systems: (a) Rf (0.02 mM); (b) Rf (0.02 mM) + FNN (0.14 mM).

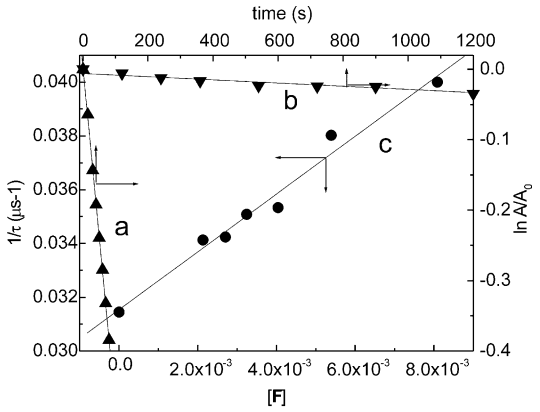


Fig. 5. First order plots for the consumption of (a) 9,10-DMA and (b) FVN, both in MeOH. A and A_o represent the absorbances of 9,10-DMA and FVN at the observation wavelengths at times $t = t$ and $t = 0$, respectively. (c) Stern–Volmer plot for the quenching of $O_2(^1\Delta_g)$ phosphorescence by FVN in MeOD.

the phototransformation of **F**. Although the interaction with excited singlet Rf (step (3), Scheme 2) occurs with a rate constant close to the diffusion limit, relatively high concentrations of **F**—higher than 30 mM as a mean value—are necessary to partially hinder triplet excited state generation (for example 50% inhibition), for which a quantum yield of population of 0.6 in MeOH has been reported [3] [step (4), Scheme 1]. Hence, the interactions of **F** with ^{1}Rf* should be disregarded under work conditions.

It is known that ^{3}Rf* in solution upon aerobic light irradiation generates mainly $O_2(^1\Delta_g)$ (6) and to a lesser extent $O_2^{\bullet-}$ (reaction not included in Scheme 2), with reported quantum yields of 0.49 and 0.009, respectively [27]. $O_2^{\bullet-}$ can also be formed through an indirect pathway represented by process (5) followed by process (7). In previous papers we demonstrated for other hydroxy-aromatic derivatives such as Trolox and 4-hydroxypyridine, that the quenching of ^{3}Rf* [process (5)] produces $Rf^{\cdot-}$, and subsequently the species $O_2^{\bullet-}$ is generated [process (7)] [26,28]. A rate constant value of 1.4×10^8 M^{-1} s^{-1} has been recently reported for k_7 [process (7)] [27], a reaction that at the same time regenerates ground state Rf.

Whether $O_2^{\bullet-}$ or $O_2(^1\Delta_g)$ are the dominant species in the present Rf-photosensitised system will be highly dependent on the competition for the quenching of the species ^{3}Rf*, between $[O_2(^3\Sigma_g^-)]$ and F. Triplet Rf is quenched by **F** with a $^3k_{qapp}$ values between of between 0. 3 and 2.3×10^8 M^{-1} s^{-1} (Table 1), depending on the involved **F** [process (5)]. In the presence of oxygen this process could compete with the generation of reactive oxygen species such as $O_2(^1\Delta_g)$ [process (6)]. It is currently accepted that the quenching of ^{3}Rf* by $O_2(\Sigma_g^-)$, to produce $O_2(^1\Delta_g)$, occurs with a rate constant k_{ET} of 1/9 of the difussional value [29]. A value of approximately 8×10^8 $M^{-1}s^{-1}$ for the bimolecular rate constant for process (6) in H_2O–MeOH can be estimated as a mean value in pure MeOH and H_2O [24]. Employing an averaged concentration of 1.2 mM for the dissolved oxygen in air saturated H_2O–MeOH solution [30], it appears that at 1 mM **F**—a concentration similar to those employed in typical oxygen uptake experiments—the rates of formation of $O_2(^1\Delta_g)$ (6) should be ca. 4 times higher for the cases of of CHL and FNN and around 30 times higher in the case of FVN. In other words: the generation of $O_2(^1\Delta_g)$ will compete with process (5) in the cases of CHL and FNN and will be highly favoured in the case of FVN.

Rf is a recognised quencher of $O_2(^1\Delta_g)$ with $k_t = 6\times10^7 M^{-1}s^{-1}$ [31]; nevertheless, due to the low concentration employed in the practise ([Rf] = 0.02 mM) the photooxidation of the very pigment will be a secondary process, in competitive kinetic terms.

In the absence of experimental evidence demonstrating any measurable interaction between $O_2(^1\Delta_g)$ and CHL or FNN, we consider that in these cases the oxygen consumption should be attributed to $O_2^{\bullet-}$, i.e. the second oxygen-active species generated by Rf. This proposition, strongly supported by the SOD-inhibitory effect of oxygen consumption, is in agreement with the $^3k_{qapp}$ values determined and with the relative values for Rf decomposition (Table 1): as depicted in Scheme 2, the quenching of ^{3}Rf* by CHL and FNN generates $Rf^{\cdot-}$ which by means of an electron-transfer process produces the species $O_2^{\bullet-}$. Hence, the most reactive **F** towards ^{3}Rf* (CHL and FNN) efficiently generate $O_2^{\cdot-}$ followed by the concomitant oxygen consumption due to CHN and FNN oxidations. The mentioned electron transfer process [step (7)] regenerates ground state Rf, with a reported rate constant value of 1.4×10^8 M^{-1} s^{-1} [27]. The recovery of the pigment represents a crucial step in living organisms, in which it is well known that $O_2^{\bullet-}$ is a key intermediate in the oxygen redox chemistry [32]. FVN, which poorly inhibits pathway (6), is oxidized by $O_2(^1\Delta_g)$.

Acknowledgements

Thanks are given to CONICET, ANPCT, SECyT UNRC and SECyT UNSL, all from Argentina, for financial support.

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