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Stability of Flavonoids in the Presence of Riboflavin-photogenerated Reactive Oxygen Species: A Kinetic and Mechanistic Study on Quercetin, Morin and Rutin

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ABSTRACT

Kinetic and mechanistic aspects on the stability of the flavones (FL) quercetin (Que), morin (Mor) and rutin (Rut), in methanolic solution and in the presence of reactive oxygen species (ROS) generated by visible light-promoted riboflavin (Rf, vitamin B_2) photoirradiation were studied. The system was chosen as a model for the evaluation of the *in vivo* protective effect of biological targets by the flavones. The overall picture includes the vitamin as an endogenous natural photosensitizer. A systematic study on the effect of ROS on FL photostability shows that under work conditions Que is oxidized by singlet molecular oxygen ($O_2(^1\Delta_g)$), superoxide radical anion ($O_2^{\bullet-}$) and hydrogen peroxide; Mor is degraded by $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ whereas Rut only reacts with $O_2(^1\Delta_g)$. Que and Rut, with an extremely poor overall rate constant, are mainly physical quenchers of $O_2(^1\Delta_g)$. Mor, with $O_2(^1\Delta_g)$-interception ability slightly lower than the recognized synthetic antioxidant trolox (Tx), behaves as a typical sacrificial scavenger provided that *ca* 80% of the collisions with $O_2(^1\Delta_g)$ cause its own degradation, whereas this parameter reaches around 50% in the case of Tx.

INTRODUCTION

It is well recognized that the oxidative damage of biologically relevant molecules in living tissues, induced by reactive oxygen species (ROS), plays an important role in a considerable number of pathological problems (1,2). Flavonoids are a family of polyphenol derivatives found in vegetables as well as in popular beverages such as red wine and tea (3). The main physiological benefits of these compounds have been largely attributed to their antioxidant properties, being particularly active against ROS (4,5). Following this line, it is a common temptation to straightforwardly correlate antioxidant protection with flavonoid content. Nevertheless, an essential requirement to ensure the effectiveness of flavonoids as antioxidants is their own stability in the presence of ROS. In this context, the main aim of this work was the evaluation of the reactivity and potential degradation of three flavones (FLs), taken as representative antioxidant protectors, in the presence of photogenerated ROS. The chosen FLs were quercetin (Que), morin (Mor) and rutin (Rut). Their respective chemical structures are shown in Scheme 1. The oxidative species were produced by photoirradiation of riboflavin (Rf, vitamin B_2) with light of wavelength higher than 450 nm, a spectral region where the FLs are transparent. This combination reasonably mimics a natural picture in a living/biological system, in which the sensitizer, the oxidizable targets and the photoprotectors are simultaneously present in a given environment illuminated by daylight. Rf is one of the endogenous visible light absorbers which has been postulated as a possible sensitizer for the *in vivo* photo-oxidative degradation of several biologically relevant substrates (6,7). Upon photoirradiation in MeOH the pigment generates the oxidative species singlet molecular oxygen ($O_2(^1\Delta_g)$), process [11] in Scheme 2) with a quantum yield of 0.49 (8) and superoxide radical anion ($O_2^{\bullet-}$) (process [3] in Scheme 2) with a quantum yield of 0.009 (9). Rf is synthesized by green plants and participates in a variety of enzyme-catalyzed oxidation–reduction reactions (10), being widely distributed also in human tissues, both in the free and in the conjugated form (11).

In the present work we discuss the results of a systematic kinetic study on the interaction of Rf-photogenerated ROS with some FLs, toward the elucidation of the oxidative mechanisms that could account for the reactive interactions ROS–FLs. In this way we evaluate the stability of the chosen natural FLs in comparison with Trolox C (Tx), a well-recognized water-soluble analog of the natural antioxidant α-tocopherol (12,13).

MATERIALS AND METHODS

Materials. The flavones Que, Mor and Rut, Rf, sodium azide (NaN_3), superoxide dismutase (SOD, from bovine erythrocytes, 2500–7000 U mg^{-1} protein), methionine (Met), eosin and monodeuterated methanol (MeOD) were purchased from Sigma. Water was triply distilled and methanol (HPLC quality) was provided by Sintorgan (Argentina).

Methods. Open or teflon-stopped 1 cm quartz cells were employed working under air- or nitrogen-saturation conditions, respectively. Ground-state absorption spectra were registered in an Agilent 8453 diodo-array spectrophotometer.

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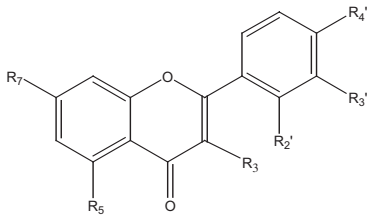

Flavonols	R_3	R_5	R_7	$R_{2'}$	$R_{3'}$	$R_{4'}$
Quercetin	OH	OH	OH	H	OH	OH
Morin	OH	OH	OH	OH	H	OH
Rutin	Rutinoside	OH	OH	H	OH	OH

Scheme 1. Chemical structure of the flavonoids quercetin, morin and rutin.

$Rf + hv \rightarrow {}^1Rf^* \rightarrow {}^3Rf^*$	[1]
${}^1Rf^* + FL \rightarrow Rf + FL$ or P(2)	[2], rate constant ${}^1k_q$
${}^3Rf^* + O_2({}^3\Sigma_g^-) \rightarrow Rf^{\bullet+} + O_2^{\bullet-}$	[3]
${}^3Rf^* + FL \rightarrow Rf^{\bullet-} + FL^{\bullet+}$	[4], rate constant ${}^3k_q$
$Rf^{\bullet-} + H^+ \leftrightarrow RfH^\bullet$	[5]
$2\ RfH^\bullet \rightarrow Rf + RfH_2$	[6]
$RfH_2 + O_2({}^3\Sigma_g^-) \rightarrow RfH_2^{\bullet+} + O_2^{\bullet-}$	[7]
$RfH_2^{\bullet+} + O_2^{\bullet-} \rightarrow Rf + H_2O_2$	[8]
$O_2^{\bullet-} + FL \rightarrow P(12)$	[9]
$H_2O_2 + FL \rightarrow P(10)$	[10]
${}^3Rf^* + O_2({}^3\Sigma_g^-) \rightarrow Rf + O_2({}^1\Delta_g)$	[11], rate constant k_{ET}
$O_2({}^1\Delta_g) \rightarrow O_2({}^3\Sigma_g^-)$	[12]
$O_2({}^1\Delta_g) + FL \rightarrow O_2({}^3\Sigma_g^-) + FL$	[13], rate constant k_q
$O_2({}^1\Delta_g) + FL \rightarrow P(14)$	[14], rate constant k_r
	Being $k_t = k_r + k_q$

Scheme 2. Major kinetic processes in the visible light irradiation of an air-equilibrated solution of an electron donor molecule (FL) in the presence of riboflavin (Rf).

Stationary aerobic photolysis of solutions containing any FL (0.5 mM) and Rf (0.02 mM) were carried out in a PTI unit provided with a high-pass monochromator and a 150-W Xe lamp, irradiating with 450 ± 5 nm, or in a home-made photolyzer for nonmonochromatic irradiation (150 W quartz-halogen lamp). In the latter case, cut-off filters (450 nm) ensured that the light was only absorbed by the sensitizer.

The Rf-sensitized photo-oxygenation rates of the FLs and Met were evaluated from the initial slopes of oxygen consumption *vs* irradiation time, employing the specific oxygen electrode Orion 97-08. Anaerobic photodecomposition rates of Rf were determined by evaluation of the initial slopes of Rf consumption (decrease of absorbance at 446 nm) *vs* irradiation time.

The reactive rate constant, k_r, for the reaction of $O_2({}^1\Delta_g)$ with each FL (process [14]) was determined as described previously (14) using the expression $slope/slope_R = k_r\ [FL]/k_{rR}\ [R]$, for which the knowledge of the reactive rate constant for the photo-oxidation of a reference compound, R, at similar concentration, is required, and where slope and $slope_R$ are the respective slopes of the first-order plots of FL and R consumption, or oxygen consumption by the same compounds, under sensitized irradiation. Oxygen uptake in water was monitored with a 97-08 Orion electrode. Employing Eo as a sensitizer, it was assumed that the reaction of $O_2({}^1\Delta_g)$ with each FL is the only way of oxygen consumption. The reference R was FFA, with a reported pH-independent k_{rR} value in water of $1.2 \times 10^8\ M^{-1}\ s^{-1}$ (15).

The overall quenching rate constant of deactivation of $O_2({}^1\Delta_g)$ by each FL (k_t, the sum of k_q plus k_r, processes [13] and [14], respectively, Scheme 2) was determined using a previously reported system (16). Briefly, a Nd:YAG laser (Spectron) was used for the excitation (532 nm) of the sensitizer Eo ($A_{532} = 0.3$), and the emitted radiation ($O_2({}^1\Delta_g)$ phosphorescence at 1270 nm) was detected at right angles using an amplified Judson J16/8Sp germanium detector, after passing through two Wratten filters. The output of the detector was coupled to a digital oscilloscope and to a personal computer for the signal processing. Usually, 16 shots were needed for averaging, so as to achieve a good signal-to-noise ratio, from which the decay curve was obtained. Air-saturated solutions were employed in all the cases. In the dynamic determinations, MeOD, instead of MeOH, was used as a solvent in order to enlarge the lifetime of $O_2({}^1\Delta_g)$ (15). The $O_2({}^1\Delta_g)$ lifetimes were evaluated in the presence (τ) and in the absence (τ_0) of the quencher, and the data were plotted as a function of concentration, according to a simple Stern–Volmer treatment, $1/\tau = 1/\tau_0 + k_t\ [FL]$.

RESULTS

Decrease in the photoprotective effect by a flavone

It is well known that several amino acids, including Met, are photo-oxidized when exposed to visible light in the presence of ROS generators in general and Rf in particular (17–20). We employed this fact to test the photoprotection of the FL Mor toward the oxidative damage of Met, taking the amino acid as a relevant oxidizable biological target.

The experiment was performed as follows: the visible light irradiation of a pH 7 aqueous solution containing 0.5 mM Met, and Rf ($A_{445} = 0.5$) as a photosensitizer, produced oxygen consumption (Fig. 1, trace "a"), due to the mentioned oxidation processes operated by the reaction of ROS with the amino acid. When the same experiment was carried out in the presence of 0.5 mM Mor the rate of oxygen uptake clearly decreases, in a process that could be interpreted as a sort of photoprotection exerted by the FL (trace "b"). Finally, a pH 7 aqueous solution of Rf ($A_{445} = 0.4$) plus 0.5 mM Mor, in the absence of Met, was photoirradiated with visible light for 5 min. Thereafter, Met was dissolved at a concentration of

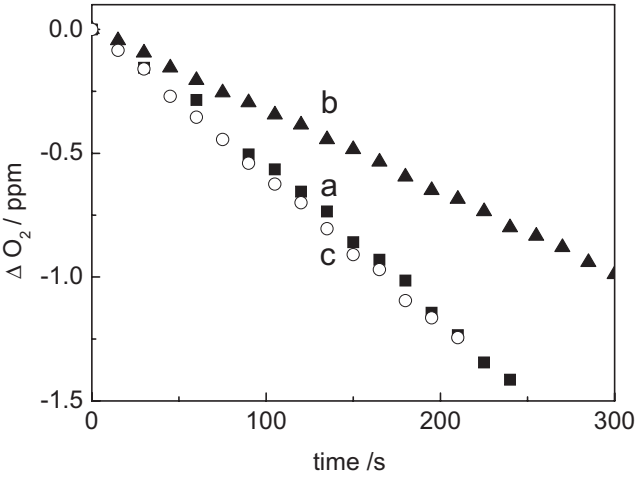

Figure 1. Oxygen consumption as a function of the photoirradiation time of the following pH 7 aqueous solutions: methionine 0.5 mM + riboflavin 0.04 mM (a); methionine 0.5 mM + riboflavin 0.04 mM + morin 0.5 mM (b); the same as run (b) but the mixture riboflavin 0.04 mM + morin 0.5 mM was previously photoirradiated for 5 min.

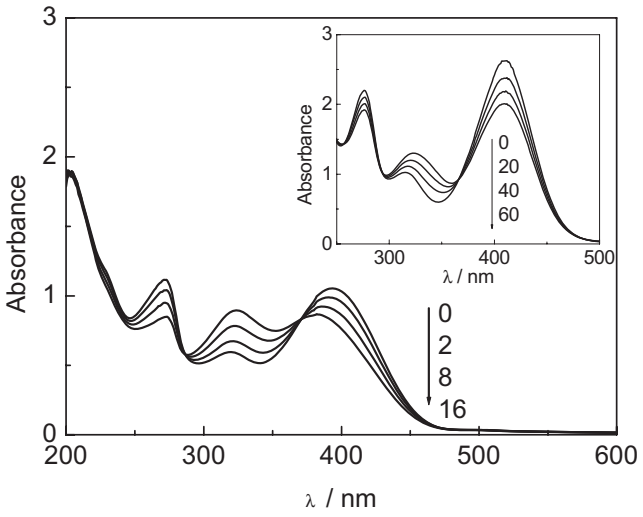

Figure 2. Spectral changes in quercetin 0.06 mM + riboflavin 0.04 mM *vs* riboflavin 0.04 mM in air-equilibrated aqueous solution (pH 7) after photoirradiation. Numbers on the spectra represent photoirradiation time in minutes. Inset: spectral changes in morin 0.12 mM + eosin 0.04 mM *vs* eosin 0.04 mM in air-equilibrated methanolic solution, in the presence of 10 mM KOH. Numbers on the spectra represent photoirradiation time in seconds.

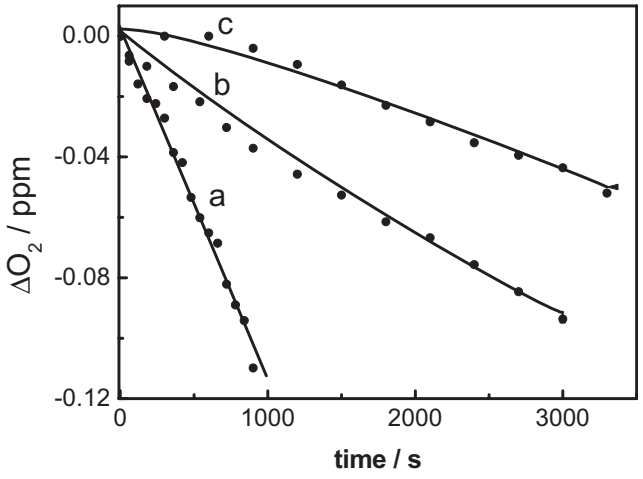

Figure 3. Oxygen consumption as a function of photoirradiation for the following solutions: quercetin 0.5 mM + riboflavin 0.04 mM (a); morin 0.5 mM + riboflavin 0.04 mM (b); rutin 0.5 mM + riboflavin 0.04 mM (c).

0.5 mM and the solution was again photolyzed. The rate of oxygen consumption increased as shown in trace "c." The rate is now indistinguishable, within the experimental error, from the initial trace "a" of Met, in the absence of Mor. The experimental conditions in the photolysis of solutions employed for traces "a," "b" and "c" were identical.

A comparison of the respective rates of oxygen uptake observed in the solutions containing Met and Met + Mor strongly suggests that the photoprotection exerted by the flavonoid on the biological target varies with the degree of exposition of the FL to sensitized photoirradiation. This fact, in the context of the stability of FLs as biological photoprotectors, could indicate a loss of the antioxidative properties of Mor due to photoirradiation, and deserves to be carefully investigated. It was done through a comparative evaluation of the degradability of three flavones Mor, Que and Rut in the presence of ROS photogenerated by vitamin B_2.

Possible reaction steps of Rf upon visible light photoirradiation

When Rf absorbs visible light, and in the presence of electron donating molecules, several ROS can be formed in solution, as depicted in Scheme 2 (21,22). The meaning of the different steps is self-defined within the context of photochemical reactions.

Phototransformations of the FLs due to Rf-sensitized irradiation

The visible light irradiation of methanolic or aqueous solutions of the individual 0.06 mM FL (Mor, Rut and Que) in the presence of Rf (A_{445} = 0.4) produces chemical transformations in the FL, as depicted by the absorbance evolution at different irradiation times. Figure 2 shows the case of Que in pH 7 water. Qualitatively similar changes were observed for Mor and Rut. From parallel experiments on similar photoirradiated solutions, oxygen uptake was observed (Fig. 3), and the respective rates for the three FLs are shown in Table 1.

The results herein shown clearly indicate that either Rf electronic excited states or ROS produced through these states, or even both processes operating simultaneously, are responsible for the photodegradation of the FLs. On this basis, we carried out a systematic kinetic study in order to evaluate and characterize the nature, mechanism and extent of the possible processes involved in the Rf-sensitized degradation of FL.

Table 1. Rate constants for the overall (k_t) and reactive (k_r) quenching of $O_2(^1\Delta_g)$ by the flavones quercetin (Que), morin (Mor) and rutin (Rut) in methanol; k_r/k_t quotients, reactive rate constants in methanol in the presence of 10 mM KOH (MeOH/OH^-), relative rates of oxygen uptake in buffered pH 7 aqueous solution upon photosensitized irradiation in the respective presence of eosin (Eos) and riboflavin (Rf) and pK values for the first ionization of a phenolic group (pK_{a1}) in the flavonoid molecule.

Compound	k_t (MeOD) $\times 10^8$ M^{-1} s^{-1}	k_r (MeOH) $\times 10^8$ M^{-1} s^{-1}	k_r/k_t (MeOH)	k_r (MeOH/OH^-) $\times 10^8$ M^{-1} s^{-1}	$\Delta O_2/\Delta t$ (relative), pH 7 water	
					Eos	Rf
Que pK_{a1} = 6.7*	0.031	0.011	0.35	Spontaneous decomposition	0.06	1
Mor pK_{a1} = 3.5*	0.39	0.3	0.77	4.4	1	0.5
Rut pK_{a1} = 7.1*	0.009	0.001	0.11	1.1	0.01	0.25
Trolox C pK_a = 11.9*	1.25†	0.64†	0.52†	5.5†		

*From reference (30); †in MeCN-EtOH 4:1 (vol/vol), from reference (12).

The interaction of Rf electronically excited states with FL

The photoirradiation of nitrogen-saturated Rf aqueous-methanolic solutions of Rf in the presence of Que and Mor in the sub-mM concentration range, typically 0.5 mM, produces spectral changes that may be attributed to the reactive interactions between Rf electronically excited states and FL ground state (data not shown). These spectral perturbations were practically undetectable in the system Rut-Rf.

The reported lifetime of $^1Rf^*$ in water and methanol is 5 ns (7). A FL concentration in the sub-mM range, similar to those employed in all experiments performed in this work, is not enough to intercept $^1Rf^*$, even assuming a diffusion-controlled value for the rate constant ^1k_q of process [2] in Scheme 1. Hence, the interaction $^1Rf^*$-FL must be disregarded under work conditions.

It is known that the photodegradation of Rf in solution, in the absence of oxygen and under visible light irradiation, predominantly proceeds through $^3Rf^*$ (6) for which a lifetime of 15 μs has been reported (16) and the rate of the process can be evaluated by the absorbance decrease in the Rf absorption spectrum at 445 nm (Fig. 4, inset). In the individual presence of 0.5 mM Mor and Que, the rate of Rf decomposition increased compared with the rate in the absence of FL, whereas in the presence of 0.5 mM Rut this rate suffers a delay (Fig. 4). These results strongly suggest the occurrence of some sort of interaction between $^3Rf^*$ and FL. In principle, and according to Fig. 4, this interaction could be different for Rut than for Mor and Que.

The FL–ROS interaction

In order to evaluate the potential participation of Rf-photogenerated ROS in the degradation of the FLs, oxygen consumption experiments in the presence of specific ROS interceptors were carried out. The representative case of Que is shown in Fig. 5. The presence of 10 mM NaN_3 decreased the rate of oxygen consumption for the three FLs. The enzyme SOD at a concentration of 1 μg mL^{-1} also decreased the rate of oxygen consumption only in the cases of Que and Mor, whereas the run in the presence of Rut was not affected. The dissolution of 1 μg mL^{-1} CAT only modified the rate of oxygen uptake for Que, again as a delay. No effect at all was observed for any FL by the presence of 10 mM mannitol. Similar experiments with ROS interceptors have been formerly employed to confirm/discard the participation of $O_2(^1\Delta_g)$, $O_2^{\bullet -}$, H_2O_2 and $OH^\bullet$, respectively, in a given oxidative event (23–25). The enzyme SOD dismutates the species $O_2^{\bullet -}$ (reaction [15]), whereas CAT decomposes H_2O_2 (reaction [16]), mannitol deactivates the species $OH^\bullet$ (reaction [17]) and NaN_3 quenches $O_2(^1\Delta_g)$ (reaction [13], Scheme 2), with NaN_3 instead of FL.

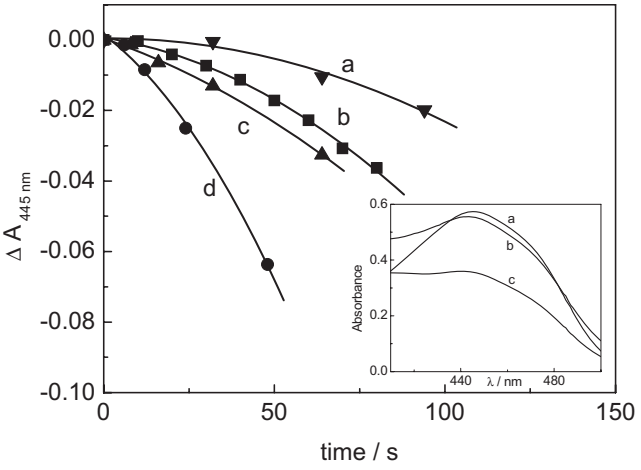

Figure 4. Absorbance decrease of 0.04 mM riboflavin at 445 nm in nitrogen-saturated methanolic solutions in the absence (b) and in the presence of: 0.6 mM rutin (a); 0.6 mM morin (c) and 0.6 mM quercetin (d). Inset: absorption spectra of Rf (*ca* 0.04 mM) in nitrogen-saturated solutions: (a) nonphotolyzed; (b) photolyzed 200 s in the presence of 0.5 mM quercetin; (c) photolyzed 200 s (for details, see Materials and Methods section).

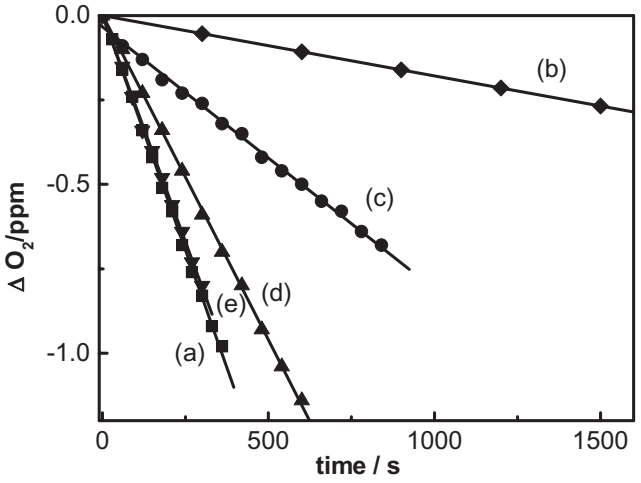

Figure 5. Oxygen uptake as a function of photoirradiation time of methanolic solutions containing 0.04 mM Rf plus: 0.5 mM quercetin (a); 0.5 mM quercetin + 1 μg mL^{-1} SOD (b); 0.5 mM quercetin + 2 mM NaN_3 (c); 0.5 mM quercetin + 1 μg mL^{-1} CAT (d); 0.5 mM quercetin + 10 mM mannitol (e).

$$2O_2^{\bullet -} + 2H^+ + SOD \rightarrow O_2(^3\Sigma_g^-) + H_2O_2 \quad [15]$$

$$2H_2O_2 + CAT \rightarrow 2H_2O + O_2(^3\Sigma_g^-) \quad [16]$$

$$OH^\cdot + \text{mannitol} \rightarrow \text{deactivation} \quad [17]$$

All experiments involving possible $O_2(^1\Delta_g)$-mediated processes were made in methanolic solutions in order to facilitate the adequate dissolution of FL up to required concentrations. The exclusive $O_2(^1\Delta_g)$-generator dye Eos (processes [1] + [11], with Eos (A_{515} = 0.5) instead of Rf) was employed as a dye sensitizer. Rf was not used in these kinetic determinations in order to avoid interferences by other oxidative species. Eos is one of the sensitizers most frequently employed in $O_2(^1\Delta_g)$ reactions, producing the oxidative species with a quantum yield of 0.30 in MeOH (26). In order to search for possible interactions of the FLs with Eos electronically excited states, argon-saturated methanolic solutions of Eos (A_{515} = 0.5) + 0.5 mM of the individual FLs were photoirradiated. No spectral changes could be observed after relatively prolonged

irradiation time, strongly suggesting the absence of interactions by processes [2] and [4], with Eos instead of Rf.

Photoirradiation with visible light of aerated mixtures of Eos (A_{515} = 0.6) plus a FL 0.12 mM, in MeOH and in MeOH in the presence of 10 mM KOH (alkaline MeOH), produces changes in the absorption spectrum of the particular FL (Fig. 2, inset), as well as oxygen consumption. These changes were not observed in Ar-saturated solutions. The presence of a FL in the sub-mM concentration range quenches the IR phosphorescence emission of $O_2(^1\Delta_g)$, as detected by TRPD experiments (see below). It is well known that phenols and polyphenols interact with $O_2(^1\Delta_g)$ through processes that are highly dependent on the degree of ionization of the OH groups (27). The rate constant k_t for the overall interaction of $O_2(^1\Delta_g)$–FL was independently determined in MeOD and in MeOD in the presence of 10 mM KOH, through a Stern–Volmer treatment (Fig. 6). The phosphorescence quenching experiments unambiguously demonstrate the existence of an $O_2(^1\Delta_g)$–FL interaction, which may be physical in nature (process ([13]) and/or reactive (process [14]). The k_t values, as determined by TRPD (Table 1), do not depend on the type of sensitizer or on potential interactions of the substrate with excited states of the sensitizer involved in $O_2(^1\Delta_g)$ generation.

The overall rate constant values (k_t) for the FLs were determined in MeOH and in alkaline MeOH, and are shown in Table 1. According to the respective absorption spectra, the OH groups are ionized in alkaline MeOH. Que was not employed in alkaline medium due to its spontaneous decomposition.

The reactive rate constant values (k_r) (process [14]) were obtained by monitoring oxygen consumption upon visible light irradiation of mixtures Eos (A_{515} = 0.6) plus 0.5 mM FL, following a previously described method (14) (Fig. 6, Table 1). The calculated k_r/k_t ratios represent a measure of the efficiency of the degradation pathway *via* $O_2(^1\Delta_g)$ reaction.

Simple relative rates of oxygen consumption upon Eos-sensitized photoirradiation of the FLs, in pH 7 water are also included in Table 1. The comparison of these rates with the relative rates of oxygen consumption upon Rf-sensitized photoirradiation in the same medium strongly suggests that the oxidative mechanisms involved in both processes must be different from each other. The xanthene dye only generates $O_2(^1\Delta_g)$ (15) whereas in the Rf-sensitized process the oxygen uptake could be due to a combined action of several ROS.

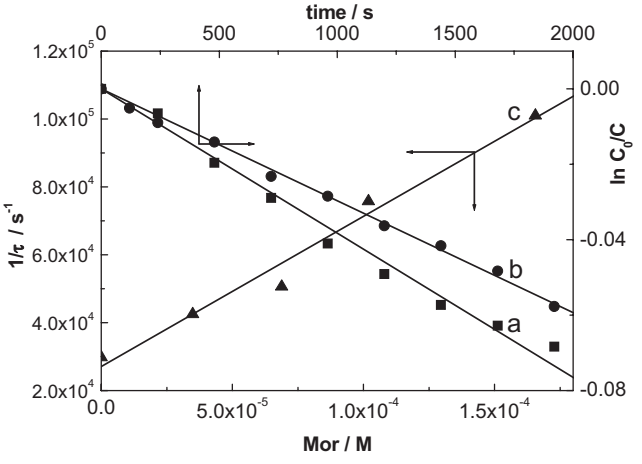

Figure 6. First-order plot of oxygen uptake in the eosin-sensitized photo-oxidation of furfuryl alcohol 0.5 mM (a) and morin 0.5 mM (b) in methanolic solutions, and Stern–Volmer plot for the quenching of $O_2(^1\Delta_g)$ phosphorescence by morin in MeOD solution, with eosin as a photosensitizer (c).

DISCUSSION

Although the importance of Rf as a natural endogenous photosensitizer and the abundance of different FLs in living organisms is well known (7,28), practically there are no studies involving the conjunctive visible light-induced photochemistry of these compounds. In this sense, we think that the employment of Rf as a dye sensitizer represents an adequate choice, given that the vitamin is a source for the *in vivo* generation of several important ROS, constituting an interesting model for the evaluation of the stability of FLs under natural conditions.

The Rf-sensitized photo-oxidation of Met is known to occur through the interaction with $O_2^{\bullet-}$ and $O_2(^1\Delta_g)$ (17,19). It is also known that the quenching of $O_2(^1\Delta_g)$ is an entirely chemical process, with a rate constant $k_t = k_r = 2.1 \times 10^7$ $M^{-1}s^{-1}$ in water. The delay in the rate of oxygen uptake by the system Rf + Met in the presence of 0.5 mM Mor must be ascribed to a physical deactivation of ROS by the FL (Fig. 1) and to the deactivation of $^3Rf^*$ by Mor. Nevertheless, as it can be appreciated from Fig. 4, the presence of Mor 0.6 mM minimally affects the anaerobic degradation rate of Rf, a fact that can be associated with a slight interaction of the electronically excited vitamin with the FL under aerobic conditions. Around 20% of the Mor–$O_2(^1\Delta_g)$ collisions (Table 1) deactivate the oxidative species without destruction of the FL. On the other hand, *ca* 80% of such collisions are reactive in nature, and the photoprotective effect exerted by Mor on Met photo-oxidation is practically cancelled upon enough exposure of the FL to the action of Rf-generated ROS. The loss of the photoprotective effect by the FL is just a question of photoirradiation time.

From the stationary photolysis experiments, the photodecomposition of the three FLs, Que, Mor and Rut, was detected in aerobic medium. The same was true in Ar atmosphere only for Que and Mor. As already stated in the Results section, the degradation of FL must be attributed, in anaerobic conditions, to reactive interactions between ^{3}Rf* and FLs. It was confirmed by the results shown in Fig. 4 for the effect of the presence of FL on the photodecomposition of Rf. Que and Mor accelerate the Rf degradation through reactive interaction with ^{3}Rf*, whereas Rut exerts a protection against Rf degradation presumably through a physical quenching of ^{3}Rf*. On this basis, different yields for the generation of the diverse ROS should be expected from ^{3}Rf* for each FL, in the presence of dissolved $O_2(^3\Sigma_g^-)$. Following this line, Rut appears as an inefficient producer of RfH , the $O_2^{\bullet-}$ precursory species. Therefore, the negative result observed for Rut in the oxygen uptake experiments employing SOD as a selective quencher could be simply due to the absence of $O_2^{\bullet-}$ in the medium.

We had previously reported that the quenching of $^3Rf^*$ by electron-donating compounds such as phenols and polyphenols, including flavonoids, occurs through an electron transfer reaction, represented by step [4] in Scheme 2, with relatively

high values of rate constants ^3k_q, in the range of 10^8–10^9 $M^{-1}s^{-1}$ (16,22). The thermodynamic feasibility of the electron transfer process can be evaluated by means of the Gibbs free energy for electron transfer, $\Delta_{ET}G_0 = E_{0(FL/FL+)} - E_{0(Rf/Rf-)} - E_{Rf*} + C$, where $E_{0(Rf/Rf-)}$ is the standard electrode potential of the acceptor Rf (−0.80 V), E_{Rf*} is the ^{3}Rf* energy (2.17 eV), C is the coulombic energy term (−0.06 V) (22) and $E_{0(FL/FL+)}$, is the standard electrode potential of the FLs, less than 1 V for the three FLs herein studied (29). The so-calculated $\Delta_{ET}G_0$ value, lower than −0.4 ev in the three cases, indicates that process [4] may be thermodynamically operative and consequently the species $Rf^{\bullet -}$ could be spontaneously formed by electron transfer from ^{3}Rf* (process [4]). We should take into account that the quantum yield for direct $O_2^{\bullet -}$ generation, through reaction [3], is practically negligible (9). Under aerobic conditions and after an effective electron transfer process, a cascade of reactions can occur (Scheme 2), most of them generating ROS. At neutral pH values the species $RfH^{\bullet}$ [step 6] is produced. The bimolecular decay of $RfH^{\bullet}$ is known to proceed through a disproportionation reaction yielding Rf and fully reduced Rf (RfH_2) (process [6]). In the presence of $O_2(^3\Sigma_g^-)$, RfH_2 is reoxidized, giving rise to $RfH_2^{\bullet +}$ and $O_2^{\bullet -}$ (process [7]), and eventually Rf and H_2O_2 (process [8]).

In aerobic medium, results of oxygen uptake in the presence of specific ROS inhibitors indicate that, under work conditions, Que is oxidized by $O_2(^1\Delta_g)$, $O_2^{\bullet -}$ and H_2O_2; Mor reacts with $O_2(^1\Delta_g)$ and $O_2^{\bullet -}$ whereas for Rut only the reaction with $O_2(^1\Delta_g)$ was detected. Somewhat controversial are the reports on the ability of flavonoids to inactivate $O_2^{\bullet -}$ (28). For example, various natural and synthetic flavonols were found to be efficient superoxide scavengers, whereas other authors reported opposite results for Que and kaempferol (30). A rapid decomposition of Que by enzymatically generated $O_2^{\bullet -}$ has been reported by Ueno *et al.* (31) and more recently by us (29), whereas Rut proved to be an inefficient scavenger of the oxidative species under similar experimental conditions.

The quenching of $O_2(^1\Delta_g)$ by hydroxyaromatic compounds in general and flavonoids in particular has been extensively reviewed (28). Hydroxy-substituents promote the electron donor ability of the aromatic structure toward the electrophilic species $O_2(^1\Delta_g)$. The rate constants k_t and k_r of the studied FLs increase with the ionization of the OH groups, a property well described for hydroxy-aromatic compounds (27,32). This behavior has been explained on the basis of a mechanism involving an intermediate complex possessing charge-transfer character (27,33,34).

The $O_2(^1\Delta_g)$-mediated photo-oxidation quantum efficiency φ_r ($\varphi_r = k_r\,[FL]/(k_d + k_t\,[FL])$) (27) is not easy to evaluate, particularly in natural environments, because its determination includes the knowledge of the concentration of the photo-oxidizable substrates represented by the FLs in this case. A simpler and useful approach is the evaluation of the k_r/k_t ratio (Table 1), which indicates the fraction of overall quenching of $O_2(^1\Delta_g)$ by the substrate that effectively leads to a chemical transformation. In the present case, k_r/k_t values range from 0.11 to 0.77 for the three FLs. In the context of FL stability, low k_r/k_t values indicate a sort of self-protection of the substrates against $O_2(^1\Delta_g)$-mediated oxidation. The prevalence of the physical process is a desirable possibility as the final result is the elimination of the oxidative species without considerable loss of the scavenger.

Mor is the most labile of the studied FLs. Around 80% of the $O_2(^1\Delta_g)$–Mor collisions effectively oxidize FL. These results are in line with our previous reports on the $O_2(^1\Delta_g)$-quenching ability of dihydroxybenzenes (35). We found that cathechol—a structure present in ring B of Que and Rut—only quenches $O_2(^1\Delta_g)$ in a physical fashion. On the other hand, the quenching of $O_2(^1\Delta_g)$ by the resorcinol structure—similar to that of Mor in ring B—is mostly reactive, producing the effective oxidation of the quencher. These facts are clearly reflected by the respective k_r/k_t values in Table 1. Finally, an additional reason for the high $O_2(^1\Delta_g)$ rate constant values found for Mor is the existence of an ionized phenolate structure for this FL under work conditions. Mor is the only among the studied FLs possessing a very low pK value for the first phenolic group ionization (30) (Table 1).

The FL molecules herein studied present two centers (Scheme 1) susceptible to attack by $O_2(^1\Delta_g)$: the already described ring B and the phenylchromen-4-one moiety, containing ring A (Scheme 1). Que and Mor possess OH substitution in position 3 at the chromen moiety, whereas Mor has a rutinosid residue in that position. All three FLs are OH-substituted in position 7. The substitution pattern in ring B, especially the presence of a OH group in that position, has been recognized as a determinant factor promoting $O_2(^1\Delta_g)$–flavonoid interaction (36). This fact is neatly reflected by the low rate k_t and k_r values of Rut (Table 1).

Considering the structure–reactivity relationship reports of FL as quenchers of ROS in general, three main points are well established, through several studies (for a review, see Ref. [28]): (1) ortho-dihydroxy (cathechol) structure in the ring B is important for high $O_2^{\bullet -}$ scavenging activity; (2) there is no unanimity about the role of the presence of an OH group in position 3; (3) a C2–C3 double bond enhances the antioxidant activity by stabilization of the flavonoid radical through electron delocalization across the molecule. In synthesis, the maximal overall antioxidant activity is shown by flavonoids with a free OH group in position 3, a cathechol moiety in the B-ring and a double in C2–C3, such as Que. Our results are coincident with these observations in the sense that Que, with the higher relative rate for oxygen consumption under Rf photosensitization (Table 1), is the most degradable FL.

In an attempt to standardize the persistence of the antioxidant capacity of the FLs Que, Mor and Rut in the presence of photogenerated ROS, their respective stabilities were compared with that of the recognized synthetic antioxidant Tx. It is known that Tx interacts with different radicals, including Rf-generated $O_2^{\bullet -}$ with relatively high rate constants, to form the neutral trolox radical (12,30,37). Furthermore, the rate constants for the reaction of Tx with $O_2^{\bullet -}$ is very similar to that reported for several flavonoids, including Que, Mor and Rut, determined by pulse conductivity in aqueous solution (30). In this sense Que and Mor behave in a fashion similar to Tx with respect to $O_2^{\bullet -}$ under Rf-sensitized photoirradiation.

Regarding the $O_2(^1\Delta_g)$ interaction, the data in Table 1 indicate that the rate constants for both physical and chemical quenching of the oxidative species are much higher for Tx than for either of the studied FLs. It is necessary an increase in concentrations of *ca* 2 orders of magnitude for Rut, *ca* 40 times for Que and approximately three times for Mor, with

respect to Tx concentration, to quench $O_2(^1\Delta_g)$ with a similar rate than the synthetic antioxidant. Considering the stability towards $O_2(^1\Delta_g)$-mediated degradation, the balance between rate constants for physical and chemical deactivation of the oxidative species indicates that Que and Rut, the poorest $O_2(^1\Delta_g)$ quenchers, behave mainly as physical deactivators, with Rut being the most longstanding photoprotector in view that only around 10% of the collisions produce its effective photodegradation. In this context, Mor, the more promising of the FLs for the overall deactivation of $O_2(^1\Delta_g)$, is paradoxically a typical sacrificial scavenger, provided that *ca* 80% of the collisions with $O_2(^1\Delta_g)$ cause its own degradation, whereas this parameter reaches around 50% in the case of Tx.

As a conclusion we can say that the visible light photoirradiation of Rf in the individual presence of Que, Mor and Rut, in aqueous and methanolic solutions, generates ROS that degrade each FL by particular mechanisms and to different extents. This fact clearly reduces the characteristic photoprotective effect attributed to FLs. According to oxygen uptake experiments the photodegradation rate of the FLs is Que > Mor > Rut.

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