# Generation, Spectroscopic Characterization by EPR, and Decay of a Pyranine-Derived Radical

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The pyraninoxyl radical is readily formed from the $MnO_2$-promoted oxidation of pyranine. The free radical can be formed in high concentrations (mM), and presents a characteristic EPR spectrum that indicates a high spin-density delocalization. It is relatively stable under nitrogen (half-life *ca.* 50 min) but readily decays in presence of $O_2$. In spite of its high stability, the radical readily reacts with antioxidants (phenols and ascorbic acid) with a partial recovery of the parent pyranine. High concentrations of the pyraninoxyl radical (*ca.* 9 μM) are present when pyranine is exposed to a free radical source (10 mM 2,2′-azobis[2-amidinopropane], 37°). The fact that these radicals readily react with antioxidants (ascorbic acid and caffeic acid) supports the proposal that protection by antioxidants of peroxyl radical-promoted pyranine bleaching is mainly due to the occurrence of a repair mechanism.

**Introduction.** – The protection afforded to a target molecule damaged by peroxyl radicals by the addition of a given compound is generally related to its capacity to scavenge the damaging radicals. This simple relationship between reactivity of the additive and afforded protection is the basis of a large number of methods aimed at evaluating the antioxidant capacity of pure compounds and complex mixtures [1–3]. These methods are based on competitive experiments in which the target molecule (TH) and the tested additive (antioxidant, XH) compete for a ROS (*i.e.*, peroxyl radicals, hydroxyl radicals, superoxide, singlet oxygen, or hypochlorite) or RNS (*i.e.*, peroxynitrite) [4][5]. When a source of radicals is employed to damage the target molecule, this direct relationship between the additive reactivity and the afforded protection is based on a simplified mechanism comprising *Processes 1* and *2:*

$$R^{\cdot} + TH \rightarrow RH + T^{\cdot} \quad (1)$$

and

$$R^{\cdot} + XH \rightarrow RH + X^{\cdot} \quad (2)$$

In this very simplified reaction scheme [6], *Reaction 1* leads to the target molecule consumption by a radical source ($R^{\cdot}$). If the rate of radical production is kept constant, it is predicted that, under ideal (zero-order limit) conditions, the rate of TH

consumption in absence ($r^0$) and in presence of competitive molecules ($r$) are related by:

$$\frac{r^0}{r} = 1 + \frac{k_2}{k_1}\frac{[XH]}{[TH]} \tag{3}$$

leading to a *Stern–Volmer*-like behavior. This simple relationship requires that: *i*) removal of R$^\bullet$ radicals by self-reaction must be negligible in presence of TH; *ii*) the stoichiometry of the process, determined by the fate of radicals T$^\bullet$ and X$^\bullet$, must be constant; *iii*) damage of TH by X$^\bullet$ radicals is negligible; assuming that *Reaction 4* can be disregarded.

$$X^\bullet + TH \rightarrow XH + T^\bullet \tag{4}$$

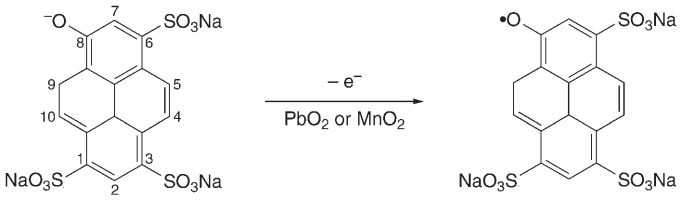
*iv*) repair of TH by XH is negligible. This amount to assume that *Process* 5

$$T^\bullet + XH \rightarrow TH + X^\bullet \tag{5}$$

does not significantly contribute to the observed protection.

In previous works [6][7], we have shown that condition *iii* is frequently not fulfilled, leading to a strong downwards curvature when $r^0/r$ values are plotted as a function of the additive concentration. Even more, when c-phycocyanin (c-Pc) a blue-green pigment is employed as target molecule, some additives such as pyranine (=trisodium 8-hydroxypyrene-1,3,6-trisulfonate; see **1** in the *Scheme*) and naphthalen-2-ol, increase its bleaching rate elicited by peroxyl radicals [1]. This result has been attributed to a nearly quantitative intervention of *Reaction 4*.

*Scheme*




**1** **2**

Pyranine is a hydrophilic pyrene derivative that also shows a peculiar behavior when it is employed as target molecule [7][8]. Its rate of consumption by peroxyl radicals follows zero-order kinetics even at very low concentrations (0.1 μM), implying a very fast reaction with the initiator radicals. In spite of this, its bleaching is almost totally prevented by a large number of additives, implying that the protection is considerably larger than that expected from the $k_2/k_1$ ratio. This anomalous result has been attributed to a significant repair of pyraninoxyl radicals (see **2** in the *Scheme*) by added phenols [7]. This requires a relevant role of *Reaction 5*.

The large unpaired electron delocalization in pyrenoxyl radicals (radicals from pyrenols) can lead to relatively stable free radicals. Pyranine-derived radicals (or pyraninoxyl radicals) have been produced by laser flash photolysis and detected by UV/VIS spectroscopy [9], and a persistent and oxygen-insensitive free radical has been produced in the oxidation of 2,7-di(*tert*-butyl)-1-hydroxypyrene [10][11]. In the present communication, we present data supporting the proposal that the apparently anomalous behavior of pyranine in presence of peroxyl radicals is mostly related to the slow rate of pyraninoxyl radicals' self-reactions.

**Materials and Methods.** – *Oxidation of Pyranine by $PbO_2$ and $MnO_2$.* Pyranine (2 mM) in phosphate buffer (Pi), 100 mM, pH 7.0, was oxidized by $PbO_2$ (25 mg/ml) or activated $MnO_2$. After addition of the oxidant, the soln. was hand-shaken and filtered. UV/VIS and EPR spectra of the filtrated solns. were recorded with *HP-8453* diode array and *Bruker EMX-1572* series spectrometers, resp., under aerobic, and anaerobic and $O_2$-saturated solns. ($N_2$-purged).

*Pyranine Oxidation Promoted by Peroxyl Radicals.* Pyranine (200 μM) was incubated at 37° in the presence of 10 mM 2,2′-azobis[2-amidinopropane] (AAPH) in the cavity of the EPR spectrometer. Spectra were recorded as a function of the incubation time.

**Results.** – *Characteristics of Pyranine-Derived Radicals.* Oxidation by activated $MnO_2$ or $PbO_2$ have been employed to produce persistent radicals, such as the radical anion 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate] ($ABTS^{\bullet-}$) [12] and 2,7-di(*tert*-butyl)pyren-1-oxyl radicals [10]. Addition of a small amount (25 mg/ml) of either $MnO_2$ or $PbO_2$ to a pyranine solution produces an instantaneous noticeable change in the UV/VIS spectra, and the concomitant appearance of a strong EPR signal, as is shown in *Fig. 1,a*.

A large amount of the pyranoxyl radical is obtained, as assessed by a noticeable decrease of the UV/VIS pyranine spectra and the intensity of the EPR signal. Calibration of this signal with TEMPO (=2,2,6,6-tetramethylpiperidin-1-oxyl) as standard indicates that 2.1 mM of radical is produced in the anaerobic oxidation of 2.3 mM of pyranine. The change in UV/VIS spectra is shown in *Fig. 2*.

In *Fig. 2*, a decrease of the pyranine band (450 nm), and the appearance of a new and narrower band attributable to the formed radical (centered at 457 nm) is observed. Comparison of this band intensity with the size of the EPR signal leads to $\varepsilon_{457} = 33{,}800 \pm 200\ M^{-1}cm^{-1}$. This value compares favorably with that reported from flash-photolysis experiments ($\varepsilon_{445} = 40{,}000\ M^{-1}cm^{-1}$) [9].

The EPR spectrum shows a complex pattern mainly due to the six H-atoms at the pyrene core of the radical structure. However, an analysis, performed by computer simulation using the EasySpin [13] software, suggested that there is a seventh hyperfine coupling (hfc) constant from an H-atom that could be involved in H-bonding between the radical and $H_2O$. This hfc, along with the lowest hfc of the H-atoms in the molecule, provides a triplet character to the whole hyperfine structure in the spectrum. The $g_o$ value obtained was $2.00783 \pm 0.00001$, and the reaming parameters are collected in the *Table*. Thus, simulation of the spectrum (*Fig. 3,a*) under $N_2$ matches to 94.4% that expected from the structure of the radical shown in the *Scheme*, while the simulation under air (*Fig. 3,b*) renders the same spectrum affected by broader lines due to $O_2$, with a 96% of correspondence to the experimental spectrum.

To visualize the spin distribution, we performed spin-density calculations by using density functional theory. The density map calculated shows that the free electron is

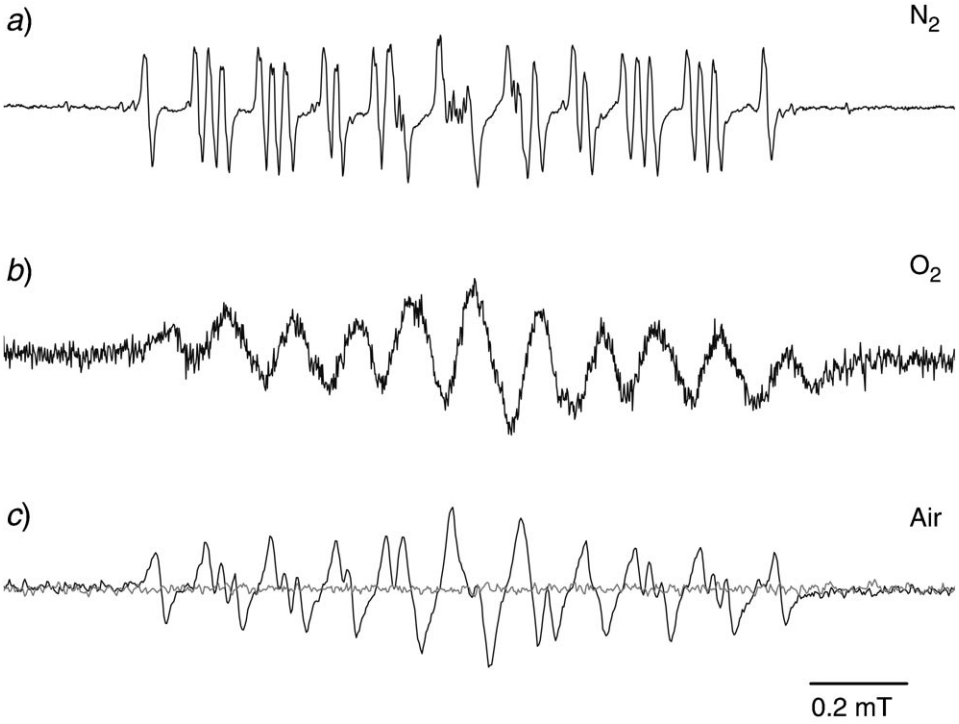



Fig. 1. *Experimental EPR spectra corresponding to* a) *ca. 1.2 m*M of *pyraninoxyl radicals obtained under $N_2$ atmosphere*; b) *under $O_2$ and after 20 min of its preparation*; c) *radical prepared under air. Gray line:* signal after addition of 500 μM of caffeic acid in buffer Pi, pH 7.

highly delocalized among the O-atom and C-atoms C(5), C(9), C(7), C(2), C(4), and C(10) in agreement with the hyperfine structure of the EPR spectrum.

The electronic structure of the radical was calculated employing Gaussian 98, (Revision A.9) [14]. A calculation of level ub3lyp/6-31G(d) was performed to obtain the molecular geometries and the spin densities. Relevant parameters are collected in the *Table*. The simulated spectrum is shown in *Fig. 3* and nicely matches to that obtained experimentally.

After formation, the pyraninoxyl radicals slowly decay, particularly in $N_2$-purged solutions. The lost of spin does not follow a simple mono-exponential decay (see *Fig. 4*), but, half-life time is close to 50 min, independently of the initial free spin concentration. In fact, the data given in *Fig. 4* show a very similar decay at 0.27 and 2.5 mM initial concentrations. On the other hand, the decay is considerably faster when the radicals are produced in the presence of $O_2$ ($O_2$-saturated solution; *Fig. 5*). Under these conditions, the lost of EPR signal intensity is almost complete in few minutes. Under air-saturated solutions, 50% of the signal intensity is lost in *ca.* 14 min (*Fig. 5*, initial concentration 1.2 mM).

The rather long lifetime of the radicals under $N_2$ indicates that self-reactions are slow and/or reversible.

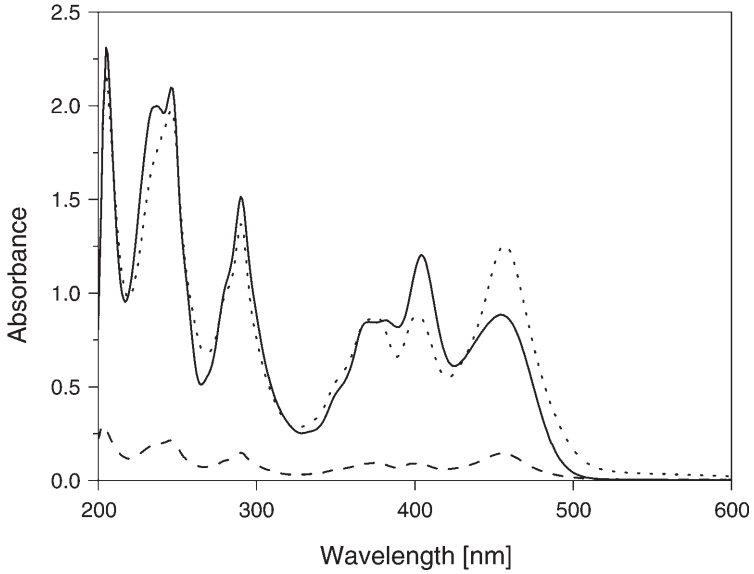



Fig. 2. Solid line: *Aliquot corresponding to a 6.7 μM of pyranine.* Dashed line: *Aliquot of pyraninoxyl radicals from a pyranine solution oxidized by $MnO_2$.* Dotted line: *10 Times concentrated pyraninoxyl radical solution.*

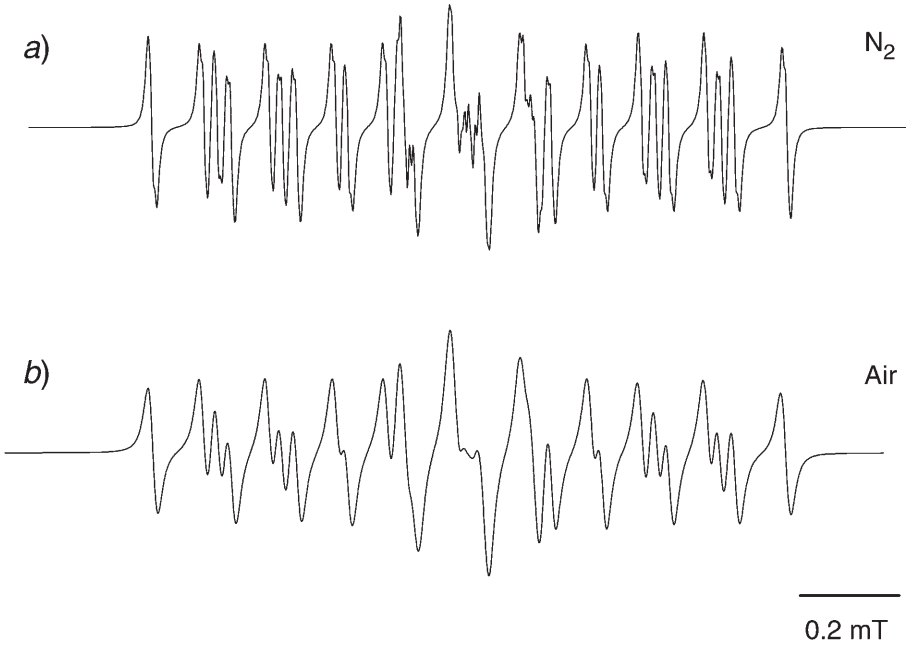



Fig. 3. *Simulated spectra for the radical obtained under $N_2$ (a) and air (b).* Concentration: 1.2 mM.

Table. *Experimental hfc Constants and Spin Densities for Pyranoxyl Radicals.* $\alpha_H$ Values were calculated in buffer Pi, pH 7.0.

| Parameter | O–C(8) | H–C(7) | H–C(5) | H–C(4) | H–C(2) | H–C(10) | H–C(9) |
|---|---|---|---|---|---|---|---|
| $\alpha_H$ [mT] (nitrogen) | – | 0.10646 | 0.13533 | 0.52489 | 0.16225 | 0.37828 | 0.00666 |
| $\alpha_H$ [mT] (air) | – | 0.10488 | 0.13525 | 0.52387 | 0.16117 | 0.37865 | 0.00666 |
| Calculated spin density on C | 21.32 | 10.8 | 9.7 | 22.9 | 10.9 | 16.7 | 6.9 |

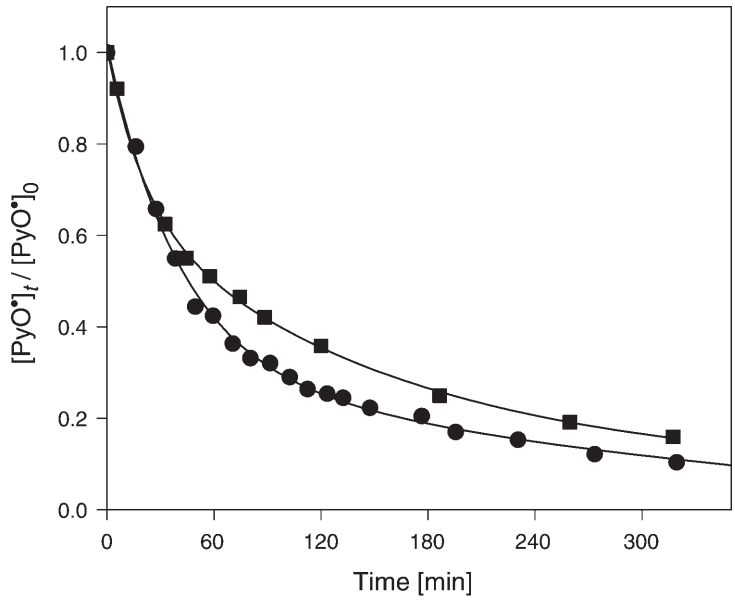



Fig. 4. *Normalized concentration decay of pyraninoxyl radicals formed from $MnO_2$ oxidation as a function of time under a $N_2$ atmosphere for two different starting concentrations.* ■: 2.5 mM and ●: 270 μM. The indicated fit lines correspond to bi-exponential decays.

Furthermore, the fact that the decay is not mono-exponential in spite of the fact that the half-life time is almost concentration-independent implies that the kinetics of the decay is complex. This complexity is not due to the presence of traces of $Mn^{II}$ ions in the solution, since EDTA (=ethylenediaminetetraacetic acid; 300 mM) addition does not modify the profile of the EPR signal intensity decay.

In spite of their stability, pyraninoxyl radicals readily react with good H-atom or electron donors. Thus, the EPR signal is totally and instantaneously quenched by addition of an excess of caffeic acid or ascorbic acid. In particular, addition of 500 μM of caffeic acid (*Fig. 1,c*) or ascorbic acid to a 60 μM solution of pyraninoxyl radical totally quenches the EPR signal and the UV/VIS spectrum of the radical, recovering almost quantitatively the UV absorption at 450 nm, which corresponds to a characteristic band of pyranine (data not shown). Also, a partial recovery (*ca.* 8%) of the initial pyranine fluorescence (excitation 450 nm; emission at 511 nm) takes place. This low recovery could be, at least partially, due to pyranine fluorescence quenching by the remaining

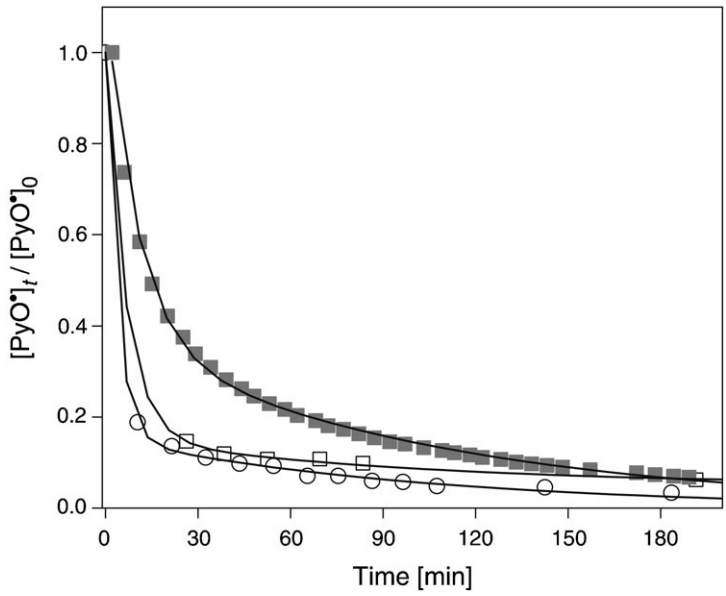



Fig. 5. *Decay of pyraninoxyl radical concentration* (normalized) *formed from $MnO_2$ oxidation as a function of time under $O_2$-saturated atmosphere for two different starting concentrations.* □: 2.5 mM and ○: 270 μM. ■: Decay in air-saturated solution. Initial concentration 1.2 mM.

ascorbic (or caffeic) acid and residual $Mn^{II}$ ions present in the solution. These results are compatible with a repair process such as:

$$PyO^{\bullet} + AOH \rightarrow PyOH + AO^{\bullet} \quad (6)$$

where $PyO^{\bullet}$ and PyOH represents the pyraninoxyl radicals and pyranine, respectively, and AOH an antioxidant molecule (such as caffeic acid). The process is followed by fast reactions of the antioxidant-derived radical. Under the present experimental conditions, the cross reactions between $PyO^{\bullet}$ and $AO^{\bullet}$ radicals could be relevant, precluding a total recovery of the initial parent pyranine.

*Reaction of Pyranine with Peroxyl Radicals.* Aerobic thermolysis of AAPH (10 mM, 37°) in the presence of pyranine (500 μM) leads to an accumulation of a free radical species, as detected by EPR. The spectrum of this radical is identical to that shown in *Fig. 1,c*. The concentration of this radical readily reaches a steady state, where the estimated steady state concentration is *ca.* 9 μM. This implies that the rate of formation (given by the rate of free radicals production [1]) is equal to the removal rate (by self-reaction and/or reaction with freshly produced peroxyl radicals). If the rate of production of radicals is taken as 0.8 μM/min, the lifetime of the radicals can be estimated to amount to *ca.* 10 min. This value, obtained in air-saturated solutions, is compatible with the data shown in *Fig. 5*.

Addition of *Trolox*® (= 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) to the system delays the accumulation of the $PyO^{\bullet}$ in a concentration-dependent way

(data not shown). This can be due to trapping of the peroxyl radicals (avoiding PyO$^{\bullet}$ formation) and/or by trapping of these radicals. This last process is supported by the fact that addition of *Trolox*® (500 μM), after reaching the steady state condition, totally suppresses the EPR signal in less than 1 min. The same is observed in the presence of caffeic acid.

**Conclusions.** – All the above results allow concluding that *i*) PyO$^{\bullet}$ radicals are readily formed by PyOH oxidation and, in particular, in free radical-mediated processes; *ii*) PyO$^{\bullet}$ radicals (μM to mM concentration range) in anaerobic conditions have half-life times of *ca.* 50 min. Their decay is considerably faster in presence of $O_2$; *iii*) PyO$^{\bullet}$ radicals are readily reduced by ascorbic and caffeic acids.

These results support our former proposal that repair reactions by stable pyraninoxyl radicals are involved in the protection of pyranine afforded by reactive phenols [8].

We thank Dr. *Claudio Olea*, Universidad de Chile, for his help during the former detection of the pyranine-radical EPR spectrum. Special thanks are due to Dr. *Otaciro Rangel Nascimento*, Instituto de Física de São Carlos, Universidade de São Paulo-Brasil, for his help on simulating the EPR spectra and helpful discussions. *C.A.* thanks to the *World Bank CONICYT: Programa Bicentenario de Ciencia y Tecnología and DICYT – Usach*.

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*Received June 12, 2007*