ARTICLES

Role of Hydroxyl Radicals and Singlet Oxygen in the Formation of Primary Radicals in Unsaturated Lipids: A Solid State Electron Paramagnetic Resonance Study

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Primary radicals were generated by UV photolysis of samples of trilinolein, at 77 K and under a controlled atmosphere. The resulting EPR spectra clearly show that the amount of radicals is dependent on the purity of the lipid, the exposure to visible light in the presence of a photosensitizer and oxygen, and, finally, the presence of an antioxidant. These solid state EPR experiments indicate that if all of the elements for the production of singlet oxygen (Rose Bengal, molecular oxygen, and visible light) are not present, primary radicals are practically not generated. They also point out the various steps of the oxidation mechanism: formation of singlet oxygen, which reacts with the lipid to form a hydroperoxide; and photolytic formation of the hydroxyl radical, which reacts with the frozen lipid to generate primary lipidic radicals. This constitutes a new method for investigating lipid oxidation and studying the influence of photosensitizers and molecules that are likely to react with singlet oxygen.

Keywords: *Radicals; EPR; lipids; hydroxyl radicals; singlet oxygen*

INTRODUCTION

The oxidation of fatty acids is one of the most fundamental reactions in food chemistry (Frankel, 1998). Volatile compounds formed during lipid oxidation cause rancidity, decreasing the quality of foods containing lipids. Rancidity is often the reason for short shelf life of foods. Rancidity in edible oils and fats is a serious problem for the food industry because of the increasing use of polyunsaturated vegetable and fish oils, the discontinuing of the use of synthetic antioxidants, and the fortifying of some foods with transition metal ions. In addition, products issued from lipid oxidation have been implicated in many vital biological reactions (McBrien and Slater, 1982). Considerable effort has, therefore, been made to control lipid oxidation, for example, by using natural antioxidants (Löliger et al., 1996). In this context, it is particularly important to understand the mechanism of lipid oxidation and to identify the reaction intermediates (Borseth and Melo, 1993).

The autoxidation of unsaturated fatty acids is a chain process occurring autocatalytically through free radical intermediates.

initiation

$$\mathrm{LipH} + \textit{initiator} \rightarrow \mathrm{Lip}^{\bullet} + \mathrm{H}^{\bullet}$$

propagation

$$\mathrm{Lip}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{LipOO}^{\bullet}$$

$$\mathrm{LipOO}^{\bullet} + \mathrm{LipH} \rightarrow \mathrm{LipOOH} + \mathrm{Lip}^{\bullet}$$

termination

$$\mathrm{Lip}^{\bullet} + \mathrm{Lip}^{\bullet} \rightarrow \mathrm{Lip\text{-}Lip}$$

Another important process in the oxidation of lipids proceeds through activated species of oxygen, for example, singlet oxygen. Singlet oxygen is an important reagent that has drawn much attention from organic and biological chemists. It can be produced, for example, from oxygen by photo-oxidation in the presence of a type II sensitizer.

$$^1\mathrm{Sens} \rightarrow {}^1\mathrm{Sens}^*$$

$$^1\mathrm{Sens}^* \xrightarrow{\text{intersystem crossing}} {}^3\mathrm{Sens}^*$$

$$^3\mathrm{Sens}^* + {}^3\mathrm{O_2} \rightarrow {}^1\mathrm{Sens}^* + {}^1\mathrm{O_2}$$

$$^1\mathrm{O_2} + \mathrm{Lip\text{-}H} \rightarrow \mathrm{Lip\text{-}OOH}$$

The revival of interest in the field of lipid oxidation in the past two decades can be largely attributed to the accumulating evidence that free radicals and reactive oxygen species participate in tissue injuries and degenerative diseases.

Radical intermediates in lipid oxidation are short-lived species that cannot be directly observed in a liquid

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phase by electron paramagnetic resonance (EPR) spectroscopy (Davies, 1987). They can, however, be directly detected in a frozen medium (Davies, 1987). In pioneering studies, Sevilla and co-workers (Zhu et al., 1990; Yanez et al., 1987; Sevilla et al., 1981) showed that low-temperature EPR spectra can be very informative as to the nature of radicals successively formed during the initiation, propagation, and termination phases of the lipid autoxidation process. Here, we use a similar approach to explore the generation of primary radicals involved in lipid oxidation and show how minor components, for example, photosensitizers (Rodgers and Powers, 1981), and carotenoids (Foote, 1976; Burton and Ingold, 1984; Frank et al., 1991) can affect the sensitivity of lipids toward oxygen.

MATERIALS AND METHODS

Materials. Trilinolein, β-carotene, Rose Bengal, and triphenylphosphine were obtained from Fluka (Buchs, Switzerland). Iatrobeads 6RS 8060 (porous fine silica gel) was obtained from Iatron Laboratories Inc. (Tokyo, Japan).

Treatment of Trilinolein with Triphenylphosphine. A mixture of 400 mg of trilinolein and 5 mg of Ph_3P in 6 mL of *n*-hexane was stirred at room temperature for 5 min. After removal of the solvent under vacuum, the lipid was passed through a cotton pad to remove Ph_3P.

Hydroperoxide Titration. Hydroperoxides were measured according to the colorimetric method based on the oxidation of ferrous to ferric ion and the determination of the latter as ferric thiocyanate (Rossell, 1994).

Sample Preparation. Samples were prepared in Suprasil tubes (Wilmad 726-PQ,) equipped with a homemade O-ring stopcock allowing the maintenance of a vacuum of 10^{-5} Torr.

General Procedure for Samples Devoid of Oxygen. *Procedure 1 (Nonchromatographed Lipid).* Trilinolein (0.08 g) was transferred to a sample tube inside a glovebox (nitrogen atmosphere). The sample was then very carefully degassed and the tube closed under 10^{-5} Torr.

Procedure 2a (Lipid Chromatographed on an Iatrobeads Column with Low-Polarity Eluant). A column was prepared, inside a glovebox, with 1.5 g of Iatrobeads in 10 mL of hexane; 0.2 mL of trilinolein was applied to the column and eluted with a hexane/ether mixture (95:5). The solvent was evaporated, for 3 h, on a vacuum line, and 0.08 g of the purified lipid was transferred— inside a glovebox—to the sample tube. After degassing, the tube was closed under 10^{-5} Torr.

Procedure 2b (Lipid Chromatographed on an Iatrobeads Column with High-Polarity Eluant). Trilinolein (0.2 mL) was chromatographed as in procedure 2a. A second elution was then performed using a hexane/ether mixture (50:50). The solvent was evaporated, and the lipid was transferred into the sample tube and degassed as in procedure 2a.

General Procedure for Samples Containing Dissolved Oxygen. *Procedure 3.* Inside a glovebox, 0.05 mL of oxygen was injected over a very short time ($\sim$3 s) into 0.045 g of degassed trilinolein (not treated on Iatrobeads) contained in a sample tube. The tube was immediately cooled to 77 K and closed under 10^{-5} Torr.

Procedure 4. This was the same as procedure 3, but with trilinolein purified on Iatrobeads.

Samples Containing Rose Bengal. *Procedure 5.* Degassed, chromatographed trilinolein (0.08 g; as in procedure 2a) was added—inside a glovebox—to 5.2 mg of Rose Bengal contained in a tube previously purged with nitrogen. The tube was then degassed and closed under 10^{-5} Torr.

Samples Irradiated with Visible Light in the Presence of Rose Bengal and Oxygen. *Procedure 6.* Degassed, chromatographed trilinolein (0.08 g) was transferred (as in procedure 2a)—inside a glovebox—into a sample tube containing 2 mg of degassed Rose Bengal. Then, under visible light irradiation, 20 mL of oxygen was passed through the mixture for 15 min. The sample was then degassed and closed under 10^{-5} Torr.

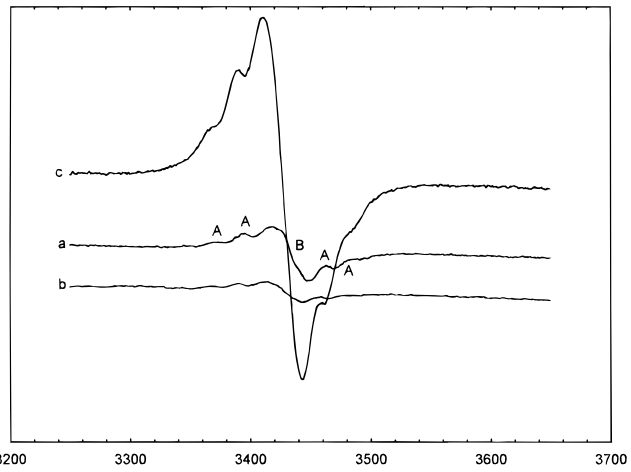

Figure 1. EPR spectrum obtained after UV photolysis at 77 K of (a) a degassed sample of trilinolein, (b) a degassed sample of trilinolein previously chromatographed on an Iatrobeads column, and (c) a degassed chromatographed sample of trilinolein that had been previously exposed to visible light in the presence of molecular oxygen and Rose Bengal.

Samples Irradiated with Visible Light in the Presence of Rose Bengal, Oxygen, and an Antioxidant. *Procedure 7.* A solution of β-carotene in benzene (8 mg/mL; 0.09 mL) was added to a sample tube containing Rose Bengal and trilinolein (as for procedure 6). The sample was then exposed to visible light irradiation in the presence of a flow of oxygen according to procedure 6. Benzene was evaporated on a vacuum line, and the sample was degassed and closed under 10^{-5} Torr.

Instrumentation. UV irradiation was carried out in situ in the EPR cavity at 77 K, generally for 44 min, with a high-pressure Hg lamp (OSRAM, 500 W). Exposure of the sample to visible light was performed outside the EPR cavity with an OSRAM lamp (100 W), the radiation of which was filtered through a water cell. Samples were degassed by using several freeze−thaw cycles on a vacuum line.

EPR spectra were recorded on a Varian E-9 spectrometer (100 kHz field modulation, insert Dewar with variable temperature attachment or finger Dewar for experiments at 77 K). The following EPR settings were used: modulation amplitude, 1 G; gain, 800; scan width, 400 G; time response, 1 s; scan time, 8 min; microwave power, 8 mW. The amplitude of the most intense central line of the spectrum was used for characterizing the abundance of trapped radicals. Particular care was taken to perform these measurements under reproducible conditions.

RESULTS

Nonchromatographed Trilinolein. EPR spectra recorded with a trilinolein sample that had not been chromatographed on Iatrobeads (procedures 1 and 3) are in total accordance with those previously reported by Sevilla and co-workers (Yanez et al., 1987). We will summarize them here: UV irradiation, at 77 K, of a carefully degassed sample of trilinolein (procedure 1) led to the EPR spectrum shown in Figure 1a as long as the temperature was kept below 110 K. This spectrum is composed of two sets of signals, which have been identified in previous studies (Yanez et al., 1987): (i) Several lateral lines, marked A, exhibit hyperfine splittings of $\sim$20 G with several protons; these lines have been attributed to radicals localized on the saturated part of the fatty acid chain ($R-CH_2^{\bullet}CHCH_2-R'$). (ii) A rather broad central line exhibits no clearly resolved

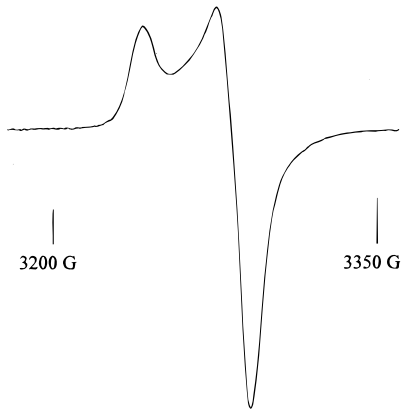

Figure 2. Frozen solution EPR spectrum of the LipOO• radical.

structure; this central signal results from the overlap of A components and of a signal, marked B, due to allylic and pentadienyl radicals. These two last species, delocalized on several carbon atoms, are formed by the homolytic scission of a C−H bond located in an α position to one or two double bonds (e.g., RCH═CH •CHCH═CHR′). Raising the temperature led to a modification of the lines in accordance with an increase in the mobility of the radicals. Only the delocalized radicals remained stable at 175 K. It is worthwhile noting that treating a trilinolein sample with triphenylphosphine provoked a drastic decrease (92%) of the intensity of its EPR spectrum obtained following procedure 1.

UV photolysis of trilinolein in the presence of traces of dissolved oxygen (procedure 3) resulted in observing, below 110 K, the above-described intense spectrum with A and B signals; however, heating the sample provoked a drastic modification of the sample: around 135 K signals A and B were replaced by the characteristic spectrum (Figure 2) of the peroxyl radical ROO• (axial **g** tensor whose anisotropy $g_{\|} - g_{\perp}$ is equal to 0.03). A further increase in temperature gave rise to signals corresponding to delocalized species (see above).

Trilinolein Chromatographed on Iatrobeads. Using the above-mentioned titration method, hydroperoxides were shown to be considerably reduced in trilinolein during Iatrobeads treatment, that is, from 18.4 mequiv of O_2/kg in commercial trilinolein to 0.3 mequiv of O_2/kg after chromatography on an Iatrobeads column.

The EPR spectrum obtained after UV irradiation of trilinolein chromatographed on Iatrobeads and containing no dissolved oxygen (procedure 2a) is shown in Figure 1b. It is clear that this treatment drastically reduced the formation of radicals. Subsequent increase of the temperature did not give rise to new signals. Carrying out this experiment in the presence of dissolved oxygen (procedure 4) did not increase the spectrum. Furthermore, the signals due to ROO• were not observed when the temperature was raised. On the contrary, intense EPR signals were recorded following UV irradiation at 77 K of a sample obtained by successive elution with ethanol/ether 95:5 and ethanol/ether 50:50 (procedure 2b). The ratio of EPR intensities obtained with a sample collected after the elution with ethanol/ether 50:50 to that obtained with a sample collected after elution with ethanol/ether 95:5 amounts to 10.

When a sample of trilinolein chromatographed on Iatrobeads (procedure 2a) was exposed at room temperature to visible light in the presence of molecular oxygen, it was not possible, after subsequent degassing and UV photolysis at 77 K, to generate an EPR spectrum having an intensity that exceeded 50% of that measured with nonchromatographed trilinolein (procedure 1). A considerable change was observed when Rose Bengal was added to the solution containing the lipid chromatographed on Iatrobeads (procedure 6): after visible irradiation in the presence of oxygen, degassing, and exposition to UV irradiation, a particularly intense spectrum was recorded (Figure 1c). In the absence of one of the three factors—visible radiation, Rose Bengal, or molecular oxygen—the intensity of the signal drastically decreased. Intensities of the EPR spectra recorded after 44 min of UV irradiation at 77 K are shown in Table 1. A decrease in intensity of >50% was observed when, in the presence of these three factors, β-carotene was added to the lipid (procedure 7). It was also checked that the intensity of the EPR spectrum obtained by UV photolysis of a nonchromatographed lipid (procedure 2a) was practically not affected by the presence of β-carotene in the sample.

Table 1. EPR Intensities Recorded after UV Irradiation at 77 K[a]

chromatography on Iatrobeads	treatment before UV photolysis	intensity[b] (mm)	relative intensities[c]
−	none	43 ± 5	2.5
+	none	17 ± 5	1
+	visible irradiation	18.5 ± 5	1.08
+	O_2 flow	12 ± 5	0.7
+	addition of RB[d]	16 ± 5	1.06
+	visible irradiation + addition of RB	22 ± 5	1.3
+	visible irradiation + O_2 flow	22.5 ± 5	1.3
+	O_2 flow + addition of RB	12 ± 5	0.7
+	visible irradiation + O_2 flow + addition of RB	255 ± 5	15

[a] UV photolysis during 44 min at 77 K. [b] Amplitude of the intense central line. [c] The value measured for the sample chromatographed on Iatrobeads, without further treatment, was used as reference (intensity = 1). [d] RB, Rose Bengal.

DISCUSSION

The experiments performed with a nonchromatographed lipid in the presence of oxygen confirm the previously proposed mechanism (Yanez et al., 1987): dissolved O_2 molecules are not sufficiently mobile at 77 K to attack the primary radicals produced by UV photolysis. This reaction becomes possible only at 135 K, as shown by the EPR detection of ROO• radicals at this temperature. As discussed below, the present investigations with a chromatographed lipid allow a better understanding of the mechanism of formation of primary radicals which are at the origin of the sensitivity of lipids toward oxygen.

Role of Hydroperoxides. Eluting a lipid deposed on an Iatrobeads column with ethanol/ether 95:5 was reported to remove oxidized species from the lipid (Prior and Löliger, 1994). Accordingly, we measured a very small amount of hydroperoxides in trilinolein chromatographed in such a way. Thus, the very low concentration of primary radicals observed with a chromatographed trilinolein indicates that the absence of hydroperoxides prevents the formation of primary radicals at 77 K. In the same way, a very small EPR signal is obtained with

trilinolein treated with triphenylphosphine, which is known to reduce hydroperoxides into alcohols (Rowley, 1979).

Eluting a lipid deposed on an Iatrobeads column using successively ethanol/ether 95:5 and ethanol/ether 50:50 is expected to yield a sample enriched in hydroperoxides. In accordance with the hypothesis that hydroperoxides are a prerequisite for the formation of photolytic radicals at 77 K, intense EPR signals are indeed recorded with a trilinolein chromatographed in this way (procedure 2b).

Role of Hydroxyl Radicals. As outlined above, the presence of hydroperoxides is necessary to generate primary radicals during photolysis at 77 K. The hypothesis for the formation of primary radicals is that UV photolysis of ROOH molecules generates hydroxyl radicals which are sufficiently reactive and mobile, even in the solid state at 77 K, to capture a hydrogen atom from the lipid.

$$\mathrm{Lip} + {}^1\mathrm{O}_2 \rightarrow \mathrm{LipOOH}$$

$$\mathrm{LipOOH} \xrightarrow{\mathrm{UV/77\ K}} \mathrm{LipO}^{\bullet} + {}^{\bullet}\mathrm{OH}$$

$$\mathrm{LipH} + {}^{\bullet}\mathrm{OH} \xrightarrow{77\ \mathrm{K}} \mathrm{Lip}^{\bullet} + \mathrm{H_2O}$$

Alkoxy radicals that are simultaneously formed during UV photolysis are much less mobile and reactive than hydroxyl radicals. For this reason alkoxy radicals probably do not contribute to reactions that generate primary radicals at 77 K. Besides, due to their large *g*-anisotropy, they are generally not detected in nonoriented systems (Symons, 1969, 1974). Alkoxy radicals, however, can form carbon-centered radicals throughout intramolecular reactions (Rota et al., 1997). These reactions were mainly described in solution. Although there are some indications related to intramolecular reactions of alkoxy radicals in the frozen state (Toriyama and Iwasaki, 1979), it cannot be inferred from the present results whether such rearrangements contribute to the formation of observed carbon-centered radicals at 77 K.

Peroxyl radicals can also be produced during UV irradiation of hydroperoxides, although in a smaller amount than hydroxyl and alkoxyl radicals due to the higher bond dissociation energy of RO_2-H with respect to RO−OH, that is, 90 and ∼44 kcal/mol, respectively (Kochi, 1973). However, EPR signals recorded after irradiation of trilinolein at 77 K do not reveal the presence of peroxyl radicals in the lipid medium. Moreover, as peroxyl radicals are less mobile and reactive than hydroxyl radicals, it can be assumed that they are not participating in the formation of primary radicals.

Role of Singlet Oxygen. Exposure of a chromatographed lipid to oxygen, even in the presence of visible light, produces only a very few primary radicals. Moreover, only a very low concentration of primary radicals is observed when a mixture of purified lipids and Rose Bengal is exposed to oxygen in the dark. In contrast to these results, intense signals (Figure 1c) are observed when all of the conditions required for the production of singlet oxygen are fulfilled: the presence of a photosensitizer (Rose Bengal), molecular oxygen, and visible light.

Singlet oxygen is known to react with lipids according to a concerted "ene" addition mechanism to produce hydroperoxides (Foote, 1976), whereas this reaction is forbidden for triplet oxygen (Rawls and van Santen, 1970). The intense EPR signals recorded in the presence of Rose Bengal, molecular oxygen, and visible light can therefore be attributed to a high concentration of hydroperoxides in the sample formed by reaction of the lipid with singlet oxygen. The fact that this increase in intensity was due to ROOH and not to additional oxidative reaction during irradiation with visible light in the presence of photosensitizer and oxygen was confirmed by recording the signal before and after UV irradiation: the very low (0.09) intensity ratio $I_{\mathrm{before-UV}}/I_{\mathrm{after-UV}}$ indicates that the radicals trapped at 77 K result from the homolytic scission of the RO−OH bonds.

The present interpretation of the EPR results is consistent with a previous paper (Chan, 1977) showing oxygen uptake and formation of hydroperoxides during photosensitized oxidation of unsaturated lipids.

Quenching Effect of *β*-Carotene. *β*-Carotene is very well-known as a potent quencher of singlet oxygen (Krinsky, 1989; Yanishlieva et al., 1998) and of excited sensitizers (Fujimori and Livingston, 1957). It is also reported to react with radicals involved in lipid oxidation (Yanishlieva et al., 1998). Accordingly, experiments carried out under conditions of photosensitized oxidation and showing an important reduction of EPR signal intensity in the presence of *β*-carotene can be interpreted by (i) *β*-carotene quenching singlet oxygen and/or its precursor, the excited triplet sensitizer, which impairs the formation of hydroperoxides and ultimately the generation of primary radicals during UV photolysis, and (ii) *β*-carotene scavenging the hydroxyl radicals at 77 K. The second mechanism is, however, rather unlikely as we have shown that under our experimental conditions *β*-carotene, immobilized at 77 K, does not react with OH radicals. As a matter of fact, EPR signals of similar intensities were recorded at 77 K during photolysis of hydroperoxide-containing lipids (nonchromatographed trilinolein) in the absence and in the presence of *β*-carotene.

Conclusion. A method based on EPR spectroscopy in the solid state was developed to follow the early stages of lipid oxidation initiated by hydroperoxides. Chemical damage produced by hydroxyl radicals formed from lipid hydroperoxides were evidenced by this method as well as the role that singlet oxygen, one of numerous initiators of lipid oxidation, is likely to play in this process.

The new method could be applied to investigate the influence of various parameters (oxygen, temperature, visible light) as well as photosensitizers (chlorophyll, riboflavin) and quenchers of singlet oxygen (carotenoids) on lipid oxidation.

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