Radicals Generated in Autoxidized Methyl Linoleate by Light Irradiation

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ABSTRACT

The structure of free radicals generated in the autoxidation of methyl linoleate (ML) was studied by the spin trapping technique using deuterated nitrosodurene, $(CD_3)_4C_6HNO$, as the spin trap. The secondary alkyl radicals were trapped after irradiation of ML with UV light. The formation rate of secondary alkyl radicals increased upon shortening the wavelength of irradiation light and was closely correlated with the peroxide value of autoxidized ML when a UV light longer than 250 nm was employed. When hydroperoxides separated from autoxidized ML were added to ML, the relationship between the formation rate of secondary alkyl radicals and the amounts of added hydroperoxides was nearly linear. These results suggest that secondary alkyl radicals are generated by proton abstraction of the active radicals, such as RO· and HO·, which are produced by the photolysis of hydroperoxides with UV light. The spin trapping technique can be applied to the study of lipid oxidation and/or photolysis of autoxidized lipid.

INTRODUCTION

The mechanisms and products of unsaturated lipid autoxidation have been studied extensively. At present, the autoxidation of lipids is generally regarded as a free radical reaction. The occurrence of transient free radicals such as ROO·, RO· and R· during the autoxidation of lipids has been postulated, but the evidence for the participation of such radicals are, for the most part, based on indirect observations, e.g., the consumption of oxygen, and the formation of peroxides and conjugated dienes. Direct detection of free radicals in oils by electron spin resonance spectroscopy (ESR) has also been attempted by several investigators, and the signals have been recorded for ozonized linolenic acid (1), UV irradiated unsaturated methyl esters (2) and triglycerides (3), and γ-ray irradiated triglycerides (4). However, all signals obtained in these experiments were broad without resolved hyperfine splittings, so no information about the structures of the radical species was given.

The introduction of the spin trapping technique with ESR has offered a possible direct technique that may provide the evidence necessary to confirm the involvement of these labile intermediates. The technique has been applied very successfully to studies on free radical mechanisms in various chemical and photochemical reactions (5–10), and recently, also to the study biological free radical systems (11–20). This technique involves the capture of transient free radicals with spin traps, resulting in the formation of persistent nitroxide radicals (spin adducts) which can be easily identified by ESR. Two types of compounds, nitrones and nitroso compounds, are most commonly utilized as spin traps. The formation of spin adducts can be represented by the following reactions:

$$R\cdot + R'\text{-}N{=}O \longrightarrow RR'\text{-}N\text{-}O\cdot$$

$$R\cdot + R'\text{-}CH{=}N^{+}(O^{-})\text{-}R'' \longrightarrow R'\text{-}CH(R)\text{-}N(O\cdot)\text{-}R''$$

where R′-N=O and R′-CH=N^+ (O^-)-R″ represent nitroso compounds and nitrones, respectively. The trapped radicals in these reactions usually give discernable hyperfine ESR spectral patterns that permit identification of the initial radicals trapped.

We examined the spin trapping technique to investigate autoxidation of methyl linoleate (ML) and detected the adducts of secondary alkyl radicals with the spin trap, deuterated nitrosodurene. Since light causes the generation of various radicals, a UV source for the irradiation was adopted to generate transient radicals. The results, including some conditions appropriate to the spin trapping experiments, are reported here.

EXPERIMENTAL PROCEDURES

Materials

As the substrate, ML was prepared from the methyl ester of safflower oil by urea-adduct formation technique. Trace amounts of impurities, such as tocopherols and peroxides, were eliminated by silicic acid column chromatography and vacuum distillation just before experiments. The purity of ML, checked by gas liquid chromatography (GLC), was 99.5%.

ML hydroperoxides were prepared from autoxidized ML by bubbling dry air at 40 C and purified by silicic acid dry column chromatography (21). The purity of ML hydroperoxides was confirmed by thin layer chromatography (TLC) (plate: Wakogel B-5, solvent system: hexane/diethyl ether, 7:3) and POV (AOCS official method, found: 6.19×10^3 meq/kg, theoretical: 6.14×10^3 meq/kg).

Spin Traps

Phenyl N-tert-butylnitrone (Eastman Kodak Co.) was used as received. Deuterated nitrosodurene, 2,3,5,6-tetrakis (trideuteriomethyl) nitrosobenzene, was synthesized according to the method described by Terabe et al. (22)

ESR Measurements

A branched pyrex tube attached to a cylindrical quartz cell (Fig. 1) was designed for ESR measurement. One ml of a spin trap solution in benzene was injected into the arm (B in Fig. 1) of the tube, and the sample ester was poured into the main chamber (A in Fig. 1). After degassing both the sample ester and the spin trap solution by the freeze-pump-thaw technique using a high-vacuum system, the tube was sealed and both liquids were mixed thoroughly at room temperature. Then the mixed solution was moved to the quartz cell (id 4 mm) and the ESR spectrum was immediately recorded at room temperature on a Varian E-4 spectrometer with 100 kHz magnetic field modulation.

Irradiation of ML

For irradiation of ML, a 500-W xenon lamp equipped with various filters (Toshiba glass filters) was used. The filters, VY-42, UV-35, UV-31, UV-27, UV-25, completely cut off

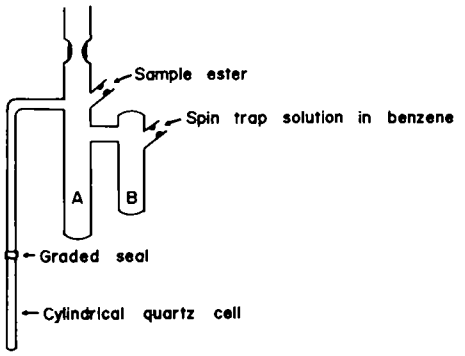

FIG. 1. A branched pyrex reaction tube.

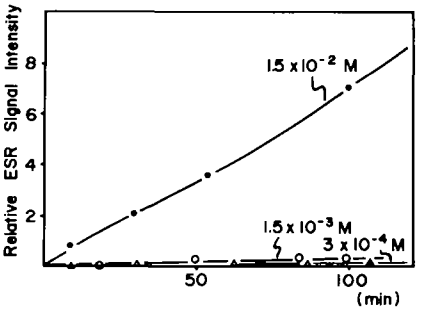

FIG. 3. Increase of the ESR signal intensity with time at 3 DND concentrations at room temperature. The intensities were measured in the dark without light irradiation.

the light shorter than 400, 330, 290, 250, and 230 nm, respectively. A solution was irradiated in situ in the cavity of the spectrometer.

Peroxide Value

Peroxide value was measured colorimetrically by the method of Takagi et al. (23).

RESULTS AND DISCUSSION

ESR Spectrum of the Spin Adduct

A typical ESR spectrum of the spin adduct recorded after irradiating the solution of ML containing deuterated nitrosodurene (DND) is shown in Figure 2. Hyperfine structure of the spectrum is a doublet (1:1) of triplets (1:1:1), indicating that the spin interacts strongly with one nitrogen atom and one hydrogen atom. Thus, the trapped radicals are obviously secondary alkyl radicals, R_1–$\dot{C}$H–R_2. The splitting constants, a_N=13.8G and a_H=6.0 G, and the g-value, 2.0062, are in agreement with the values reported for the spin adducts of several alkyl radicals (22).

The solution of ML and DND in benzene originally showed no ESR signal in the dark. Therefore, the trapped radicals were generated photochemically. The use of another spin trap, phenyl N-tert-butylnitrone (PBN), did not exhibit any signal due to the spin adduct after irradiation. Since the rate constant of spin trapping of DND (2.0×10^8 $mol^{-1}dm^3$ s^{-1}) is more than 10^4 times larger than that of PBN (1.0×10^4 $mol^{-1}dm^3s^{-1}$) (24), the difference of the spin trapping abilities between two spin traps, DND and PBN, seems to be mainly attributable to the rate constants. Thus, PBN was inadequate for the irradiated ML system under study. Consequently, DND was used as the spin trap in subsequent experiments.

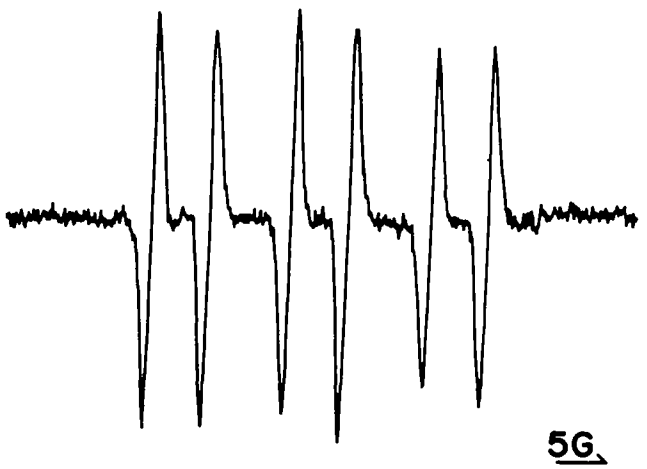

FIG. 2. ESR spectrum of the spin adduct generated from the secondary alkyl radical and DND. Hyperfine splitting constants: a_N= 13.8 G, a_H= 6.0 G.

Conditions Favorable to Trap Radicals Generated in the Irradiated ML

In order to find out the concentration of DND in ML that is suitable for trapping radicals, the effect of the concentration of DND on radical formation was examined. The results are illustrated in Figure 3. In high concentrations of DND, such as 1.5×10^{-2} M, the marked increase in signal intensity with time was observed at room temperature without irradiation. This phenomenon implies the acceleration of radical production by DND at high concentrations. At concentrations lower than 1.5×10^{-2} M, however, the increase of the intensity was very small in the dark and was ignored in the concentrations for measurements. Therefore, DND usually was added at a concentration of 1.5×10^{-3} M in subsequent experiments.

Light Specific to Radical Generation

To clarify the wavelength specificity to radical formation, the degassed solutions containing ML and DND in benzene were irradiated with the light from a 500-W xenon lamp through some filters which cut off the light shorter than the wavelength indicated. The changes of signal intensities as a function of irradiation time are shown in Figure 4. The figure clearly demonstrates that the light shorter than 400 nm gives rise to radical generation and that, apparently, the shorter the wavelengths, the more radicals produced.

Dependence of ESR Signal Intensity on POV

It can be readily assumed from our results that the radical generation by light irradiation includes species having absorption shorter than 400 nm. The species would be closely related to the degree of autoxidation of ML. The following experiments have been done to determine the relationships between the signal intensity and the autoxidation.

The autoxidation of ML was promoted by irradiation with a 15-W fluorescent lamp at 25 C ± 1 in a thermally controlled incubator.

No ESR signal was observed by only mixing the autoxidized ML with the solution of DND in benzene in the dark. Irradiation to the mixed solution with light longer than 250 nm caused the appearence of the signal due to spin adducts. An increase in the signal with irradiation time was observed for some autoxidized solutions. For each sample, the signal intensity increased linearly within a short irradiation time

(5 min) and the slopes of the lines are indicative of the degree of autoxidation. The relationship between POV of ML and its signal intensity after irradiation for 5 minutes is illustrated in Figure 5. This good correlation led to the suggestion that peroxides, presumably hydroperoxides, which are the main oxidation products of unsaturated fatty acids in the early stage, in ML play an important role in generation of the alkyl radicals. Trapping experiments were then carried out for ML, to which a small amount of hydroperoxide was added.

Effect of Addition of Hydroperoxides on ESR Signal Intensity

Spin trapping with DND was done with the ML solutions containing known amounts of methyl linoleate hydroperoxides. The spectra were measured after irradiation with light longer than 250 nm. Each ML with added hydroperoxides also showed an increase in signal intensity with time of irradiation and the increasing rate was closely related to the amount of added hydroperoxides. The relationship between the amount of added hydroperoxides and signal intensity after irradiation for 5 min is illustrated in Figure 6. Showing a nearly linear plot, these results strongly imply that the decomposition of hydroperoxides by light leads to the formation of secondary alkyl radicals. It is generally supported by evidence from O-O bond energy considerations and product analysis that the photocatalytic decomposition of hydroperoxides results in the formation of the radicals such as RO· and HO· (25).

$$ROOH \longrightarrow RO\cdot + HO\cdot \qquad [I]$$

After photolysis (I) of hydroperoxides, the following chain reactions can be assumed when DND is present in the reaction mixture:

$$RO\cdot + RH \longrightarrow ROH + R\cdot \qquad [II]$$

$$HO\cdot + RH \longrightarrow H_2O + R\cdot \qquad [III]$$

$$R\cdot + DND \longrightarrow R\text{-}DND\cdot \qquad [IV]$$

$$RO\cdot + DND \longrightarrow RO\text{-}DND\cdot \qquad [V]$$

$$HO\cdot + DND \longrightarrow HO\text{-}DND\cdot \qquad [VI]$$

However, the radicals which could be trapped with DND were only alkyl radicals derived from Equations II - IV. The absence of the spin adduct signals of RO· and HO· might be due to relatively small rate constants of the spin trapping Equations V, VI compared with the proton abstracting reactions (II, III) which had not yet been confirmed and with the instability of these spin adducts (20).

Methyl linoleate without any addition of hydroperoxides also gave signals (Fig. 6). Purified by silicic acid column chromatography, followed by vacuum distillation just before the experiments, the substrate ML gave a small POV (0.5 meq/kg) and it was impossible to get the ML completely free from peroxides. Therefore, the trace amount of hydroperoxides present in the substrate ML might cause the formation of spin adduct. However, if the signal intensity of the ML that is free from hydroperoxides is estimated by extrapolation to zero POV in Figure 6, the intensity does not fall to zero but remains at a considerable value, ca. 96% of the value of the ML (POV = 0.5 meq/kg). This result implies the participation of some mechanisms other than the decomposition of hydroperoxides in the radical formation in ML by UV irradiation.

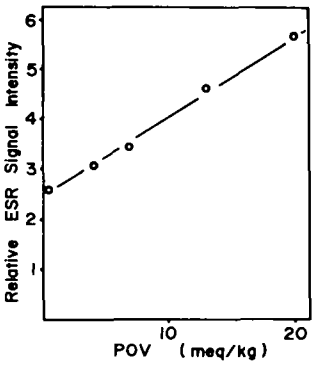

FIG. 4. Change of ESR signal intensity of spin adducts with the time of irradiation using 4 filters, VY-42, UV-35, UV-31 and UV-25.

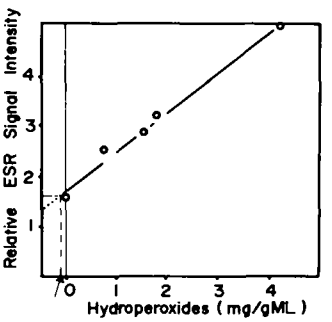

FIG. 5. Relationship between POV and signal intensity after 5 min irradiation.

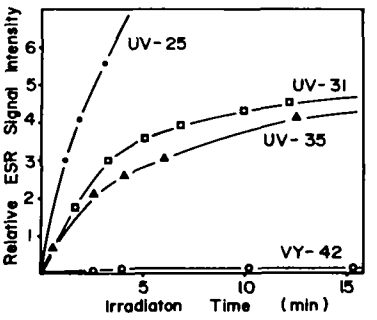

FIG. 6. Relationship between ESR signal intensity and amounts of added hydroperoxides to ML. ESR signal was measured after irradiation with the light longer than 250 nm for 5 min. Arrow shows position of zero POV.

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⁂Analysis of Lipid Classes and Lipofuscin Substances by High Performance Liquid Chromatography

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ABSTRACT

The fractionation and analysis of the lipid classes and fluorescent substances of animal tissues by high performance liquid chromatography (HPLC) using a combination of fluorescence and flame ionization detectors is described. The lipid classes and fluorescent substances are extracted from rat kidney and liver tissue by a new method that involves preextraction of nonlipid and aqueous-soluble fluorescent substances with hot dilute (0.05 N) acetic acid. The lipid classes and organic-soluble fluorescent substances are extracted from the residual tissue in three extractions: the first with chloroform/methanol, 1:1, v/v; the second with chloroform/methanol, 1:2, v/v; and the third with methanol. The fractionation of these compounds by HPLC is carried out with a column 0.2 × 45 cm, packed with a special adsorbent prepared by reacting silicic acid with ammonium hydroxide. The eluent is passed through a fluorescence detector, which provides a profile of the fluorescent compounds, and then to a flame ionization detector for analysis of the lipid classes. The method is demonstrated on rat blood serum, liver and kidney tissue.

INTRODUCTION

The lipid classes are generally analyzed by thin layer chromatography (TLC), either directly by charring and photodensitometry (1-5) or by application of conventional analytical methods to compounds recovered from chromatoplates (6-9). The quantification of lipofuscin substances is based on spectrophotofluorometric analysis applied directly to tissue extracts (10), after fractionation by silicic acid through column chromatography (11), Sephadex chromatography (12) or TLC (13). Although these methods are used widely for the analysis of lipofuscin substances and lipid classes, the fact that they are applied in many ramifications atests to the need for simple, more precise procedures. In this paper we report the application of HPLC to the analysis of lipid classes and fluorescent substances in animal tissues simultaneously by a combination of fluorescence and flame ionization detectors.

EXPERIMENTAL

Standards

Retinol and retinyl palmitate, which was used as a standard for retinyl esters, were purchased from Sigma Chemical Company, St. Louis, MO. The lipid class standards used in this study were available in our laboratory from the Lipid Preparation Project, in which we were active. The identity and purity of each preparation was monitored by TLC. Neutral lipids used as standards were cholesterol (CH), cholesteryl palmitate (CE), tripalmitin (TG), dipalmitin (DG), and palmitic acid (FFA). Phospholipid standards included rat liver phosphatidylcholine (PC), lysophosphatidylcholine (LPC), beef brain phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and phosphatidylserine (PS), egg sphingomyelin (SPH), soybean phosphatidylinositol (PI), and beef kidney diphosphatidylglycerol (DPG).

Solvents

Solvents used in the extraction of the tissues as well as for liquid chromatography, including the regeneration of the columns, must be rigorously purified to remove substances that interfere in the analyses of both the fluorescent substances and lipid classes. Purification is performed by passing each solvent; i.e., Skellysolve B, methylene chloride, chloroform and methanol through individual columns of silicic acid followed by distillation in an all-glass still.

Tissues

Blood serum, liver and kidney tissue used as specimens in this work were obtained from male Sprague-Dawley rats fed a rat chow diet. The animals were killed by exsanguination by withdrawal of the blood from the retroocular plexes; the kidneys and livers were excised and used immediately.

Tissue Extraction

A new extraction procedure that we developed for brain (14) and plant tissue (15) was modified for extraction of the lipid classes and fluorescent substances from liver and kidney tissues. Freshly excised tissue (0.5 g) is placed in a 15-ml centrifuge tube containing 5 ml of hot dilute (0.05 N) acetic acid in a boiling water bath (ca. 95 C) for 30 min. After the heat treatment, the tissues are homogenized for 1 min with a Tekmar tissuemizer, Model SDT