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Hydroperoxide characterisation as a signature of the micelle/monomer balance in radiation-induced peroxidation of arachidonate

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Abstract

Archidonate peroxidation has been studied using $HO^{\cdot}$ radicals radiolytically generated as initiators of this process. Irradiated aqueous solutions of arachidonate (between 0.01 and 25 mM at pH 10.5) have been characterised by means of conjugated dienes measurement (234 nm-absorption spectroscopy) and hydroperoxide detection (high-performance liquid chromatography coupled with a chemiluminescence detection). Radiation-induced peroxidation of arachidonate gives a different trend of peroxide products, depending on the degree of substrate interaction; endoperoxide and hydro-endoperoxide being favored at low concentrations (monomer/oligomer) and monohydroperoxide at high concentrations (micellar form).The experimental ratios $G_{(Hydro2)}/G_{(Hydro1)}$ increase significantly only for arachidonate concentrations higher than 1 mM, i.e. in micellar medium. However, between 0.1 and 1 mM in arachidonate, *G*-values (for conjugated dienes, Hydro2 and Hydro1) remain nearly constant, meaning that the physical arrangement of the solution changes: Aggregation occurs. The experimental yields of conjugated dienes formation indicated that $G_{Dienes} > G_{HO}$ for [arachidonate] > 2.5 mM, indicating that a chain propagation process had occurred. Radiolytic yields and structural identification (HPLC-MS analysis) of peroxidation products allowed us to propose a mechanism for the formation of both hydroperoxides.

Keywords: *Hydroperoxides, hydroxyl free radicals, peroxidation, radiolysis, arachidonate monomers, arachidonate micelles*

Introduction

Aerobic systems can be submitted to oxidative stress because of an imbalance between antioxidant and pro-oxidant mechanisms, which may lead to oxidative damages involving lipids, proteins and DNA [1]. A central feature of oxidant injury is lipid peroxidation. It is well known that lipid peroxidation is involved in certain pathologies (cardiovascular and neurodegenerative diseases, …) and ageing [2,3].

In vivo, arachidonate serves as the precursor of the prostaglandins, prostacyclins, thromboxanes and leukotrienes [4,5,6], potent intercellular mediators that control a variety of complex processes. Previous works [7] primarily elucidated that auto-oxidation of arachidonic acid *in vitro* results in the formation of bicyclic endoperoxide prostaglandin-like compounds. It is now well established that non-enzymatic peroxidation of arachidonic acid by free radicals and reactive oxygen species, produce an endoperoxide intermediate leading to isoprostane species [8].

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Isoprostanes are currently useful markers of lipid peroxidation and diseases (ischemia-reperfusion syndromes, Alzheimer's disease, ...) [9].

Since lipid molecules are amphiphilic, at a sufficient concentration called critical micellar concentration (cmc) they tend to aggregate, giving structured molecular aggregates, named micelles [10]. The most accepted model for micelle microstructure [11] consists of an apolar core containing the hydrophobic chains surrounded by a polar shell including hydrophilic headgroups and some solvent molecules. This phenomenon creates regions containing high local concentrations of unsaturated moieties and presenting a microenvironment different from the aqueous phase. Nevertheless, even when slightly soluble in the form of sodium salts, fatty acids arrange themselves into oligomers. These small aggregates can then be observed in solution, even for fatty acid concentrations lower than the cmc [10].

Such molecular aggregates (micelles) have a valuable advantage. They make it possible to modelise, *in vitro*, using radiolysis, biomembranes and lipoproteins [12,13,14,15]. While they are structurally less complex than the dual chain molecules generally encountered in studies of biological phospholipids, fatty acids provide reasonable model compounds susceptible to undergo processes associated with lipid degradation. Several model studies have demonstrated the decisive influence of certain parameters like the dose rate [13,16], the concentration in lipids [17,18,19], the fatty acid nature [16], the number of double bonds [20], the ionic strength of the medium [17] and the pH [18]. Steady-state study of radiation-induced peroxidation of linoleic acid in aqueous homogeneous medium as well as in micelles have been reported by a number of worker. Sodium fatty acid micelles have been studied [21,22]. Aside from providing characterisation of some parameters that govern the chain process and establishing the effects of aggregation on peroxidation degradation, it has been shown that in these systems $HO^{\cdot}$ radical (but not $O_2^{\cdot -}$ anion [23]) is responsible for initiation of one-electron oxidation process in the hydrocarbon chain. Such investigations demonstrate that the reaction rates, which determine the overall direction of radical reactivity, can be strongly affected by the nature of lipid aggregation. Previous studies [24,25] have shown that a lower rate constant of $HO^{\cdot}$ with linoleic acid was observed upon micelle formation. It was suggested that the clustering of monomers with relatively unreactive headgroups at the micelle surface presents a barrier, which slows down the interactions between $HO^{\cdot}$ and fatty acid sites for H-atom abstraction.

In this paper, using irradiated arachidonate solutions as a lipid model, several peroxidation indexes have been measured. In addition, the effect of arachidonate concentration (in terms of molecular aggregation, i.e. from monomers to micelles) on the yield of peroxidation as well as on the nature of oxidation products are presented. HPLC-MS analysis allowed us to identify the molecular structure of several oxidation products. Finally, the reactivity of hydroxyl radicals towards arachidonate is discussed in terms of generated products and mechanisms of hydroperoxide formation.

Materials and methods

Chemicals

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), purchased from Sigma (St. Louis, Mo.), was stored under argon atmosphere in order to limit autoxidation. Since peroxidation levels were negligible, arachidonic acid was used without further purification. By taking account of the slow spontaneous autoxidation of arachidonic acid, only freshly made solutions were prepared. Arachidonic acid, at concentrations ranging between 0.01 and 25 mM, was dissolved in water (Maxima Ultra-pure water, ELGA, High Wycombe, U.K.; resistivity: 18.2 MΩ) and the pH was adjusted to 10.5 with sodium hydroxide in order to deprotonate quantitatively the carboxylic groups and to form sodium arachidonate. The standard hydroperoxide 15-(S)-HPETE (hydroperoxieicosa-5Z,8Z,11Z,13E-tetraenoic acid) was purchased from Biomol (Plymouth Meeting, Penn.).

Irradiations

Arachidonate solutions were irradiated under air at 25°C with increasing doses up to 392 Gy with an IBL 637 irradiator (CIS Bio international, Gif-sur-Yvette, France) composed of ^{137}Cs γ-ray sources (activity 222 TBq). The γ-ray dose rate was 9.8 Gy min^{-1}. The dosimetry was done according to the Fricke method [26,27]. Irradiations were carried out in tubes that were cleaned using TFD4 soap (Franklab SA, St.-Quentin-en-Yvelines, France), then rinsed thoroughly. All vessels used for irradiation were heated at 400°C for 4 h after washing, in order to remove all the remaining organic pollutants.

Within the nanosecond time scale, γ-irradiation of water leads to the formation of three radical moieties: $HO^{\cdot}$, $H^{\cdot}$ and hydrated electrons e_{aq}^{-}. However, in our experimental conditions (at pH 10.5 in the presence of oxygen), only two radical species are quantitatively formed: $HO^{\cdot}$ and $O_2^{\cdot -}$ with the radiolytic yields (*G*-values expressed in moles per Joule) of 0.28 and 0.34 μmol J^{-1}, respectively, [27]. Although both are oxyradicals, their thermodynamic and kinetic properties are very different. In our experimental conditions (absence of any metallic species), indirect oxidizing properties of $O_2^{\cdot -}$ (Haber-Weiss reaction leading to $HO^{\cdot}$ generation) cannot be observed. In addition,

at pH 10.5, perhydroxyl radical ($HO_2^{\cdot}$, pKa ($HO_2^{\cdot}/O_2^{\cdot -}$) = 4.8 [28]) and the basic form of $HO^{\cdot}$ ($O^{\cdot -}$, pKa($HO^{\cdot}/O^{\cdot -}$) = 11.9 [29]) are negligible compared to $HO^{\cdot}$ species. Hence, it can be assumed that, in our experimental conditions, the only initiating species are hydroxyl radicals.

Analysis

For estimation of conjugated dienes, the UV-visible absorption spectra were recorded by means of a Beckman DU 70 spectrophotometer. The difference between the absorbance at 234 nm [30] of the peroxidised fatty acid and that of the corresponding non-peroxidised lipid was taken as a measure of diene conjugation. A molar extinction coefficient of 28,000 $M^{-1}cm^{-1}$ was used for calculation of conjugated dienes concentration [30].

Hydroperoxides were detected by High Performance Liquid Chromatography in reversed-phase associated with a detection by chemiluminescence [31]. The injection and separation procedures used in this work have already been described [32]. Briefly, 100 µl of each irradiated sample was taken and dissolved into 400 µl of methanol. About 25 µl of this solution was injected (isocratic pump with a flow rate of 1.5 ml.min^{-1} from Spectra-Physics SA, Darmstadt, Germany). Separation of the hydroperoxides was accomplished on a C18 reversed-phase column (Kromasil, Eka Chemicals, Sweden; 250 ×4.6 mm I.D., 5 µm) associated with a C8 reversed-phase column (Kromasil Eka Chemicals, Sweden; 150 × 4.6 mm I.D., 5 µm) with methanol (94%) and ammonium acetate (6%, 10 mM at pH 5) as eluent. The column was operated at 40° C and 1600 p.s.i. (11 MPa) pressure. Hydroperoxides were detected by chemiluminescence: In a post-column reaction, hydroperoxides react with microperoxidase (Sigma, St. Louis, Mo.) (10 mgl^{-1}) and isoluminol (Sigma, St. Louis, Mo.) (55 mgl^{-1}) dissolved in borate buffer (0.1 moll^{-1} at pH 9.2) [31]. The eluent was passed through a fluorimeter (spectroflow 980, Applied Biosystems, Inc., Foster City, Calif.) used as a photon detector with the excitation source turned off. Hydroperoxide concentrations were deduced from calibration curves developed with authentic 15-(S)-HPETE. The standard is eluted with a retention time of 3.4 min. Unirradiated arachidonic acid samples contained small quantities of hydroperoxides and the concentrations corresponding to the radiation-induced oxidation were corrected for this. Unchanged arachidonate was eluted with a retention time of 5.1 min and analysed by a UV-visible spectrophotometric detector (Pharmacia, Uppsala, Sweden) operating at 205 nm. However, under our experimental conditions, this quantification was only possible between 0.01 and 1 mM, where the relative quantity of arachidonate, that had disappeared during the irradiation, was significantly measurable.

In addition to HPLC/Chemiluminescence identification of hydroperoxide, samples have been analysed by HPLC/mass spectrometry, in order to determine the masses of every oxidation products. Samples were separated on a C18 reversed-phase column (Supelco Discovery HS-C18, 150 × 2,1 mm I.D., 5 µm), with the following gradient (total time: 25 min): From 20% methanol / 80% ammonium acetate (10 mM in water) to 100% methanol in 8 min, then hold at 100% for 5 min, go down at 20% methanol / 80% ammonium acetate in 3 min, and finally hold during 9 min. The flow rate was 250 µl min^{-1} and 50 µl of each sample was injected. Products were first analysed by a photodiode array detector (Surveyor, Thermo Finnigan, Les Ulis, France), scanning from 200 to 400 nm, and immediately sent to the electrospray ionisation source of the ion trap mass spectrometer (LCQ Advantage, ThermoFinnigan, Les Ulis, France), working in negative mode. The apparatus was tuned with a solution of non-oxidised arachidonate (1 mM in water, pH 10.5, diluted by 5 in methanol) by optimising the intensity of the parent ion at m/z 303.5 ($[M-H]^-$). Parameters were set as following: Spray voltage = 4.5 kV, capillary temperature = 250°C, relative sheath gas flow = 20 and relative auxiliary gas flow = 5 (units as given by the manufacturer). The mass spectrometer was used in both full MS mode, by scanning between 50 and 500 Da.

Results

Hydroperoxides

Hydroperoxides formed by radiation-induced peroxidation were detected by HPLC associated with chemiluminescence detection. Whatever the initial arachidonate concentration (0.01–25 mM) and for all the radiation doses (up to 392 Gy), the chromatograms (Figure 1) indicate the presence of two peaks corresponding to two different kinds of hydroperoxides. The first hydroperoxide peak (named Hydro1) was observed at a retention time of 2.9 min while the second one (named Hydro2) has the same retention time (3.4 min) as the standard 15-(S)-HPETE, used for calibration. As shown in Figure 1, both peak areas increase with the radiation dose. However, the ratio [Hydro2]/[Hydro1] is clearly linked to the fatty acid concentration. Indeed, Hydro1 is predominant below 2.5 mM, while Hydro2 is predominant above. At 2.5 mM in arachidonate, both peaks have almost the same area, meaning that [Hydro1] ≈ [Hydro2]. The presence of both types of hydroperoxides in the same HPLC conditions has already been observed in the case of

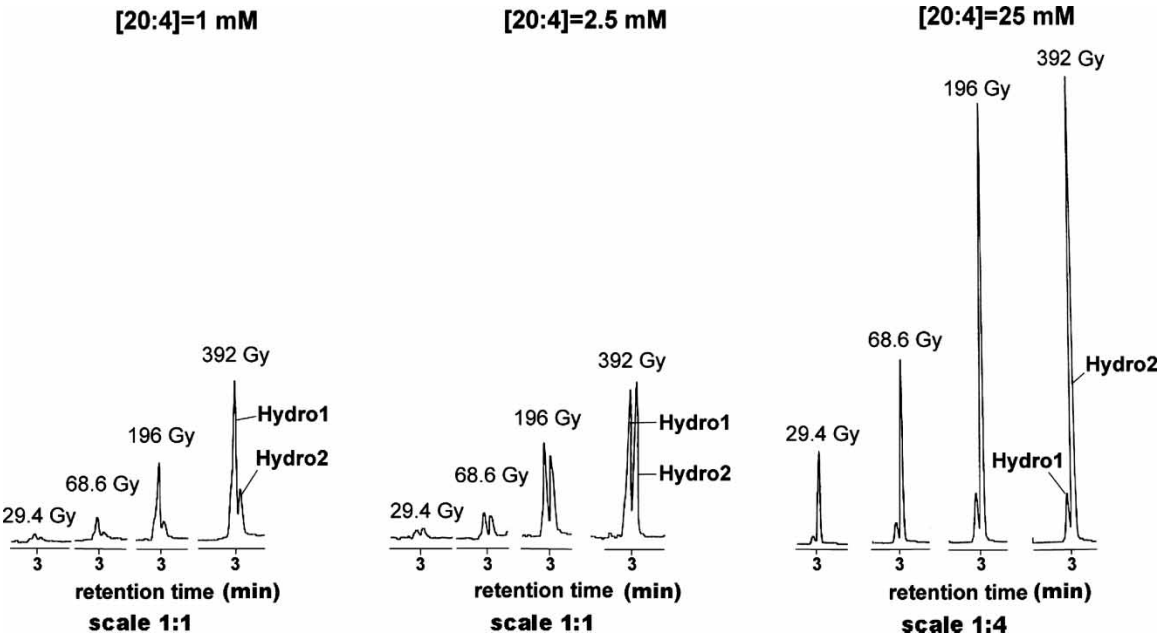


Figure 1. Reverse-phase HPLC chromatograms recorded by chemiluminescence for three different concentrations of aerated aqueous solutions of arachidonate (1, 2.5 and 25 mM, pH 10.5) irradiated at four radiation doses (29.4, 68.6, 196 and 392 Gy, dose rate $I = 9.8\,\mathrm{Gy\,min^{-1}}$). Calibration was done using 15-(S)-HPETE as a standard. For drawing facilities, chromatograms at 25 mM in arachidonate are represented under a minimised scale (1:4)

other fatty acids, namely linoleic acid [32] and γ-linolenic acid [33].

Characterisation of the oxidation products

Remarkably, whatever the initial arachidonate concentration and the radiation dose, the total concentration of hydroperoxides (Hydro1 in addition to Hydro2) is lower than that of conjugated dienes (data not shown). This observation implies the existence of dienic compounds other than hydroperoxidic ones.

Aqueous solutions of arachidonate (1 mM) have been irradiated at 400 Gy and analysed by high performance liquid chromatography / mass spectrometry (HPLC/MS) for characterisation of the oxidation end-products. Figure 2 shows the trace chromatograms for every detected ions. The first trace, at m/z 303.5, corresponds to the parent ion of arachidonate ($[M\text{-}H]^-$). Then, oxidation products resulting from formal addition of oxygen atoms (from 1 to 5) to arachidonate are presented at m/z 319.5, 335.5, 351.5, 367.5 and 383.5 Da. The other traces are those of various oxidation products detected by HPLC/MS (m/z 296.5, 352.5, 353.5, 361.5 and 369.5). For some traces, several peaks are detected, indicating the occurrence of various isomers: In this case, a mean retention time was taken into account.

Table I summarises the parent ion of the oxidation products of arachidonate detected by HPLC/MS, along with the wavelength of maximal absorption for every peak and the corresponding shifts in mass related to the mass of arachidonate parent ion.

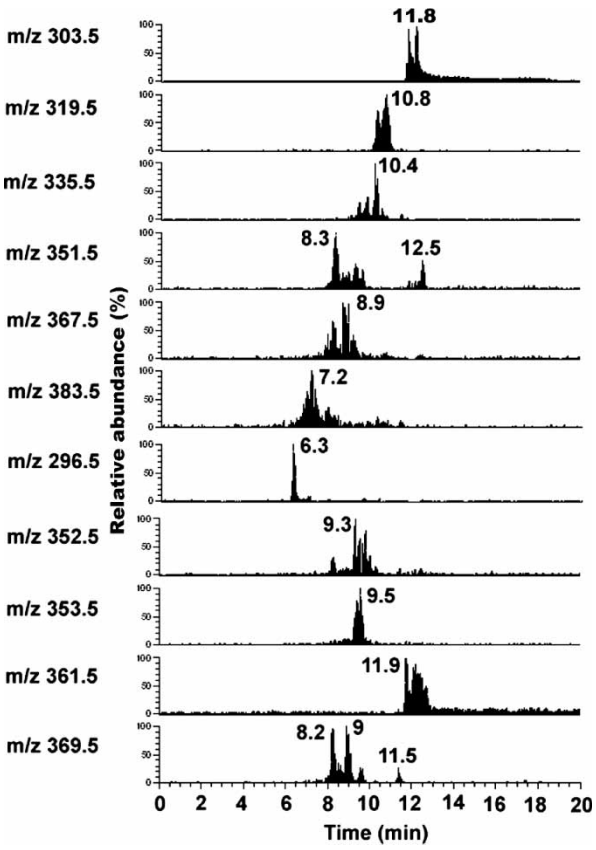


Figure 2. Trace chromatograms of an aqueous solution of arachidonate (1 mM, pH 10.5), irradiated at 400 Gy (dose rate $I = 9.8\,\mathrm{Gy\,min^{-1}}$), detected in full MS mode from 50 to 500 Da, after HPLC separation and negative electrospray ionisation. The arachidonate parent ion $[M\text{-}H]^-$ is detected at m/z 303.5; *x*-axis: Time; *y*-axis: Relative intensity for each trace.

Table I. Parent ions of the oxidation products of arachidonate identified by HPLC/MS; retention times are those found in Figure 2. A peak assignment is presented, along with UV/visible data for each peak and the corresponding shift in mass related to the mass of the arachidonate parent ion.

Parent ion (Da)	Rt[a] (min)	Peak Assignment	UV[b] (nm)	Shifts in mass (Da)
303.5	11.8	Arachidonate*	210–220	0
319.5	10.8	–	230–240	+16
335.5	10.2	Hydro 2	230–240	+ (2 × 16)
351.5	8.3/12.5	–	220–230 / ∅	+ (3 × 16)
367.5	8.9	Hydro 1	230–240	+ (4 × 16)
383.5	7.2	–	∅	+ (5 × 16)
296.5	6.4	–	220–240	+ (4 × 16) − 71
352.5	9.3	–	230–250	+49
353.5	9.5	–	230–250	+16 + (2 × 17)
361.5	11.9	–	∅	+58
369.5	8.2 / 9.0 / 11.5	–	220–230 / 220–250 / 270–300	+ (2 × 16) + (2 × 17)

[a] Reversed-Phase-HPLC retention time using column and mobile phase as in Figure 2. When several peaks are detected at a single mass/charge ratio, a mean retention time is considered.[b] UV maximum absorption; ∅ = no absorption. *non-oxidised product.

In the first part of Table I (top) are presented oxidation products whose masses correspond to a formal addition of oxygen atoms to arachidonate during the oxidation (from 1 to 5 oxygen atoms). Hydro2 was previously found to have the same retention time than 15-(S)-HPETE (Figure 1) and is then a monohydroperoxidic compound: It is detected at m/z 335.5 ($[M + 32\text{-}H]^-$) by HPLC/MS. To characterise specifically Hydro1, an ethanolic solution of arachidonate (100 μM), containing only monomeric molecules, has been analysed by HPLC/MS: Only one peak at m/z 367.5 was detected, showing that Hydro1 (specifically generated from monomers) results from the addition of two dioxygen molecules on arachidonate. Hence, Hydro1 could be a dihydroperoxide or a cyclic hydroperoxide. These two products are more hydrophilic than Hydro2 and should have a shorter retention into a reversed-phase column, that was experimentally verified.

The ion at m/z 319.5 could be the result of hydroxylation of arachidonate in solution. Comparatively to Hydro1 and Hydro2, the chemical structure of this ion is closer to the arachidonate one: The end-carbonyl chain, responsible for interactions with the silica stationary phase of the column, is preserved and thus retention times of m/z 303.5 (arachidonate) and 319.5 (hydroxyl) do not differ a lot. This phenomenon is observed for each product on the top of Table I: As the number of oxygen atoms added to arachidonate increases, the molecular structure of the considered ion becomes more different and the retention time is shorter, traducing a weaker retention into the reversed-phase column. Products that have an odd number of oxygen atoms, detected at m/z 351.5 and 383.5 could differ from arachidonate by the presence of a combination of hydroxyl and hydroperoxide chemical functions.

The second part of Table I (bottom) presents all the other ions detected by HPLC/MS. The lowest in mass, at m/z 296.5 could correspond to a fragmentation of Hydro1 in solution, leading to a loss of the terminal alkyl-chain (C_5H_{11}). This assumption is compatible with its low retention into the HPLC column ($tr = 6.4$ min). Two other products are detected at m/z 353.5 and 369.5. They could, respectively, come from m/z 351.5 and Hydro1 (m/z 367.5) by the opening of an endoperoxide chemical function, leading to two hydroxyl functions.

Precisions can be provided by looking at UV data. Table I shows that most of the products have an absorption band in the 230–240 nm range, meaning that they contain a conjugated diene function. However, some products do not absorb at all between 200 and 400 nm (m/z 351.5, 383.5 and 361.5) and another one, which absorbs between 270 and 300 nm, seems to be trienic. These results show that some oxidation products detected by HPLC/MS are not hydroperoxidic nor dienic compounds. For example, the peak detected at m/z 351.5 ($tr = 12.5$ min) does not absorb between 200 and 400 nm and could contain, according to its mass, a hydroxyl and an endoperoxide functions. A similar hypothesis could be proposed for the peak at m/z 383.5, which could contain a hydroxyl and two endoperoxide functions without being dienic.

Radiolytic yields

The quantification of conjugated dienes and of both hydroperoxides and the kinetic studies of their formation pointed out variations in the chemical behaviour of arachidonate solution regarding concentrations of fatty acid. The initial radiolytic yields of formation of conjugated dienes (G_{Dienes}) and of both hydroperoxides (G_{Hydro2} and G_{Hydro1}) and the initial radiolytic yields of fatty acid consumption ($G_{(-LH)}$) corresponding to the initial slopes of the markers' evolution as a function of the radiation dose (data not shown) have been determined. Figure 3 displays the

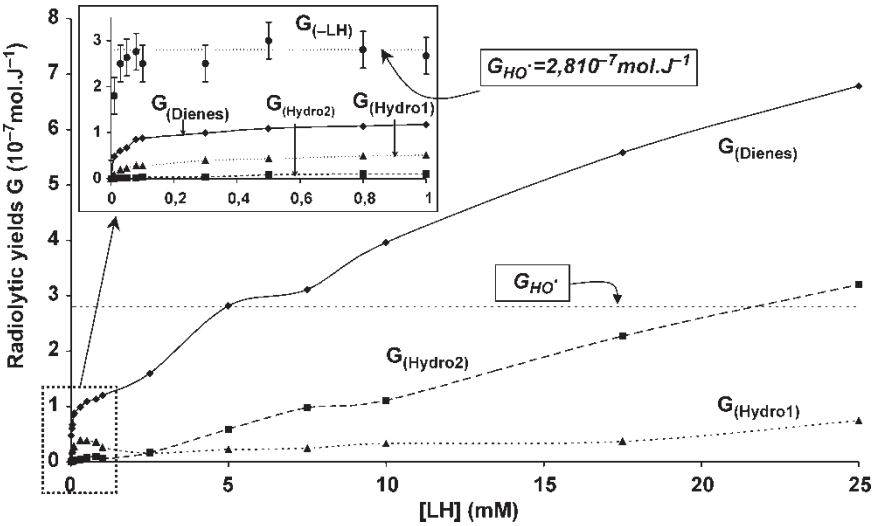


Figure 3. Initial radiolytic yields of formation of conjugated dienes, Hydro2 and Hydro1 as a function of the initial arachidonate concentration. *Insert*: Initial yields of arachidonate consumption and zoom on initial yields of oxidation products for low arachidonate concentrations, from 0.01 to 1 mM. Uncertainties range from 5 to 9%, at the 95% confidence level (2σ, $n = 3$), but error bars are not represented for sake of clarity.

variation of G_{Dienes}, G_{Hydro2} and G_{Hydro1} as a function of arachidonate concentration. In the insert in Figure 3 are reported the initial yields of arachidonate consumption for arachidonate concentrations lower than 1 mM, where variations in fatty acid concentration during irradiation are measurable.

[Arachidonate] ≤ 2.5 mM. For arachidonate concentrations lower than 2.5 mM (insert of Figure 3), i.e. for *G*-values lower than G_{HO} ($2.8\ 10^{-7}$ mol J^{-1}), two phases can be distinguished. First, for arachidonate concentrations between 0.01 and 0.1 mM, the *G*-values increase as a function of arachidonate concentration. Besides, $G_{(-LH)}$ remains almost constant and close to $G_{HO^{\cdot}}$, meaning that, at these concentrations (0.01–1 mM), all hydroxyl radicals react with arachidonate. This experimental behaviour can be explained by kinetics in homogeneous medium.

In a mechanistic point of view, in aqueous medium containing arachidonate (LH), hydroxyl radicals $HO^{\cdot}$ are expected to add to a double-bond (giving $HO-LH^{\cdot}$) according to reaction (1) and/or to abstract a hydrogen atom from a C—H bond according to reaction (2): A bis-allylic one or a hydrogen atom from CH_2 groups along the chain (giving $L^{\cdot}$).

$$HO^{\cdot} + LH \rightarrow HO - LH^{\cdot} \quad (1)$$

$$HO^{\cdot} + LH \rightarrow L^{\cdot} + H_2O \quad (2)$$

It can be noted that $HO^{\cdot}$ reacts differently whether the fatty acid is in monomeric or in micellar form: Reaction (1) is predominant in monomeric medium and reaction (2) occurs preferentially in micellar medium [24,25]. Both types of arachidonate carbon-centered free radicals ($HO-LH^{\cdot}$ and $L^{\cdot}$) react with oxygen at nearly the same rate ($k = 10^9 M^{-1} s^{-1}$), giving arachidonate peroxyl radicals $LOO^{\cdot}$ (reaction (3)) [14]. Then the oxidation reaction proceeds *via* abstraction of the bis-allylic hydrogen atoms from arachidonate by the peroxy radicals (reaction (4)) affording hydroperoxides (LOOH) [20].

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (3)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (4)$$

Finally, biradicalar termination reactions involve $LOO^{\cdot}$ (as example, reaction (5)) and $L^{\cdot}$ radical species. In addition, superoxide anions, that are present in radiation experiments, could play a role in biradicalar chain termination.

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{non-radicalar product(s)} \quad (5)$$

On the basis of the reaction mechanism (reactions (1–5)) and the steady-state approximation for all free radicals, a relation giving the yield of formation of peroxidation products, expressed as $G_{Products}$ can be derived for a homogeneous medium [19],

$$G_{Products} = \frac{k_4}{\sqrt{2k_5}}[LH]\sqrt{\frac{G_{HO^{\cdot}}}{I}} \quad (6)$$

where k_4 and k_5 are the rate constants of reactions (4 and 5), respectively, and where I is the dose rate. Equation (6) means that $G_{Products}$ is [LH]-dependent, i.e. it increases with [LH], which is in agreement with our experimental results for arachidonate

concentrations ≤ 0.1 mM. It can be concluded that, for [20:4] ≤ 0.1 mM, arachidonate is present mostly in its monomeric form, since all reactions (1–5) are bimolecular steps.

In a second phase, for arachidonate concentrations between 0.3 and 2.5 mM, the increase in *G*-values is inhibited, and therefore equation (6) cannot be applied. Consequently, the simple mechanism composed of reactions (1–5) is not able to explain the results in this concentration range. Actually, this mechanism does not take into account the heterogeneity of the solution due to aggregation phenomenon.

[Arachidonate] > 2.5 mM. For concentrations higher than 5 mM (Figure 3), *G*-values of conjugated dienes become higher than G_{HO}, implying a chain propagation reaction occurring mostly in aggregates and micelles. G_{Hydro2} becomes higher than G_{HO} only for the highest arachidonate concentration (25 mM). Nevertheless, G_{Dienes} and G_{Hydro2} increase with arachidonate concentration and both curves exhibit the same profile. Contrarly to Hydro1, the concentration of which remains constant, Hydro2 can be assumed as one of the numerous species produced during the chain propagation reaction occurring in aggregates and micelles.

Above 2.5 mM, G_{Hydro1} remains constant (Figure 3). In this case, Hydro1 production could be assumed to be independent of the aggregation phenomenon since increasing micelles quantity has no effect on the yield of Hydro1 formation.

Hydroperoxides ratio as a signature of the balance micelles/monomers

The balance of hydroperoxide production during $HO^{\cdot}$-induced oxidation of arachidonate can give useful indications on the physical arrangement of the fatty acid in solution. $G_{(Hydro2)}/G_{(Hydro1)}$ ratio illustrates the various steps of aggregation. In Figure 4 are reported $G_{(Hydro2)}/G_{(Hydro1)}$ values as a function of the initial arachidonate concentration (logarithmic scale). A clear change in the slope of the curve appears above 2.5 mM, exhibiting the formation of micelles above the cmc. The same type of phenomenon is observed, in the insert of Figure 4, above 100 μM. In the latter case, the variation of the slope accounts for a progressive aggregation of the fatty acid in solution, leading to small aggregates (oligomers) and starts around 100 μM as discussed previously. Thus, the determination of the $G_{(Hydro2)}/G_{(Hydro1)}$ ratios gives three concentration ranges corresponding to the three possible physical arrangements for arachidonate, i.e. monomers, aggregates (oligomers) and micelles.

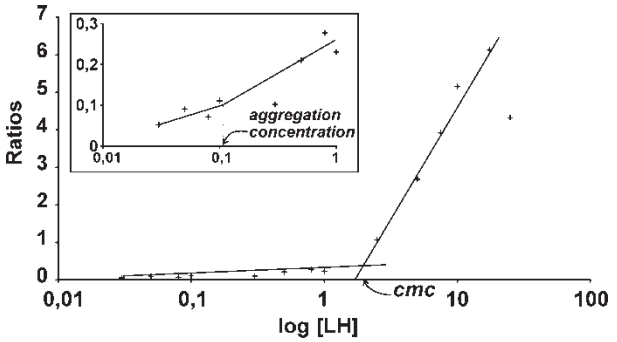


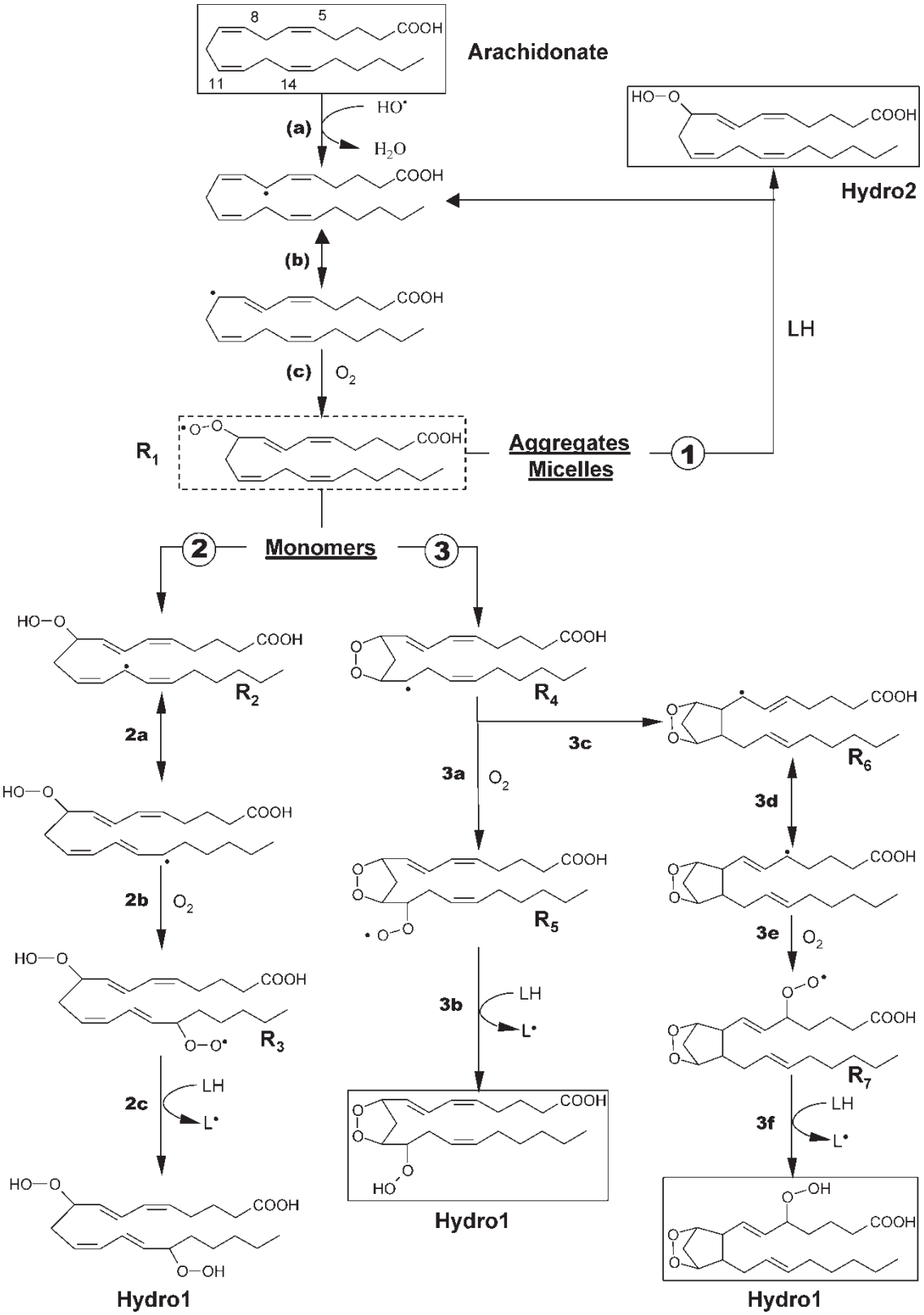
Figure 4. $G_{(Hydro2)}/G_{(Hydro1)}$ ratio as a function of the initial arachidonate concentration (logarithmic scale). *Insert*: Zoom for low arachidonate concentration, from 0.01 to 1 mM (logarithmic scale). *Crossings* between solid lines allowed determining the two arachidonate concentrations (experimental) where aggregation phenomena occur (aggregation concentration: 100 μM and cmc: 2 mM in our conditions).

Reaction scheme

As shown previously, two different kinds of hydroperoxides are formed in monomeric and aggregated arachidonate aqueous solutions. Scheme 1 could be proposed to illustrate the formation of both hydroperoxides: Hydro2 (characterised as a monohydroperoxide product) and Hydro1 (characterised as a cyclic hydroperoxide or an aliphatic dihydroperoxide product). Hydroxyl free radicals initiate the oxidation of arachidonic acid by removing a H-atom for example on the C_7 bis-allylic position (reaction (a)). The resulting carbon-centered radical rearranges (mesomeric effect (b)) and reacts with oxygen to give peroxyl free radical R_1 (dashed box, reaction (c)).

Then, two different pathways may occur. The first one (pathway 1) concerns reactions leading finally to Hydro2. These reactions would be predominant in large aggregates and micelles. The reaction of the peroxyl radical R_1 with an adjacent LH molecule giving a carbon-centered radical $L^{\cdot}$ and Hydro2 would be responsible for the chain process propagation. The second one concerns the reactions giving finally Hydro1 (pathways 2 and 3). These reactions would occur in fatty acid diluted solutions where monomers are the predominant forms. Pathway 2 leads to a dihydroperoxide, while pathway 3 leads whether to a monocyclic hydroperoxide or to a bicyclic hydroperoxide. These reactions could occur only if the peroxyl radical R_1 is located in β position of a double bond, as shown by Porter and co-workers [7].

According to pathway 2, peroxyl radical R_1 abstracts a H-atom on the C_{13} bis-allylic position intramolecularly, giving a carbon-centered radical $L^{\cdot}$ (R_2) and a hydroperoxide function on C_9. This radical rearranges (mesomeric effect (2a)) and reacts with oxygen (reaction (2b)) to give a peroxyl free radical located on C_{13} (R_3). The dihydroperoxide is obtained through reaction (2c) by abstraction of a H-atom from another LH molecule (intermolecularly).



Scheme 1. Reaction pathways for the formation of hydroperoxide species upon H-atom abstraction on the C_7 bis-allylic position of arachidonic acid. Pathway 1 is proposed for high arachidonate concentrations ($\geq$ 2.5 mM) and pathways 2 and 3 for low arachidonate concentrations ($<$ 2.5 mM).

According to pathway 3, peroxyl radical R_1 located on C_9 adds intramolecularly on the β-double bond (for example C_{11}) giving an endoperoxide and a new carbon-centered radical R_4. Then, R_4 can evolve according to two different pathways. First, reactions (3a) and (3b) lead, respectively, to peroxyl radical R_5 by addition of molecular oxygen and to the corresponding hydroperoxide obtained by abstracting a H-atom from another LH molecule (intermolecularly), giving a monocyclic hydroperoxide. Second, reaction (3c) leads to a cyclic carbon-centered radical R_6 by addition of the carbon-centered radical (located on C_{12}) to the C_8 of the same molecule, creating an intramolecular bond. Then, R_6 (located on C_7) rearranges (mesomeric effect (3d)) and reacts with oxygen to give peroxyl free radical R_7 (reaction (3e)). Reaction (3f) finally leads to a bicyclic hydroperoxide by abstraction of a H-atom from another LH molecule.

To summarise, it can be supposed that, in aggregates and micelles, the reaction (1) of the peroxyl radical R_1 abstracting a H-atom from an adjacent LH molecule would exhibit an important rate because of the proximity of the unsaturated acyl chains, while in diluted solutions (monomers), this reaction would be slow, allowing peroxyl radical R_1 to react on itself (unimolecular reactions), affording three different types of Hydro1.

Discussion

Various data concerning fatty acids cmc have been published. Gebicki and Allen [12] determined the cmc of sodium linoleate solutions by surface tension measurements. The cmc was estimated 1.7 mM. Al Sheikhly and Simic [14] studied the radiation-induced oxidation of linoleate in monomeric and micellar solutions. The estimated cmc was 2.3 mM. Nevertheless, oxygen consumption measurements pointed out that the uptake of oxygen remains constant around the cmc (2.3 mM) and also between 40 and 100 μM. This observation was explained by the aggregation of fatty acid described by reactions (7–9) [14].

$$LH + LH \rightarrow (LH)_2 \quad (7)$$

$$(LH)_2 + LH \rightarrow (LH)_3 \quad (8)$$

$$(LH)_n + LH \rightarrow (LH)_{n+1} \quad (9)$$

The slowdowns in oxygen consumption below the estimated cmc show the considerable tendency of fatty acid hydrophobic backbone to produce dimers, trimers and small aggregates, even at very low concentrations. Other works, using fluorescent probes to determine the cmc of lipids, as developed by Chattopadhyay and London [34], confirmed this early aggregation ability: An arachidonate aggregation concentration value of 73 μM was published [35]. Thus, aggregation of fatty acid molecules seems to be a progressive phenomenon, beginning early with the formation of small aggregates (oligomers) progressively leading to structured micelles above the cmc *via* a physical rearrangement.

Besides the distinction between Hydro1 and Hydro2 as specific oxidation products of monomers and micelles, respectively, several observations indicate that fatty acid concentration has a clear effect on $G_{(Hydro2)}/G_{(Hydro1)}$ ratio. Indeed, as seen on Figure 4, the ratio-values increase significantly only around concentrations, where arachidonate molecules rearrange to give oligomers (range 100–400 μM) and micelles (around 2.5 mM). Hence, the determination of the nature and of the concentration of the hydroperoxides produced can be used to probe the physical arrangement of a fatty acid aqueous solution.

In addition to chemical markers of the arachidonate aggregation, our results provide a complete reaction scheme for understanding the early phases of the oxidation initiated by $HO^{\cdot}$ free radicals. This allowed us to propose a hypothetical mechanism (Scheme 1) for the formation of these compounds. In low hydrogen donating capacity medium (monomers), peroxyl radical can react predominantly on itself (pathways 2 and 3). In aggregates, these reactions are inhibited because of the predominant hydrogen donating reaction ($LOO^{\cdot}$ + LH—reaction (4)) due to the aggregation of fatty acid molecules. Another reason for the competitive inhibition of endocyclisation (pathway 3) in micelles could be the spatial structure of the fatty acid molecules inside the aggregates.

Our study also pointed out the great variety of oxidative products generated by radiation-induced oxidation of arachidonate, as previously described by works on autoxidation of arachidonate conducted by Porter and co-workers [7,36]. These authors demonstrated that *β*-scission and endocyclisation are unimolecular events, while H-atom transfer is critically, dependent, on the total hydrogen atom donating ability of the medium. Our results are in agreement with such considerations. Given that arachidonate molecule contains four double bonds, H-atom abstraction by $HO^{\cdot}$ can occur on three sites (bis-allylic positions C_7, C_{10} and C_{13}). As a consequence, $HO^{\cdot}$-induced oxidation of arachidonate would lead to six monohydroperoxide isomers (5, 8, 9, 11, 12 and 15-HPETE), while three different types of species, namely dihydroperoxide, monocyclic and bicyclic hydroperoxides would be formed in diluted solutions (monomers).

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