

## Chapter 16

# **Pulse radiolysis studies of free radical processes in peptides and proteins**

Chantal HOUÉE-LEVIN and Krzysztof BOBROWSKI

### **Introduction**

The story of free radical reactions in biological systems began many years ago, when Harman postulated that they played a prominent role in ageing [1]. Since the sixties, the relevance to biological as well as industrial processes became more and more clear every year. It is now current to invoke free radicals in ordinary life, in cooking, in prevention of ageing processes, etc. and the scientific knowledge gave a basis to these assertions [2]. It is now beyond doubt that free radicals processes in proteins are involved in all steps of life, going from conception to death induction. They are believed to be part of the cellular defence against oxidative stress and at the same time responsible of severe damage like atherosclerosis [3] Protein free radicals are also enzyme active sites [4].

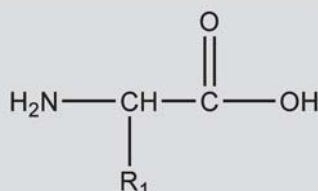
The studies by the methods of radiolysis provided a wealth of knowledge about the kinetic and thermodynamic controls of radical reactions, the importance of which is no more questioned. Indeed, it is known that the chemical events initiated by ionizing radiation, are the same as those that take place in normal and deleterious events of every day's life. In this review we focus on some of the major knowledge that was acquired by the use of pulse radiolysis and steady-state gamma radiolysis of aqueous solutions of amino acids, peptides and proteins (**Inset**). The potential role of pulse radiolysis (Chapter 2) for studying biomolecules has been acknowledged rather early. In most cases, pulse radiolysis

method has been very valuable in identification of radicals, establishing their structures and exploring their reactivity (Chapter 1). We would like also to show how the reactions that were evidenced took their place in biological events, thus how a synergy between pure chemistry and biology was established.

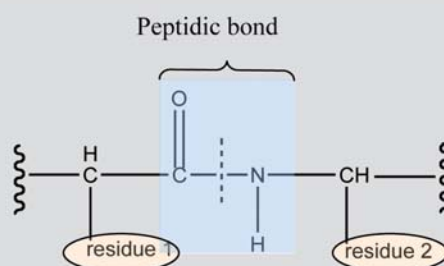
### Inset : Protein structure

A protein is a polymeric chain. (A) Monomers are amino acids. (B) In polymerization, residues are linked by the peptidic bond, which is an amide function.

A/ An amino acid.  $R_1$  will be the residue 1 in scheme B.



B/ The protein

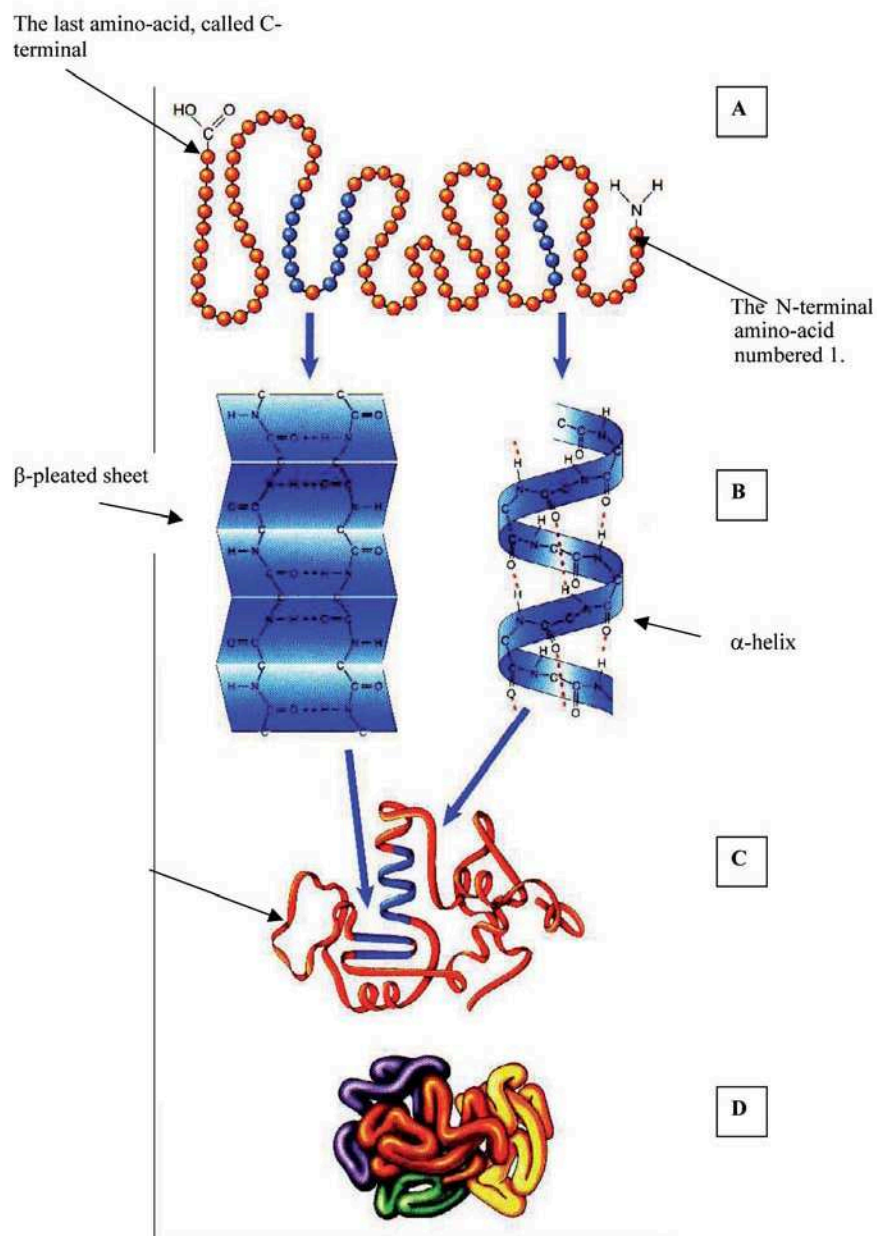


However this polymeric chain is folded. The folding has a tremendous importance in the action of the protein and in its recognition by biological partners and/or by the degradation pathways. The different levels of structure are presented in **Figure 1**. (For more details, see for instance [\[1\]](#)).

[\[1\]](#) Voet D., Voet J.G., Biochemistry 2nd edition, John Wiley New York.

### The simplest amino-acid, glycine

One of the more recent results concerns  $\cdot\text{OH}$ -induced oxidation of glycine, the simplest amino acid [\[5\]](#). Two main radical products  $^+\text{H}_2\text{N}^+-\text{CH}_2-\text{CO}_2^-$  and aminyl radicals  $\text{HN}^+-\text{CH}_2-\text{CO}_2^-$  have been identified and their subsequent reaction pathways including decarboxylation with parallel formation of  $\cdot\text{CH}_2\text{NH}_2$  and  $\beta$ -fragmentation into the respective



**Figure 1 :** The several levels of structure of a protein.

imine and carboxyl radical  $\text{CO}_2^{\cdot-}$ , respectively. The possible initiation of amino acid decarboxylation by C-centred radicals are considered to be of general significance and interest in chemical and biological systems.

### From amino acids to proteins

It was, however, soon recognized that in aqueous solutions peptides and proteins (**Inset**) and free radicals derived from them behaved differently from amino acids and free radicals derived from them. These differences arise from the following:

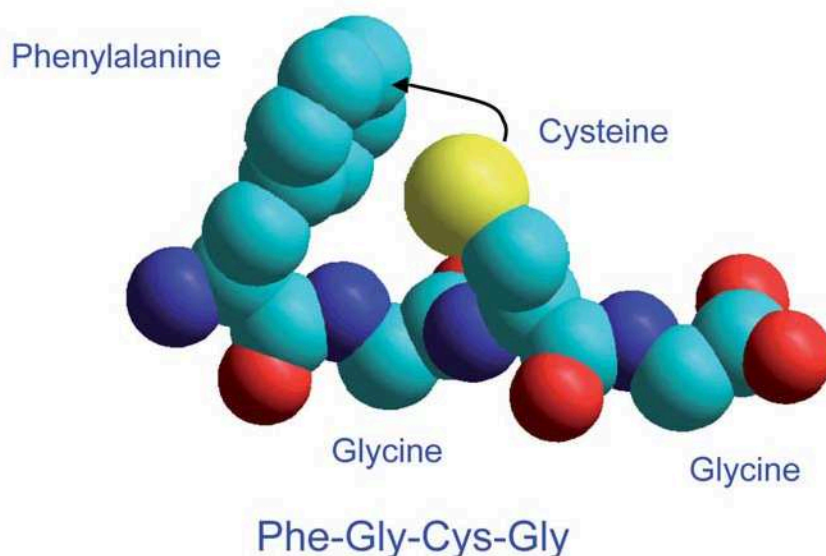
- The folding (**Fig. 1**) renders some amino acids not available to free radicals. Indeed, since the reactions are fast, their control is kinetic and not thermodynamic. One can imagine that the amino acids that are on the surface are more reactive than those in the interior.
- Some amino acids are charged; thus the protein creates an electrostatic field around itself, which orients the reactivity of the charged radicals.
- The reduction potentials of amino acids are sensitive to the environment.
- Functional groups present in side chains of neighbouring amino acids can be involved in reaction mechanism and thus may affect reaction pathway of radicals formed.

Thus kinetics as well as thermodynamics aspects of the free radical reactions are modified. Some examples are detailed in what follows.

#### *Thiyl radicals*

Thiyl radicals are important reactants in several enzymes and form *in vivo* during conditions of oxidative stress [6]. They have been considered for a long time as rather unreactive species. However, recently several reactions of thiyl radicals with biomolecules have been described (catalysis of *cis-trans* isomerization of unsaturated fatty acids, addition to the pyrimidine bases C5-C6 double bonds, and hydrogen abstraction from polyunsaturated fatty acid, thymine and peptide C<sub>α</sub>-H and side chain C-H bonds) [7]. More recently, the intramolecular addition of peptide cysteine thiyl radicals (CysS<sup>•</sup>) to phenylalanine (Phe) yielding alkylthio-substituted cyclohexadienyl radicals was demonstrated in the peptides Phe-Cys and Phe-Gly-Cys-Gly (**Fig. 2**) [8].

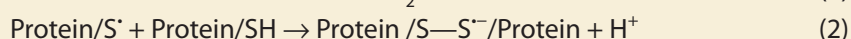
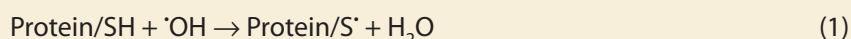
This addition reaction might be of great biological significance since the intramolecular addition of CysS<sup>•</sup> to aromatic ring of Phe can compete with addition of O<sub>2</sub> to CysS<sup>•</sup>, assuming biologically relevant tissue concentration of O<sub>2</sub>. It presents a possible free radical pathway to thioether-containing peptide and protein cross-links.



**Figure 2 :** In the peptide Phe-Gly-Cys-Gly (formed by Phenylalanine, Glycine, Cysteine and Glycine amino acids linked by peptidic bonds), the sulphur atom is very close to the phenyl ring. An intramolecular addition of the CysS<sup>•</sup> radical on the aromatic ring of Phe (marked with an arrow) leads easily to the alkylthio-substituted cyclohexadienyl radicals. Atom colors: Cyan: carbon; blue: nitrogen; red: oxygen; yellow: sulphur. H atoms are not shown.

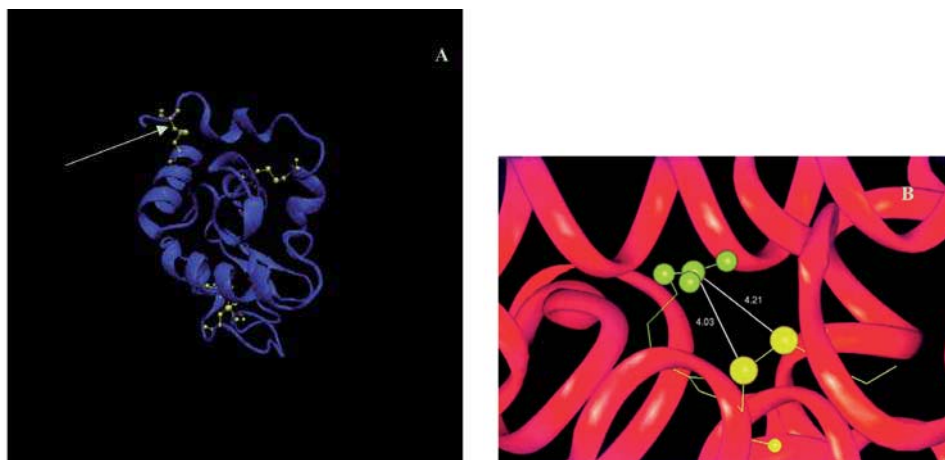
### Disulfide radicals

Disulfide radical anions might play an important role in oxidative stress. In cellular media, they can be formed by oxidation of protein thiol functions (by <sup>•</sup>OH radicals, for instance, Chapter 1), followed by dimerization:



These disulfide radical anions are believed to be strong reductants. Thus they might counterbalance the action of oxidizing free radicals.

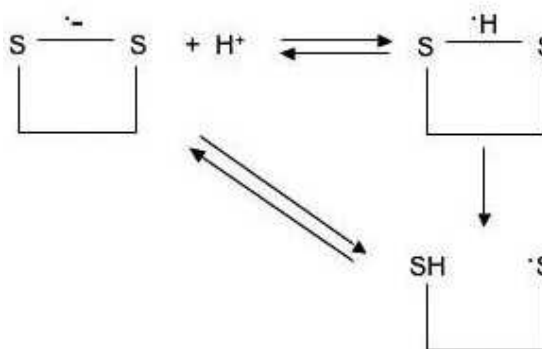
To study their properties, the easiest way is to form them by one-electron reduction of disulfide bonds. Among radicals from water radiolysis, hydrated electron is the most powerful reductant. It reacts with almost all amino acids and especially with the disulfide groups. Using less powerful reductants such as COO<sup>•-</sup> radicals, some selectivity in the attack appears. An example is displayed in **Figure 3**.



**Figure 3 :** *Hen egg white lysozyme has 4 disulfide bridges. However, only one of them is easily reduced A) marked with an arrow. The detail of its structure is shown in B) The disulfide radical anion is stabilized by interaction with the charged end of an Arginine residue (in green). In white: the distances (in Å) between the central carbon atom and the sulphur atoms. In red: the polypeptidic chain.*

In hen egg white lysozyme, out of the four disulfide bridges, one is much more easily reduced than the others (Fig. 3 A and B) [9]. A study by quantum chemistry could justify this selectivity by the stabilization of the resulting disulfide free radical by the positively charged end of arginine. The distances between sulphur atoms and the central carbon of arginine confirm the strong interaction between these groups (Fig. 3).

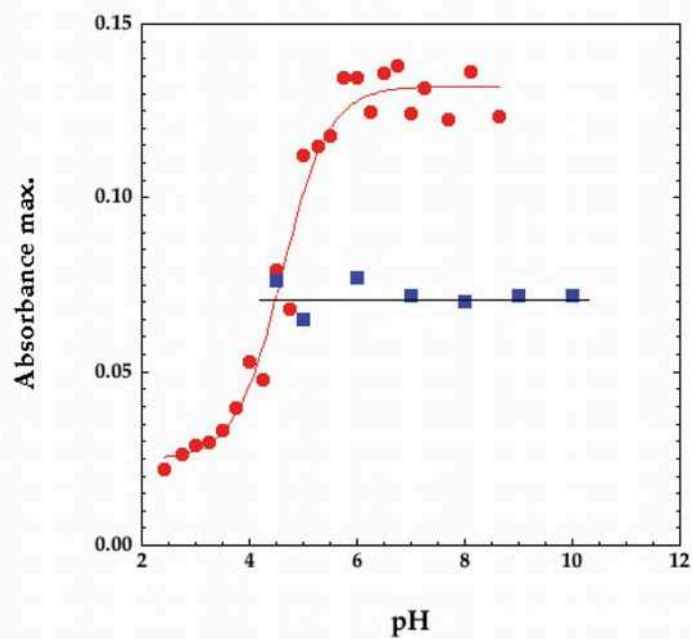
Pulse radiolysis studies demonstrated that all disulfide radicals do not have the same chemical properties. An example is given by the study of protonation equilibrium (Scheme 1).



**Scheme 1 :** *Protonation-deprotonation equilibrium of disulfide radical anion.*

The resulting protonated disulfide radical undergoes cleavage of the S-S bond. Since the thiyl radical is a strong oxidant, the protonation equilibrium appears like a switch

between oxidant/reductant radical. The  $pK_a$  values of some of these radicals, measured by pulse radiolysis, are between 5 and 6 (**Fig. 4**). However in thioredoxin, it is below 3 [10].



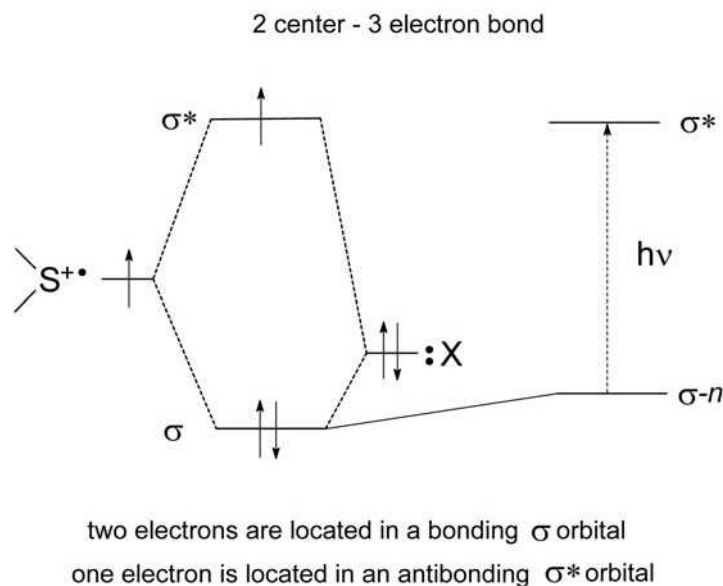
**Figure 4 :** Titration curves for hen egg white lysozyme and thioredoxin disulfide radical. Red : lysozyme; blue : thioredoxin.

It means that sulphur free radical from this protein are mostly reductant, which enlightens the role of thioredoxin in restoring sites which were oxidized and thus as protection against oxidative stress.

### *One-electron oxidation of methionine*

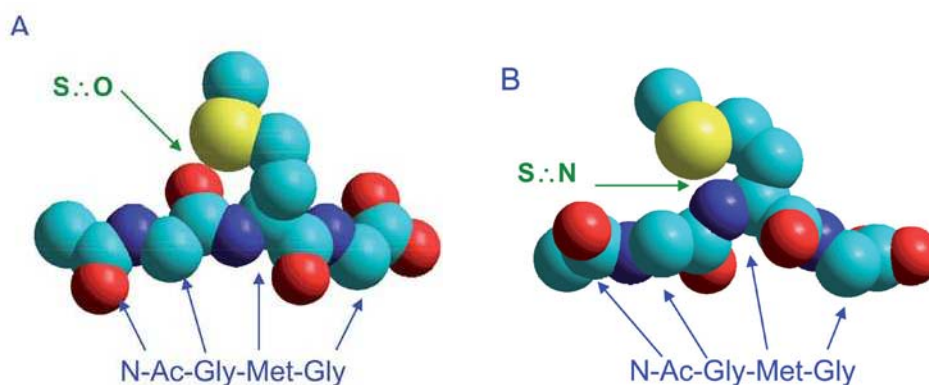
Methionine (Met) is one of the sensitive sulphur-containing amino-acids toward one-electron oxidation. However its ease of oxidation is also modulated by the structure. The one-electron oxidation of Met in peptides yields sulfide methionine radical cations ( $\text{MetS}^{+\bullet}$ ) which convert into intermediates that obtain catalytic support from neighbouring groups containing electron rich heteroatoms (S, N, O) and thus stabilize electron deficient sulphur centres in  $\text{S}:\cdot\text{S}$ ,  $\text{S}:\cdot\text{N}$ , and  $\text{S}:\cdot\text{O}$ -three-electron bonded complexes (**Fig. 5**) [11].

Interaction with particular peptide or protein domains would likely involve N- and C-terminal nucleophilic functionalities ( $\text{NH}_3^+$ ,  $\text{COO}^-$ ), and nucleophilic functionalities in the side chains of amino acid residues (Asp, Glu, Lys, Val, Thr). However, very often heteroatoms in peptide bonds are the only nucleophiles present in the vicinity of the  $\text{MetS}^{+\bullet}$ . It was



**Figure 5** : Electronic energetic diagram of the molecular orbitals of three–electron bonds in sulphur radicals and optical transition. X: S, N, or O atom.

recently shown that such interactions play an important role in oligopeptides of the form N-Ac-Gly-Met-Gly and N-Ac-Gly-Gly-Gly-Met-Gly-Gly-Gly [12]. Pulse radiolysis studies with UV/Vis spectrophotometrical and conductometric detection showed for the first time that MetS<sup>+</sup> in peptides can be stabilized through bond formation with either the oxygen or the nitrogen atoms of adjacent peptide bonds (Fig. 6).



**Figure 6** : Structure of N-Ac-Gly-Met-Gly peptide showing interactions of the sulfur atom (S) in Met with the oxygen atom (O) (A) and the nitrogen atom (N) (B) (marked with arrows) located in the N-terminal adjacent peptide bond that lead to the S:O and S:N-bonded radicals, respectively. Atom colors : Cyan: carbon; blue: nitrogen; red: oxygen; yellow: sulphur. H atoms are not shown.



Moreover, formation of radical transients with S:O bonds is kinetically preferred, but on longer time scale they convert into transients with S:N bonds in a pH dependent manner. Ultimately transients with S:N bonds transform intramolecularly into C-centred radicals located on the  $\alpha$ C moiety of the peptide backbone. Another type of C-centred radicals located in the side chain of Met-residue,  $\alpha$ -(alkylthio)alkyl radicals, are formed *via* deprotonation of MetS<sup>•+</sup>. C-centred radicals are precursors for peroxy radicals (ROO<sup>•</sup>) that might be involved in chain reactions of peptide and/or protein oxidation. Stabilization of MetS<sup>•+</sup> through formation of S:O- and S:N-bonded radicals might potentially accelerate oxidation and autooxidation processes of Met in peptides and proteins. Considering that methionine sulfoxide, which is the final product coming from all radicals centred on sulphur, is restored by the enzyme methionine sulfoxide reductase into MetS, stabilization of MetS<sup>•+</sup> appears as a protection against an eventual peroxidation chain that would develop from a carbon centred radical.

The amyloid  $\beta$  peptide ( $\beta$ AP), a 39- to 43-amino acid-long peptide is the major constituent of the neuritic plaques and neurofibrillary tangles in brain. Their progressive formation is characteristic for the development of the Alzheimer disease. The integrity of Met35 is very important for the constitution of the senile plaque. In the structure of  $\beta$ AP, Met35-S<sup>•+</sup> formation can be facilitated by a pre-existing close sulphur-oxygen (S-O) interaction between the Met35 sulphur and the carbonyl oxygen of the peptide bond C-terminal to isoleucine (Ile31) that might lower one-electron reduction potential of MetS<sup>•+</sup>/Met couple. The first experimental evidence that Met is more easily oxidized than in other peptides and proteins comes from one-electron oxidation of  $\beta$ AP1-40 using azide radicals (N<sub>3</sub><sup>•</sup>) produced by pulse radiolysis [13]. One-electron oxidation of the natural peptide was compared to that of the non-natural peptide of reverse sequence ( $\beta$ AP40-1). Circular dichroism showed that whereas A $\beta$ 1-40 is highly structured, A $\beta$ 40-1 has no regular structure. It appeared that the oxidation patterns of these two compounds are different: in  $\beta$ AP (1-40) Met35 is mostly the target of free radicals, whereas in  $\beta$ AP40-1, Tyr10 gets dimerized. This observation underlines the role of structure in driving the free radical reactions and seems to be indeed relevant to explain specificity of the A $\beta$ 1-40 in the development of Alzheimer disease.

The pathogenesis of another well-known neurodegenerative disease (Jacob Creutzfeld disease) seems to be strongly linked to the presence of prion proteins in the brain. These macromolecules contain multiple Met residues, some of them in close vicinity. Such structure should favour stabilization of MetS<sup>•+</sup> as intramolecular (S:S)<sup>•+</sup> complexes. Since weak intramolecular non-bonded S...O and S...N interactions have been recently suggested in proteins [14], stabilization of MetS<sup>•+</sup> through formation of S:N- and/or S:O-complexes might potentially accelerate oxidation processes in proteins. The first experimental evidence

for the stabilization of  $\text{MetS}^{+\bullet}$  as intramolecular complex with N-atom in adjacent amide group was obtained during one-electron oxidation of calmodulin ( $\text{CaM-Ca}_4$ , wild type), studied on the microsecond time domain by pulse radiolysis [15]. Calmodulin is a regulatory “calcium sensor” protein that contains nine Met residues. Stabilization of  $\text{MetS}^{+\bullet}$  by peptide bonds might be a general phenomenon in proteins.

There are different reaction pathways of decay of the  $\text{S}:\text{N}$ -complex depending on the structure of the peptide. In model peptides containing N-terminal glutamic acid residue ( $\gamma\text{-Glu-Met}$ ,  $\gamma\text{-Glu-Gly-Met-Gly}$ ), an intramolecular electron transfer from the carboxylate group to the electron-deficient center at the nitrogen within  $\text{S}:\text{N}$ -bond followed by homolytic bond-breakage of the carbon-carboxylate bond leads to formation of  $\alpha$ -amino radicals and  $\text{CO}_2$  [16]. On the other hand, in model peptides  $\text{Thr-(Gly)}_n\text{-Met}$ ,  $n = 0-4$ , the intermediary  $\text{S}:\text{N}$ -bonded radicals do not decarboxylate but undergo homolytic cleavage of the  $\text{C}_\alpha\text{-C}_\beta$  bond of Thr, yielding the highly toxic acetaldehyde [17]. Once again, the fate and the consequences of peptide oxidation depend strongly on the stabilization of  $\text{MetS}^{+\bullet}$ .

### **Intramolecular long range electron transfer in peptides and proteins**

Intramolecular long-range electron transfer (LRET) plays a major role in many biological processes, including fundamental energy storage processes such as photosynthesis and respiration, redox-mediated enzyme catalysis, and in various pathologic processes such as radiation damage, oxygen toxicity and cellular aging. Most of these processes occur on ground-state potential energy surfaces, making pulse radiolysis an effective and truly unique tool for these studies. The typical LRET pulse radiolysis experiment begins with the rapid selective oxidation or reduction of one-redox site on a macromolecule (formation of the donor-acceptor complex) followed by the intramolecular LRET. The advantage of pulse radiolysis is that using either an oxidizing or a reducing radical can generate the donor-acceptor complex. These radicals have redox potentials covering the approximate range from  $-1.1$  to  $+1.8$  V (vs. SCE) [18]. The entire subject of LRET is much too wide to be covered in detail. Numerous comprehensive reviews are available which present and discuss this topic in a more detailed manner (see for instance [19] and references therein). Therefore, a few selected important achievements emerging in this field will be highlighted.

Most LRET processes in biological systems are nonadiabatic. In quantum-mechanical electron-transfer theory, the rate constant for nonadiabatic ET from a donor to acceptor can be expressed as the product of the square of an electronic coupling matrix element ( $H_{\text{DA}}$ ) and a nuclear Franck-Condon factor (FC):  $k_{\text{ET}} = (2\pi/\hbar)[H_{\text{DA}}]^2(\text{FC})$ . The  $[H_{\text{DA}}]$  is a measure of the

coupling or the interaction between the orbitals of the donor (D) and the acceptor (A) and is influenced by the distance and structure of the medium separating the electron donor and acceptor. The role of the distance, standard free energy change, and reorganization energy was examined. Influence of secondary structural features of the peptide bridge has been probed by applying flexible oligoglycine bridges, conformationally more rigid oligoproline bridges and helical bridges (for review see [20] and references therein) Pulse radiolysis studies of simple model synthetic peptides have demonstrated intramolecular ET involving radicals located on the side chains of aromatic, histidine and sulfur containing (methionine, cysteine) amino acid residues.

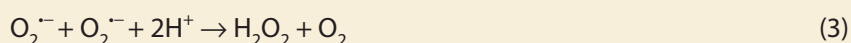
Elaboration of LRET mechanism by resolving the parameters that determine specific rates of LRET has stimulated pulse radiolysis studies in proteins. Examples include generation of metastable electron donor and acceptor complexes in (1) native and mutant proteins, (2) proteins with the directed single-site specific mutations, (3) native and mutant multi-site redox proteins, (4) proteins with the site specific modification with transition metal complexes covalently attached to a specific surface amino acid residues.

### **Pulse radiolysis investigations related to oxidative stress**

The methods of gamma and pulse radiolysis provided the basis for the understanding of oxidative stress. Indeed, one cannot imagine how the biological importance of short-lived transients could have been demonstrated otherwise and how their chemical properties could have been studied.

#### *Control of superoxide radical anion steady state*

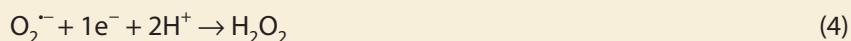
A striking example is control of the steady state level of superoxide radical anion ( $O_2^{\cdot-}$ ). Although superoxide anions are not very reactive, they play a prominent role in oxidative stress by triggering formation of peroxynitrite. The control of their steady state level is thus vital for cells. In most living organisms the well-known metalloenzyme superoxide dismutase (SOD), present in almost all aerobic cells catalyzes the disproportionation of  $O_2^{\cdot-}$  into  $H_2O_2$  and  $O_2$ :



SODs are differentiated mainly by the redox-active metal in the active site and by the localization: copper-zinc (in the cytosol), manganese (in mitochondria) and iron. The iron and manganese SODs are structurally similar. However, some bacteria can live without the Cu-Zn SOD, whereas no organisms can live without Mn-SOD. It stresses the vital importance of these

enzymes close to the place where  $O_2^{\bullet-}$  are produced. The mechanisms by which Mn-SOD reacts with  $O_2^{\bullet-}$  are of interest as knowledge of the kinetic parameters, and the reaction pathways may allow the synthesis of model compounds with specific chemical features. The kinetics of the dismutation of  $O_2^{\bullet-}$  by *Escherichia coli* MnSOD were measured by pulse radiolysis, and shown to fit a mechanism involving the rapid reduction of  $Mn^{3+}$ SOD by  $O_2^{\bullet-}$  followed by both the direct reoxidation of  $Mn^{2+}$ SOD by  $O_2^{\bullet-}$  and the formation of a  $Mn^{II}SOD(O_2^{\bullet-})$  complex [21].

The second system was discovered in prokaryotic cells a few years ago [22]. It is the recently characterized non-heme iron superoxide reductase (SOR) that catalyzes the reduction of  $O_2^{\bullet-}$  into  $H_2O_2$  by an intracellular reductant:



For studies about the mechanisms of action of this enzyme, pulse radiolysis also played a prominent role. Current research on improving knowledge about these enzymes, creating more performant ones by site directed mutagenesis or testing the ability of destruction of superoxide by metal complexes also uses pulse radiolysis as a method of screening.

Haem peroxidases are globular proteins with an iron-porphyrin complex as a prosthetic group. These enzymes are widespread among prokaryotes and eukaryotes. They catalyze the oxidation of substrates by organic peroxides or hydrogen peroxide. During the past decades, considerable scientific effort has been put into elucidation of the mechanisms of reactions catalyzed by these enzymes. Pulse radiolysis technique has made an important contribution by providing information on the redox states of the enzymes and their interconversion, as well as on the properties of the free radical intermediates involved [23].

*Pulse radiolysis investigations of the reaction of superoxide radical anions ( $O_2^{\bullet-}$ ) with radicals derived from various amino acids*

The following results were all obtained using pulse radiolysis.

**Methionine.** The reaction of superoxide radical anions ( $O_2^{\bullet-}$ ) with sulfide radical cation-nucleophile complexes might represent an efficient sulfoxide-forming process in peptides and proteins containing methionine under conditions where significant amounts of sulfide radical cation complexes and superoxide are formed simultaneously. The rate constant for the reaction of  $O_2^{\bullet-}$  with the  $(S\cdot:N)^+$  complex was found to be *ca.* 3-fold slower as compared to that of the reaction with the  $(S\cdot:S)^+$  complex. This drop in reactivity may, in part, reflect the lower probability of  $O_2^{\bullet-}$  to encounter S-atom in the  $(S\cdot:N)^+$  complex as

compared to the symmetrical (S...S)<sup>++</sup> complex. It is important to note that the reactions of O<sub>2</sub><sup>•−</sup> with the sulfide radical cation complexes proceed 2.5 to 8-fold faster than the reaction of O<sub>2</sub><sup>•−</sup> with superoxide reductases SOD [24]. From a biological point of view, it means that sulfide radical cation-O<sub>2</sub><sup>•−</sup> reactions might represent a potential source for sulfoxide formation when the system is exposed to high concentrations of reactive oxygen species (ROS).

**Tryptophan.** The reaction of O<sub>2</sub><sup>•−</sup> with the semi-oxidized tryptophan neutral radical (Trp<sup>•</sup>) generated from tryptophan (Trp) by pulse radiolysis has been studied in a variety of functionalized Trp derivatives and in lysozyme [25]. These studies demonstrate that the reactivity of O<sub>2</sub><sup>•−</sup> with the Trp<sup>•</sup> radical is significantly dependent on the charge in the vicinity of the Trp residue from which it originates, by the electrostatic field generated by neighbouring groups and/or other charged cell constituents (e.g. DNA and lipids). The kinetics of O<sub>2</sub><sup>•−</sup> reaction with the semi-oxidized tryptophan neutral radicals (Trp<sup>•</sup>Lyz) has been investigated at various pHs and conformational states. It was found that at pH lower than 6.2, the apparent bimolecular rate constant is about  $2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  but drops to  $8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  or less above pH 6.3 and in CTAC micelles. Interestingly, at all pHs the rate constants of the reaction of O<sub>2</sub><sup>•−</sup> with Trp<sup>•</sup> radicals are more than an order of magnitude greater than rate constants characterizing the intermolecular recombination of Trp<sup>•</sup> radicals. The well-established LRET from Tyr residues to Trp radicals-leading to the repair of the Trp<sup>•</sup>Lyz radicals is inhibited by the Trp<sup>•</sup>Lyz + O<sub>2</sub><sup>•−</sup> reaction. In conclusion, the reaction of O<sub>2</sub><sup>•−</sup> with reactive radicals derived from amino acids in proteins may have important consequences for the turnover of proteins involved in metabolic reactions.

## Conclusion

The studies summarized here brought important knowledge at several levels. In fundamental science, the evidence of phenomena such as Long range Electron Transfer, LRET, has stimulated experiments and discussions all over the world. It was discovered that LRET concerns all polymers. In DNA, it may explain the localization of base lesions induced by oxidative stress. As for biology, it helped to understand the chemical basis of initiation and development of the inflammation processes that take place in all diseases. Moreover this fundamental research had consequences on everyday life: a search for evidences of the importance of antioxidants in food in relation to quality and duration of life was undertaken. The results were so positive that the content of antioxidants is now a criterion of quality of food. The redox reactions of the sulphur amino acids have triggered a research on new anti-inflammatory drugs, and some of them (such as N-acetyl cysteine) are currently used in many pathologies. There is no doubt that in the close future other comprehension and applications of the radiolysis results will appear.

## References

- [1] Harman D., Aging: a theory based on free radical and radiation chemistry, *J. Gerontol.*, 1956, 298-300.
- [2] Houée-Levin C., Sicard-Roselli C., Bergès J., *Chimie et Biochimie radicalaires*, Belin coll. Ellipses, 2005.
- [3] Aruoma O. I., Halliwell B., *Molecular biology of free radicals in human diseases*, OICA international, London, 1998.
- [4] Stubbe J. A., Van der Donck W. A., Protein radicals in enzyme catalysis, *Chem. Rev.*, 1998, **98**, 705-762.
- [5] Bonifačić M., Armstrong D. A., Carmichael I., Asmus K.-D.,  $\beta$ -Fragmentation and other reactions involving aminyl radicals from amino acids, *J. Phys. Chem. B*, 2000, **104**, 643-649.
- [6] Jourdeuil D., Jourdeuil F. L., Feelisch M., Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide. Evidence for a free radical mechanism, *J. Biol. Chem.*, 2003, **278**, 15720-15726.
- [7] Ferreri C., Kratzsch S., Landi L., Brede O., Thiol radicals in biosystems: effects on lipid structures and metabolisms, *Cell. Mol. Life Sci.*, 2005, **62**, 834-847.
- [8] Nauser T., Casi G., Koppenol W. H., Schöneich C., Intramolecular addition of cysteine thiol radicals to phenylalanine in peptides: formation of cyclohexadienyl type radicals, *Chem. Commun.*, 2005, 3400-3402.
- [9] Bergès J., Kassab E., Conte D., Adjadj E., Houée-Levin C., Ab initio calculations on arginine-disulfide complexes modelling the one-electron reduction of lysozyme. Comparison to an experimental reinvestigation, *J. Phys. Chem.*, 1997, **101**, 7809-7817.
- [10] El Hanine, Lmoumène C., Conte D., Jacquot J.-P., Houée-Levin C., Redox properties of protein disulfide bond in oxidized thioredoxin and lysozyme. A pulse radiolysis study, *Biochemistry*, 2000, **39**, 9295-9301.
- [11] Bobrowski K., Holcman J., Formation and stability of intramolecular three-electron S: $\cdot$ N, S: $\cdot$ S, and S: $\cdot$ O bonds in one-electron-oxidized simple methionine peptides. Pulse radiolysis study, *J. Phys. Chem.*, 1990, **93**, 6381-6387.
- [12] Schöneich C., Pogocki D., Hug G. L., Bobrowski K., Free radical reactions of methionine in peptides: mechanisms relevant to  $\beta$ -amyloid oxidation and Alzheimer's disease, *J. Am. Chem. Soc.*, 2003, **125**, 13700-13713.
- [13] Kadlčík V., Sicard-Roselli C., Kodicek M., Houée-Levin C., One-electron oxidation of beta-amyloid peptide: sequence modulation of reactivity, *Free Rad. Biol. Med.*, 2004, **37**, 881-891.
- [14] Iwaoka M., Takemoto S., Tomoda S., Statistical and theoretical investigations of directionality of nonbonded S $\cdots$ O interactions. Implications for molecular design and protein engineering, *J. Am. Chem. Soc.*, 2003, **124**, 10613-10620.
- [15] Nauser T., Jacoby M., Koppenol W.H., Squier T.C., Schöneich C., Calmodulin methionine residues are targets for one-electron oxidation by hydroxyl radicals: formation of S: $\cdot$ N three-electron bonded radical complexes, *Chem. Commun.*, 2005, 587-589.

- [16] Bobrowski K, Schöneich C., Decarboxylation mechanism of the N-terminal glutamyl moiety in  $\gamma$ glutamic acid and methionine containing peptides, *Radiat. Phys. Chem.*, 1996, **47**, 507-510.
- [17] Pogocki D., Ghezzi-Schöneich E., Schöneich C., Conformational flexibility controls proton transfer between methionine hydroxy sulfuranyl radical and the N-terminal amino group in Thr-(X)<sub>n</sub>-Met peptides, *J. Phys. Chem. B*, 2001, **105**, 1250-1259.
- [18] Wardman P., Reduction potentials of one-electron couples involving free radicals in aqueous solution, *J. Phys. Chem. Ref. Data*, 1989, **18**, 1637-1755.
- [19] Pecht I., Farver O., Pulse radiolysis: A tool for investigating long-range-electron transfer in proteins, in "Photochemistry and radiation chemistry. Complementary methods for the study of electron transfer", Wishart J. F., Nocera D. G. (eds.), *Adv. Chem. Ser.*, 1998, **254**, ACS, Washington, D.C., 65-69.
- [20] Houée-Levin C., Sicard-Roselli C., Radiation chemistry of proteins, in "Radiation chemistry: present status and future prospects", Jonah C., Rao B.M. (eds.), Elsevier, 2001, 53-84.
- [21] Hsu J.L., Hsieh Y., Tu C.K., O'Connor D., Nick H.S., Silverman D.N., Catalytic properties of human manganese superoxide dismutase, *J. Biol. Chem.*, 1996, **271**, 17687-17691.
- [22] Jenney F.E. Jr., Verhagen M.F.J. M., Cui X., Adams, M.W.W., Anaerobic microbes: oxygen detoxification without superoxide dismutase, *Science*, 1999, **286**, 306-309.
- [23] Candeias L.P., Gebicka L., Investigation of mechanisms of peroxidase-catalyzed reactions by radiation-chemical techniques, *J. Radioanal. Nucl. Chem.*, 1998, **232**, 29-34.
- [24] Bonifacic M., Hug G.L., Schoeneich C., Kinetics of the reaction between sulfide radical cation complexes [SS]<sup>+</sup> and [SN]<sup>+</sup> and superoxide or carbon dioxide radical anion, *J. Phys. Chem. A*, 2000, **104**, 1240-1245.
- [25] Santus R., Patterson L.K., Hug G.L., Bazin M., Mazière J.C., Morlière P., Interactions of superoxide anion with enzyme radicals : kinetics of reaction with lysozyme tryptophan radicals and corresponding effects on tyrosine electron transfer, *Free Rad. Res.*, 2000, **33**, 383-391.





## Chapter 17

# **Radiation-induced damage of membrane lipids and lipoproteins**

Monique GARDES-ALBERT

### **Introduction**

If radiation-induced damages to DNA have long been considered as the only critical events for the cell machinery, it is now admitted that cell membranes are also significant biological targets of ionizing radiation [1]. Lipids are the major constituents of biomembranes and of lipoproteins. Their amphiphilic structure, namely hydrophilic polar heads and hydrophobic hydrocarbon tails, allowing the build up of dense networks of intermolecular bonds, favours the packing of membrane lipids into bilayers. In addition, proteins are inserted into the lipidic matrix where they are associated with lipid domains. The cell membrane is not a simple barrier defining the boundaries of the cell, since it modulates signals from the extracellular medium into the cell and it also controls intercellular communications [2].

Ionizing radiations such as  $\gamma$ -rays, are able to directly ionize the lipid/protein network of biomembranes, but also to ionize the water molecules surrounding all the cell constituents. Molecules of  $H_2O$  which are present in both intra- and extracellular compartments, undergo radiation-induced decomposition into free radical species. The proportion of directly ionized biological targets versus indirectly-mediated damages, via free radicals from water radiolysis, remain unclear and controversial, often depending on the studied systems. In this chapter, it will be focused on the indirect effects coming from the reactions of lipids and lipoproteins

with oxygenated free radicals produced by radiation-induced decomposition of water. These effects are related to numerous works devoted to free radical-induced damages on aqueous lipidic model systems (micelles, liposomes, lipoproteins) submitted to ionizing radiation. Moreover, the mechanism of formation of free radicals from water radiolysis being well known, together with the radiolytic yields of production of each radical species (Chapter 1), it is relatively easy to obtain from radiation dose effects, kinetic results on the chemical reactions of these free radicals with biological targets (such as lipids) dissolved in water [3]. For these reasons, water radiolysis can be seen as a powerful tool for studying radical mechanisms on defined biological systems.

Lipid peroxidation has been found as the main damage of membrane lipids and lipoproteins, initiated by oxygenated free radicals. A key feature of this phenomenon is its propagating capacity into the lipidic network by a chain reaction, due to the tight packing of the lipid molecules, leading to lipid hydroperoxides as major initial products. In addition, radiation-induced oxidative fragmentation of lipids has been described [4], together with changes in the physical state (fluidity/rigidity, permeability, ...) and as a function of biomembranes [1,2]. In the present review, recent knowledge about the chemical nature and mechanism of free radical-induced lipidic damages will be examined, from simple models of lipid aggregates submitted to  $\gamma$ -rays. The consequences of the presence of new peroxidized lipidic products and short-chain fragments into the cell membranes will be discussed. Oxidative stress phenomena can be indeed responsible for the *in vivo* peroxidation of lipoproteins leading to atherosclerosis [5].

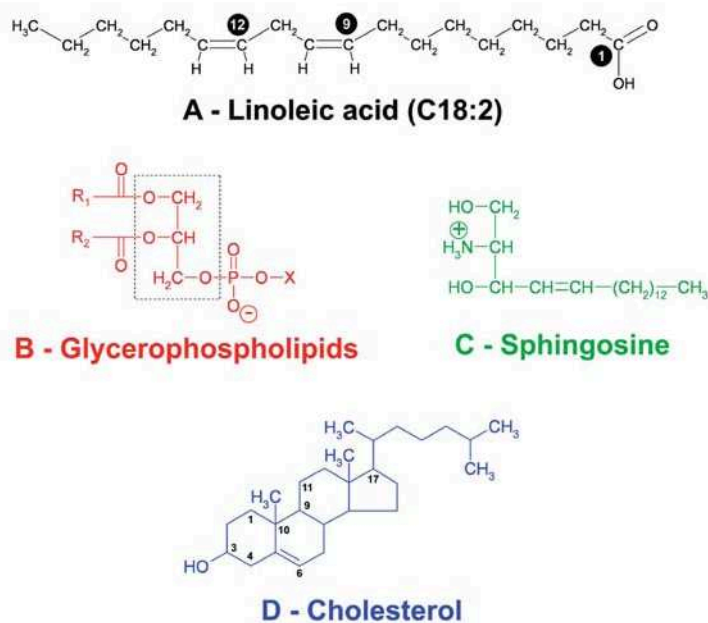
### Composition of membrane lipids

Fatty acids, glycerophospholipids and sphingolipids, are the three major classes of lipids entering in the composition of biological membranes. Fatty acids are carboxylic acids (RCOOH) with long-chain hydrocarbon side groups, usually ranged between 14 and 20 carbon atoms. In **Table 1** are listed some common biological fatty acids. In higher plants and animals, the predominant fatty acid residues are those of the C16 and C18 species [6] named palmitic, oleic, linoleic and stearic acids (**Tab. 1**). Over half of the fatty acid residues of plant and animal lipids are often polyunsaturated (containing two or more double bonds). They are symbolized by PUFAs (Poly Unsaturated Fatty Acids). For example, 18:2 means that the hydrocarbon chain contains 18 carbon atoms and 2 carbon-carbon double bonds (see linoleic acid in **Tab. 1** and **Fig. 1A**). Almost all fatty acid double bonds have the *cis*-configuration as it can be seen for linoleic acid as an example, in the **Figure 1A**.

**Table 1.** Names and formulas of fatty acids.

Name <sup>1</sup>	Symbol <sup>2</sup>	Structure
<b>Unsaturated fatty acids</b>		
Palmitoleic acid (9-Hexadecenoic acid)	16:1	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Oleic acid (9-Octadecenoic acid)	18:1	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$
Linoleic acid (9,12-Octadecadienoic acid)	18:2	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_2(\text{CH}_2)_6\text{COOH}$
$\gamma$ -Linolenic acid (6,9,12-Octadecatrienic acid)	18:3	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_3(\text{CH}_2)_3\text{COOH}$
Arachidonic acid (5,8,11,14-Eicosatetraenoic acid)	20:4	$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_4(\text{CH}_2)_2\text{COOH}$
<b>Saturated fatty acids</b>		
Palmitic acid (Hexadecanoic acid)	16:0	$\text{CH}_3(\text{CH}_2)_{14}\text{COOH}$
Stearic acid (Octadecanoic acid)	18:0	$\text{CH}_3(\text{CH}_2)_{16}\text{COOH}$

1) Common name (systematic name in brackets). 2) Number of carbon atoms : number of carbon-carbon double bonds. The systematic name of fatty acids derives from the total number of carbon atoms of the chain (for example, "hexadeca" for 16 atoms, "octadeca" for 18 atoms,...), the other numbers designating the carbon atoms bearing the carbon-carbon double bonds. For example, linoleic acid is the 9,12-Octadecadienoic acid which has 2 double bonds beared on the 9th and 12th carbon atoms, the 1<sup>st</sup> being attributed to the carboxylic acid function (Fig. 1A).



**Figure 1 :** Structures of some membrane lipids. A) Linoleic acid as an example of PUFA. B) General structure of glycerophospholipids, R1, R2 being long chain fatty acid residues and X a polar group; the glycerol backbone is inside the dashed line. C) Formula of sphingosine, the simplest member of the sphingolipids family. D) Cholesterol which can be esterified to long chain fatty acid on its C3 OH group.

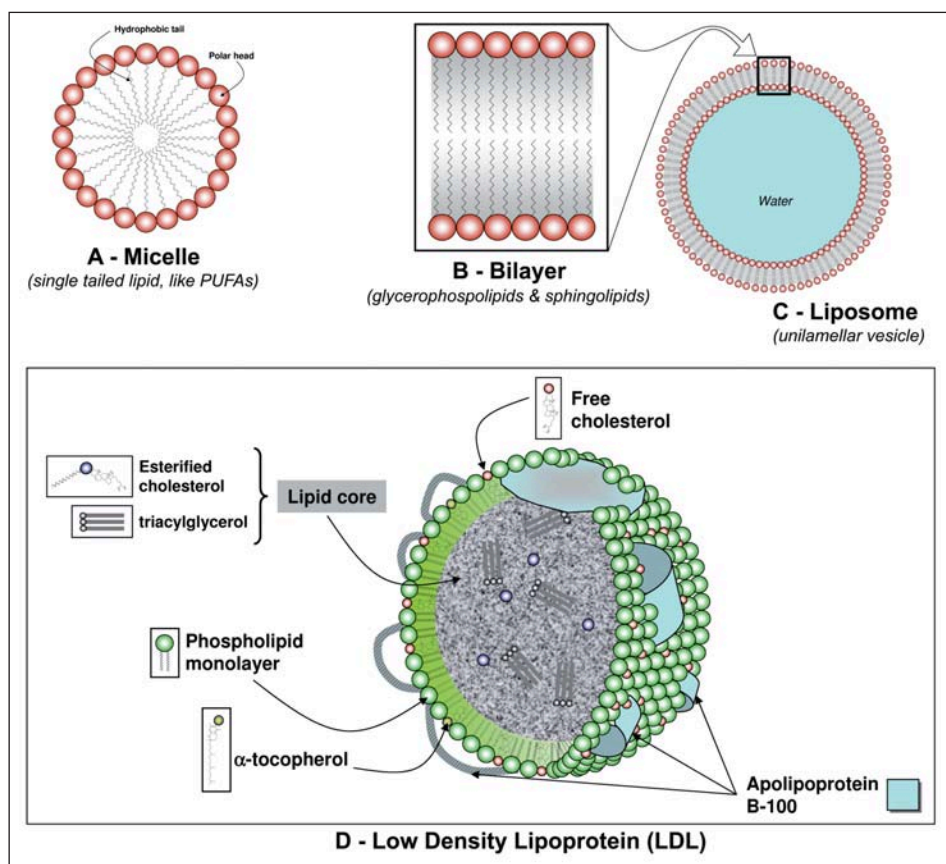
Glycerophospholipids are, with sphingolipids, the major lipid components of biological membranes. Glycerophospholipids (general formula in **Figure 1B**) derive from glycerol ( $\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{OH}$ ) by esterification to two long-chain fatty acid residues (R1 and R2 in **Figure 1B**), the third alcohol function being transformed into a phosphoryl-X group. X is a polar group such as an alcohol or an amine. Sphingolipids are derivatives of the amino alcohol sphingosine (**Fig. 1C**). In this latter class of lipids, there are several different families such as ceramides, sphingomyelins and sphingoglycolipids with complex head groups of up to four sugar residues.

Cholesterol (**Fig. 1D**), which is the metabolic precursor of steroid hormones, is a major component of plasma membranes. Its fused ring system brings a greater rigidity than other membrane lipids. Cholesterol can be esterified on the C3 OH-group to long-chain fatty acids to form cholesteryl esters which are major components of lipoproteins.

### Lipid aggregates and model systems

The common feature of lipids is their amphiphilic structure, characterized by a hydrophobic part (the non-polar aliphatic acid residue(s)) and a hydrophilic part (for example, the polar carboxylic acid function in fatty acids or the phosphoryl head in glycerophospholipids, ...). When lipids are dissolved in water, they spontaneously aggregate, the non-polar tails being associated by weak Van der Waals interactions. Single tailed lipids as PUFAs tend to form micelles (**Fig. 2A**) which are spheroidal aggregates where the polar carboxylate heads are in contact with water. Micelles appear in aqueous solution when the fatty acid concentration surpasses the critical micelle concentration (symbolized by cmc), namely the upper limit concentration above which monomers aggregate. The cmc depends on the nature of the fatty acid and on the solution conditions (pH, temperature, ...). For example, at room temperature, the value of linoleate cmc is close to  $2 \times 10^{-3} \text{ mol l}^{-1}$  in aqueous solution at pH = 10.5 [7]. This means that at concentration below  $2 \times 10^{-3} \text{ mol l}^{-1}$ , linoleate anions cannot form micelles but only very small aggregates (oligomers) and monomers, whereas above  $2 \times 10^{-3} \text{ mol l}^{-1}$  they are mainly associated into micelles. The basic pH (10.5) of the aqueous medium is necessary in order to deprotonate the carboxylic acid function of PUFAs giving carboxylate groups  $\text{COO}^-$ , namely polar charged heads (surrounded by polar water molecules). PUFAs micelles in aqueous solution are the simplest lipidic models allowing a mechanistic approach of lipid peroxidation under ionizing radiations (see section "Quantitative determination of hydroperoxides").

Glycerophospholipids and sphingolipids are biological lipids which have two large hydrophobic tails, one of them being often a PUFA chain. They exhibit a very low cmc,



**Figure 2:** Lipid aggregates. A) Micelle, spheroidal aggregate of single tailed fatty acid, the polar heads being in contact outside with water molecules. B) Bilayer, two dimensional fluid, composed of glycerophospholipids or sphingolipids, each molecule possessing two hydrophobic tails. C) Liposome, spheroidal vesicle filled with water, bounded by a single bilayer. D) LDL, lipids/protein/antioxidants aggregate, carrier of cholesterol in blood plasma.

generally lower than  $10^{-6} \text{ mol l}^{-1}$ , and tend to form bilayers (Fig. 2B). Lipid bilayers are two dimensional fluids in which lipid molecules can diffuse laterally but not across the bilayer [6]. Liposomes are vesicles filled with water, constituted of several bilayers (multilamellar vesicles, MLV), which can rearrange to form unilamellar vesicles bounded by a single bilayer (Fig. 2C). According to the size distribution, one considers the small unilamellar vesicles (SUV) with a diameter of about 50 nm and the large unilamellar vesicles (LUV) whose diameter is superior to 100 nm. The liposome size distribution depends largely on the method of production of the vesicles, the sonication (agitation by ultrasonic vibrations) giving mainly SUVs, whereas the extrusion (submission to high pressures) generates LUVs. SUVs being highly curved, the unsaturated fatty acyl chains have a better exposure to water and consequently SUVs are highly oxidizable *via* radiation-induced water free radicals. By contrast, LUVs (high diameters) characterized by a tight packing of phospholipid molecules are less penetrable by water

molecules and consequently less oxidizable than SUVs [8]. Liposomes can be considered as rather good model systems since they reflect the arrangement of lipid bilayers of cell membranes.

However, to get still closer the living membranes, it is necessary to add proteins into the lipid matrix. Lipoproteins are suitable candidates to study lipid peroxidation and protein oxidation under ionizing radiation, allowing to specify lipid/protein interactions. Low Density Lipoproteins (LDLs) (**Fig. 2D**) isolated from human plasma, are composed of a single big protein (apolipoprotein B-100) surrounded by lipids (choline phospholipids, cholesteryl esters and triacylglycerols). In addition, antioxidants (mainly  $\alpha$ -tocopherol and  $\beta$ -caroten) are located in the lipidic part. LDLs are now considered as very good membrane models allowing to determine the reciprocal influence of the oxidation of both lipid and protein moieties at the molecular level, and to specify the role of antioxidants under oxidative stress conditions, specially those of water radiolysis [5, 9]. It can be noticed that other membrane models such as erythrocyte membranes have been investigated under radiation exposure, providing interesting results on the post-irradiation effects to the lipid and protein moieties, together with structural changes in the membrane arrangement (see for example [1]).

### Water radiolysis

When liposome or micelle suspensions in diluted aqueous medium, are irradiated by  $\gamma$ -rays (from a radioactive source of  $^{137}\text{Cs}$  or  $^{60}\text{Co}$ ), they are attacked by the free radicals generated by water radiolysis. Indeed, there is no direct ionisation of the lipids by the  $\gamma$ -rays if their concentration remains lower than  $10^{-2} \text{ mol l}^{-1}$ , whereas the molecules of water (in high concentration of  $55 \text{ mol l}^{-1}$  in aqueous solutions) are the only molecular targets to be ionized (Chapter 1).

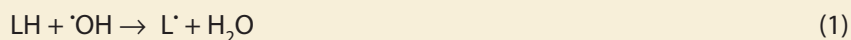
The radiolysis of water leads to the formation of the three radical species  $\cdot\text{OH}$  (hydroxyl radical),  $\text{H}\cdot$  (hydrogen atom) and  $\text{e}_{\text{aq}}^-$  (hydrated electron), within the nanosecond time scale [3]. In aerated medium (that is in the presence of dioxygen,  $\text{O}_2$  concentration dissolved in water being  $2 \times 10^{-4} \text{ mol l}^{-1}$ ), the free radicals  $\text{H}\cdot$  and  $\text{e}_{\text{aq}}^-$  are replaced by  $\text{HO}_2\cdot$  and  $\text{O}_2^{\cdot-}$  radical species, respectively, which are related by an acid/base equilibrium ( $\text{pK}_a(\text{HO}_2\cdot/\text{O}_2^{\cdot-}) = 4.8$ ). The radiolytic yields (G-values expressed in moles per Joule) of each radical species are well known:  $2.8 \times 10^{-7} \text{ mol J}^{-1}$  and  $3.4 \times 10^{-7} \text{ mol J}^{-1}$ , respectively for  $\cdot\text{OH}$  and  $\text{O}_2^{\cdot-}$  free radicals at  $\text{pH} = 7$  (Chapter 1) [3].

Hydroxyl ( $\cdot\text{OH}$ ) and superoxide ( $\text{O}_2^{\cdot-}$ ) radical species are thus the protagonists of the oxidative stress initiated by water radiolysis to diluted biological targets [10]. But they exhibit very different properties against lipids. Hydroxyl free radical is the strongest oxidant

species because its one-electron reduction potential is very high ( $E^\circ \cdot\text{OH}/\text{H}_2\text{O} = 2.34 V_{\text{NHE}}$  at pH = 7, versus the potential of normal hydrogen electrode (NHE) as reference, under standard conditions) and its second order rate constants are diffusion controlled, namely close to the upper limit of  $10^{10}$ - $10^{11} \text{ l mol}^{-1} \text{ s}^{-1}$  (Chapter 1). In contrast, superoxide free radical is not an efficient initiator of lipid oxidation. Indeed, even if its one-electron reduction potential is high enough ( $E^\circ \text{O}_2^{\cdot-}/\text{H}_2\text{O}_2 = 0.93 V_{\text{NHE}}$  at pH = 7), its second order rate constants against lipids are very low, generally lower than  $10^2 \text{ l mol}^{-1} \text{ s}^{-1}$  [11]. In other words, in aqueous medium, superoxide free radicals do not attack lipids. However, it has been shown that their protonated form,  $\text{HO}_2^\cdot$ , can react more rapidly with PUFAs ( $10^3 \text{ l mol}^{-1} \text{ s}^{-1}$  [12]) than does  $\text{O}_2^{\cdot-}$ . Note that at pH = 7,  $\text{HO}_2^\cdot$  radicals represent less than 1% of the total amount of  $\text{O}_2^{\cdot-}$  radicals ( $\text{pK}_a (\text{HO}_2^\cdot/\text{O}_2^{\cdot-}) = 4.8$ ) in the irradiated medium. Hence, it can be assumed that at pH = 7, the predominant initiating species are hydroxyl radicals.

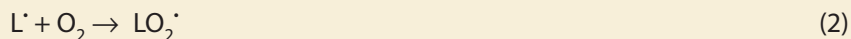
### Radical-chain mechanism of lipid peroxidation

Hydroxyl radicals are able to initiate the one-electron oxidation of unsaturated acyl chains symbolized here by LH (as linoleic acid (18:2), for example), by abstracting a single H-atom from the aliphatic chain leading to a carbon-centred radical  $\text{L}^\cdot$  (alkyl radical) (reaction (1)).

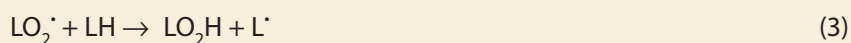


The removed hydrogen atom is preferentially located on a methylene group ( $\text{CH}_2$ ) between two adjacent  $\text{C}=\text{C}$  double bonds (bis-allylic position) (see Fig. 3 for linoleic acid). Indeed, such C-H bonds are weaker than others along the saturated carbon chain and they represent critical target sites [13,14].

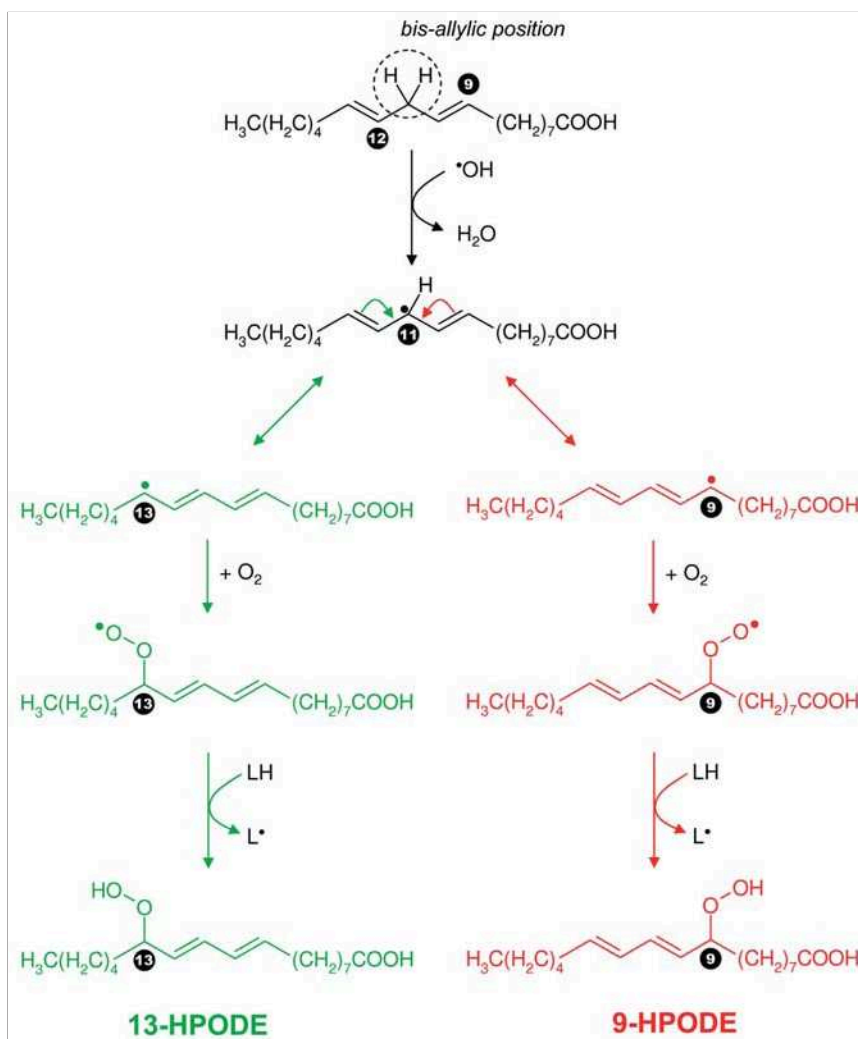
The fate of the carbon-centred radical  $\text{L}^\cdot$  (which is a resonant pentadienyl radical in the case of linoleic acid, Fig. 3) is to lead, in a very rapid reaction with dioxygen, to the formation of a peroxy radical,  $\text{LO}_2^\cdot$  (reaction (2)).



In the case of linoleic acid, two isomeric peroxy radicals are formed (Fig. 3). Such peroxy radical species are expected to abstract H-atoms from other fatty acid molecules (reaction (3)) giving lipid hydroperoxides  $\text{LO}_2\text{H}$  (9 and 13-hydroperoxyoctadecadienoic acid in the case of linoleic acid (Fig. 3)), which are the initial non-radical products formed during lipid peroxidation.



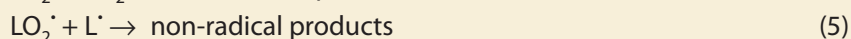
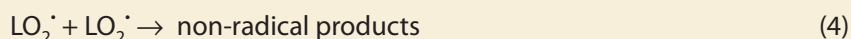




**Figure 3:** Mechanism of peroxidation of linoleic acid (18:2) initiated by  $\cdot\text{OH}$  free radicals from water radiolysis. LH symbolizes another molecule of linoleic acid in micelles. 9- and 13-HPODE are the main hydroperoxides formed, possessing a conjugated dienic structure.

The resulting alkyl free radical  $\text{L}^\cdot$  can in turn react with dioxygen (reaction (2)), propagating the radical-chain mechanism (reactions (2) and (3)) inside the lipid aggregates. These radical-chain reactions amplify the peroxidation process since one single  $\cdot\text{OH}$  radical can lead to several alkyl radicals ( $\text{L}^\cdot$ ), peroxy radicals ( $\text{LO}_2^\cdot$ ) and hydroperoxide molecules ( $\text{LO}_2\text{H}$ ). To stop the radical-chain mechanism, two radical species have to react together (for example reactions (4) and (5), reaction (4) being the most probable in an oxygenated medium) leading to non-radical products.



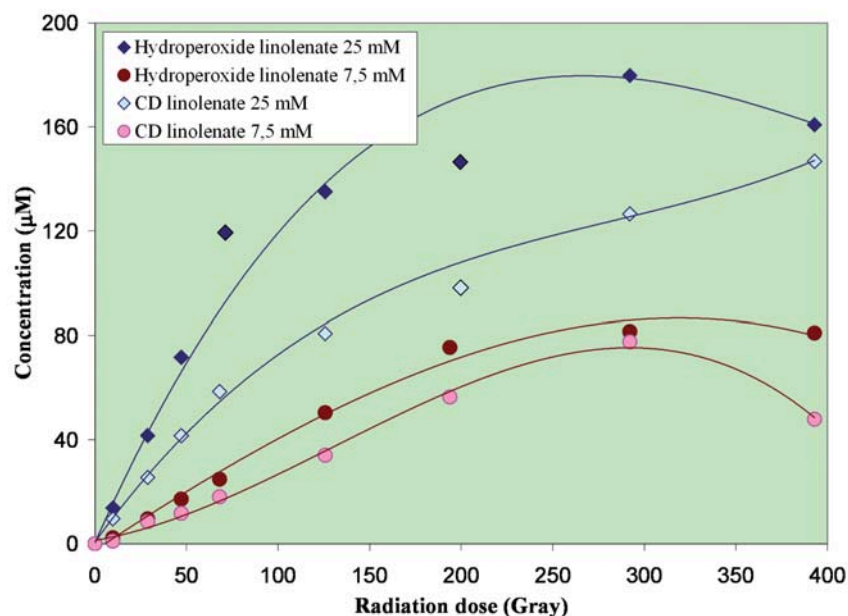


From this free radical-chain mechanism it appears that hydroperoxides ( $\text{LO}_2\text{H}$ ) are the major early reaction products of lipid peroxidation. Their structure are very diversified since it depends on the number of allylic hydrogen atom(s) to be abstracted along the acyl chain of the PUFA (see next section). However, the lipid degradation process does not stop after the formation of hydroperoxides. Indeed, hydroperoxides are not very stable products (apart from irradiation) and they undergo spontaneous decomposition (oxidative cleavage) that generates numerous breakdown products (see section "Oxidative fragmentation of lipids").

### Quantitative determination of hydroperoxides, initial markers of lipid peroxidation

#### *In micelles*

Hydroperoxides ( $\text{ROOH}$ ) are the next non radical products to be formed in aerated medium, after  $\cdot\text{OH}$ -attack on the unsaturated carbon chain. Hence their quantification as a function of radiation doses is particularly of interest. They can be specifically titrated by reverse-phase HPLC (High Pressure Liquid Chromatography) using detection by chemiluminescence [15,16]. As it can be shown on a simple lipidic model such as linolenate (18:3) micelles, increasing radiation doses lead to increasing hydroperoxide concentrations (Fig. 4). This  $\text{ROOH}$  formation is linolenate concentration-dependent, *i.e.* at a given radiation dose, hydroperoxide concentration is higher in the presence of 25 mM than of 7.5 mM linolenate (Fig. 4). The highest part of hydroperoxides comes generally from the H-abstraction on the bis-allylic positions of PUFAs (see section above). Thus conjugated dienes are simultaneously formed on the unsaturated carbon chain. Hence conjugated dienes (CD) are also initial markers of lipid peroxidation. They can be determined by spectrophotometric absorption at 234 nm (maximum absorption wavelength), with a molar extinction coefficient of  $28000 \text{ l mol}^{-1} \text{ cm}^{-1}$  [17]. As it can be seen in Figure 4, the concentration of conjugated dienes formed in linolenate micelles are increasing with the radiation dose, and they are, like hydroperoxides, linolenate concentration-dependent. However, for a given linolenate concentration, the CD concentration is lower than that of hydroperoxides whatever the radiation dose, meaning that some hydroperoxides do not possess a dienic structure.

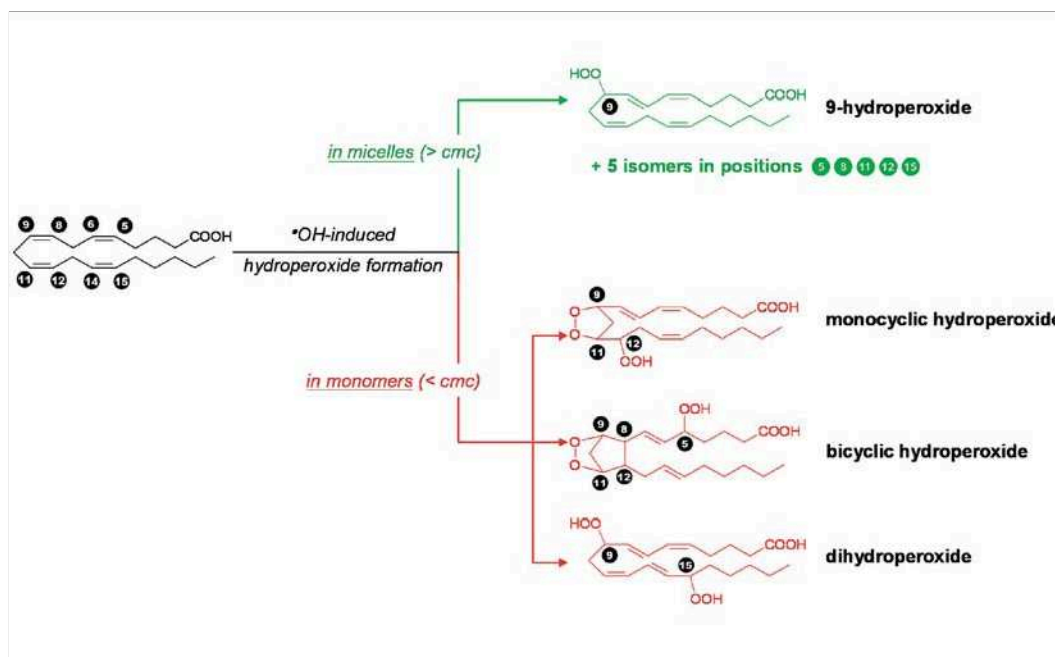


**Figure 4:** Formation of hydroperoxides and conjugated dienes (CD) as a function of the radiation dose (dose rate =  $9.8 \text{ Gy} \cdot \text{min}^{-1}$ ) in linolenate (18:3) aqueous micellar solutions at pH = 10.5. Influence of the concentration of linolenate (25 mM and 7.5 mM) on the peroxidation process [16].

The radiolytic yields of formation of hydroperoxides ( $G(\text{hydro})$ ) are determined from the slope of the initial tangent of the curves (concentration versus radiation dose, as in Fig. 4). An important lipid concentration dependency is observed in micellar medium where, above the cmc, the yields of oxidized products formation increase steeply with PUFA concentration. This means that a chain-mechanism is involved in the formation of hydroperoxides and conjugated dienes. As an example, in the case of a 25 mM linolenate concentration (Fig. 4), the  $G$ -values are  $20.0 \times 10^{-7} \text{ mol J}^{-1}$  and  $9.3 \times 10^{-7} \text{ mol J}^{-1}$  for hydroperoxides and conjugated dienes, respectively. These yields are considerably larger than  $G_{\text{OH}} = 2.8 \times 10^{-7} \text{ mol J}^{-1}$ , since the hydroxyl radicals are initiators of the oxidation chain mechanism.

It has been demonstrated, not only with linolenate (18:3) aqueous solutions but also with linoleate (18:2), and more recently with arachidonate (20:4) solutions [18], that the hydroperoxides were composed of two different kinds of molecules according to the concentration of PUFA in the irradiated medium. Indeed, for a given PUFA, above the critical micellar concentration, namely when micelles are formed, one type of hydroperoxide predominates, whereas when monomers (or very small aggregates) are dispersed in solution (below the cmc), other types of hydroperoxide are formed. This phenomenon is illustrated in Figure 5 for arachidonate. We can see that monohydroperoxides are produced in micelles whereas in monomers, either a cyclic hydroperoxide or an aliphatic dihydroperoxide is obtained [18].

Such differences seem to arise from the mechanism of their formation which privileges a self-rearrangement in monomers, instead of an intermolecular reaction in aggregates. The ratio of “micellar” hydroperoxide concentration over “monomer” hydroperoxide concentration appears to be a signature of the balance micelles/monomers in aqueous medium [18].



**Figure 5:** Proposed structures of hydroperoxides formed in arachidonate (20:4) aqueous solutions ( $\text{pH} = 10.5$ ) submitted to  $\gamma$ -rays. Monohydroperoxides (6 isomers in positions 5, 8, 9, 11, 12, 15) are formed in micellar medium (high arachidonate concentration) whereas monocyclic and bicyclic hydroperoxides, together with dihydroperoxides are produced in dispersed monomers (low arachidonate concentration) [18].

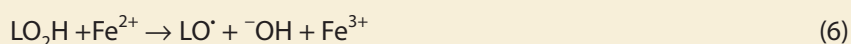
### In liposomes

In the case of liposomes of PLPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine) submitted to free hydroxyl radicals attack (generated by  $\gamma$ -radiolysis of water), phosphatidylcholine hydroperoxides are the initial markers of lipid peroxidation. Since the molecule of PLPC contains only one bis-allylic position located on the linoleyl acyl chain, the  $\cdot\text{OH}$ -induced hydroperoxides are characterized by the OOH groups at positions 9 and 13 on the linoleyl chain, as in the case of linoleate micelles (Fig. 3). Moreover, these hydroperoxides exhibit a conjugated dienic structure. A G-value of total hydroperoxide formation has been determined to be equal to  $6 \times 10^{-7} \text{ mol J}^{-1}$ , in  $\gamma$ -irradiated small unilamellar vesicles (SUVs) of PLPC  $250 \mu\text{mol l}^{-1}$  [19]. Such a G-value which is 2 fold the G-value of  $\cdot\text{OH}$ -formation, also indicates a chain-mechanism of hydroperoxide production. In addition, other products such as hydroxides, epoxides and

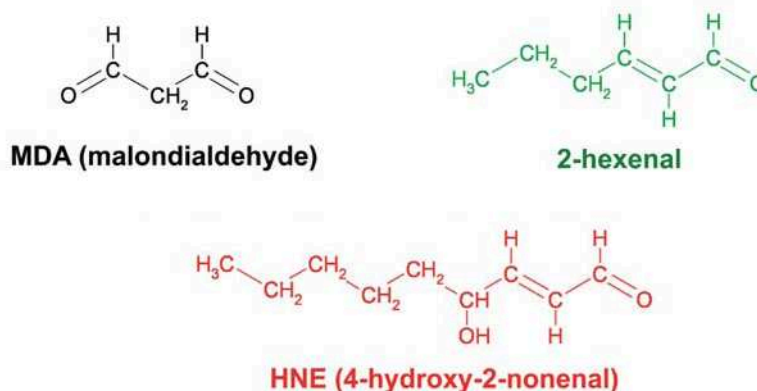
fragments have been identified using HPLC/MS analysis. In LUVs (high diameters), because of the tight packing of PLPC molecules,  $\cdot\text{OH}$  radicals have to react first with the polar head before reaching the linoleyl chains, and for this reason kinetic results are very different between LUVs and SUVs peroxidation, LUVs being less oxidizable than SUVs [8].

### Oxidative fragmentation of lipids

Lipid hydroperoxides are not very stable products and their decomposition can lead to the formation of a lot of breakdown products such as short-chain aldehydes (**Fig. 6**), epoxides, ketoaldehydes, and alkanes (pentane, hexane, for example). These hydroperoxide decomposition may result from the action of traces of reducing metal cations ( $\text{Fe}^{2+}$ ,  $\text{Cu}^{+}$ ) which catalyses the cleavage of hydroperoxides according to "Fenton like reactions" (reaction (6)), in which  $\text{LO}_2\text{H}$  acts like  $\text{H}_2\text{O}_2$ . The alkoxyl radicals  $\text{LO}\cdot$  so formed are able to abstract H-atom leading to hydroxylated products ( $\text{LOH}$ ) or to generate epoxy-allyl radical by a self-addition on a double bond or still to undergo a scission giving aldehydic products [13]:



Consequently, in addition to hydroperoxides, a lot of secondary oxidized lipidic compounds, mainly short-chain aldehydes, may appear and represent late markers of lipid peroxidation. As an example, malondialdehyde (MDA, **Fig. 6**) is known to be the most abundant lipid peroxidation aldehyde whose determination by 2-thiobarbituric acid (TBA) is one of the most common assays in lipid peroxidation studies [20]. However, it can be noticed that the TBA assay method [21] is not specific of MDA titration since it also can detect a variety of peroxides and secondary degradation products of lipid peroxidation called



**Figure 6:** Short-chain aldehydic products resulting from the oxidative fragmentation of lipid hydroperoxides.

2-thiobarbituric acid-reactive substances (TBARS). Hence, the determination of TBARS must be used as a global index of lipid peroxidation. Aldehydic molecules are involved in cytotoxic processes because they can easily react with DNA, protein, and phospholipids leading to covalent adducts. In the case of the 4-hydroxy-2-nonenal (HNE, **Fig. 6**) which is produced during peroxidation of PUFAs such as linoleic and arachidonic acids, HNE is known to react with amino acids (cystein, histidine, lysine) and with cellular target proteins, inactivating enzymes [20].

However, hydroperoxides decomposition is not the only process undergoing short-chain lipid fragments. Indeed,  $\gamma$ -radiolysis of multilamellar liposomes of glycerophospholipids such as cardiolipin, phosphatidylglycerol and phosphatidylinositol, has been characterized by the formation of phosphatidic acid resulting from the cleavage of the phosphatidylglycerol moiety, as the main product [4]. Hence the polar heads of glycerophospholipids appear to be targeted by  $\cdot\text{OH}$ -attacks. The determination of phosphatidic acid has been performed using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry [4]. The formation of carbon-centred free radicals, in  $\beta$ -position with respect to the phosphoester bond and containing a hydroxyl group, seems to be a key step of this type of fragmentation process. A similar mechanism has been proposed to explain the formation of lysophosphatidylcholine in  $\gamma$ -irradiated liposomes of PLPC (LUVs) [8]. Radiation-induced free radical fragmentation of lipids in their polar moiety seems to play an important role as signaling molecules in living systems [4].

### Peroxidation of low density lipoproteins

It has well been established that PUFAs contained in phospholipids and cholesteryl esters (see composition of LDLs in section "Lipid aggregates and model systems", and in **Fig. 2D**) are targeted sites of radiation-induced oxygen free radicals attack in LDL particles. Mechanisms of lipid peroxidation of LDLs, initiated by oxygen free radicals such as  $\cdot\text{OH}$ ,  $\cdot\text{OH}/\text{O}_2^{\cdot-}$ ,  $\text{O}_2^{\cdot-}/\text{HO}_2^{\cdot}$  have been proposed, based on the determination of the radiolytic yields of consumption of  $\alpha$ -tocopherol and  $\beta$ -carotene (endogenous antioxidants of LDL), and of formation of conjugated dienes and TBARS (for a review see [5]). However, as we have already seen, TBARS are not specific markers of lipid peroxidation (section above). Recently, the determination of defined hydroperoxides such as hydroperoxides of phosphatidylcholines (linoleate, arachidonate and docosahexaenoate) and of cholesteryl esters (linoleate and arachidonate) have been developed using HPLC coupled with a chemiluminescence detection [9]. These hydroperoxide formations are kinetically characterized by three steps, namely (1) at low radiation doses, the inhibition step resulting from the presence of  $\alpha$ -tocopherol and  $\beta$ -carotene, able to protect LDL by scavenging radiolytic-induced oxygenated free radicals, (2) at higher radiation doses, the propagation step resulting from the lipid peroxy radicals

formation ( $\text{LOO}^\bullet$ ) by a chain-mechanism, leading to hydroperoxides of phosphatidylcholines and of cholesteryl esters, and (3) the chain-termination and decomposition phase. The kinetic behaviour of conjugated dienes is similar to those of hydroperoxides, the propagation step beginning at the end of the inhibition step, when there is no more antioxidants. As an example of quantification of lipid markers for LDLs  $3 \text{ g l}^{-1}$ , the radiolytic yield of CD formation ( $3.4 \times 10^{-7} \text{ mol J}^{-1}$ ) is found slightly higher than  $G_{\text{OH}}$  ( $2.8 \times 10^{-7} \text{ mol J}^{-1}$ ) due to the presence of a short chain reaction, and the G-value of hydroperoxide formation is 10 times lower than the yield of CD production, meaning that conjugated dienes other than hydroperoxides (such as alcohols, epoxides and endoperoxides) are taken into account [9]. Besides, it has been shown that the phosphatidylcholines located on the outer surface of LDLs were more accessible than the cholesteryl ester molecules (in the lipidic core), to the free radicals attack.

In addition, the protein moiety contained in LDLs (apoB-100) is also targeted by  $^\bullet\text{OH}$  radicals, since western blot immunoassays allow to detect carbonyl groups ( $\text{C}=\text{O}$ ) into protein side chains [9]. These assays involve the derivatization to 2,4-dinitrophenylhydrazone of the carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH). It has been shown that carbonylated apoB-100 appeared at low radiation doses, during the inhibition phase of CD and hydroperoxides, meaning that the protein was not protected by the endogeneous antioxidants which are localized in the lipid part of LDLs and that the polypeptidic chain was attacked by  $^\bullet\text{OH}$  free radicals. This apoB-100 carbonylation was radiation dose-dependent, its radiolytic yield being relatively low ( $1.8 \times 10^{-8} \text{ mol J}^{-1}$ ) because  $^\bullet\text{OH}$  free radicals simultaneously react on all other molecular components of LDLs (lipids, antioxidants, ...). Moreover, the fragmentation of the carbonylated apoB-100 may occur, but this process which only begins during the lipid peroxidation propagation phase, seems to be related to the interactions between the oxidized parts of lipids and protein. Hence, whereas radiolytic-induced apoB-100 carbonylation appears to be independent on lipid peroxidation, in contrast oxidative fragmentation seems to be dependent on it. All these results obtained from radiolysis conditions represent an accurate and rigorous approach of the oxidative stress phenomena which can be responsible of the *in vivo* peroxidation of LDLs leading to atherosclerosis [5].

## Conclusion

Effects of ionizing radiation on lipid molecules have been understood by studying model systems which are simpler than the real biological membranes, such as PUFA micelles and liposomes. The formation of lipid oxidative modifications of PUFAs appears as a dynamic process initiated by hydroxyl free radicals generated by water radiolysis, amplified by a propagating-chain mechanism involving alkyl and peroxy free radicals, and leading not only to hydroperoxides but also to a lot of other lipidic oxidized end-products. Kinetic data, such

as radiolytic yields of production of hydroperoxides and conjugated dienes which are early products of lipid peroxidation, have allowed us to establish the reaction schemes of their formation. Knowing that hydroperoxides and other lipid oxygenated products are more hydrophilic than the initial hydrocarbon chains of PUFAs, their appearance can lead to morphological changes in the bilayers, together with disturbances in membrane functions. Moreover, hydroxyl free radicals may initiate the oxydative processes not only within the hydrophobic moiety of lipids but also in the hydrophilic polar heads leading to fragments such as phosphatidic acid and lysophosphatidylcholine residues. Such fragments seem to regulate a variety of cellular functions and to stimulate various signalling pathways. Hence, membrane constituents which are molecular targets of ionizing radiations have to be considered as important factors the damages of which are susceptible to largely control the viability of the cells.

### Acknowledgments

I am especially indebted to Fabrice Collin for his talented designs of lipid aggregates and molecular mechanisms and for his judicious comments. I would like to acknowledge Prof. Dominique Bonnefont-Rousselot for her precious collaboration all along the story of oxidized lipoproteins (EA 3617, *Faculté de Pharmacie, Université Paris 5*).

### References

- [1] Benderitter M., Vincent-Genod L., Pouget J.P., Voisin P., The cell membrane as a biosensor of oxidative stress induced by radiation exposure: a multiparameter investigation, *Radiat. Res.*, 2003, **159**, 471-483.
- [2] Albanese J., Dainiak N., Modulation of intercellular communication mediated at the cell surface and on extracellular, plasma membrane-derived vesicles by ionizing radiation, *Experimental Hematology*, 2003, **31**, 455-464.
- [3] Spinks J.W.T., Woods R.J., Water and inorganic aqueous systems, in "Introduction to Radiation Chemistry", 3rd ed., Wiley, New York, 1990, p. 243-313.
- [4] Shadyro O.I., Yurkova I.L., Kisel M., Brede O., Arnhold J., Formation of phosphatidic acid, ceramide, and diglyceride on radiolysis of lipids: identification by MALDI-TOF mass spectrometry, *Free Radical Biology and Medicine*, 2004, **36**, 1612-1624.
- [5] Bonnefont-Rousselot D., Gamma radiolysis as a tool to study lipoprotein oxidation mechanisms, *Biochimie*, 2004, **86**, 903-911.
- [6] Voet D., Voet J.G., Lipids and membranes (Chapter 11), in "Biochemistry", 2nd ed., Wiley & Sons, INC., New York, 1995, p. 277-329.
- [7] Hauville C., Rémita S., Théron P., Rouscilles A., Couturier M., Jore D., Gardès-Albert M., Determination of the yield of radiation-induced peroxidation of sodium linoleate in aqueous monomeric and micellar solutions, *Radiat. Res.*, 1998, **150**, 600-608.