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Review

Thiyl radicals in biosystems: effects on lipid structures and metabolisms

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Abstract. Thiyl radicals are intermediates of enzyme- and radical-driven biochemical processes, and their potential as reactive species in the biological environment has been somehow underestimated. From organic chemistry, however, it is known that thiyl radicals isomerize the double bonds of unsaturated fatty acids to a mixture with very dominating *trans* isomers. Recently, this reaction has been particularly studied for biosystems, focusing on the effect of thiyl radicals on the natural all-*cis* double bonds of unsaturated phospholipids, which undergo a conversion to the unnatural *trans* form. In this paper we report briefly the role of thiyl radicals in biosystems, describe the main features of the radical-induced *cis-trans* isomerization process under both in vitro and in vivo conditions, and reflect on some consequences for membrane structures, lipid metabolism and enzymatic reactions.

Key words. Thiyl radicals; *cis-trans* isomerization; unsaturated fatty acids; *trans* lipids; lipid metabolism; membrane phospholipid.

Introduction

This review will focus on some aspects of thiyl radical reactivity towards lipids and the biological implications that have emerged in the last years. In particular, the studies of thiyl radical-catalysed *cis-trans* isomerization process, which causes a structural change of the naturally occurring *cis* unsaturated fatty acid residues in lipid molecules, will be described. Data available on thiyl radical reactivity in solution and in liposomes will be presented. This biomimetic model has enabled study of the effect of different thiol compounds and the factors which influence the isomerization process and exploration of the properties of membranes when *cis* or *trans* lipids are present. Recent achievements in the formation of thiyl radicals from thiols during normal metabolism will be described as well as their relationship to detection of *trans* isomers of mono- and polyunsaturated fatty acid residues in membrane phospholipids. It must be pointed out that the occurrence of *trans* lipids was first reported in nutrition research and was correlated to the consumption of *trans* fatty acids from foods [1]. As a matter of fact, the most relevant findings on the consequences of *trans* lipids on cell metabolism and human health came from these studies. Chemical and biochemical research on *trans* lipids formed by sulfur-centered radicals aim at giving a different perspective of the occurrence of these isomers in the biological environment, suggesting that their origin can be due to both exogenous and endogenous paths, namely dietary supplementation and thiyl radical damage, respectively.

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Thiyl radicals as intermediates in biochemistry

Functionalized aliphatic thiols (RSHs) are contained in living organisms in considerable amounts. Typical levels of intracellular non-protein thiol (principally glutathione, GSH) are in the order of 5 mM, although values of around 10 mM have been reported [2]. The total level of protein SH may approach or exceed that of GSH. For example, metallothionein in proteins with a low molecular mass found in the cytosol of eukaryotic cells are rich in sulfur (23–38% of amino acids are cysteines). Therefore, they represent a significant portion of total cell protein thiols [3]. The thiol group of the main sulfur-containing amino acid, cysteine, is found predominantly in the unionized state under physiological conditions, being the thiol pK_a in the range of 8–9. It is worth pointing out that for proteins the value of pK_a may vary substantially, as reported for example in case of thioredoxin [3a]. However, an increase in pH will increase the fraction of ionized molecules. Once formed, the thiolate specie RS^- is one of the most reactive functional groups found in proteins. It can react as a nucleophile and attack a disulfide bond ,R_1-S-S-R_2, displacing one sulfur atom and forming a new disulfide bond, R-S-S-R_1 or R-S-S-R_2. Such disulfide bond formation is a versatile oxidation that is used biologically in a diversity of processes such as enzyme catalysis, protection against oxidative damage, stabilization of extracellular proteins and regulation of biological activity. This reaction exchanges redox equivalents between different thiol/disulfide pairs and serves to oxidize one thiol (RSH) while reducing another disulfide (R_1-S-S-R_2) [4, 5]. Recently, its implication in signal transduction and regulation of redox transcription factors was shown [5]. The oxidative folding of proteins and the catalysis given by protein disulfide isomerases (PDIs), as chaperone-like activity, are also based on the regeneration of disulfide bonds by oxidation [5a, 5b].

Several investigations have focused on the reactivity of thiol groups based on the production of sulfur-centered radical species, RS$^\bullet$. Thiyl radical generation turned out to be important for several biological mechanisms, such as the enzymatic functioning of ribonucleotide reductase and pyruvate formate lyase [6, 7]. It must be taken into account that different sulfur moieties are present in biomolecules, that is, RSHs, disulfide (RS-SR), thioester [RS-C(O)R′] and thioether functionalities (RSR′), from which pathways can be described for the generation of thiyl radicals.

The most relevant pathway is from thiols, which is well known as the 'repair' reaction: a thiol RSH is a good hydrogen atom donor towards most C-centered radicals generated from the breakage of a C-H bond, which can occur through enzyme activity, radiation or toxic agents [8]. The repair of radical DNA species shown in equation 1 was mostly investigated in radiation studies of biomolecules [9]. Among others, this reaction provides a rationale for the pharmacological application of thiols as radiation-protecting substances, certainly in sense of repair reactions instead of primary shielding [10–12].

$$DNA^\bullet + RSH \rightarrow DNA + RS^\bullet \quad (1)$$

An example is given by GSH as the dominant intracellular antioxidant, which exerts its activity in the aqueous phase by enzymatic peroxide reductions as well as by repairing radical species, such as tyrosyl (PhO$^\bullet$) or peroxyl (ROO$^\bullet$) radicals, as shown in equation 2.

$$PhO^\bullet \text{ (or } ROO^\bullet) + RSH \rightarrow PhOH \text{ (or } ROOH) + RS^\bullet \quad (2)$$

It is worth pointing out that the role of thiols as repairing agents is counterbalanced by the formation of thiyl radical species which can damage other biomolecules, such as amino acids [12a], carbohydrates [12b] and, as will be discussed in this review, lipid molecules**.** That is why thiols can be considered a double-edged sword in the biological environment [13].

From disulfide functionalities thiyl radical species can be generated as depicted in equations 3 and 4. In the first case the sulfur-sulfur bond can be directly broken under free-radical conditions. In the second case single-electron reduction processes generate disulfide radical anion species, which are in equilibrium with thiolate and thiyl radicals [14].

$$X^\bullet + RS\text{-}SR \rightarrow RSX + RS^\bullet \quad (3)$$

$$RS\text{-}SR + e^- \rightarrow (RSSR)^{\bullet-} \rightarrow RS^\bullet + RS^- \quad (4)$$

From all functionalities, including thioether and thioester groups, thiyl radicals can be generated by ultraviolet (UV) photolysis, which causes direct homolysis of the C-S bond as shown for thioethers in equation 5 [15].

$$R\text{-}S\text{-}CH_2R^\bullet \xrightarrow{h\nu} RS^\bullet + {}^\bullet CH_2R \quad (5)$$

Thiyl radicals can be also formed by single-electron oxidation. Hence heavy metal ions such as Fe^{3+} , Cu^{2+} or others are able to oxidize thiols according to equation (6) [16, 17, 17a], and at natural pH, thiyl radicals in the deprotonated state can be formed via a complex intermediate. The presence of several iron-sulfur proteins in the biological environment, having different types of coordination between cysteine residues and iron centers [18], could make this thiyl radical generation path an interesting subject of investigation.

$$RSH + Fe^{3+} \xrightarrow{-H^+} (RS^-)\text{-}Fe^{3+} \rightarrow RS^\bullet + Fe^{2+} \quad (6)$$

Thiyl radicals were considered to be less reactive species because of their deactivation due to dimerization to disul-

fides (eq. 7). However, in vivo the steady-state level of $RS^{\bullet}$ is normally expected to be low, and radicals are unlikely to meet.

$$2\ RS^{\bullet} \rightarrow R\text{-}S\text{-}S\text{-}R \quad (7)$$

An efficient quenching of thiyl radicals in the biological environment is given by ascorbate anions in the aqueous phase which regenerate thiol compounds and form ascorbate radical anions, as shown in equation 8 [14].

$$RS^{\bullet} + AscH^{-} \rightarrow RSH + Asc^{\bullet -} \quad (8)$$

The reactivity of sulfur-centered radicals is also well known in organic chemistry, and there are two processes efficiently carried out by these species: reversible addition to double bonds and H-atom abstraction from activated positions such as bisallylic hydrogen [13, 19–21]. Only recently have these two reactions been taken into consideration for biologically related mechanisms, and in this context lipids have become an interesting target since their structures have reactive sites, such as C=C double bonds and bis-allylic positions.

Lipid *cis-trans* isomerization by thiyl radicals in homogenous systems

Early work on the thiyl radical-catalysed isomerization of alkenes was carried out by Walling [22]. The reaction was applied by Sgoutas and Kummerow [23] in the case of fatty acid methyl esters (FAMEs), although the conditions were typical of an organic transformation. More recently, *cis-trans* isomerization has been reported as part of a biologically related process with mono- and polyunsaturated fatty acid (MUFA and PUFA) derivatives, including unsaturated phospholipids. The groups involved in this review contributed to the reconsideration of this reaction and of its biological implications [24, 25].

A variety of thiol compounds have been used for the generation of thiyl radicals, ranging from hydrophobic molecules such as phenylthiol, PhSH or MECH, i.e. [3-(2-mercaptoethyl)quinazoline-2,4(1H,3H)dione] [24, 26], to amphiphilic thiols such as 2-mercaptoethanol [25, 27], which allowed phospholipid reactivity to be compared in homogenous and heterogenous (aqueous) systems. Finally, the use of other hydrophilic thiols, such as 2-mercaptoethylamine [26, 28], and more biologically relevant compounds, such as cysteine, glutathione, methionine or some sulfur-containing proteins [25, 27, 29], allowed a radical stress in biomimetic and cellular systems to be more specifically considered.

A summary of the main biosynthetic pathways of saturated, MUFA and PUFA (n-6 and n-3 series) residues is given in figure 1, where the IUPAC (International Union of Pure and Applied Chemistry) nomenclatures presenting the length of the carbon atom chain, the number and the position of double bonds are paired with trivial names, when available, that are still largely used in lipid chemistry and biochemistry.

For the generation of thiyl radicals, the initiation step is represented by H-atom abstraction from a thiol moiety RSH carried out by generation of initiating radical species. Several initiation methodologies are available, including thermal decomposition of azocompounds or peroxides, radiolysis and photolysis. All of these methods can be successfully used, and the information obtained by

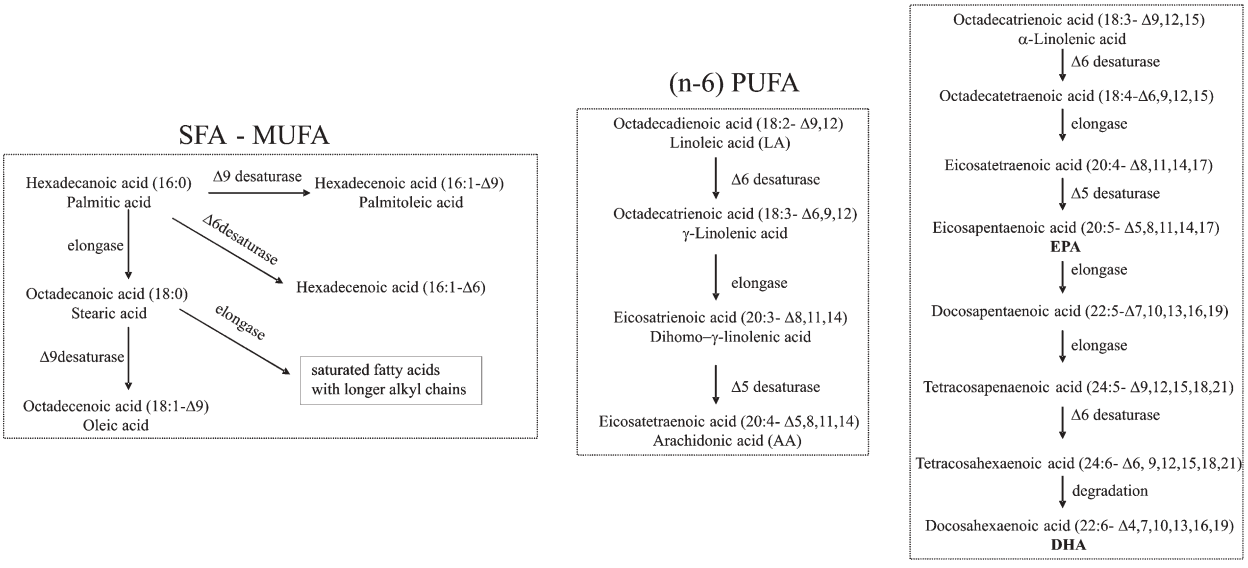
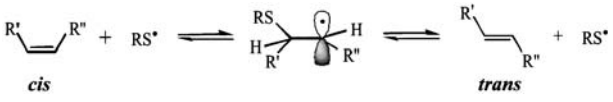


Figure 1. Biosynthesis and nomenclatures of saturated, mono- and polyunsaturated fatty acids.

the different techniques can be interfaced in a complementary way to understand several damaging contributions. For example, gamma radiolysis can be particularly useful as a generation path of a low, continuous and controllable flux of radicals, rather than a bolus addition. This holds in particular for investigating processes caused by oxidative stress and other radical-generating conditions in a complex situation such as the cell environment.

The reactivity of the double bond in the case of MUFA and PUFA derivatives or in the case of phospholipids was first considered in a homogenous system, where these substrates are completely dissolved. This means that reactions were studied in alcoholic solutions. Alcohols are indeed good solvent systems for lipids.

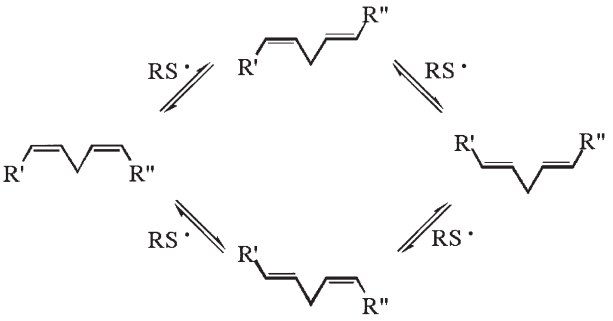
The mechanism shown in scheme 1 operates in the *cis-trans* isomerization process and includes the thiyl radical addition to a monounsaturated compound with formation of a radical intermediate, where the carbon-carbon bond can rotate. Upon fragmentation of the thiyl radical moiety, *cis* or *trans* double bond is generated together with thiyl radical, which continues the cycle. Because the *trans*-constellation is energetically preferred by about 0.6–1 kcal mol^{-1}, in the equilibrium state a mixture of dominating *trans* olefin (about 80%) and *cis* isomer (about 20%) is reached independently starting with pure *cis* or *trans* isomer [25–28].



Scheme 1. Mechanism of thiyl radical-catalysed *cis-trans* isomerization.

For example, for a monounsaturated FAME a *cis/trans* ratio of 13/87 was found at 22 °C, which is in agreement with an enthalpy difference of 1 kcal/mol between the two isomers at this temperature [25, 27]. It has been demonstrated that *trans* isomers are also the favoured kinetic products since the fragmentation process from the radical intermediate tends to give *trans* isomers rather than *cis* alkenes [30]. Competition experiments using different monounsaturated compounds showed that in solution all double bonds are equivalent and isomerization is a random process without preference for the position of the double bond along the structure [27, 28, 31, 32].

PUFA substrates in homogeneous solution gave a statistical isomerization of the double bonds within error limits. PUFA *cis-trans* isomerization is a more complex process which proceeds via a step-by-step mechanism shown in scheme 2 [26, 28, 33, 34]. Considering a standard substrate containing two double bonds, the first step is the formation of mono-*trans* isomers. Then, by progressive formation of the intermediate mono-*trans* products in the reaction medium, thiyl radicals also start to add to these compounds, and di-*trans* isomer is formed.



Scheme 2. Isomerization mechanism with more than one double bond.

Figure 2 shows the time course of a standard isomerization of linoleic acid methyl ester (9c,12c-18:2) in solution carried out by 2-mercaptoethanol under radiolytical conditions.

In the case of phospholipid substrates of natural sources such as lecithin, different fatty acyl chains can be present in positions 1 and 2 of L-glycerol. Figure 3 shows the general structure of L-α-phosphatidylcholine where R_1 and R_2 are the fatty acyl chains, together with the percent composition of a commercial egg lecithin, which contains oleate (9c-18:1), linoleate (9c,12c-18:2) and arachidonate (5c,8c,11c,14c-20:4) as the main unsaturated residues.

In figure 4 time courses of the disappearance of *cis* fatty acid residues in egg lecithin isomerization by 2-mercaptoethanol in alcoholic solutions are shown, where the double-bond content of the different fatty acids is normalized to 100% for a better comparison [30, 31].

It is easy to see that isomerization in homogeneous systems randomly involves all double bonds, with arachidonate residues containing four unsaturations, which is twice more reactive than linoleate and so on. The inset of

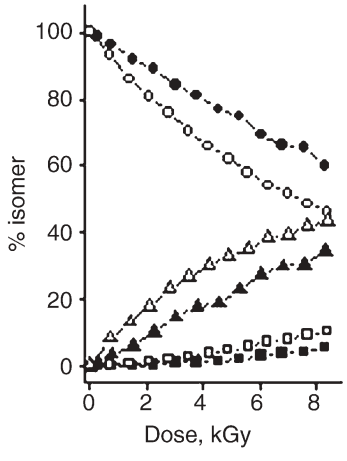
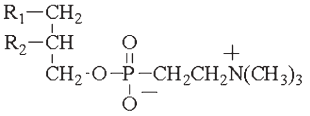


Figure 2. Time course of *cis/trans* isomerization by γ-radiolysis of methyl linoleate with 75 mM 2-mercaptoethanol in alcohol at 22 °C with (black) or without (white) oxygen: (●, ○) 9*cis*,12*cis*-18:2; (▲, Δ) 9*cis*,12*trans*-18:2 and 9*trans*,12*cis*-18:2 (□, ■); 9*trans*, 12*trans*-18:2. Reproduced with permission from [30]. Copyright 2001 Am. Chem. Soc.



Palmitoyl	32.0%
Stearoyl	14.1%
Oleoyl	27.0%
Vaccenoyl	1.2%
Linoleoyl	20.0%
Arachidonoyl	4.8%

Figure 3. L-α-phosphatidylcholine and a commercial egg yolk lecithin composition. R_1 and R_2 are fatty acid residues whose relative percentages are indicated in the square.

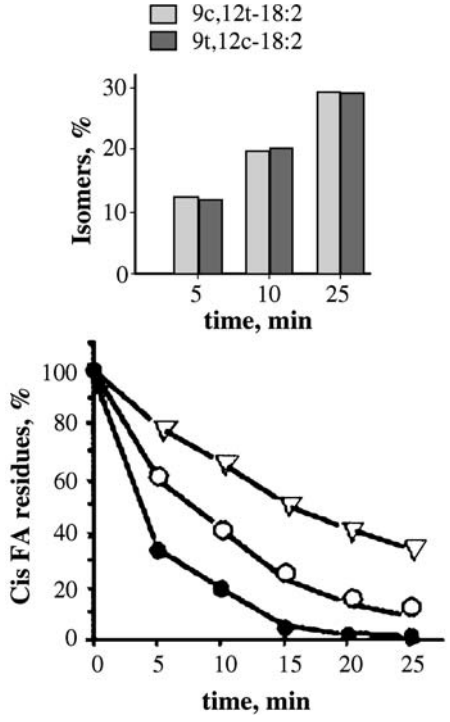


Figure 4. Egg lecithin fatty acid isomerization time course by ultraviolet (UV) photolysis in alcohol in the presence of 7 mM 2-mercaptoethanol at 22 °C: (●), arachidonate residues; (○), linoleate residues; (▽), oleate residues. Inset: formation of linoleic mono-*trans* isomers, Reproduced with permission from [31]. Copyright 2004 Am. Chem. Soc.

figure 4 reports the relative amounts of mono-*trans* isomers of linoleic acid formed in solution: they are equal, as expected from a random process.

In the case of PUFA substrates thiyl radicals are ABLE to react not only with double bonds but also with activated positions, such as bisallylic H atoms, carrying out a hydrogen abstraction as shown in scheme 3.

Scheme 3. H-atom abstraction from a bisallylic position by thiyl radicals.

Indeed, this reaction demonstrated the damaging role of thiyl radicals to biomolecules for the first time, and it was supported by the formation of a well-defined optical absorption at 280 nm attributable to a bisallylic radical. It must be pointed out that no product study was provided [21], as for example the isolation of the corresponding conjugated dienes. From the product isolation and characterization of PUFA isomerization reactions in the presence of alkanethiols, geometrical *trans* isomers were found to be nearly exclusively formed from an alkyl thiyl radical-catalysed process on polyunsaturated substrates [26, 33, 34]. The different information derived from time-resolved spectroscopy and product studies indicate the need for further investigation into this intriguing mechanism.

The role of oxygen in these reactions was also considered, and the isomerization of lecithin in alcohol was carried out in the presence of $[O_2] = 2.34 \times 10^{-4}$ mol dm^{-3} [33]. Thiols are known to inhibit lipid peroxidation [10–12]. Indeed, no lipid consumption due to oxidative degradation could be detected, whereas the isomerization occurred efficiently in quantitative yield (see fig. 2). The importance of lipid damage for biological and medical implications brought to the development of isomerization studies in model membranes, as described in the next section.

Organized systems: *cis-trans* isomerization of unsaturated fatty acid residues in membrane lipids

Liposomes have been used in biologically related studies of thiyl radical effects on lipid structures, since they are widely accepted as a model of membrane lipid assembly [36]. Both multilamellar (MLV) and small or large unilamellar (SUV, LUV) vesicles are frequently used as models for reactions involving lipids, such as peroxidation processes and effect of antioxidants against this chain reaction [37, 38]. Since the choice of biomimetic models is very important to extrapolate results obtained in organic solution to biological conditions, lipid isomerization was first modelled in MLV vesicles. Then, large unilamellar vesicles obtained by an extrusion technique (LUVET) were found to be very useful in these experiments [39]. Vesicles with a diameter of 100 nm form an almost transparent suspension which was also suitable for studies under photolytic conditions.

Fatty acid double bonds have a precise disposition in the hydrophobic core of this membrane model, which is governed by the supramolecular arrangement of polar heads and apolar fatty acid tails. Figure 5 represents a sketch of a vesicle made by different unsaturated fatty acid residues, which are packed in the bilayer so that the higher the carbon atom number of the double bond in the molecule, the deeper the double-bond location with respect to the bilayer borders. This means that the double bonds are not reached at the same time and are not equivalent when a radical species diffuses from the aqueous

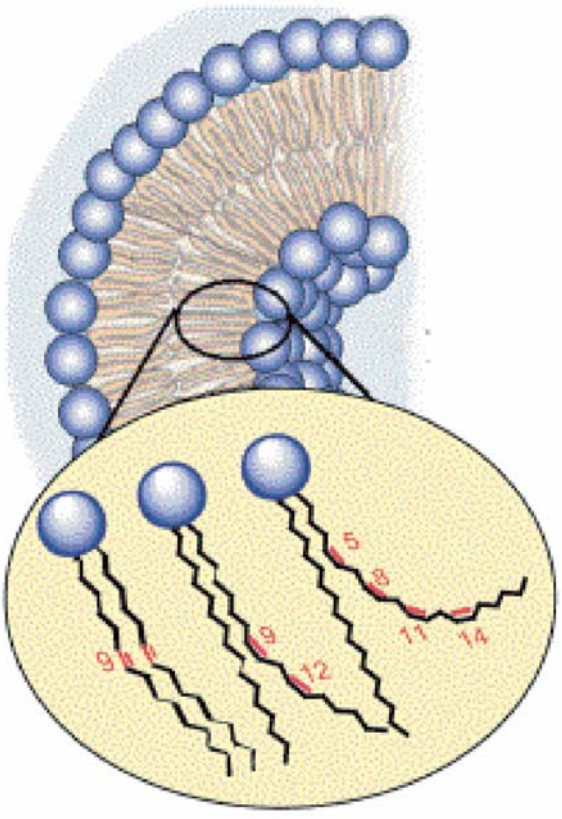


Figure 5. Membrane model with different fatty acid residues.

phase to the lipid bilayer. So it could be expected that the thiyl radical reactivity and isomerization process occurred in a different fashion, compared with the solution experiments shown in the previous section.

In this scenario two other aspects come into play and are related each other:

1) thiol properties involved in the partition between the two vesicle compartments (aqueous and lipid): Hydrophilic, lipophilic and amphiphilic compounds can thus exhibit different behavior. In particular, biologically relevant compounds such as cysteine, glutathione, lipoic and dihydrolipoic acids [27, 32, 33] are the most important in correlating radical processes in cellular systems.

2) The initiation step and the compartment where the initiator meets the thiol compound to effect H-atom abstraction: As previously indicated, different thiol compounds and initiators were used in order to take into account their properties [37, 38, 40].

For this latter aspect, initiation in the aqueous compartment was obtained from the thermal decomposition of a hydrophilic azocompound [37, 38] or, alternatively, by direct photolysis of thiol compound dissolved in the aqueous compartment. Also, γ-irradiation is a well-known methodology for generating radicals in the aqueous compartment [9]. In this case, conditions can be chosen to select the radical species formed during radiolysis. For example, many studies were done with $^{\bullet}OH$ radicals as the prevalent species, which can be obtained by simply saturating the reaction medium with N_2O prior to irradiation, as depicted in equations (9) and (10) [41].

$$H_2O \leadsto e_{aq}^- + {}^{\bullet}OH + {}^{\bullet}H \quad (9)$$

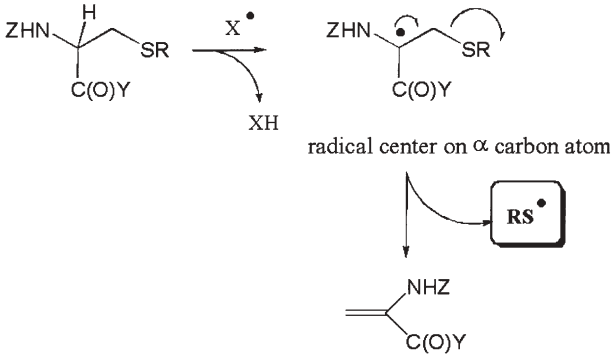
$$e_{aq}^- + N_2O \xrightarrow{H^+} N_2 + {}^{\bullet}OH \quad (10)$$

It must be also taken into account that $H^{\bullet}$ atoms and hydroxyl radicals are very reactive species and react not only with S-H bonds but also abstract hydrogen from organic molecules (R′H), thus forming carbon-centred radicals. However, in biological conditions, these radicals are long lasting and are finally repaired by thiols, thus resulting in thiyl radical formation (eqs 11 and 12).

$$OH^{\bullet} + R'H \rightarrow R'^{\bullet} + H_2O \quad (11)$$

$$R'^{\bullet} + RSH \rightarrow R'H + RS^{\bullet} \quad (12)$$

The formation of thiyl radicals in the aqueous compartment is a biologically related situation since it can mimic a radical stress. As far as the influence of the nature of the thiol is concerned, the hydrophilic cysteine and glutathione are not able to enter the lipid bilayer. Consequently, their corresponding thiyl radicals cannot cause lipid isomerization, as was observed with initiation given by carbon-centred radicals generated from water-soluble azocompounds [27, 33]. On the other hand, when initiation was obtained from γ-radiolysis in water, it was evidenced that there are different operative mechanisms to form thiyl radicals, and lipid isomerization can also be caused by water-soluble sulfur-containing compounds. An example is given in scheme 4, where the α-H bond is abstracted by an initiating radical $X^{\bullet}$ with the generation of a C-centred radical in the α-position [41a, 41b] and the



Scheme 4. Mechanism of formation of diffusible thiyl radical species from hydrophilic S-alkyl cysteines.

subsequent β-fragmentation of the C-S bond causes a release of low-molecular-weight thiyl radicals [27].

This reactive and highly diffusible thiyl radical species can enter the lipid bilayer and cause isomerization of the double bonds. More recently, the attack of H atoms formed during water radiolysis (see eq. 9) to sulfur residues in proteins with formation of low-molecular-weight thiol compounds [41c] was connected with an alternative mechanism of formation of diffusible thiyl radicals. In a biomimetic model constituted by RNase A, as the sulfur-containing protein, and *cis*-unsaturated liposomes thiyl radicals were able to transfer the damage from the aqueous to the lipid compartment. The use of *trans* lipids formed in membranes as markers of sulfur-containing protein degradation was proposed for the first time [29].

To study the outcome of lipid isomerization given by diffusible thiyl radicals, 2-mercaptoethanol was used, which is an amphiphilic thiol and can be partitioned equally well in water and lipid compartments [42]. This thiol was also used in homogenous systems as previously shown, and by comparison with its behavior in solution, the experiments in vesicles could indicate an effect of lipid supramolecular organization. Lipid vesicles made of natural lecithin, such as soybean or egg yolk lecithin, were used, and generation of thiyl radicals was carried out both under radiolysis and photolysis conditions [33, 34]. An example of isomerization outcome in vesicles is reported in figure 6 for egg lecithin, where the reactivity of arachidonate residues was much higher than other fatty acids present in the mixture. Comparing figure 6 with figure 4 referred to the solution experiment, the different behavior of lipids in vesicles is evident.

In the inset of figure 6 linoleic mono-*trans* isomers are shown, which were formed in different amounts in favour of 9t,12c mono-*trans* isomer. Comparing these data with those given in figure 4, a regioselective process is noticed in vesicles, thus providing evidence that different locations of double bonds within the lipid bilayer influenced fatty acid reactivity in the thiyl radical-based isomerization. The double bonds located closest to the membrane polar region are the most involved in the reaction with thiyl radicals diffusing in the lipid bilayer. Therefore, in the case of linoleic acid, the double bond in position 9 is more reactive than that in position 12. Considering all residues of egg lecithin, arachidonic acid showed a higher reactivity compared to oleic and linoleic acids (fig. 6), and arachidonic double bonds in 5 and 8 positions are transformed fastest by thiyl radicals. These experiments in model membranes allowed different arachidonate regioisomers to be characterized, using in particular gas chromatography/mass spectrometry (GC/MS) and ^{13}C nuclear magnetic resonance (NMR) [35], which can be a very useful analytical tools in the complex approach of lipid recognition in biological samples. An approach

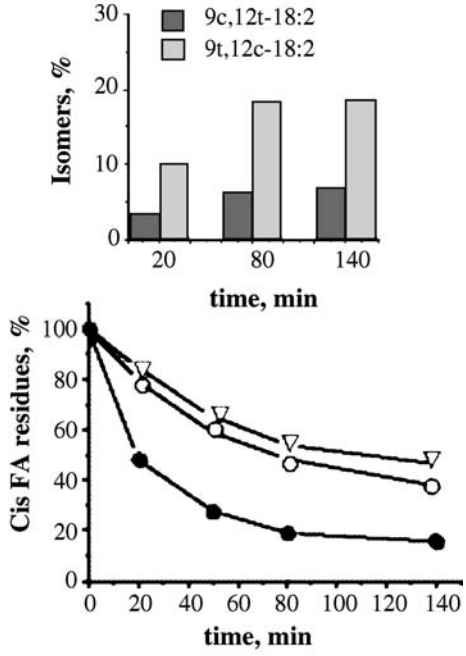


Figure 6. Egg lecithin isomerization time course in LUVET by UV photolysis in the presence of 7 mM 2-mercaptoethanol at 22 °C: (●), arachidonate residues; (○), linoleate residues; (▽), oleate residues. Inset: formation of linoleic mono-*trans* isomers. Reproduced with permission from [31]. Copyright 2004 Am. Chem. Soc.

was proposed for distinguishing *trans* isomers formed by radical processes from the *trans* isomers derived from dietary contribution. It starts from the consideration that, during lipid biosynthesis, desaturase enzymes selectively form *cis* unsaturation. In the case of arachidonic acid, as shown in the biosynthetic pathways of figure 1, two double bonds (positions 11 and 14) originate from linoleic acid, the precursor taken from the diet, whereas the two other double bonds (positions 5 and 8) are formed in vivo. It is evident that positions 5 and 8 can only have a *cis* configuration, unless these positions are involved in an isomerization process and transformed into *trans* isomers.

Through the previously discussed biomimetic model of vesicles, the double bonds in 5 and 8 positions were converted to *trans* configuration by thiyl radicals, and this was confirmed by model studies carried out with erythrocyte membranes [35].

Based on these observations, it can be concluded that a careful analysis of membrane lipids helps to understand the origin of *trans* lipids, which can be important for lipidomics researches. Indeed, lipidomics gives attention to each type of lipid modification present in tissues [43], and lipid *trans* isomers found in biological samples can be meaningful also for their relationship with endogenous processes and radical stress.

It was also investigated whether autoxidation competed with isomerization in vesicles, which should not be the

case since it is well known that thiols are inhibitors of peroxidation processes by trapping peroxyl radicals (see, eq. 2). As shown in homogeneous systems and in vesicles, in the presence of oxygen at a concentration of 1.34×10^{-4} M, which is three times more than that of a well-oxygenated tissue, i.e. 40 µM [44], unsaturated lipids were quantitatively recovered after isomerization, with no consumption and with conversion to *cis* and *trans* geometrical isomers [34].

It is worth mentioning that thiyl radicals can give a reversible addition to oxygen. For example, $GS^{\bullet} + O_2$, which forms $GSOO^{\bullet}$, has an equilibrium constant $K = 3200\ M^{-1}$ (rate constants for the forward and reverse reactions are $2.0 \times 10^9\ M^{-1}\ s^{-1}$ and $6.2 \times 10^5\ s^{-1}$, respectively) [44a]. However, under the experimental conditions used, this reaction did not affect the most prevalent process, i.e. *cis*-*trans* isomerization.

The influence of *trans* configuration on lipid and membrane properties

The thiyl radical-catalysed conversion from *cis* to *trans* geometry can have a profound influence in the biological environment because of the change of the lipid molecular shape, as shown in figure 7 for a monounsaturated compound. The *cis* geometry confers a kink in the carbon atom chain, whereas the *trans*-unsaturated carbon atom chain is straight and is more similar to that of saturated lipids. It can be expected that the *cis*-*trans* isomerization affects the physical properties of the bilayer (e.g. microviscosity and thermal phase behaviour). Some of these changes were investigated with partially hydrogenated egg phosphatidylcholines, since during partial hydrogenation some *trans* isomers are produced depending on the degree and conditions of the processes [45]. For a *trans* content of 10–20 mol%, the differences in thermal phase behaviour were not found to be dramatic, whereas *trans* liposomes were more resistant to oxidation, probably because the bilayer became more resistant to the penetration of initiating radical species and oxygen. It is worth

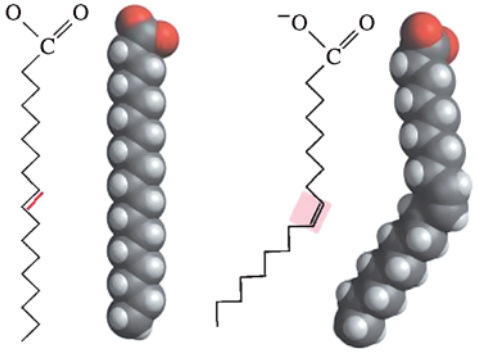


Figure 7. *Trans* and *cis* monounsaturated fatty acids.

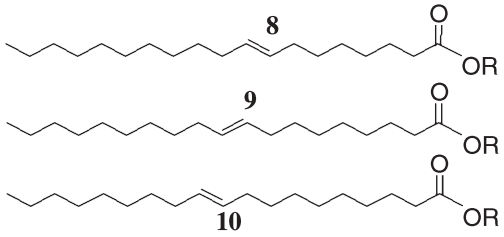


Figure 8. Geometrical and positional *trans* isomer derivatives: in the middle, elaidic acid (9t-18:1) and two positional isomers (8t-18:1 and 10t-18:1).

noting that, during partial hydrogenation, *trans* isomers are formed but they are a mixture of geometrical and positional isomers, with unshifted and shifted double bonds respect to the original position of the natural compounds. This is shown with the example of elaidic acid (9t-18:1) in figure 8, which is the geometrical *trans* isomer of oleic acid and two positional isomers with the *trans* double bond in positions 8 or 10. It should be mentioned that in thiyl radical-catalysed isomerization, the position of the double bond does not shift to adjacent positions, so that only the geometry at the C=C bond changes.

A recent report confirmed that *trans* unsaturated fatty acids are less efficiently oxidized than the corresponding *cis*, correlating this effect with possible protection from oxidation of lipoproteins [46]. This study was performed in vesicles composed of linolelaidic acid (9t,12t-18:2) residues, which were oxidized four times slower than vesicles prepared from linoleic acid (*cis*) residues. It was also shown that liposome resistance to the oxidation is temperature-dependent. But at higher temperature the differences of oxidation between *cis* and *trans* isomers diminished, although never becoming identical.

The difference between geometrical *cis* and *trans* phospholipid isomers is remarkable, as in the example of L-α-phosphatidylcholine made of C16:0/C18:1 *cis*-Δ^9 and C16:0/C18:1 *trans*-Δ^9, which have transition temperatures of –3 and 35 °C, respectively [47]. By summing the structural and physical differences, it can be expected that the properties of the bilayer be deeply influenced by the double-bond geometry.

Since the role of polyunsaturated residues in natural membranes is to lower the gel-to-liquid crystal phase transition temperature, and to dispose double bonds in order to ensure a chain motion [48], a change of *cis* to *trans* double bonds could have a dramatic effect on membrane properties such as fluidity and permeability.

The effect of lipid isomerization on membrane properties was observed in liposomes made of dipalmitoyl-, dioleoyl- and dielaidoyl-phosphatidylcholines. In particular, an example of permeability measurements is given in figure 9, using the spontaneous release of the hydrophilic and polar fluorescent dye carboxyfluorescein, encapsu-

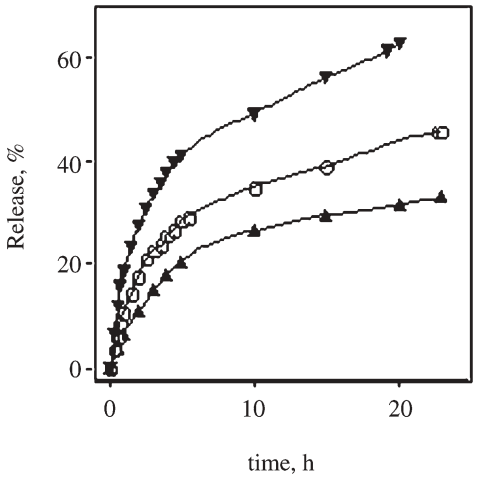


Figure 9. Permeability measurements. Rate of carboxyfluorescein release from multilamellar vesicles made of phosphatidylcholine at 37 °C: DOPC (*cis*,▲), 80:20 DEPC:DOPC (*trans*:*cis*, ○), DPPC (*saturated*, ▼). Reproduced with permission from [29]. Copyright 2002 Elsevier.

lated in MLV vesicles at a self-quenching concentration [32].

Leakage into the surrounding medium was monitored as an increase of fluorescence intensity due to the dilution of the water-soluble probe. From the graph in figure 9 it can be seen that vesicles containing 80% of *trans* isomers (DEPC) were less permeable than vesicles containing 100% *cis* isomer (DOPC). The saturated vesicles (DPPC) were the less permeable, as expected. The more ordered lipid structures, such as those formed by *trans* isomers, decrease solute permeability, and the tight packing of lipids changes the barrier properties of membranes. Recently, *cis* and *trans* differences were again addressed with studies on permeability and fluidity of membrane models [49]. It can be argued that membranes composed only of *trans* lipids (or saturated and *trans* lipids) cannot be considered biologically related, since it is known that *trans* geometry never prevails over the *cis* isomer in nature. Although structurally the *trans* isomer and the corresponding saturated lipid are quite similar in terms of orientation and steric hindrance of the lipid chains, their functions can be dramatically different. For example, extrapolating from the fluidity data obtained in vesicles, saturated chains should result in a more dramatic change compared to the incorporation of the same quantity of monounsaturated *trans* isomers. It could be hypothesized that the strongest feedback for fluidity control systems in cells therefore results from saturated fatty acids, and this subject should attract interest for further work in metabolic responses connected with the presence of different fatty acid residues in membranes.

Furthermore, it can be hypothesized that permeability changes influence membrane protein activity. An example is the bovine brain phospholipid exchange protein, tested with liposomes made of dimiristoyl-, dioleoyl- and dielaidoyl-phosphatidylcholines [50].

Finally, we must mention the adaptation mechanism used by some bacteria to resist changes in their environment and growth in the presence of membrane-disrupting compounds. It has been shown that this resistance mechanism is based on *trans* lipid formation and its reducing effect on the permeability of cell membranes. Interestingly, isomerization is obtained through the activity of a *cis-trans* isomerase enzyme (cti) which converts membrane lipids does not require ATP or other cofactors such as NAD(P)H or glutathione, and works in the absence of de novo synthesis of lipids [51]. It is worth pointing out that bacterial cti is the only enzymatic system known to change the double-bond geometry of phospholipids, whereas such enzymatic isomerization is unknown for eukaryotes.

Recognition of lipid *cis* and *trans* isomers

The improvement of analytical techniques, which allow lipid *cis* and *trans* isomers to be separated, has been extremely important for study of the thiyl radical effect

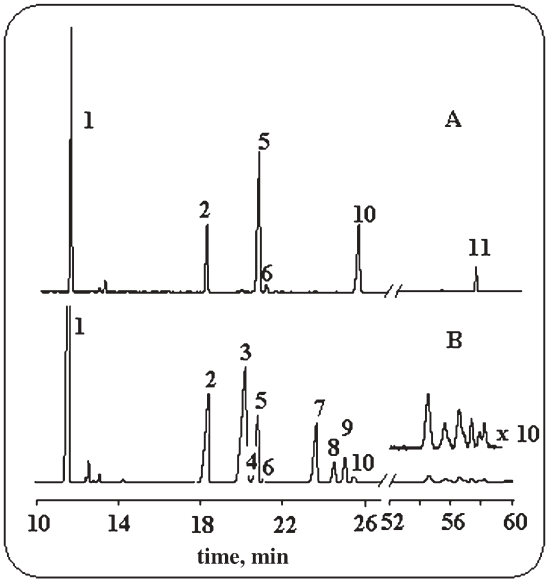


Figure 10. GC chromatograms of egg lecithin isomerization in solution under UV photolysis in the presence of 7 mM 2-mercaptoethanol. (*A*) Starting FAME composition of a commercial egg lecithin obtained after transesterification; (*B*) fatty acid composition (FAME) after 30 min of photolysis. Peak labels: (1) methyl palmitate (16:0); (**2**) methyl stearate (18:0); (**3**) methyl elaidate (9t-18:1); (**4**) methyl *trans*-vaccenate (11t-18:1); (**5**) methyl oleate (9c-18:1); (**6**) methyl vaccenate (11c-18:1); (**7**) 9t,12t-18:2; (**8**) 9c,12t-18:2; (**9**) 9t,12c-18:2; (**10**) methyl linoleate (9c,12c-18:2); (**11**) methyl arachidonate (5c,8c,11c,14c-20:4**).** In trace *B* an enlargement (10×) is given of the mixture of mono- and di-*trans* isomers of arachidonic acid. Reproduced with permission from [31]. Copyright 2004 Am. Chem. Soc.

on lipid geometry. In particular, GC detection is an efficient tool under conditions described as standard procedures in the Official Methods of the American Oil Chemists Society [52]. As a representative example, in figure 10 the analysis of mixtures of 18:0, 18:1 and 18:2 fatty acid methyl esters obtained before and after reaction with thiyl radicals is shown.

In particular, separation of the four isomers of linoleic acid is very effective. The GC detection is coupled with MS for determination of molecular mass and fragmentation patterns, which are very important for lipid identification [53]. For lipid mixtures it is sometime necessary to insert an additional chromatographic separation by means of argentation/thin layer chromatography (Ag/TLC), which is based on the ability of *trans* isomers to give Ag complexes with different elution coefficients [54]. However, it has been shown that detection of eicosenoic acid derivatives is better carried out by other GC conditions. Actually, the use of two parallel GC runs has been suggested in the case of biological samples [32]. The characterization of lipid structures is also performed by NMR spectroscopy, which is effective for tissue lipid analyses [21, 55, 56].

In vitro and in vivo systems: *cis-trans* isomerization of membrane lipids

Model membrane studies envisaged thiyl radical-catalyzed *cis-trans* isomerization of unsaturated phospholipids as a possible endogenous path of *trans* lipid formation. However, whether the isomerization process might be feasible in cells remained to be established. This aspect is indeed very crucial for the origin of *trans* lipids, since there is an enormous amount of research done in the field of nutrition with animal and human subjects, which showed that *trans* lipids from the diet are processed in vivo and are incorporated in tissues [1]. The simple examination of biological samples could not be conclusive, since in the subjects an overlap of diet and stress contributions can occur. Therefore, appropriate in vitro as well as in vivo models must answer the question: Do thiyl radicals form *trans* lipids in membranes? These models consist of *trans*-free systems examined under free-radical stress under appropriate conditions in order to check the integrity of the membrane lipid geometry. This topic still needs to be thoroughly investigated, and preliminary indications came from in vitro studies of human monocytic leukemia cell line THP-1 incubated with 10 mM thiols [57] such as 2-mercaptoethanol, 3-(2-mercapthoethyl)-quinazolin-2,4(1H,3H)-dione (MECH) [58] and glutathione. After a 24 h incubation period, oleic, linoleic and arachidonic acid residues of membrane phospholipids were analysed. The thiol supplementation was found to induce isomerization up to ca. 6% *trans* lipids, considering the total amount of main saturated and unsaturated fatty acid components. When thiol supplementation was coupled with radical stress to cells a more consistent formation of *trans* fatty acid residues in membrane phospholipids was reached (up to 15.5%). The fatty acid isomers formed in cells are geometrical *trans* isomers, as is shown by comparison with appropriate references in the case of C18 fatty acid residues (fig. 11).

Since thiyl radicals are known to specifically convert double bonds in geometrical fashion, it could be hypothesized that isomerization occurs in THP-1 cells by physiologically produced radicals, which abstract an H-atom from the thiol compounds added in millimolar concentration to the medium.

These results showed for the first time that, through a radical-based isomerization process, thiols are harmful compounds under circumstances of *cis* lipid geometry in eukaryotic cells. When radical stress is coupled with decreased defence systems, thiyl radical overproduction can occur, which then involves lipid geometry. Further work is needed to elucidate whether this transformation has consequences for cells. It is worth noting that endogenous *cis-trans* isomerization of lipids has quite

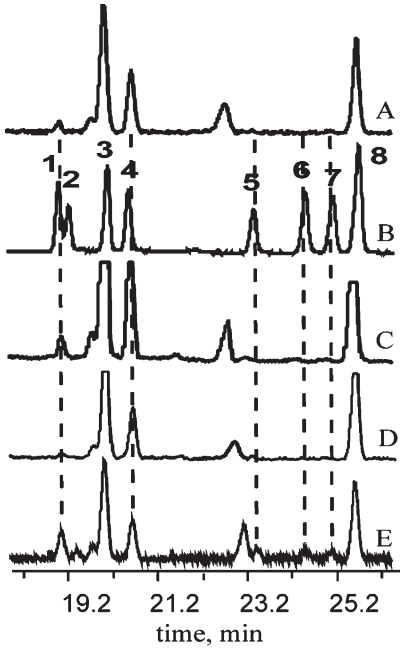


Figure 11. GC chromatograms regarding C18 unsaturated fatty acids isolated from THP-1 membranes: (*A*) control cells after 24 h incubation without thiol; (*C*) after 24 h incubation with 10 mM glutathione; (*D*) after 1 kGy of irradiation dose without thiol; (*E*) after 1 kGy of irradiation dose in the presence of 10 mM glutathione. For comparison the figure also shows (*B*) a reference isomeric mixture of C18 unsaturated fatty acid methyl esters, composed in the following order starting from peak **1**: (**1**) methyl elaidate (9t-18:1); (**2**) methyl *trans*-vaccenate (11t-18:1); (**3**) methyl oleate (9c-18:1); (**4**) methyl vaccenate (11c-18:1); (**5**) 9t,12t-18:2; (**6**) 9c,12t-18:2; (**7**) 9t,12c-18:2; (**8**) methyl linoleate (9c,12c-18:2).

different connotations from exogenous supplementation of chemically manipulated fats. As far as diet is concerned, prevention by avoiding *trans* fat supplementation has been suggested, and actually by 2006 in the USA *trans* content must be included in the nutrition facts for all foods. On the other hand, diet is also involved in the prevention of radical damage, and appropriate nutrients with known radical-trapping ability, such as vitamin C, vitamin A and carotenoids, were found to be the most effective inhibitors of lipid isomerization in model membranes [32].

Radical *cis-trans* isomerization produces unnatural *trans* lipids that resemble saturated lipids. It is known that *trans* lipids incorporated in cell membranes can influence their properties and functions [59–61]. As far as the occurrence of lipid isomerization in vivo is concerned, it can be also expected that both chemical and enzymatic systems, known to quench radical species, can be active as defence mechanisms against this transformation [32]. Based on the relevance of *cis* geometry for lipid structures and the relative ease of converting *cis* to the thermodynamically more stable *trans* structure, we cannot exclude the existence, yet unknown, of a specific enzymatic surveillance system of lipid double-bond geometry in eukaryotic cells. All these points are subjects of further work, which aims at discovering the role of thiyl radicals in biosystems, assessing the importance of regulation systems for *cis* lipid geometry in cells. Results in cells suggest that *trans* isomers cannot only be derived from dietary contribution, as endogenous radical formation is able to produce double-bond conversion under physiological conditions. It is also worth adding that formation of *trans* arachidonate isomers occurred in human platelets exposed to NO_2• radical, a toxic free radical found in biological systems, which gives further support to the hypothesis of in vivo isomerization [62].

The effects of thiyl radicals on enzymatic reactions and lipid metabolism in living systems

Glutathione is known as a cofactor of some enzymes during eicosanoid metabolism, but there is little evidence for direct involvement of thiyl radicals during these activities [63, 63a]. On the other hand, data on in vitro formation of geometrical *trans* lipids by isomerization processes occurring in the biological environment supports involvement of thiyl radicals in lipid metabolism.

Indications of the effect of *trans* lipids in living organisms come from different studies, but clearly these isomers have an 'exogenous' origin, i.e. they were supplied by the diet. However, they have been shown to interfere with different pathways.

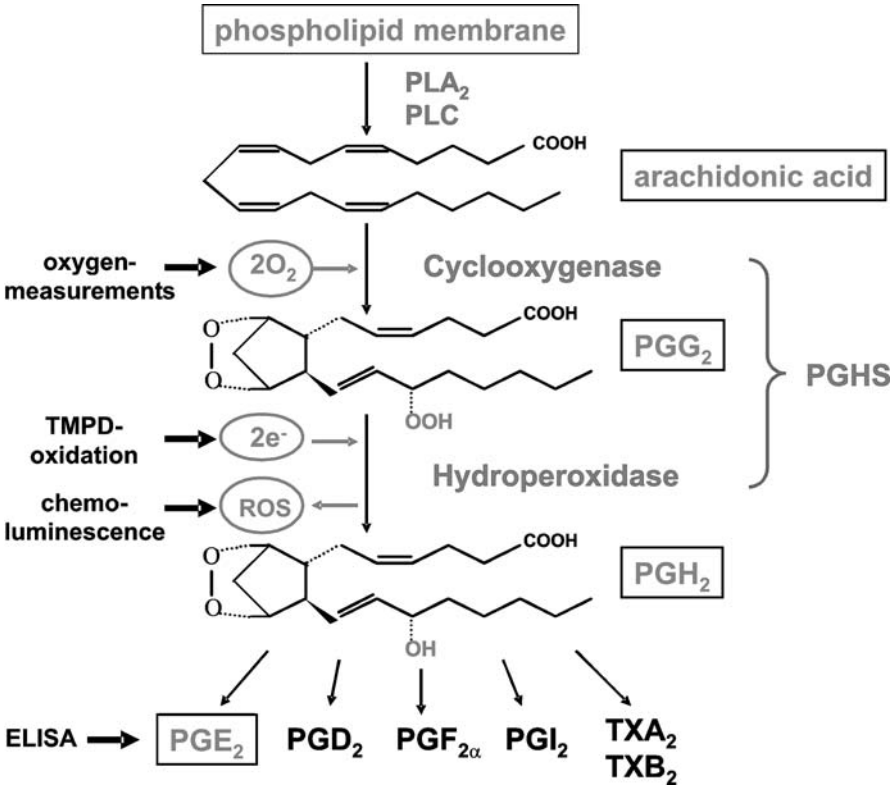


Figure 12. Main steps of arachidonic acid metabolism and biological assays.

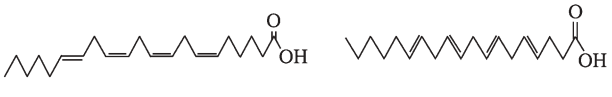
In fact, as already noted, the structural changes of the unsaturated fatty acid double bond can result in inhibition of biochemical transformations and influence the course of physiological processes in vivo [64]. Recently, the mono-*trans* isomer of arachidonic acid in position 14 (14t-20:4; structure **1** in fig. 12) was found to inhibit synthesis of thromboxane B_2 (TBX_2, see fig. 13) and, therefore, to prevent rat platelet aggregation [65]. Also, a preliminary report on the activity of all-*trans* arachidonic acid **2** (fig. 12) in rabbit platelet aggregation showed that inhibition is given by this unnatural isomer [66].

Other data gathered on *trans* lipids showed that they also interact with lipid enzymes and continue the conversion like the natural isomers, but give rise to different molecules. Thus, the mono-*trans* isomer **1** has been found to react with cytochrome P450 epoxygenase, a monooxygenase enzyme present in rat liver microsomes, resulting in the corresponding epoxide, which is an unnatural compound [67].

Assays on the in vitro desaturation or elongation of mono-*trans* isomers of linoleic acid by rat liver microsomes showed that 9c,12t-isomer was better desaturated, whereas 9t,12c-isomer was better elongated [68]. A general inhibition of the metabolic conversion of linoleic acid to arachidonic acid and to other (n-6) PUFAs has been confirmed by a recent study on dietary supplementation of hydrogenated fats to piglets [69].

Recently, arachidonic acid statistically isomerized from 10 to 30% into its mono-*trans*-isomers was studied for its effect on prostaglandin H_2-synthase (PGHS) activity and the efficiency of the eicosanoid synthesis pathway (fig. 13) under in vitro conditions [70, 71]. Vitamin D_3-differentiated HL-60 cells with a monocytic character were used, and intracellular PGHS activity was stimulated by lipopolysaccharide (LPS). Influence on the metabolism was checked by measuring the efficiency of different steps of the arachidonic cascade, such as oxygen consumption, TMPD oxidation, chemoluminescence and prostaglandin E_2 (PGE_2) as final product (black arrows). It was found that the extent of arachidonic acid metabolism and in particular PGE_2 expression is dramatically decreased.

The examples given demonstrate the potential effects of *trans*-unsaturated fatty acids in living systems. Certainly, under real physiological conditions, pathway and conversion rates of thiol radical generation (balance) should be thoroughly characterized.



1 **2**

Figure 13. Mono-*trans* isomer of arachidonic acid **1** (14t-20:4) and all-*trans* arachidonic acid **2** (5t,8t,11t,14t-20:4).

Conclusions

The field of thiyl radical chemistry linked to biological processes is in constant development, as has been shown with the research reviewed in this article. Research topics can be further expanded in different directions, ranging from membrane behaviour to pharmacological mechanisms and molecular basis of biological processes. A better comprehension of thiyl radical generation and reactivity during cell metabolism is promising for the consequences on several cellular mechanisms that still need to be clarified, dealing in particular with the *cis* lipid geometry, its structural role and signalling activity.

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