Cis−Trans Isomerization of Polyunsaturated Fatty Acid Residues in Phospholipids Catalyzed by Thiyl Radicals

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Abstract: Phospholipids containing *trans*-unsaturated fatty acid residues are the major products of the thiyl radical attack on L-α-phosphatidylcholine from soybean lecithin in homogeneous solution or in liposomes (LUVET). Thiyl radicals act as the catalyst for the cis−trans isomerization, and the number of catalytic cycles depends on the reaction conditions. The presence of ∼0.2 mM oxygen does not influence the reaction outcome but accelerates the efficiency of cis−trans isomerization in homogeneous solution. Under these conditions, the PUFA peroxidation is found to be unimportant. A detailed study of the isomerization of methyl linoleate including product studies indicates the formation of a small amount of conjugated dienes that act as inhibitors. Indeed, *all-trans*-retinol substantially retarded the isomerization process.

Introduction

Unsaturated fatty acids present in the cell membranes play an essential role in adaptation responses, which are correlated to both the geometry and the number of the double bonds. Some bacteria enzymatically convert unsaturated lipids from cis to trans isomers without a shift of the double bond, as a strategy for protecting themselves from increases in the ambient temperature or from the presence of high concentrations of toxic substances.[1,2] The number of double bonds has recently been correlated to thermotolerance in plants[3] as well as to salt stress tolerance in bacteria.[4] The regulation of the fatty acid unsaturations affects the physical properties of the membrane bilayer (e.g., microviscosity and relative motional freedom of the lipid molecules), and a fine-tuning is needed in order to acclimate these organisms to changes of environmental conditions.

Enzymatic pathways for the cis−trans isomerization of unsaturated fatty acid residues in mammals are unknown. However, trans isomers can arise in cell membranes from exogenous supplies as the food intake. Meat and milk contain ∼2−8% *trans*-fatty acid residues[5] due to the biohydrogenation of lipids by bacteria occurring in the first stomach of ruminant animals.[6] *trans*-Fatty acids are produced in much higher proportion (∼25−45%) in the manufacturing of vegetable and fish oils by heat, deodorization, and catalytic hydrogenation processes;[7] mono-*trans*-linoleic and α-linolenic acids are the most commonly found, di-trans isomers being in minor quantities.[8] The effect of *trans*-unsaturated fatty acids supplied by the diet has recently raised some concern in human health and nutrition since they have been correlated with higher serum lipoprotein cholesterol and triglyceride levels and an increasing risk of heart diseases.[9] It is known that the various *trans*-fatty acids are inhibitors of the desaturation and chain-elongation enzymes.[11,16] Linolelaidic acid can also interfere with the metabolism of linoleic acid and the biosynthesis of prostaglandins.[17,18] However, the relationship between *trans*-fatty acids

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(8) In these industrial processes, both configurational and positional isomers can be obtained. Therefore, the analytical conditions used for such components are of crucial importance in order to separate and correctly identify them. For example, see: (a) Kramer, J. K. G.; Fellner, V.; Dugan, M. E. R.; Sauer, F. D.; Mossoba, M. M.; Yurawecz, M. P. *Lipids* **1997**, *32*, 1219−1228. (b) Lie Ken Jie, M. S. F.; Mustafa, J. *Lipids* **1997**, *32*, 1019−1034. (c) Ratnayake, W. N. M. In *Trans Fatty Acids in Human Nutrition*; Sébédio, J.-L., Christie, W. W., Eds.; The Oily Press: Dundee, Scotland, 1998; Chapter 4; pp 115−161.

(9) In a series of studies performed in several countries[10] the presence of trans isomers in human milk,[11] blood,[12, 13] and adipose tissues[14] has been found to be correlated with dietary intake. In this respect, a significant difference among countries has been pointed out, ranging from values of 12−20% in North America to 1−2% in Mediterranean countries.[15]

(10) van Poppel, G. *Lancet* **1998**, *351*, 1099.

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(12) Siguel, E. N.; Lerman, R. *Am. J. Cardiol.* **1993**, *71*, 916−920. Willett, W.; Stampfer, M. J.; Manson, J. E.; Colditz, G. A.; Speizer, F. E.; Rosner, B. A.; Sampson, L. A.; Hennekens, C. H. *Lancet* **1993**, *341*, 581−585.

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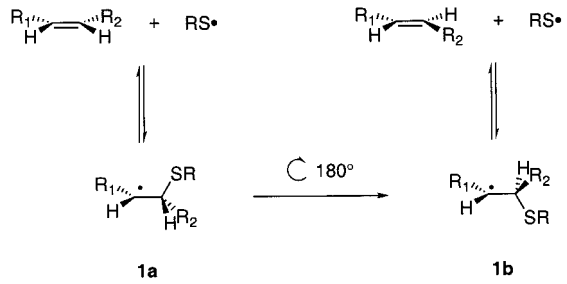
Scheme 1

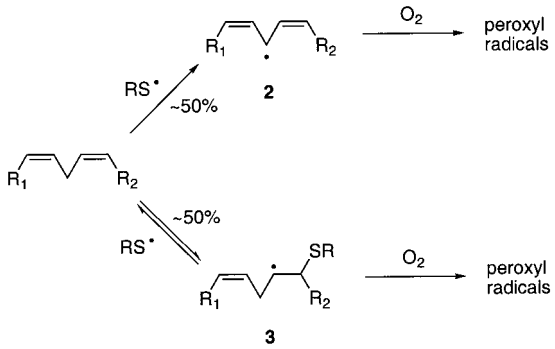


and pathological conditions is far to be understood, and up to now, the presence and effects of *trans*-fatty acid residues in mammal cells have only been correlated to the dietary intake.[21]

Radical-based damage of biologically relevant molecules has increasingly attracted the interest of researchers from different scientific fields, from chemistry to medicine. Radical processes involving membrane phospholipids are mainly referring to polyunsaturated fatty acid (PUFA) peroxidation.[22] More recently the homolytical cleavage of lysophospholipids[23] and the cis−trans isomerization of unsaturated fatty acid residues have been reported.[24−26]

We have shown that phospholipids containing *trans*-unsaturated fatty acids are the only products of the thiyl radical attack on natural dioleoylphosphatidylcholine.[24] The mechanism that we conceived for this transformation includes the addition of thiyl radicals to the cis double bond of an oleate moiety to give the intermediate radical **1a**, half-rotation about the carbon−carbon bond to give **1b**, and ejection of the thiyl radical by β-scission (cf. Scheme 1).[27] We have also modeled the occurrence of such a reaction in cell membranes using lipid vesicles, naturally occurring thiols, and aerobic conditions,[24] showing that the isomerization in lipid bilayer aggregates is still an effective process. Thiyl radicals can attack the double bond of the membrane lipids randomly causing a change in the configuration of the hydrocarbon tails.

Scheme 2



We suggested that the geometrical isomerization of unsaturated lipids could be an endogenous process. The biological consequences are predictable as they are similar to the alterations already observed when *trans*-unsaturated fatty acids taken from the diet are included into membranes.[9] Therefore, we pursued our interest toward polyunsaturated lipids since the event of an endogenous process could bring about an even more important biological effect. In fact, since the trans lipid geometry resembles that of saturated lipids,[28] the geometrical isomerization can be considered equivalent to a decrease of the number of unsaturations in the cell membrane, thus causing an impairment of the cell adaptation systems.[3]

Schöneich et al. studied the reaction of polyunsaturated fatty acids with a variety of thiyl radicals by pulse radiolysis technique.[29] A variety of kinetic information was obtained based on the buildup of pentadienyl-type radical **2** formed according to Scheme 2. For example, rate constants in the range $(0.6-3.1) \times 10^7$ M^{-1} s^{-1} are obtained for the reactions of biologically relevant thiyl radicals with linoleic acid. On the basis of several assumptions, the authors proposed that ~50% of the thiyl radicals abstracts the bisallylic hydrogen whereas the remaining RS• add to the double bond to form the radical adduct **3**. In the absence of oxygen, the latter pathway is reversible, whereas in the presence of oxygen, both radicals **2** and **3** react to form the corresponding peroxyl radicals (Scheme 2). However, as the authors themselves pointed out, product studies are necessary in order to address more properly the reaction mechanism.[29]

Schwinn et al. reported the thiyl radical-induced cis−trans isomerization of methyl linoleate in methanol.[25] On the basis of the previous mechanistic considerations,[29] they postulated that the trans isomers of linoleate moieties represent the fate of the pentadienyl radical, which reacts with thiols in different conformations.[25a] The same group later revised their conclusions[25b] and, on the basis of our preliminary communication,[24] suggested a mechanistic scheme for the cis−trans isomerization of methyl linoleate similar to that reported in Scheme 1. Balazy and coworkers have shown that the reaction of $^{\bullet}NO_2$ radicals with arachidonic acid produces a mixture of isomers, having one trans

(14) Aro, A.; Kardinaal, A. F. M.; Salminen, I.; Kark, J. D.; Riemerama, R. A.; Delgado-Rodriguez, M.; Gomez-Aracena, J.; Huttunen, J. K.; Kohlmeler, L.; Martin, B. C.; Martin-Moreno, J. M.; Mazaev, V. P.; Ringstad, J.; Thamm, M.; van't Veet, P.; Kok, F. J. *Lancet* **1995**, *345*, 273−278.

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(17) Kinsella, J. E.; Bruckner, G.; Mai, J.; Shimp, J. *Am. J. Clin. Nutr*. **1981**, *34*, 2307−2318.

(18) It is worth pointing out that a few data about the fate of *trans*-polyenes have been reported so far, and most of the studies have been carried out in vivo and in vitro using only animals as models.[19] Studies on mice using isotopically labeled 18:2 isomers showed that the metabolites are incorporated into liver, plasma, heart, and brain lipids.[20]

(19) Sébédio, J.-L.; Chardigny, J.-M. In *Trans Fatty Acids in Human Nutrition*; Sébédio, J.-L., Christie, W. W., Eds.; The Oily Press: Dundee, Scotland, 1998; Chapter 6; pp 191−215.

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(26) Jiang, H.; Kruger, N.; Lahiri, D. R.; Wang, D.; Vatèle, J.-M.; Balazy, M. *J. Biol. Chem.* **1999**, *274*, 16235−16241.

(27) Chatgilialoglu, C.; Ballestri, M.; Ferreri, C.; Vecchi, D. *J. Org. Chem.* **1995**, *60*, 3826−3831.

(28) The similarity between a mono-trans and a saturated fatty acid was confirmed by steady-state fluorescence polarization studies of vesicles composed of phospholipids from the liver mitochondria of rats fed with elaidic acid. See: Wolff, R. L.; Entressangles, B. *Biochim. Biophys. Acta* **1994**, *1211*, 198−206.

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and three cis double bonds.[26] They proposed a reversible addition of $^{\bullet}NO_2$ radicals to arachidonic moieties analogous to the mechanism shown in Scheme 1.

We report herein the reaction of thiyl radicals with mono- and polyunsaturated phospholipids in the naturally occurring mixture of purified L-α-phosphatidylcholine from soybean lecithin. The geometrical isomerization has been studied both in solution and in lipid vesicles (LUVET), which model the cell membrane. The isomerization of methyl linoleate has been revisited, and product studies allow proposing a detailed mechanistic scheme. The effectiveness of the cis−trans isomerization under aerobic conditions and the inhibition of this chain process by conjugated dienes will also be addressed in some details.

Results and Discussion

Generation of Radicals in *t*-BuOH. Thiyl radicals were produced by the reaction of an alkyl radical with benzenethiol (PhSH) or β-mercaptoethanol ($HOCH_2CH_2SH$). Alkyl radicals were generated by either thermal decomposition of azobis(isobutyronitrile) (AIBN) and azobis(dimethylvaleronitrile) (AMVN) at 71 and 54 °C, respectively (eq 1),[30] or γ-irradiations of N_2O-saturated *t*-BuOH.

$$R{-}N{=}N{-}R \xrightarrow{\Delta} N_2 + 2R^{\bullet} \qquad (1)$$

Radiolysis of *t*-BuOH leads to the species e_{sol}^{-} and $^{\bullet}CH_2C(CH_3)_2OH$ as shown in eq 2. In N_2O-saturated solutions,

$$t\text{-BuOH} \rightsquigarrow e_{sol}^{-},\ {}^{\bullet}CH_2C(CH_3)_2OH \qquad (2)$$

e_{sol}^{-} is transformed into the $HO^{\bullet}$ radical (eq 3). Hydrogen abstraction from *t*-BuOH by $HO^{\bullet}$ produces $^{\bullet}CH_2C(CH_3)_2OH$ (eq 4, $k_4 = 6.0 \times 10^8\ M^{-1}\ s^{-1}$), and therefore, we can consider the G value of $^{\bullet}CH_2C(CH_3)_2OH$ radical to be $\sim$0.65 μmol J^{-1}.[31] The $^{\bullet}CH_2C(CH_3)_2OH$ in turn reacts with the $HOCH_2CH_2SH$ to give the corresponding thiyl radical (eq 5, $k_5 \cong 5 \times 10^7\ M^{-1}\ s^{-1}$).[33]

$$e_{sol}^{-} + N_2O \xrightarrow{H^+} N_2 + HO^{\bullet} \qquad (3)$$

$$HO^{\bullet} + (CH_3)_3COH \rightarrow {}^{\bullet}CH_2C(CH_3)_2OH + H_2O \qquad (4)$$

$$^{\bullet}CH_2C(CH_3)_2OH + RSH \rightarrow (CH_3)_3COH + RS^{\bullet} \qquad (5)$$

(30) At these temperatures, the half-life times are longer than 4 h. Taken from the half-life time vs temperature curves reported by Akzo Chemie.

(31) Based on the total $G = 0.61$ μmol J^{-1} in water (eq 6) and $G(^{\bullet}CH_2OH) = 0.67$ μmol J^{-1} in methanol,[32] the assumption of $G[^{\bullet}CH_2C(CH_3)_2OH] = 0.65$ μmol J^{-1} seems reasonable.

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(33) It is known[34] that in water the $^{\bullet}CH_2C(CH_3)_2OH$ abstracts hydrogen from the $HOCH_2CH_2SH$ with a rate constant of $k_5 = 8.2 \times 10^7\ M^{-1}\ s^{-1}$. The rates of thiol trapping of alkyl radicals are solvent dependent.[35] The rate constant for the reaction of a primary alkyl radical with octanethiol is measured to be $1.9 \times 10^7\ M^{-1}\ s^{-1}$ at 30 °C in THF.[35]

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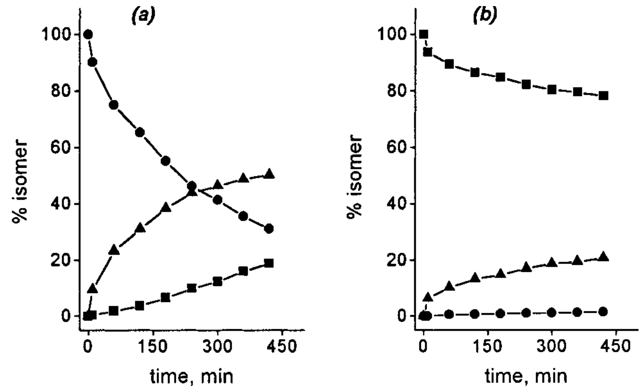


Figure 1. (a) Time profiles of methyl linoleate (0.15 M) isomerization with 75 mM PhSH and 30 mM AMVN in *t*-BuOH at 54 °C: (●) methyl linoleate; (▲) mono-trans isomers; (■) methyl linolelaidate. (b) Time profiles of methyl linolelaidate isomerization (0.15 M) with 75 mM PhSH and 30 mM AMVN in *t*-BuOH at 54 °C: (■) methyl linolelaidate; (▲) mono-trans isomers; (●) methyl linoleate.

Isomerization of Methyl Linoleate.[36] A solution of 0.15 M methyl linoleate (9*cis*,12*cis*-18:2), 0.075 M PhSH, and 0.03 M AMVN in *t*-BuOH was heated at 54 °C for 7 h. The reaction was monitored by GC (Rtx-2330 column), and its time profile is shown in Figure 1a. The disappearance of the 9*cis*,12*cis*-18:2 (solid circles) was replaced by the formation of mono-trans isomers (solid triangles) and 9*trans*,12*trans*-18:2 (solid squares). The two mono-trans isomers, i.e., 9*trans*,12*cis*-18:2 and 9*cis*,12*trans*-18:2, were found in equal amounts and are reported together. The overall yield of the four isomers decreases linearly with the time and is found to be 85% after 7 h by using methyl palmitate as internal standard. Furthermore, 6% of four conjugated dienes (i.e., *cis*- and *trans*-9,11- and -10,12-octadecadienoic acid methyl esters) were singled out, based on the comparison with a commercially available mixture. A parallel GC analysis by using a HP-5 column also showed a broad peak (or cluster of peaks) that was assigned by GC/MS to PhSH adducts of methyl linoleate. Chromatographic attempts to isolate this fraction from the other components failed. Figure 1b shows the analogous reaction in which the starting material is methyl linolelaidate. The disappearance of the 9*trans*,12*trans*-18:2 (solid squares) was replaced by the formation of mono-trans isomers (solid triangles) whereas the formation of methyl linoleate (solid circles) is close to zero. In agreement with the preceding experiment, after 7 h the yields were found to be 84 and 6% for the geometrical isomers and the conjugated dienes, respectively.

To reach the equilibrium mixture, methyl linoleate or methyl linolelaidate or a commercially available mixture of the four geometrical isomers 18:2 (9*cis*,12*cis*/9*cis*,12*trans*/9*trans*,12*cis*/9*trans*,12*trans* = 8/20/20/52) was allowed to react at 71 °C with an excess of PhSH (0.45 M) and AIBN as the initiator. The final isomeric composition is independent of the starting material and corresponds to 9*cis*,12*cis*/9*cis*,12*trans*/9*trans*,12*cis*/9*trans*,12*trans* = 4/16/16/64. By using the Boltzmann distribution expression, we obtained ΔE values of 0.87 kcal mol^{-1} between methyl linoleate and the mono-trans isomers and between the mono-trans isomers and methyl linolelaidate. These values account for the difference in the stability of the four isomers. Indeed, for *cis*- and *trans*-2-butene, $\Delta_f H°(trans) - \Delta_f H°(cis) = -1.0$ kcal mol^{-1}.[37]

(36) The following nomenclature system will be used throughout the article: methyl linoleate or 9*cis*,12*cis*-18:2, methyl linolelaidate or 9*trans*,12*trans*-18:2, and their mono-trans isomers, i.e., 9*trans*,12*cis*-18:2- and 9*cis*,12*trans*-18:2.

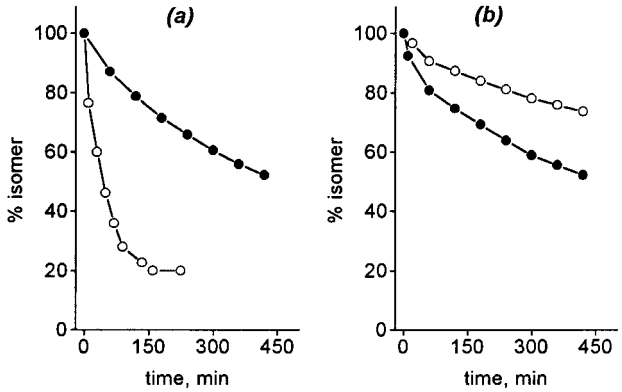
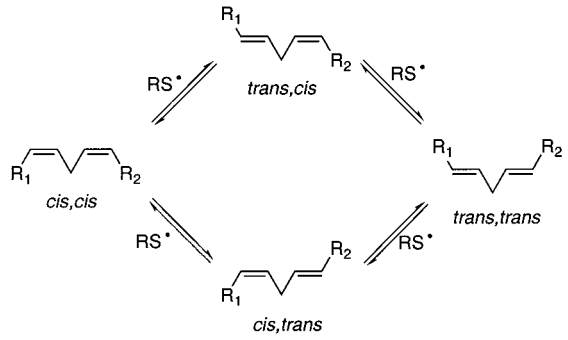


Figure 2. Time profiles of methyl linoleate (●) or methyl oleate (○) in *t*-BuOH at 54 °C. (a) Isomerization of methyl linoleate (0.15 M) with 75 mM $HOCH_2CH_2SH$ and 30 mM AMVN or methyl oleate (0.15 M) with 75 mM $HOCH_2CH_2SH$ and 30 mM AMVN. (b) Isomerization of a mixture of methyl linoleate (0.15 M) and methyl oleate (0.15 M) with 75 mM $HOCH_2CH_2SH$ and 30 mM AMVN.

Scheme 3



These experiments show that the initial disappearance of methyl linoleate is replaced by the two mono-trans isomers, which in their turn are the precursors of the di-trans isomer, i.e., a step-by-step isomerization (Scheme 3).[38a] The formation of conjugated dienes is probably due to the abstraction of bisallylic hydrogen by the PhS• radical to give the pentadienyl radical **2** (Scheme 2) followed by hydrogen abstraction from PhSH.[38b]

Figure 2a (solid circles) shows the disappearance of methyl linoleate when the above azo-initiated reaction was run in the presence of $HOCH_2CH_2SH$ (0.075 M). In comparison with the PhSH experiment (Figure 1a, solid circles), the reaction is considerably slower in accordance with the relative reactivities of PhS• and $HOCH_2CH_2S$• radicals.[24,40] However, under these conditions, the yield of the four geometrical isomers is 93% whereas the four conjugated dienes (i.e., *cis*- and *trans*-9,11- and -10,12-octadecadienoic acid methyl esters) are absent.

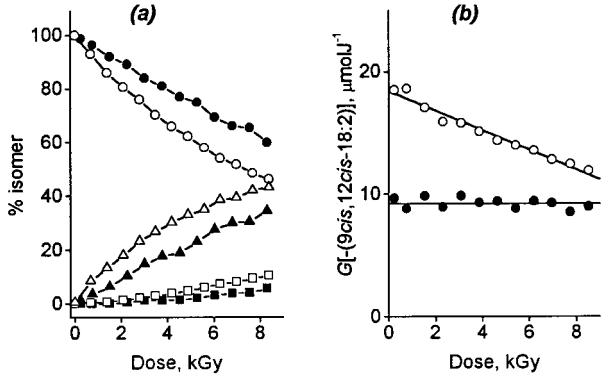


Figure 3. (a) 9*cis*,12*cis*-18:2 (●, ○), 9*trans*,12*cis*-18:2, and 9*cis*,-12*trans*-18:2 (▲, △) or 9*trans*,12*trans*-18:2 (■, □) vs dose from the γ-radiolysis (23.3 Gy min^{-1}) of methyl linoleate with 75 mM $HOCH_2CH_2SH$ in N_2O-saturated *t*-BuOH at 22 °C: (●, ▲, ■) without oxygen; (○, △, □) with 2.34×10^{-4} M oxygen. (b) *G*[-(9*cis*,12*cis*-18:2)] vs dose (●) without oxygen and (○) with oxygen.

Figure 2a also shows the time profile of the disappearance of methyl oleate under identical condition (open circles) taken from our previous work.[24] It is evident that the isomerization of methyl linoleate is much slower than the corresponding reaction of methyl oleate. To throw some light on this unexpected result, we studied the isomerization of a mixture of methyl linoleate (0.15 M) and methyl oleate (0.15 M) with $HOCH_2CH_2SH$ (0.075 M) in *t*-BuOH using AMVN (0.03 M) at 54 °C. Figure 2b shows this experiment. The efficiency of methyl linoleate isomerization is the same in the presence or absence of methyl oleate, whereas the disappearance of methyl oleate is dramatically decreased (cf. Figure 2a and b). Furthermore, in Figure 2b, it can be seen that the methyl linoleate isomerized 2 times faster than methyl oleate; that is, the three double bonds (two of linoleate and one of oleate) isomerize with the same efficiency. The above observation suggests that, during the methyl linoleate isomerization, an inhibitor is forming that retards the isomerization.

Figure 3a shows the irradiation dose profiles of the disappearance of 9*cis*,12*cis*-18:2 (solid circles), the formation of the two mono-trans isomers (solid triangles), and 9*trans*,12*trans*-18:2 (solid squares). It is worth pointing out that the data are a combination of two independent experiments, and therefore, the reproducibility is quite good. Also in the irradiation experiments, the four geometrical isomers account for at least 95% of the yield without any evidence for the formation of conjugated dienes. The disappearance of the starting material (mol kg^{-1}) divided by the absorbed dose (1Gy = 1 J kg^{-1}) gives the radiation chemical yield or *G*[-(9*cis*,12*cis*-18:2)]. Figure 3b (solid circles) shows the plot of *G*[-(9*cis*,12*cis*-18:2)] versus dose. The extrapolation to zero dose gives $G \cong 9$ μmol J^{-1}. Assuming that the *G*(RS•) is 0.65 μmol J^{-1} (eqs 4 and 5),[31] we calculated the catalytic cycle to be 14 at the initial phase. For the analogous isomerization of methyl oleate, a catalytic cycle of 350 was calculated.[24,41]

Figure 3a (○, △, □) also shows the dose profile of the isomerization from a solution saturated with a mixture of N_2O/air containing 10% oxygen.[42] Again, the data are a combination of two independent experiments indicating a high reproduc-

(37) *Handbook of Chemistry and Physics*, 77th ed.; Lide, D. R., Ed.; CRC Press: Boca Raton, FL, 1996−97.

(38) (a) The kinetics and equilibrium constant for the reaction PhS• + CH_2=CHR ⇌ $PhSCH_2C$(•)HR, are reported to be $k_f = 4.0 \times 10^3$ M^{-1} s^{-1}, $k_r = 3.3 \times 10^7$ s^{-1}, and $K = 0.0012$ M^{-1}, respectively. See: Ito, O. In *S−Centered Radicals*; Alfassi, Z. B., Ed.; Wiley: Chichester; U.K., 1999; pp 193−224. (b) The bond dissociation energies of $(CH_2$=$CH)_2CH$−H and PhS−H are 76.6 and 80.8 kcal mol^{-1}, respectively.[39,40a] Therefore, the formation of pentadienyl radical is ∼4 kcal mol^{-1} exothermic.

(39) Clark, K. B.; Culshaw, P. N.; Griller, D.; Lossing, F. P.; Martino Simões, J. A.; Walton, J. C. *J. Org. Chem.* **1991**, *56*, 5535−5539. Fort, R. C., Jr.; Hrovat, D. A.; Borden, W. T. *J. Org. Chem.* **1993**, *58*, 211−216.

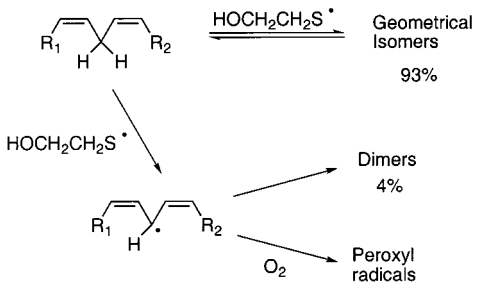
(40) For example, see: (a) Chatgilialoglu, C.; Guerra, M. In *Supplement S: The Chemistry of Sulfur-containing Functional Groups*; Patai, S., Rappoport, Z., Eds.; Wiley: London; 1993; Chapter 8; pp 363−394. (b) Chatgilialoglu, C.; Bertrand, M. P.; Ferreri, C. In *S−Centered Radicals*; Alfassi, Z. B., Ed.; Wiley: Chichester, U.K., 1999; pp 312−354.

(41) The terms "catalytic cycle" and "thiyl radical-catalyzed reaction" are more appropriate that "chain length" and "radical chain reaction", which have been used in our previous work.[24]

(42) The solubility of molecular oxygen in *t*-BuOH is determined to be 2.34×10^{-3} M at room temperature.[43] Therefore, 10% oxygen is equal to 2.34×10^{-4} M.

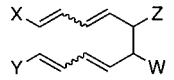
(43) Cipollone, M.; di Palma, C.; Pedulli, G. F. *Appl. Magn. Reson.* **1992**, *3*, 98−102.

Scheme 4

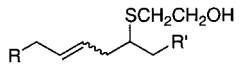


ibility. In comparison with the deoxygenated solution experiment (●, ▲, ■), the reaction is substantially accelerated. Indeed, the disappearance of 9*cis*,12*cis*-18:2 (open circles) and the appearance of the two mono-trans isomers (open triangles) and di-trans isomer (open squares) is faster at the same dose. Figure 3b (open circles) shows the *G*[-(9*cis*,12*cis*-18:2)] versus dose which gives a $G \cong 19$ μmol J^{-1} by extrapolation to zero dose and a catalytic cycle of 29 at the initial phase. The increase of catalytic cycle from 14 to 29 in the presence of 2.34×10^{-4} M oxygen[42] is not easily explained. Indeed, this behavior is opposite to the one observed for the isomerization of methyl oleate, in which the catalytic cycle was decreasing from 350 to 130 in the presence of the same amount of oxygen.[24,41]

To investigate further the dichotomy between the isomerization of methyl oleate and linoleate, we have undertaken a careful examination of the products formed in the following reaction. A 0.15 M solution of methyl linoleate (1.5 mmol) and $HOCH_2CH_2SH$ (0.075 M) in *t*-BuOH was irradiated for 7 h. Flash chromatography of the reaction mixture using *n*-hexane as the eluent gave a first fraction of the geometrical isomers in 93% yield. Further elution with 8/2 *n*-hexane/diethyl ether gave two other fractions corresponding to a mixture of dimeric conjugated dienes of type **4** (4% yield; $R_f = 0.47$) and to a mixture of thiol−



4



5

fatty acid adducts of type **5** (2% yield; $R_f = 0.16$), respectively. The mixture of dimeric conjugated dienes of type **4** suggests a coupling of the pentadienyl radicals at positions 9 and 13.[44] On the other hand, the adducts of type **5**[45] correspond to the initial addition of thiyl radicals to positions 10 and 12 followed by hydrogen abstraction from the thiol prior to β-elimination of thiyl radicals.[40]

Scheme 4 describes the reaction of methyl linoleate with $HOCH_2CH_2S^{\bullet}$ radical in the absence or presence of molecular oxygen. The main path is the formation of the geometrical isomers by the mechanism described in Scheme 3. A minor path is the hydrogen abstraction from the bisallylic position to give a delocalized pentadienyl radical.[46] The absence of octadecanoic conjugated dienes indicates that pentadienyl radicals

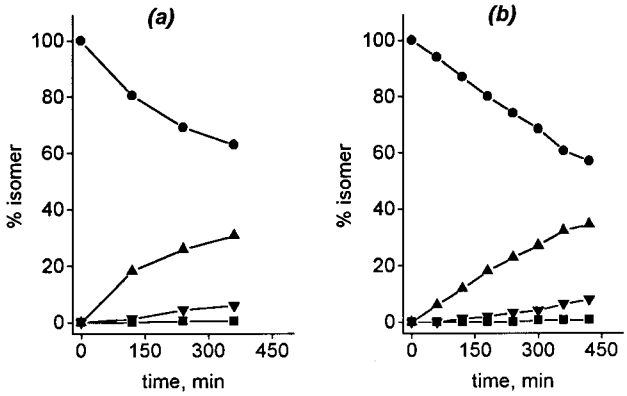


Figure 4. Time profiles of methyl linolenate (0.15 M) isomerization with 75 mM $HOCH_2CH_2SH$ in *t*-BuOH: (●) methyl linolenate; (▲) mono-trans isomers; (▼) di-trans isomers; (■) all-trans isomer. (a) 30 mM AMVN at 54 °C. (b) From the γ-radiolysis (24.9 Gy min^{-1}) at 22 °C.

prefer to combine rather than abstract hydrogen from the thiol under our experimental conditions.[46] We suggest that the formation of the dimers **4** (containing two moieties of conjugated dienes) during the isomerization process is responsible for the observed inhibition. In the presence of the molecular oxygen, the substituted pentadienyl radical should give the corresponding peroxyl radicals that lead to final products with lower inhibiting properties than the dimers **4**.

Isomerization of Methyl Linolenate. For comparison with methyl linoleate, we studied the isomerization of 0.15 M methyl linolenate (9*cis*,12*cis*,15*cis*-18:3) with $HOCH_2CH_2SH$ (0.075 M) in *t*-BuOH using either AMVN (0.03 M) at 54 °C or γ-irradiation of a N_2O-saturated solution at 22 °C. Aliquots of the solution were withdrawn at different times and examined by GC analysis. Similar results were obtained under the two experimental conditions. Figure 4 (solid circles) shows the time profile of the disappearance of methyl linolenate, the formation of the three mono-trans isomers (solid triangles up), the three di-trans isomers (solid triangles down), and the all-trans isomer (solid squares).[47a,b] It is worth pointing out that after 7 h of irradiation a 92% yield of the eight isomers was obtained. A TLC examination of the reaction mixture evidenced the formation of two secondary products, which, in analogy with the byproducts observed in the linoleate reaction, were attributed to dimers **4** and adducts **5** ($R_f = 0.57$ and 0.11, respectively, in 7/3 *n*-hexane/diethyl ether).

The plot of *G*[-(9*cis*,12*cis*,15*cis*-18:3)] versus dose obtained from Figure 4b gives a $G \cong 8$ μmol J^{-1} by extrapolation to zero dose and a catalytic cycle of 12 at the initial phase. Indeed, this behavior is similar to the one observed for the isomerization of methyl linoleate and we suggest that the mechanism reported in Scheme 4 also operates in this case.

Isomerization of L-α-Phosphatidylcholine (PC) from Soybean Lecithin in *t*-BuOH. A set of experiments similar to those

(44) Culshaw, P. N.; Walton, J. C.; Hughes, L.; Ingold, K. U. *J. Chem. Soc., Perkin Trans. 2* **1993**, 879−886.

(45) This attribution is based on COSY, NOE, and DEPT NMR experiments. The two protons attached to the double bond correlate with three signals at 1.98, 2.12, and 2.23 ppm, which integrate for 2:1:1 protons, respectively. The resonance at 1.98 ppm has been assigned to the allylic hydrogens in C-8 or C-14. The signals at 2.12 and 2.23 ppm have been assigned to the two diastereotopic allylic hydrogens in C-11. The DEPT experiment evidenced the resonances of the tertiary carbon bearing the sulfur substituent at 45.1 and 45.8 ppm, which refer to the two adducts.

(46) The bond dissociation energy of RS−H is 88.6 kcal mol^{-1}.[40] Therefore, the formation of pentadienyl radical is ~12 kcal mol^{-1} exothermic whereas the reaction of pentadienyl radical with RSH will be ~8 kcal mol^{-1} more endothermic than the corresponding reaction with PhSH.[38]

(47) (a) The GC peaks of two of the di-trans isomers, i.e., 9*cis*,12*trans*,-15*trans*-18:3 and 9*trans*,12*cis*,15*trans*-18:3, are overlapping under our best analytical conditions (cf. Figure 5b). (b) Contrary to isomerization of methyl linoleate in *t*-BuOH where the two mono-trans isomers were formed in equal amounts, the three mono-trans isomers of methyl linolenate are formed in different amounts. For example, the experiment in Figure 4b after 7 h gave a ratio of 9.9/11.8/12.7 for 9*cis*,12*cis*,15*trans*-18:3, 9*cis*,12*trans*,15*cis*-18:3, and 9*trans*,12*cis*,15*cis*-18:3, respectively.

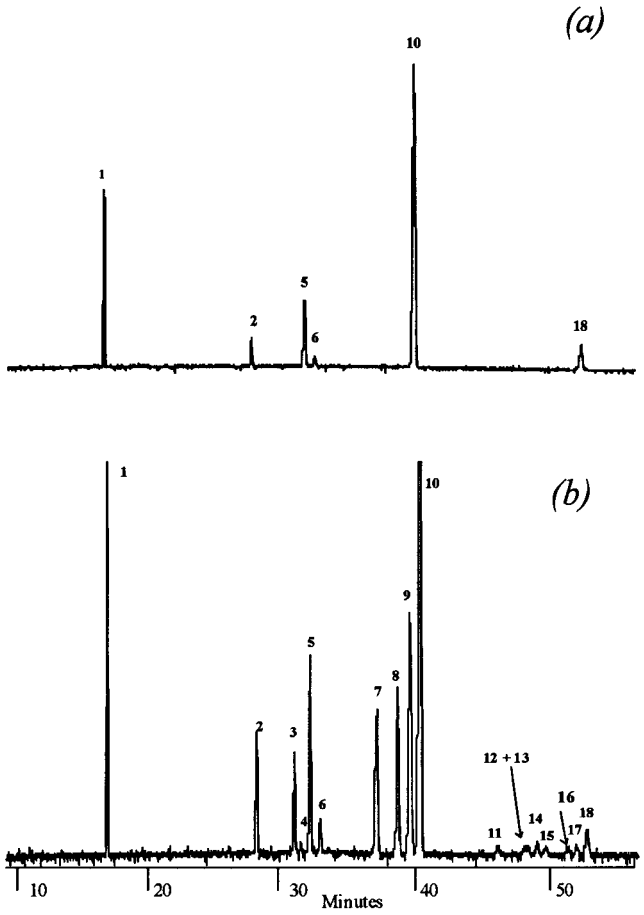


Figure 5. (a) GC chromatogram of fatty acid composition obtained by transesterification of the commercially available L-α-phosphatidylcholine from soybean lecithin. (b) GC chromatogram of fatty acid composition obtained after isomerization. Peaks: (1) 16:0, (2) 18:0, (3) 9*trans*-18:1, (4) 11*trans*-18:1, (5) 9*cis*-18:1, (6) 11*cis*-18:1, (7) 9*trans*,12*trans*-18:2, (8) 9*cis*,12*trans*-18:2, (9) 9*trans*,12*cis*-18:2, (10) 9*cis*,12*cis*-18:2, (11) 9*trans*,12*trans*,15*trans*-18:3, (12+13) 9*cis*,12*trans*,-15*trans*-18:3, and 9*trans*,12*cis*,15*trans*-18:3, (14) 9*cis*,12*cis*,15*trans*-18:3, (15) 9*trans*,12*trans*,15*cis*-18:3, (16) 9*cis*,12*trans*,15*cis*-18:3, (17) 9*trans*,12*cis*,15*cis*-18:3, and (18) 9*cis*,12*cis*,15*cis*-18:3.

with methyl linoleate or methyl linolenate was carried out with a *t*-BuOH solution of PC since it is known that phospholipids do not aggregate in this solvent.[48] The initial fatty acid composition of the commercially available L-α-phosphatidylcholine from soybean lecithin was obtained by transesterification followed by GC analysis (Figure 5a) and found to be: methyl palmitate (14.5%), methyl stearate (3.8%), methyl oleate (11.9%), methyl vaccenate (1.3%), methyl linoleate (63.5%), and methyl linolenate (6.3%). A PC/chloroform solution (3 mL; 0.15 mmol of fatty acid contents) was evaporated in a test tube under an argon stream. The thin film formed was kept under vacuum for 30 min and then a 1 mL *t*-BuOH was added. After saturation of the solution with N_2O, the $HOCH_2CH_2SH$ (0.075 mmol) was added prior to γ-irradiation. Aliquots (100 μL) of the reaction mixture were processed at different times. After transesterification[49] of the phospholipid, the various cis/trans ratios were obtained by GC analysis. An example is given in Figure 5b in which the excellent separation of all possible

(48) Barclay, L. R. C.; McNeil, J. M.; VanKessel, J.; J. Forrest, B.; Porter, N. A.; Lehman, L. S.; Smith, K. J.; Ellington, J. C., Jr. *J. Am. Chem. Soc.* **1984**, *106*, 6740−6747.

(49) The transesterification in alkaline medium is preferable, as reported by: Kramer, J. F. K.; Fellner, V.; Dugan, M. E. R.; Sauer, F. D.; Mossoba M. M.; Yurawecz, M. P. *Lipids*, **1997**, *32*, 1219−1228.

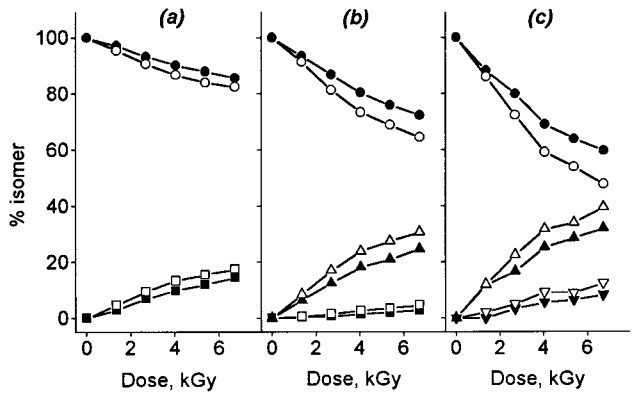


Figure 6. Fatty acid residues vs dose from the γ-radiolysis (23.3 Gy min^{-1}) of PC from soybean lecithin with 75 mM $HOCH_2CH_2SH$ in N_2O-saturated *t*-BuOH at 22 °C: (●, ▲, ■) without oxygen; (○, △, □) with 2.34×10^{-4} M oxygen. (a) 9*cis*-18:1 (●, ○) or 9*trans*-18:1(■, □). (b) 9*cis*,12*cis*-18:2 (●, ○), 9*cis*,12*trans*-18:2, and 9*trans*,12*cis*-18:2 (▲, △) or 9*trans*,12*trans*-18:2 (■, □). (c) 9*cis*,12*cis*,15*cis*-18:3 (●, ○), mono-trans isomers (▲, △), or di-trans isomers (▼, ▽).

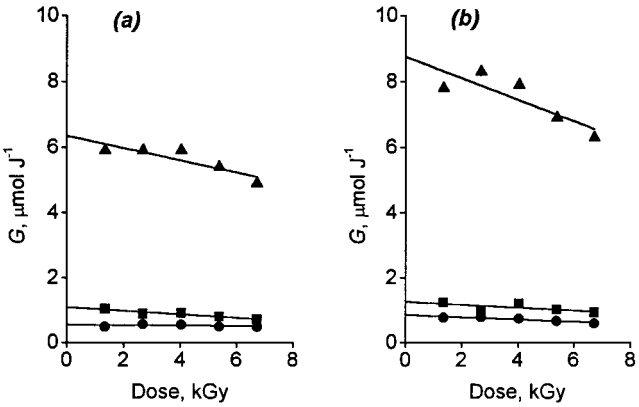


Figure 7. *G*[-(9*cis*-18:1)] (●), *G*[-(9*cis*,12*cis*-18:2)] (▲), or *G*[-(9*cis*,-12*cis*,15*cis*-18:3)] (■) vs dose. (a) Without oxygen. (b) With 2.34×10^{-4} M oxygen.

geometrical isomers of 18:1, 18:2, and 18:3 components is visible. The conversion of the initial material to the final mixture can be considered quantitative since the sum of various components accounts for a ∼95% yield.

Figure 6a shows the dose profiles of methyl oleate consumption (solid circles) and methyl elaidate formation (solid squares). Figure 6b shows the time profiles of methyl linoleate consumption (solid circles) and the formation of two mono-trans isomers (solid triangles) and di-trans isomers (solid squares). Figure 6c shows the time profiles of methyl linolenate consumption (solid circles) and the formation of three mono-trans isomers (solid triangles-up) and three di-trans isomers (solid triangles-down). The all-trans isomer if present is lower than our GC instrument sensitivity.

Figure 7a shows the plot of *G* values versus dose for oleic (circles), linoleic (triangles), and linolenic acid (squares) residues. The extrapolation to zero dose gives *G* values of 0.5, 6.4, and 1.1 μmol J^{-1}, respectively, which correspond to a total $G = 8$ μmol J^{-1}. Assuming that the $G(RS^{\bullet})$ is 0.65 μmol J^{-1} (eqs 4 and 5),[31] we calculated a catalytic cycle 12 at the initial phase. We have also considered the catalytic cycle for each component of the lipid mixture. The total molarity of C=C double bonds, based on the PC fatty acid composition (7.6% oleate, 80.6% linoleate, and 11.8% linolenate residues), can be calculated to be 0.237 M. By dividing these percentages by the corresponding *G* values (i.e., 7.6/0.5, 60.6/6.4, and 11.8/1.1),

we have again obtained an average catalytic cycle of 12, suggesting that at zero dose the attack of the RS• radical to double bonds is only a statistic.

Figure 6 (open symbols) shows the dose profile of the isomerization from a solution saturated with a mixture of N_2O/air containing 10% oxygen.[42] In comparison with the deoxygenated solution experiment (solid symbols), the reaction is considerably faster in all cases. To quantify this increase, in Figure 7b we plotted the *G* value versus dose for each of the unsaturated fatty acid residues. The extrapolation to zero dose gives *G* values of 0.9, 8.8, and 1.3 μmol J^{-1} for methyl oleate, methyl linoleate, and methyl linolenate, respectively, which corresponds to a total $G = 11$ μmol J^{-1} and to a catalytic cycle of 17 at the initial phase. Therefore, in the presence of 2.34×10^{-4} M oxygen,[42] an increase of the catalytic cycle from 12 to 17 is observed.

It is gratifying to see that the isomerization behavior is similar to the analogous reaction of methyl linoleate. The more complex structure of the starting material has no influence on the reaction mechanism. The inhibitor that contains conjugated diene moieties is also expected to be formed. Indeed, the isomerization of dioleoylphosphatidylcholine in *t*-BuOH reached the equilibrium mixture cis/trans = 20/80 in ~100 min, whereas the ratio cis/trans = 90/10 is obtained from Figure 6a under the same conditions.

Generation of Radicals in Vesicles. In the heterogeneous system (vesicles), thiols can either be incorporated into the bilayer or be dissolved in the aqueous phase. Amphiphilic $HOCH_2CH_2SH$ and glutathione (GSH) were used without any concern about the partition of thiol between hydrophobic and hydrophilic regions.[50] Radiolysis of neutral water leads to the species e_{aq}^-, HO•, and H• as shown in eq 6, where the values

$$H_2O \rightsquigarrow e_{aq}^- (0.27), HO^\bullet (0.28), H^\bullet (0.06) \qquad (6)$$

in parentheses represent the yields expressed in terms of *G* values (μmol J^{-1}).[34a] The presence of N_2O efficiently transforms e_{aq}^- into the HO• radical (eq 7, $k_7 = 9.1 \times 10^9$ M^{-1} s^{-1}). Hydrogen abstraction from *i*-PrOH by HO• and H• produces $(CH_3)_2{}^\bullet COH$ (eqs 8 and 9, $k_8 = 1.9 \times 10^9$ M^{-1} s^{-1}, $k_9 = 7.4 \times 10^7$ M^{-1} s^{-1}). The $(CH_3)_2{}^\bullet COH$ in turn reacts with the thiol to give the corresponding thiyl radical (eq 10, $k_{10} = 5.1 \times 10^8$ M^{-1} s^{-1} for $HOCH_2CH_2SH$ and $k_{10} = 1.8 \times 10^8$ M^{-1} s^{-1} for GSH).[35b]

$$e_{aq}^- + N_2O \xrightarrow{H_2O} N_2 + HO^\bullet + HO^- \qquad (7)$$

$$HO^\bullet + (CH_3)_2CHOH \rightarrow (CH_3)_2{}^\bullet COH + H_2O \qquad (8)$$

$$H^\bullet + (CH_3)_2CHOH \rightarrow (CH_3)_2{}^\bullet COH + H_2 \qquad (9)$$

$$(CH_3)_2{}^\bullet COH + RSH \rightarrow (CH_3)_2CHOH + RS^\bullet \qquad (10)$$

Isomerization of PC in LUVET. As far as the model membranes are concerned, large unilamellar vesicles (LUVET) made by the extrusion technique[51] were tested by γ-irradiation using $HOCH_2CH_2SH$ or GSH as thiols. A PC chloroform solution (3 mL; 0.15 mmol of fatty acid contents) was evaporated to a thin film in a test tube under an argon stream and then kept under vacuum for 30 min. A degassed phosphate-saline buffer was added, and multilamellar vesicles were formed by vortex stirring for 7 min under an argon atmosphere. LUVET were prepared by membrane extrusion with LiposoFast. The required amounts of $HOCH_2CH_2SH$ (7 mM) and 2-propanol (0.23 M) were added to LUVET suspensions prior to irradiation.[52] The reaction progress was monitored as already described. The rates of disappearance of oleate, linoleate, and linolenate moieties are similar to those observed in the isomerization experiments in *t*-BuOH. Figure 8a (solid symbols) shows the behavior of linoleate residues. The yield from the initial fatty acid composition to the final mixture (after 5 h of irradiation) was quantitative (by GC analysis whereas TLC showed the absence of byproducts, such as dimers and adducts). The plot of *G* disappearance versus dose for linoleic acid residue and the extrapolation to zero dose gives $G \cong 6$ μmol J^{-1}. This value is similar to the one measured in *t*-BuOH, indicating that the isomerization process in the heterogeneous system is as efficient as in homogeneous solution. In the analogous experiments of dioleoylphosphatidylcholine, a decreased efficiency of the cis−trans isomerization by the $HOCH_2CH_2S^\bullet$ radical was observed in going from the homogeneous solution to the LUVET experiment.[24] Therefore, we suggest that the formation of conjugated dienes (i.e., the inhibitor) during the isomerization process of PC in LUVET is less, compared to the analogous reaction in *t*-BuOH. However, we observed some substantial differences between the homogeneous *t*-BuOH solution and LUVET experiments: (i) the two mono-trans isomers of linoleic acid residues are formed in different amounts, the 9*trans*,12*cis*-18:2 being more abundant (Figure 8b), and (ii) the di-trans isomer (9*trans*,12*trans*-18:2) is formed in higher amounts since the initial phase (Figure 8a, solid squares).

When the reaction in LUVET was repeated in the presence of 10% oxygen, no substantial differences were observed.[53,55]

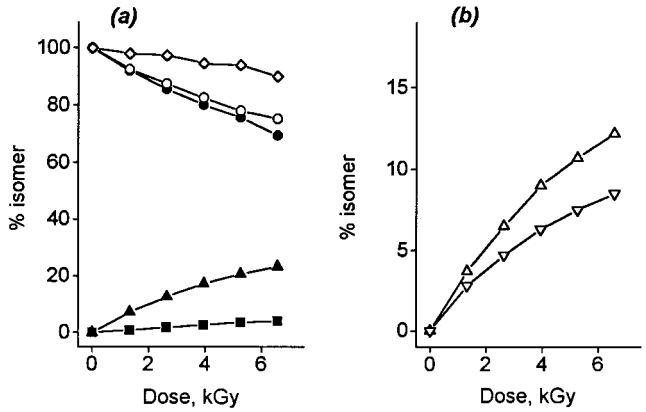


Figure 8. Irradiation (23.3 Gy min^{-1}) of PC from soybean lecithin in LUVET (0.15 M fatty acid contents) with 7 mM $HOCH_2CH_2SH$ at 22 °C. (a) Unsaturated fatty acid residues vs dose: methyl linoleate (●), mono-trans isomers (▲), or methyl linolelaidate (■) without oxygen; methyl linoleate with 1.34×10^{-4} M oxygen (○); methyl linoleate with 7 mM *all-trans*-retinol acetate (◇). (b) Time profiles of 9*cis*,12*trans*-18:2 (▽) and 9*trans*,12*cis*-18:2 (△) formation without oxygen.

(50) Newton, G. L.; Aguilera, J. A.; Kim, T.; Ward, J. F.; Fahey, R. C. *Radiat. Res.* **1996**, *146*, 206−215 and references therein.

(51) *Liposomes a practical approach*; New, R. R. C., Ed.; IRL Press: Oxford, U.K., 1990.

(52) 2-Propanol was used in order to avoid reactions of •OH and •H species with moieties other than SH in the thiols. The rate constants for the reactions of •OH and •H species with the employed thiols are known to be close to 10^{10} and 10^9 M^{-1} s^{-1}, respectively.[34b] To avoid changes in the lipid aggregates, we could not use 2-propanol as much as was necessary to capture all the •OH and •H. Therefore, on the basis of the available rate constants of the •OH and •H species with 2-propanol and the two thiols,[34b] the 2-propanol concentration was chosen (0.23 and 0.47 M for 7 mM $HOCH_2CH_2SH$ and GSH, respectively) so that ~90% of the •OH radicals and ~70% of the •H atoms reacted with 2-propanol (eqs 8 and 9).

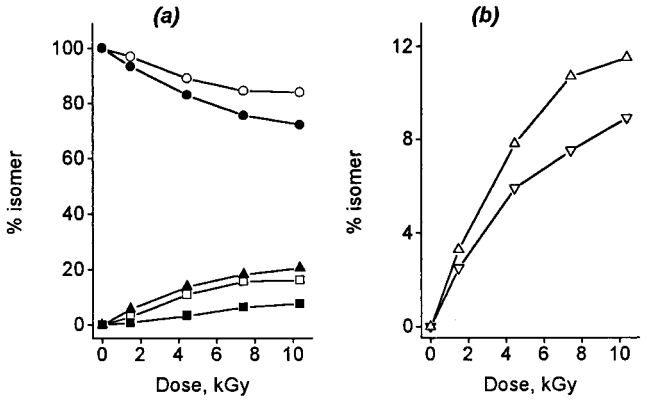


Figure 9. Irradiation (24.7 Gy min^{-1}) of PC from soybean lecithin in LUVET (0.15 M of fatty acid contents) with 7 mM GSH at 22 °C. (a) Unsaturated fatty acid residues vs dose: methyl oleate (○), methyl elaidate (□), methyl linoleate (●), mono-trans isomers (▲), or methyl linolelaidate (■). (b) Time profiles of 9*cis*,12*trans*-18:2 (▽) and 9*trans*,-12*cis*-18:2 (△) formation.

Figure 8a (open circles) shows the dose profile of the disappearance of linoleate moieties. In comparison with the experiment without oxygen, the isomerization is perhaps slightly slower. Indeed, a plot of *G* disappearance of linoleate versus dose gives $G \cong 5.6$ μmol J^{-1} (by extrapolation of the curve to zero dose), which is comparable to $G \cong 6$ μmol J^{-1} obtained without oxygen. The yield of the reaction slightly dropped (i.e., from ∼100 to ∼94%), and TLC showed traces of polar secondary products such as adducts. However, also in these experiments, the two mono-trans isomers are present in a different ratio, the 9*trans*,12*cis*-18:2 being the more abundant (similar to Figure 8b), and the rate of di-trans isomer formation is again faster. Therefore, in LUVET the efficiency of the cis−trans isomerization without or with 10% oxygen can be considered the same.

To model the biological environment, the $HOCH_2CH_2SH$ was replaced by GSH.[52] It is gratifying to see that the cis−trans isomerization is still effective (Figure 9a). For example, the plot of *G* disappearance versus dose for linoleic acid residue and the extrapolation to zero dose gives a *G* value of 4.5 μmol J^{-1}. Comparison of the two experiments with different thiols suggests that the isomerization rate follows the lipophilicity order of the two compounds[50] (i.e., $HOCH_2CH_2SH$ > GSH). Also in the experiments with GSH, we observed a similar behavior regarding the formation of mono-trans and di-trans isomers (Figure 9b). Therefore, this phenomenon is not associated with the nature of the thiol but with the supramolecular organization of unilamellar vesicles.

The initially formed alkyl radicals from 2-propanol (eqs 8 and 9) in the aqueous compartment react with thiol to form the corresponding thiyl radical that has to migrate into the lipid compartment prior to isomerization. For the amphiphilic thiol, there are no barriers of migration between the aqueous and lipid compartments. Therefore, the thiyl radical entering the hydrophobic region of the bilayer first reaches and isomerizes the 9,10-double bond. This "positional effect" is the simplest explanation for the different percentage of the two mono-trans isomers, the 9*trans*,12*cis*-18:2 being the more abundant (see Figure 8b and 9b). Due to the packing of fatty acid residues and, consequently, to a highly defined lateral diffusion,[56] thiyl radicals, after the isomerization of the 9,10-double bond, have a chance to isomerize the second double bond in the same chain, before migrating laterally.

As a consequence of the product studies described above and in view of the well-assessed capability of retinoid and carotenoid molecules to react with thiyl radicals,[57] we have tested the inhibition of the isomerization process by irradiating LUVET where 7 mM *all-trans*-retinol acetate was incorporated into the lipid bilayer. Figure 8a (open diamonds) shows the dose profile of the disappearance of linoleate moieties. The plot of *G* disappearance of linoleate versus dose gives a *G* value of ∼1 μmol J^{-1} by extrapolation of the curve to zero dose that indicates the absence of a catalytic reaction. Moreover, after 7 h of irradiation, the overall yield was found to be quantitative. In comparison with the experiment without *all-trans*-retinol acetate, the reaction is substantially retarded. Indeed, the polyene chain, intercalating into the bilayer, is able to compete with the lipid double bonds for the thiyl radical addition.

Conclusions

We have shown that phospholipids containing *trans*-fatty acid residues are the major products of the thiyl radical attack on phosphatidylcholine from soybean lecithin, both in homogeneous solution and in lipid vesicles. The presence of oxygen in concentrations higher than that of typical well-oxygenated tissues does not influence the reaction outcome, and PUFA peroxidation[22] is found to be unimportant. Our observations are in good agreement with the known role of "protection" played by thiols in autoxidation processes.[22,58] The present findings also test the hypothesis of an endogenous radical-based isomerization process occurring in the biological environment. This event does not cause lipid degradation, but a permanent modification, and deserves attention at least for two aspects: (i) the influence of *trans*-fatty acid residues on the structure and properties of human cell membranes and (ii) the perturbation of cell adaptation systems, which are based on the number of cis unsaturation present in the bilayer of bacteria and plants. Lipophilic compounds containing a polyconjugated dienic moiety inhibit the cis−trans isomerization process. In this respect, retinoids and carotenoids that are well known to participate in diverse processes, such as vision, growth, development,[59a] and anti-autoxidation,[59b] could also be protective agents for the double bond geometry.

(53) The solubility of molecular oxygen in H_2O is 1.34×10^{-3} M at 22 °C and 1 atm partial pressure.[54] Therefore, 10% of the oxygen-saturated solution is equal to 1.34×10^{-4} M which is ∼3 times higher than a typical well-oxygenated tissues, i.e., $[O_2] \cong 0.04$ mM.

(54) Battino, R.; Rettich, T. R.; Tominaga, T. *J. Phys. Chem. Ref. Data* **1983**, *12*, 163−178.

(55) Thiyl radicals have been reported to add reversibly to oxygen and thiolate. For example $GS^\bullet + O_2 \rightleftharpoons GSOO^\bullet$, $K = 3200$ M^{-1} (rate constants for the forward and reverse reactions are 2.0×10^9 M^{-1} s^{-1} and 6.2×10^5 s^{-1}, respectively) and $GS^\bullet + GS^- \rightleftharpoons GSSG^{\bullet-}$, $K = 3500$ M^{-1} (rate constants for the forward and reverse reactions are 6.2×10^8 M^{-1} s^{-1} and 1.8×10^5 s^{-1}, respectively). The disulfide radical-anion is known to react with oxygen to give superoxide ($k = 5.1 \times 10^8$ M^{-1} s^{-1}), which might be expected to oxidize the GSH slowly ($k \approx 10^3$ M^{-1} s^{-1}) to regenerate $GS^\bullet$ radical. Under our experimental conditions, these reaction are considered to be unimportant (For recent reviews, see: Wardman, P. In *S−Centered Radicals*; Alfassi, Z. B., Ed.; Wiley: Chichester, U.K., 1999; pp 289−309. Wardman, P.; von Sonntag, C. *Methods Enzymol.* **1995**, *251*, 31−45). However, work is in progress to evaluate the efficiency of cis−trans isomerization with selectively generated species such as $O_2^{\bullet-}$ and $^\bullet NO_2$.

(56) Zachowski, A. *Biochem. J.* **1993**, *294*, 1−14.

(57) Everett, S. E.; Dennis, M. F.; Patel, K. B.; Maddix, S.; Kundu, S. C.; Willson R. L. *J. Biol. Chem.* **1996**, *271*, 3988−3994. D'Aquino, M.; Dunster, C.; Willson, R. L. *Biochem. Biophys. Res. Commun.* **1989**, *161*, 1199−1203.

(58) Barclay, L. R. C.; Dakin, K. A.; Khor, J. A. Y. *Res. Chem. Intermed.* **1995**, *21*, 467−488.

(59) (a) *Handbook of Vitamins: nutritional, biochemical, and clinical aspects*; Machlin, L. J., Ed.; Marcel Dekker: New York, 1984; pp 1−43. (b) For example, see: Burton, G. W.; Ingold, K. U. *Science* **1984**, *224*, 569−573. Samokyszin, V. M.; Marnett, L. J. *Free Radical Biol. Med.* **1990**, *8*, 491−496.

Work is in progress in order to evaluate the importance of cis−trans isomerization in living systems as well as of endogenous factors involved in the protection of geometrical lipid integrity from free-radical attacks.

Experimental Section

Materials. AIBN, AMVN, AAPH, PhSH, $HOCH_2CH_2SH$, GSH, methyl oleate, methyl linoleate, linoleic acid methyl ester cis and trans isomers, linolelaidic acid methyl ester, linolenic acid methyl ester, linolenic acid methyl ester isomer mix, conjugated octadecadienoic acid methyl esters, and methyl palmitate were commercially available from Aldrich, Fluka, or Sigma and used without further purification. Lyophilized L-α-phosphatidylcholine from soybean (99%) purchased from Sigma was dissolved in chloroform (20 mg/mL) and stored in 1-mL sealed ampules at −18 °C. *tert*-Butyl alcohol, chloroform, and 2-propanol were purchased from Merck (HPLC grade) and used without further purification. Phosphate saline buffer (PBS) was prepared (Na_2HPO_4 10 mM, NaCl 0.14 M) at pH 7.2.

General Methods. GC analyses for the determination of the isomeric ratio of the unsaturated fatty acids were performed by using a Varian CP-3800 equipped with a flame ionization detector. As a stationary phase, an Rtx-2330 column (60 m × 0.25 mm of 10% cyanopropylphenyl and 90% biscyanopropylpolysiloxane) was used with helium as carrier gas. The heating was carried out at a temperature of 155 °C for 55 min followed by an increase of 5 °C/min up to 195 °C. The methyl esters were identified by comparison with the retention times of authentic samples. In few cases, GC analyses were performed on an HP 5890 Series II using a 30 m × 0.25 mm cross-linked 5% phenylsilicone capillary column (HP 5).

Continuous radiolyses were performed at room temperature (22 ± 2 °C) on 1-mL samples using a ^{60}Co Gammacell at different dose rates. The exact absorbed radiation dose was determined with the Fricke chemical dosimeter, by taking $G(Fe^{3+}) = 1.61$ μmol J^{-1}.[60] The flow monitoring of a 1:1 N_2O/air mixture prior to γ-irradiation was controlled by a flowmeter to be at 68 cm^3 min^{-1}. Occasionally the gas flowing was continued during the irradiation by means of a cannula.

Flash chromatography was performed on Merck silica gel 60 (400−230 mesh) using a nitrogen stream. ^{1}H, ^{13}C NMR spectra were recorded on a Varian VXR 400-MHz instrument using $CDCl_3$ as the solvent and the reference peak. EI MS spectra were recorded on a Finnigan MAT GCQ instrument equipped with a direct insertion probe DIP.

Isomerization of Methyl Linoleate in *tert*-Butyl Alcohol. A 0.15 M solution of methyl linoleate (43 mg) in *t*-BuOH (0.945 mL) was placed under argon in a 2.5-mL vial equipped with an open top screw cap and a Teflon-faced septum and added with methyl palmitate (27 mg; 0.1 mmol) as the internal standard. The solution was degassed with argon for 15 min, added with 0.03 M AMVN, 0.075 M PhSH, or $HOCH_2CH_2SH$ and a magnetic stirrer, and then heated at 54 °C. Alternatively, the solution was saturated with N_2O or a 1:1 N_2O/air mixture, followed by the addition of $HOCH_2CH_2SH$ prior to γ-irradiation. Aliquots of the solution were withdrawn at different times and examined by GC analysis.

The isomerization of linolelaidic acid methyl ester and linoleic acid methyl ester isomer mix was performed under similar conditions.

Product Studies in Methyl Linoleate Isomerization with $HOCH_2CH_2SH$. The isolation of the products was effected using a 10-mL scale of the above-reported experiment after 7 h of irradiation. The reaction mixture was evaporated under vacuum and flash chromatographed. Using *n*-hexane as the eluent, a first fraction was obtained corresponding to a 93% yield of the four geometrical isomers. Elution with 8/2 *n*-hexane/diethyl ether gave a second fraction, which was attributed to a mixture of dimeric conjugated fatty acids (18 mg, 4% yield; R_f = 0.47): ^{1}H NMR δ 0.88 (m, 6H), 1.05−1.48 (m, 32H), 1.56−1.63 (m, 4H), 1.92−2.05 (m, 6H, allylic Hs), 2.26−2.32 (m, 4H), 3.66 (s, 6H), 5.14−6.24 (m, 8H); ^{13}C NMR δ 13.9, 14.2 14.3 (CH_3), 22.6, 22.7, 25.0, 27.1−27.9, 29.1−29.7, 30.6, 31.6, 31.8, 32.0, 32.1, 32.7, 34.2 (CH_2), 40.6, 47.6, 47.9, 48.1, 48.3 (CH), 51.5 (CH_3), 128.6−134.3 (CH), 174.1 (C═O); MS *m*/*z* (rel abundance): 586 (M^+, 3), 555 (10), 293 (100), 261 (40), 243 (50). Further elution gave a third fraction which was attributed to a mixture of thiol−fatty acid adducts (11 mg, 2%yield; R_f = 0.16):^{1}H NMR δ 0.86 (m, 3H), 1.20−1.42 (m, 16H), 1.48−1.61 (m, 4H), 1.98 (m, 2H, recognizable as a major q with J = 6.7 Hz, allyl CH_2), 2.12 (m, 1H, allyl CH), 2.23 (m, 1H, CH allyl), 2.27 (m, 3 H, CH_2 alpha to carbonyl + OH), 2.59 (m, 1H, CHS), 2.71 (m, 2H, CH_2S), 3.64 (s, 3H, OMe), 3.67 (m, 2H, CH_2O), 5.28−5.48 (m, 2H); ^{13}C NMR δ 14.2, (CH_3), 22.2, 22.3, 25.0, 26.5−27.2, 29.1−29.6, 31.4, 31.8, 32.6, 33.8, 34.0, 34.1, 34.4, 34.5, 34.8, 34.9, 38.3 (CH_2), 45.1, 45.8 (CH), 51.4 (CH_3), 60.6, 60.7 (CH_2), 126.1, 126.3, 126.5, 128.9, 129.1, 130.5, 130.9, 131.2, 131.9, 132.1, 133.1, 133.3 (CH), 174.0 (C═O); MS *m*/*z* (rel abundance): 371 (M^++1, 3), 355 (9), 327 (100), 295 (22), 263 (9), 243 (46).

Competitive Isomerization of Methyl Oleate and Methyl Linoleate. In a Wheaton reactor equipped with a Mininert valve and a magnetic stirrer, methyl oleate (44.4 mg; 0.15 mmol), methyl linoleate (44 mg; 0.15 mmol), and methyl palmitate (27 mg; 0.1 mmol) as the internal standard were dissolved in 1 mL of *t*-BuOH. The solution was degassed with argon for 15 min, added with AMVN (7.5 mg; 0.03 mmol) and $HOCH_2CH_2SH$ (5.86 mg; 0.075 mmol), and then heated at 54 °C. At different times, an aliquot of the reaction mixture was withdrawn for the GC analysis.

Isomerization of Methyl Linolenate in *tert*-Butyl Alcohol. Conditions similar to the above-mentioned isomerization of methyl linoleate were used. After 7 h of irradiation, a 92% yield of the eight isomers was found. A TLC examination of the reaction mixture (eluent, 7/3 *n*-hexane/diethyl ether) evidenced the formation of two secondary products, which, in analogy with the byproducts isolated from the linoleate reaction, were attributed to dimers (R_f = 0.57) and to thiol−fatty acid adducts (R_f = 0.11).

The reaction mixture was evaporated and flash chromatographed. Elution with *n*-hexane gave a first fraction which contained methyl palmitate and a mixture of the eight 18:3 isomers in a 92% yield. Further elution with 9/1 *n*-hexane/diethyl ether gave a second fraction which, from the spectral characteristics, was attributed to a mixture of dimeric conjugated fatty acids (4.0 mg; 4% yield), whereas elution with 1/1 *n*-hexane/diethyl ether gave a third fraction (2 mg; 3.6% yield) which, from the spectral characteristics, was attributed to a mixture of thiol addition products.

Isomerization of L-α-Phosphatidylcholine from Soybean Lecithin in *tert*-Butyl Alcohol. A PC/chloroform solution (3 mL; 0.15 mmol of fatty acid content) was evaporated in a test tube under an argon stream and then under vacuum for 30 min. *t*-BuOH (1 mL) was added, and the solution was transferred to a vial equipped with an open top screw cap and a Teflon-faced septum where it was saturated with N_2O or a 1:1 N_2O/air mixture prior to γ-irradiation. $HOCH_2CH_2SH$ (0.075 mmol) was then added, and the solution was irradiated. Aliquots of 100 μL were withdrawn and processed at different times by partitioning between *n*-hexane (or 2/1chloroform/methanol in the case of vesicles) and brine, extraction and collection of the organic phases dried over anhydrous sodium sulfate, and evaporation of the solvent under vacuum at room temperature. The residue containing the phospholipids was treated with 0.5 M KOH/MeOH, for 10 min at room temperature and then poured into the brine and extracted with *n*-hexane. The organic layer containing the corresponding fatty acid methyl esters was examined by GC analysis in comparison with the retention times of authentic samples.

Isomerization of PC in LUVET. A chloroform solution of PC (3 mL; 0.15 mmol of fatty acid content) was evaporated to a thin film in a test tube under an argon stream and under vacuum for 30 min. Degassed PBS (1 mL) was added, and multilamellar vesicles were formed by vortex stirring for 7 min under an argon atmosphere. To obtain LUVET, the lipid emulsion was transferred into a LiposoFast (produced by Avestin, Inc.) and extruded 19 times back and forth through two polycarbonate membranes with a pore diameter of 100 nm.[61] The suspension was then transferred to a vial equipped with an open top screw cap and a Teflon-faced septum where it was saturated with N_2O or a 1:1 N_2O/air mixture prior to γ-irradiation. $HOCH_2CH_2$-

(60) Reference 32; p 100.

(61) The average diameter of the unilamellar vesicles was found to be ∼90 nm; see: Fiorentini, D.; Cipollone, M.; Pugnaloni, A.; Biagini, G.; Landi, L. *Free Radical Res.* **1994**, *21*, 329−339.

SH or GSH (0.075 mmol) and *i*-PrOH (0.23 or 0.47 M, respectively) were consecutively added and the suspension was irradiated. Aliquots of 100 μL were withdrawn and processed at different times as previously described.

Isomerization of PC in LUVETs in the Presence of Vitamin A. *all-trans*-Retinol acetate (23 mg; 0.07 mmol) was dissolved in chloroform (1 mL), and 100 μL of this stock solution (corresponding to 7 μmol of vitamin A acetate) was evaporated in a test tube under an argon stream. A chloroform solution of PC (3 mL; 0.15 mmol of fatty acid content) was then added and evaporated to a thin film under an argon stream and under vacuum for 30 min. LUVETs were prepared and irradiated as previously described.

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