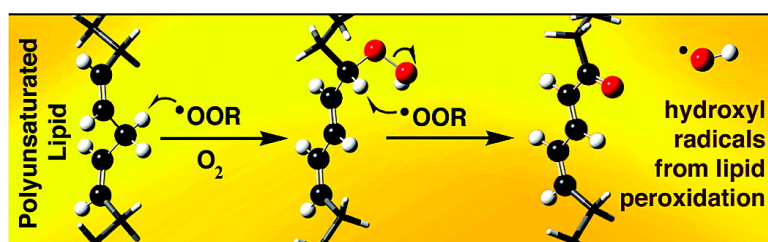


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J. Am. Chem. Soc., **2008**, 130 (30), 9634-9635 • DOI: 10.1021/ja801858e • Publication Date (Web): 03 July 2008

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Evidence for Hydroxyl Radical Generation During Lipid (Linoleate) Peroxidation

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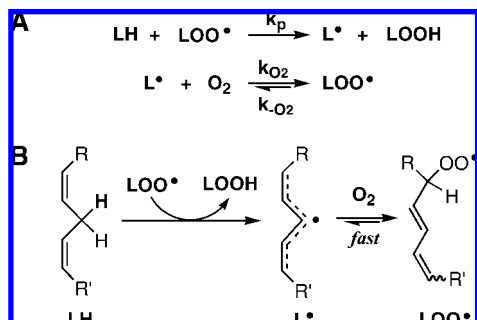
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Reactive oxygen species (ROS) are collectively responsible for most oxidative stress occurring in living organisms, and hydroxyl radicals ($\text{HO}\cdot$) are the most reactive of all ROS. Some observed oxidation products appear to be derived from reactions involving $\text{HO}\cdot$, and therefore, mechanisms for the formation of hydroxyl radicals *in vivo* have been the subject of research interest for many decades. Presumably, the most abundant source of $\text{HO}\cdot$ *in vivo* is the Fe- and Cu-catalyzed Fenton decomposition of hydrogen peroxide.¹

In this communication, we present strong experimental evidence that hydroxyl radicals are also formed during lipid peroxidation, a very common process associated with oxidative stress. Polyunsaturated lipids are prime targets for free radical induced oxidation. The primary oxidation products are mostly hydroperoxides (LOOH, Scheme 1), and the mechanisms involved in their formation are well-established.²

Scheme 1^a



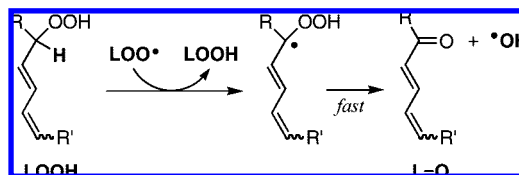
^a A: General lipid peroxidation mechanism. B: Mechanism as it applies to polyunsaturated fatty acids;^{2b} kinetic products shown, other minor isomers also expected. Only propagation steps are shown. Methyl linoleate has $\text{R}, \text{R}' = \text{CH}_3(\text{CH}_2)_4$ and $\text{CH}_3\text{OCO}(\text{CH}_2)_7$.

If free radical oxidation of lipids is allowed to continue in the absence of chain-breaking antioxidants, ketones and aldehydes are formed and these molecules are known to be biologically active.³ Despite significant interest in lipid peroxidation, the mechanism for the formation of lipid ketones (also called oxodienes) has eluded a satisfactory explanation for many years.⁴ In this contribution, we provide evidence for a simple mechanism that forms ketones from the corresponding lipid hydroperoxides (Scheme 2). Notably, the mechanism provides a pathway in which hydroxyl radicals are formed during metal-independent lipid peroxidation.

We note that the most likely reaction of peroxy radicals with hydroperoxides is the abstraction of the hydrogen atom from the $\text{LOO}-\text{H}$ bond;^{3b} however, this is effectively an identity reaction, which at most exchanges one peroxy radical for another.

At first glance, the generation of $\text{HO}\cdot$ from a lipid peroxy radical ($\text{LOO}\cdot$) may seem unlikely, but the $\text{O}-\text{O}$ cleavage of Scheme 2 is

Scheme 2. Proposed Mechanism for the Oxidation of LOOH by $\text{LOO}\cdot$ to Form a Ketone ($\text{L}=\text{O}$) and a Hydroxyl Radical



very exothermic since the breaking of the weak peroxide bond is energetically compensated by ketone formation. Reactions of this type were proposed to explain the formation of ketones in the high-temperature autoxidation of alkanes⁵ and, more recently, to explain the formation of acetophenone during the autoxidation of ethylbenzene.⁶ In theory, this mechanism could explain the formation of hydroxyl radicals from any secondary (or primary) hydroperoxide.

To explore the viability of this reaction in lipid peroxidation, we studied the oxidation of methyl linoleate (LH) initiated by the free radical initiator 2,2'-azo-bis-isobutyronitrile (AIBN). The reactions were carried out in the dark at 37 °C under air in benzene. If hydroxyl radicals are formed, they will react with benzene to yield phenol.⁷ The generation of phenol from benzene is a selective test for $\text{HO}\cdot$ since the control experiment (with no oxidizable lipid, but with a peroxy radical source) did not yield significant amounts of phenol (Figure 1, trace "CONTROL").⁸ In Figure 1, we plot the measured phenol concentration (GC/MS) as the oxidation of methyl linoleate in benzene progresses. While the yields of phenol are modest, these results are strong evidence that $\text{HO}\cdot$ is formed during lipid peroxidation. We propose that the source of $\text{HO}\cdot$ is from the reaction of $\text{LOO}\cdot$ with LOOH according to Scheme 2. The upward curvature for the formation of phenol also supports a secondary oxidation as its precursor.

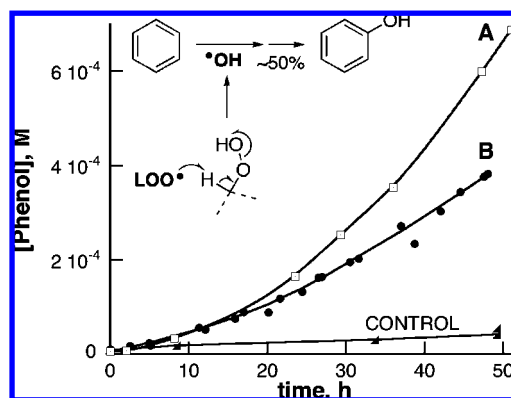


Figure 1. Phenol is formed during the 37 °C autoxidation of methyl linoleate (LH) under air in benzene due to $\text{HO}\cdot$ formation. Experimental conditions: (A) 0.372 M LH, 0.0189 M AIBN; (B) 0.189 M LH, 0.0189 M AIBN; (CONTROL) 0 M LH, 0.0189 M AIBN.

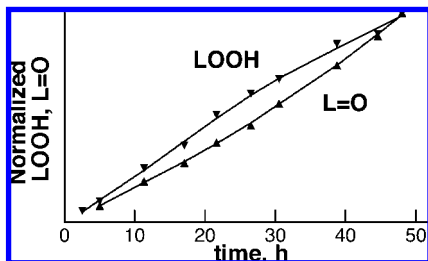


Figure 2. Normalized GC-MS signals for $m/z = 310$ and $m/z = 308$, corresponding to LOOH (major peak only, after Ph_3P reduction) and to $\text{L}=\text{O}$ (major peak). The experimental conditions are identical to trace B in Figure 1; note the similar upward curvature for $\text{L}=\text{O}$ and phenol formation.

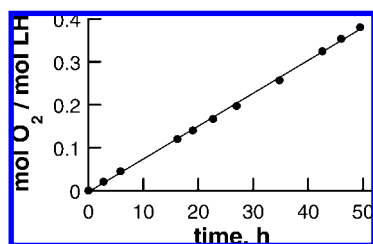


Figure 3. Oxygen consumed in function of time plotted as a fraction of oxygen consumed per LH in the system, with linear fit (same conditions as Figure 1B). See Supporting Information for more information.

We also monitored by GC-MS the formation of the major hydroperoxide peak (LOOH, $m/z = 310$ after reduction to the alcohol with Ph_3P) and the major ketone peak ($\text{L}=\text{O}$, $m/z=308$). The growth of $\text{L}=\text{O}$ and phenol both shows a similar upward curvature, but LOOH shows a slight downward curvature (Figure 2). This supports the mechanism proposed in Scheme 2. The difference between phenol and LOOH also appeases concerns that unreduced hydroperoxides could generate hydroxyl radicals in the GC/MS injector and would account for the phenol formed.

We also monitored the oxygen uptake during the autoxidation of LH (Figure 3). Under these conditions, we estimate the yield of hydroxyl radical per oxygen molecule consumed to be $\sim 1\%$ after 50 h, corresponding effectively to a 2% reaction yield, given the stoichiometric need for two oxygen molecules to produce one $\text{HO}\cdot$ radical (see Schemes 1 and 2). This yield is calculated for a level of oxidation well above naturally occurring systems; however, we think the proposed reaction is more probable in membranes due to the geminate co-localization of both reagents; in other words, LOOH and $\text{LOO}\cdot$ are “born” together in the lipid membrane. More mechanisms could be imagined for the formation of hydroxyl radicals from this complex reaction mixture, but we believe the mechanism shown in Scheme 2 is valid for many reasons, some mentioned above. Preliminary data on the autoxidation of methyl oleate (only one *cis* double bond) in benzene under air also show the characteristic upward curvature for phenol and the lipid ketone products (data not shown). Comparison of the data in Figures 1 and 3 suggests that abstraction from LH is about an order of magnitude faster than from LOOH (see Supporting Information).

We also turned to DFT calculations⁹ in an attempt to establish if computed thermodynamic energies were consistent with the mechanism proposed. We set out to compare the H-donating ability of the unsaturated lipids (LH) and the corresponding lipid hydroperoxide (LOOH). The activation energies for reaction between a model peroxy radical ($\text{MeOO}\cdot$) and LOOH and LH moieties were calculated (see Supporting Information). While the secondary reaction has a slightly higher transition state free energy,¹² the release of a hydroxyl radical remains *very* thermodynamically favorable with an exergonicity (ΔG) of -42.2 kcal/mol.

In summary, we have obtained evidence that $\alpha\text{-C-H}$ hydrogen abstraction from hydroperoxides formed during lipid autoxidation can lead to the formation of ketones and hydroxyl radicals in a long overlooked path to these intermediates.

Acknowledgment. We acknowledge generous financial support from the Natural Sciences and Engineering Research Council of Canada. M.F. is grateful for scholarships from OGS and the “Fondation Baxter & Alma Ricard”. Thanks are due to Dr. Chad Beddie for assistance with DFT calculations, Dr. Keith U. Ingold for valuable discussions, and to Dr. L. Ross C. Barclay for the very generous gift of an oxygen uptake apparatus. Some calculations were performed on the Shared Hierarchical Academic Research Computing Network (SHARCNET: www.sharcnet.ca).

Supporting Information Available: Full experimental and theoretical details; complete ref 11. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (8) In another control experiment, the oxidation of isopropylbenzene in benzene (1:1 v/v) was oxidized by AIBN (0.0189M) under air at 37 °C. After 48 h, no increase in phenol was observed, indicating that secondary hydroperoxides are necessary for the formation of hydroxyl radicals.
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JA801858E