REVIEW

Metals and Lipid Oxidation. Contemporary Issues[1]

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Lipid oxidation is now recognized to be a critically important reaction in physiological and toxicological processes as well as in food products. This provides compelling reasons to understand what causes lipid oxidation in order to be able to prevent or control the reactions. Redox-active metals are major factors catalyzing lipid oxidation in biological systems. Classical mechanisms of direct electron transfer to double bonds by higher valence metals and of reduction of hydroperoxides by lower valence metals do not always account for patterns of metal catalysis of lipid oxidation in multiphasic or compartmentalized biological systems. To explain why oxidation kinetics, mechanisms, and products in molecular environments which are both chemically and physically complex often do not follow classical patterns predicted by model system studies, increased consideration must be given to five contemporary issues regarding metal catalysis of lipid oxidation: hypervalent non-heme iron or iron-oxygen complexes, heme catalysis mechanism(s), compartmentalization of reactions and lipid phase reactions of metals, effects of metals on product mixes, and factors affecting the mode of metal catalytic action.
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Metal catalysis of lipid oxidation has been recognized for decades. Early research focussed on oxidation of food lipids, and it was in this area that there was intense research effort during the 1960s. Most attention at that time was given to determining lipid oxidation kinetics and catalytic mechanisms of the metals, including which metals, which chelates or complexes, and which valence states were most active (1-11). During the early 1970s, the feeling that the research of the 1960s had discovered all the answers led to metal catalysis of lipid oxidation being considered, for the most part, passé. However, during the late 1970s and early 1980s, the realization that metals play an important, if not critical role in oxidative cytotoxicity reawakened interest in metal catalysis (12-19). Oxidative cytotoxicity refers to pathological processes presumed to be caused by reduced forms of oxygen including H_2O_2, $O_2^{\cdot-}/HO_2^{\cdot}$ (may react directly or dismutate to H_2O_2), $HO^{\cdot}$ from reduction of H_2O_2, and lipid alkoxyl or peroxyl radicals. Production of these radicals is driven catalytically by the trace levels of iron, copper, and perhaps other redox-active metals present in tissues (14,15,18,20,21). Indeed, many scientists feel that participation of metals is *obligatory* for toxic fluxes of radicals which may overwhelm natural defense mechanisms in some diseases and in chemical and drug toxicities (18,20,22,23).

Largely due to the interest of the toxicologists, research on metal catalysis of lipid oxidation has been renewed and revitalized during the 1980s. Food scientists, too, are finding new problems with metals relative to the stability of lipids, particularly in multiphasic foods. Several contemporary issues in metal catalysis have evolved from attempts to understand and explain why oxidation kinetics, mechanisms, and products in molecular environments which are both chemically and physically complex (as in foods and tissues) often do not follow the classical patterns predicted from model system studies.

The general chemical mechanisms for metal catalysis of lipid oxidation are quite familiar (3,24-27).

DIRECT INITIATION

1) by higher valence state metals, via electron transfer and formation of lipid alkyl radicals (2,24). Iron and copper (28) are known to behave this way, as do Mn, Ni, and Co (29,30). For LH representing an unsaturated lipid molecule

$$M^{(n+1)+} + LH \rightarrow M^{n+} + L^{\cdot} + H^{+} \quad [1]$$

2) by lower valence state metals. a) *via* formation of metal-oxygen transition complexes ($M^{(n+1)+} \ldots O_2$), particularly in non-polar solvents (2,3)

$$M^{n+} + O_2 \rightarrow (M^{(n+1)+} \ldots O_2) \rightarrow \begin{cases} M^{(n+1)+} + O_2^{\cdot-} & [2a] \\ \xrightarrow{LH} L^{\cdot} + (M^{n+1)+} \ldots {}^{-}O_2H) & [2b] \\ \xrightarrow{LH} L^{\cdot} + M^{n+} + HO_2^{\cdot} & [2c] \\ \xrightarrow{LH} LO^{\cdot} + (M^{(n+1)+} \ldots OH^{-}) & [2d] \\ \xrightarrow{(M^{n+}LH)} HO_2^{\cdot} + (M^{(n+1)+}L^{-}) + M^{n+} & [2e] \end{cases}$$

b) *via* metal autoxidation, which produces reactive oxygen species, $O_2^{\cdot-}/HO_2^{\cdot}$ and H_2O_2

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\cdot-} \xrightarrow{H^{+}} HO_2^{\cdot} \quad [3]$$

$$O_2^{\cdot-}/HO_2^{\cdot} \rightarrow H_2O_2 + O_2 \quad [4]$$

Reaction of $O_2^{\cdot-}$ with double bonds of unsaturated fatty acids was proposed as early as 1962 (4), but pulse radiolysis studies have shown that it is $HO_2^{\cdot}$ rather than $O_2^{\cdot-}$ that reacts with unsaturated fatty acids (31,32), and

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Abbreviations: ADP, adenosine diphosphate; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; NHE, normal hydrogen electrode; NMR, nuclear magnetic resonance; SOD, superoxide dismutase; TBA, thiobarbituric acid.

the reaction is slow ($k = 300 - 1700\ M^{-1}\ sec^{-1}$) (31,33, 34). Reaction of $O_2^{\cdot}$ /$HO_2^{\cdot -}$ with lipid hydroperoxides has been reported (35), but a later study showed that scrupulously demetalled LOOH is not decomposed by $O_2^{\cdot -}$ (36).

More important is the reduction of H_2O_2 to yield hydroxyl radicals, HO$^{\cdot}$ (37), which initiate lipid oxidation by hydrogen abstraction ($k = 8 \times 10^9\ M^{-1}s^{-1}$ for linolenate) (32,38)

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + HO^{\cdot} + OH^- \quad [5]$$

$$HO^{\cdot} + LH \rightarrow L^{\cdot} + H_2O \quad [6]$$

INDIRECT INITIATION, PROPAGATION, OR CHAIN BRANCHING

Catalysis by some metals (Fe, Cu, Ni) occurs by oxidation (39,40) or reduction (41) of preformed lipid hydroperoxides (LOOH) to form chain-carrying LO$^{\cdot}$ and LOO$^{\cdot}$ radicals. Using iron as an example

$$LOOH + Fe^{3+} \rightarrow LOO^{\cdot} + H^+ + Fe^{2+} \quad [7]$$

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^- + Fe^{3+} \quad [8]$$

According to traditional theory, these hydroperoxide decompositions increase the rate of chain re-initiation or propagation because the rates of hydrogen abstraction by LO$^{\cdot}$ and LOO$^{\cdot}$ are much faster than the rates of *ab initio* L$^{\cdot}$ formation (24). Recent evidence, however, has suggested that secondary epoxyallylic radicals from LO$^{\cdot}$ rearrangements may be more likely chain carriers under some conditions (42).

The redox potentials of other metals such as Mn and Co are too low to effect LOOH decomposition in aqueous systems (41), but they may catalyze hydroperoxide decomposition in non-polar media by formation of metal-hydroperoxide complexes (1,40,43).

$$Co^{3+} + LOOH \rightarrow (LOOHCo)^{3+} \rightarrow Co^{2+} + LO_2^{\cdot} + H^+ \quad [9]$$

$$Co^{2+} + LOOH \rightarrow (LOOHCo)^{2+} \rightarrow Co^{3+} + LO^{\cdot} + OH^- \quad [10]$$

Inhibitory effects of metals are also known, usually when metals are present at higher concentrations (10,25, 43–46). The inhibition is thought to result from oxidation and reduction of free radicals by iron and copper (24,37, 47,48) and from metal complexation of free radicals by Co (43,49). Either mechanism results in interruption of the free radical chain and reduction in the overall oxidation rate.

$$\text{(Fe, Cu)} \quad L^{\cdot} + M^{(n+1)+} \rightarrow (L^{\cdot} \ldots M^{(n+1)+}) \text{ (stable)} \quad [11a]$$

$$\text{(Fe, Cu)} \quad L^{\cdot} + M^{(n+1)+} \rightarrow M^{n+} + L^+ \rightarrow \text{products} \quad [11b]$$

$$L^{\cdot} + M^{n+} \rightarrow M^{(n+1)+} + L^- \quad [12]$$

$$\text{(Co, Mn)} \quad R^{\cdot} + MA_n \rightarrow R\text{-}MA \quad [13]$$

where R$^{\cdot}$ may be L$^{\cdot}$, LO$^{\cdot}$, LOO$^{\cdot}$, etc.

These mechanisms have been derived from studies conducted in relatively simple chemical model systems of defined composition with controlled reaction conditions. Biological systems, on the other hand, are more complicated. They have multiple reaction environments in membranes and aqueous phases, and natural materials have complex compositions which are difficult to reproduce and control precisely. Metal catalysis of lipid oxidation in these complex biological systems is not as uncomplicated as classical mechanisms would suggest.

For example, both valence forms of metals have catalytic activity, and the factors affecting the balance or dominance between direct initiation reactions and re-initiation by hydroperoxide decompositions are poorly understood. Also, problems in the interpretation of mechanisms in complex biological systems have arisen when the reaction kinetics or the products did not obviously fit these classical mechanisms. This has been particularly true for toxicological studies which tried to determine whether HO$^{\cdot}$ produced from H_2O_2 in Fenton reactions (37,47) initiate oxidation of membrane lipids.

Clearly, new understandings of metal catalysis of lipid oxidation in biological systems must be sought. Issues needing clarification are, by their nature, complex and interdisciplinary, and solutions will require input from many different fields: metallochemistry, electrochemistry, organic chemistry, physical chemistry, biochemistry, physiology and toxicology, food chemistry. To provide a context and to stimulate renewed consideration of mechanisms along with new research approaches, the following overview of contemporary issues in metal catalysis of lipid oxidation is offered. A summary of these issues is presented in Table 1. While the discussion will focus on iron because it is the dominant redox-active metal in biological systems, most of the issues are relevant also to other redox-active metals.

HYPERVALENT IRON OR IRON-OXYGEN COMPLEXES

One of the major, and perhaps one of the most controversial, contemporary issues in regard to mechanisms of lipid oxidation is whether hypervalent iron (iron valence of +4 to +6, Table 2) or other iron-oxygen complexes form and are catalytically active in biological systems in the absence of a protein or other macromolecular prosthetic group. If they do form, what are the necessary conditions, and how do the kinetics and products of their reactions compare with those produced in classical Fe^{3+}/Fe^{2+} reactions?

Originally proposed by Hochstein *et al.* (50), the principal proponents of this theory applied to biological systems have been proposed by Aust and colleagues (51–55). Based on repeated observations that maximum lipid oxidation occurs when Fe^{2+} and Fe^{3+} are present in a 1:1 ratio, they proposed a mechanism in which the species actively catalyzing lipid oxidation in the presence of H_2O_2 is ferryl iron or mixed metal-O_2 complexes [Fe^{2+}-O_2-Fe^{3+}] rather than HO$^{\cdot}$. Recently, these authors have modified their proposed mechanism, contending that 1:1 ratios of ferric and ferrous iron facilitate redox cycling of the iron. Details of this theory and a review of their work are presented in this issue (56).

Authentic hypervalent iron complexes have long been recognized as active forms of heme proteins and porphyrin

TABLE 1

Summary of Contemporary Issues Which Need to Be Clarified Regarding Metal Catalysis of Lipid Oxidation

1. HYPERVALENT NON-HEME IRON OR IRON-OXYGEN COMPLEXES:
   - Under what conditions do they form in biological systems?
   - Do they catalyze lipid oxidation?
   - What are the characteristic kinetics and lipid products?
2. HEME CATALYSIS MECHANISM(s):
   - Hydroperoxide reduction, oxidation by hypervalent iron, or other mechanisms?
3. COMPARTMENTALIZATION OF REACTIONS AND LIPID PHASE REACTIONS OF METALS:
   - Where does metal catalysis occur in multiphase systems?
   - Are lipid phase reactions of metals significant?
   - Do catalysis mechanisms and oxidation products differ depending on phase?
4. EFFECTS OF METALS ON PRODUCT MIXES:
   - Does metal catalysis influence isomeric hydroperoxide distributions, alkoxyl radical scission reactions, intramolecular rearrangements to epoxides, etc.?
5. FACTORS AFFECTING THE MODE OF METAL CATALYTIC ACTION—direct initiation *vs* chain propagation (reinitiation), mechanism, reaction location, overall course of oxidation:
   - Chelator—partition coefficients, redox potentials, pK_a.
   - Reaction conditions—metal concentrations, pH, presence/absence of water and reducing agents, oxygen tension, type of lipid.
   - Membrane surface charge.

TABLE 2

Formulas and Nomenclatures for Hypervalent Iron Complexes

$(FeO)^{2+}$	$(FeO)^{3+}$	$(FeO_4)^{2-}$
Fe(IV)	Fe(V)	Fe(VI)
Ferryl	Perferryl	Ferrate
MIXED METAL-OXYGEN COMPLEXES:		
Fe^{2+}-O_2-Fe^{3+}		

compounds. Hypervalent iron states also have been identified and characterized spectroscopically in numerous enzymes and in iron complexes in organic solvents, but little is known about hypervalent iron in aqueous solutions. Physical chemists have expressed great skepticism that hypervalent iron occurs in non-heme small molecular complexes under physiological conditions on the grounds that hypervalent iron complexes require *macro*molecular complexers or a very high pH for formation and stabilization. Thus, hypervalent iron should not be a significant reactant since in complex biological systems decay of hypervalent states to Fe^{3+} would be essentially instantaneous, eliminating any enhanced catalytic capability.

There have been a few reports based on electron paramagnetic resonance (EPR) (57,58) and kinetic spectroscopic (59) evidence for the formation of hypervalent iron in small molecular complexes. However, the hypervalent iron indeed decayed very rapidly to its ferric form. In other reports, hypervalent iron was thought to be formed in olefin microemulsions, but only at high (10:1) hydroperoxide/iron ratios (60); 1:1 ratios yielded conventional Fenton chemistry.

Bielski (61) studied the stability and reactivity of synthetic Fe^{4+}, Fe^{5+}, and Fe^{6+} complexes in aqueous solutions, and found that all the complexes were strong oxidants at high pH. Fe^{+4} and Fe^{+5} were significantly more reactive than Fe^{+6}, due to substantial free radical character of the Fe–O bonds in the ferryl/perferryl species. Proton sources in solvent or substrate markedly increased decay rates of the hypervalent states.

While Bielski's results suggest that hypervalent iron states, *if* they form in contact with lipids in biological systems, should be particularly potent initiators of lipid oxidation, three critical issues must still be addressed to understand the practical importance of these complexes: i) Under what conditions do these hypervalent states form in small molecules under physiological conditions? What is the source of oxygen, is it dissolved molecular oxygen or hydrogen/hydroperoxides? What pH? What complexing agents? If aprotic solvents stabilize hypervalent states, may the hydrophobic lipid phases of membranes be preferential sites for hypervalent iron formation and activity? ii) Do hypervalent iron complexes actually catalyze lipid oxidation? Under what conditions? What are the rate constants? iii) How do the kinetics, mechanisms, and products differ from normal autoxidation or Fe^{2+}/Fe^{3+} catalyses? Are hypervalent iron processes distinguishable from other oxidations?

HEME CATALYSIS MECHANISM(S)

Early observations that heme compounds were catalytically active in the ferrous form and that no valence change of the iron occurred in the process led Tappel (5,62) to propose a mechanism by which lipid hydroperoxides form activated complexes through a coordinate bond between the ferrous heme and the hydroperoxide function. At the time, no distinction could be made between radical scission of the hydroperoxide,

$$\text{LOOH} + \text{Heme-Fe}^{2+} \rightarrow \text{Heme-Fe}^{2+} + \text{LO}^{\bullet} + {}^{\bullet}\text{OH} \quad [14]$$

and reductive scission followed by secondary oxidation of ions to immediately recycle the ferric to ferrous heme (5).

$$\text{LOOH} + \text{Heme-Fe}^{2+} \rightarrow \text{Hematin-Fe}^{3+} + \text{LO}^{\bullet} + \text{OH}^{-} \quad [15a]$$

$$\text{or Hematin-Fe}^{3+} + \text{LO}^{-} + {}^{\bullet}\text{OH} \quad [15b]$$

$$\text{Hematin-Fe}^{3+} + \text{OH}^{-} \rightarrow \text{Heme-Fe}^{2+} + {}^{\bullet}\text{OH} \quad [16a]$$

$$+ \text{LO}^{-} \rightarrow \text{Heme-Fe}^{2+} + \text{LO}^{\bullet} \quad [16b]$$

Tappel's mechanism is historically important, as it explained most observations for many years. Recent

advances in analytical techniques have enabled others to provide amplifications and mechanistic details to Tappel's theory. Bruice (63) has presented evidence that in aqueous solution radical scission dominates with the alkoxyl radical being released and the ·OH remaining bound to the heme compound, while in organic solvents radical scission occurs followed by secondary electron transfer such that products are equivalent to those which would be produced by reductive O–O bond cleavage. However, not all hemes follow this mechanism, so a diagnostic probe to distinguish which heme compounds decompose hydroperoxides by mechanisms yielding alkoxyl radicals has been developed using 10-hydroperoxy linoleic acid (64,65).

However, reductive scission of hydroperoxides alone does not adequately explain differences between catalytic efficiencies or the specific product mixes of different hemes (26,66). Also, recent EPR studies have shown clearly that ferric as well as ferrous hemes are active catalysts, yielding peroxyl radicals from hydroperoxides (67,68). Inconsistencies in pro-oxidant effects of hemes continue to be reported, probably because multiple mechanisms of heme catalysis exist, even for individual hemes, determined by reaction conditions and components and by the specific heme compounds involved.

Contemporary studies have provided evidence for at least four mechanisms by which heme compounds may catalyze lipid oxidation in addition to the straightforward electron transfers described in Reactions 7 and 8.

One mechanism is the formation of hypervalent iron complexes which oxidize lipids directly. In the discussion about hypervalent iron above, the qualifications "small molecule" or "non-heme" were repeatedly used because it is known clearly that ferryl iron is formed by peroxidase (69) and other heme proteins and is the active oxidizing form of these enzymes (70,71). Ferryl complexes are also formed in catalase (69,72), cytochrome P-450 (73), myoglobin (74,75), hemoglobin (57,58,76), and model porphyrins (63,77–79), although less is known about the catalytic activity of ferryl forms of these proteins.

All of these heme proteins have been shown to catalyze lipid oxidation, and by analogy to the above it seems logical to question whether hypervalent iron may be involved in this activity, formed *via* either heme-oxygen, heme-hydrogen peroxide, or heme-lipid hydroperoxide complexes. The requirement for at least one of these oxygen sources in order for catalysis of lipid oxidation to occur has been noted in tissue (80) and chemical model reaction systems (81). Observations that metmyoglobin catalysis in air was markedly accelerated as pH was increased, especially above pH 7 (81), is consistent with this hypothesis since hypervalent iron complexes are more stable at high pH (61). Hypervalent iron activity may also explain observations that hemoglobin catalyzes oxidation of linoleic acid and ester (82) and phospholipids in liposomes (83) without induction periods, and that haptoglobin inhibits hemoglobin-stimulated lipid oxidation (84). Reduction of hypervalent iron or prevention of its formation may contribute to antioxidant effects of high heme concentration (85) and the reducing agents ascorbic acid and cysteine (85).

A second mode of heme catalysis may involve an indirect mechanism in which HO· attacks lipids to initiate autoxidation chain reactions. The HO· arises from either a) the autoxidation of ferrous heme iron to produce $O_2^{\cdot-}$ and H_2O_2, which is then reduced to HO· by adventitious iron (86,87); or b) heme-catalyzed reduction of H_2O_2 added or produced *in situ* (88).

A third possible mechanism for catalysis of lipid oxidation by hemes is photosensitization, either *via* free radicals or singlet oxygen (89). Ten years ago this concept was proposed on theoretical grounds (26) and recently some experimental verification for Type I (free radical) sensitization has been published (90).

Recent observations suggest that a fourth mode of heme catalysis is also possible in systems containing H_2O_2: HO· attacks hemoglobin (or other heme proteins) and releases iron which then catalyzes lipid oxidation as non-heme iron (91,92).

Of these mechanisms, hypervalent iron states seem to account best for many characteristics of heme catalysis of lipid oxidation documented but incompletely explained in the previous literature. Nevertheless, several issues still require clarification, including which valence states of hemes are involved, whether different hemes catalyze lipid oxidation by different mechanisms, what conditions favor activity of hypervalent iron as opposed to direct reduction of hydroperoxides or any of the other mechanisms which have been proposed, and which molecular regions are involved in the electron transfers, *i.e.* whether the iron atom, porphyrin ring, or apoprotein (93) is involved.

COMPARTMENTALIZATION OF REACTIONS AND LIPID PHASE REACTIONS OF METALS

Apparent inconsistent effects of iron on oxygen radical reactions and lipid oxidation in biological systems have been observed, but this may be expected since cells, tissues, membranes, and organelles, by their nature, lack the precisely controlled conditions and defined composition of laboratory test tube reactions. For example, adenosine diphosphate (ADP), ethylenediaminetetraacetic acid (EDTA), and histidine complexation of iron may have pro- or antioxidant activity or no effect on lipid oxidation depending on the source and preparation of tissues, cells, membranes or purified lipid, the solvents used, the concentrations of metal complexes, the presence or absence of oxygen sources, and how the effects are measured. Because we need to be able to predict and control metal reactions in foods and in living tissue, we have cogent and compelling reasons to understand the molecular bases for these inconsistencies and thus to determine the physical and chemical factors which affect iron activity in complex, multiphase, and often compartmentalized systems.

One critical physical aspect which cannot be ignored is that biological systems are always at least *bi*phasic, inhomogeneous systems, *i.e.* a hydrophilic, protic aqueous phase and a hydrophobic lipid phase which may have both protic and aprotic regions. Because of the bilayer structure of the membrane, initiation of lipid oxidation must necessarily occur in the membrane interior region, where the lipid chains are located. However, in typical experimental designs, metal reagents are added to an external aqueous phase. Reactions occurring in the aqueous phase can initiate lipid oxidation only if reactive intermediates or products diffuse to the surface or into the lipid bilayer of the membrane. Because of their high reactivity, HO· generated in the aqueous phase have little, if any, chance of initiating oxidation of lipids in membranes or in lipid

phases in food products. Water-soluble chelators or free radical interceptors such as mannitol, superoxide dismutase (SOD), and catalase can be effective against reactants in the aqueous phase, but are unlikely to affect oxidations occurring in the hydrophobic regions of lipid bilayers.

This paradox highlights a third important issue regarding metals and lipid oxidation: Where does metal catalysis of lipid oxidation occur in a multiphase system? What reactions of metals occur in lipid phases and how do their mechanisms, kinetics, and products differ from aqueous phase or interface reactions of metals?

Traditional chemistry holds that catalysis in a biphasic system will occur at the phase interface or at the membrane surface. Thus, any chelators or reaction conditions which increase the binding of metals to the membrane, creating "ill-placed" metal (94), should increase the catalytic effectiveness of the metal. It is well-known, for example, that copper binds readily to membrane proteins and mediates site-specific reactions at those locations (22,95,96). Iron also has surface binding sites on proteins (97,98) and binds to the phosphoric acid moieties of phospholipids (99–101). This binding contributes to rapid iron uptake by membranes, and to reduction of lipid hydroperoxides near those sites. Zinc (102–104) and other metals can displace iron from these sites, but whether aqueous phase $HO^{\cdot}$ interceptors and metal chelators have access to and can compete with binding sites on the membrane surface remains to be determined.

Considering that lipid molecules themselves are the final targets and participants in lipid oxidation, surprisingly little attention has been given to the reactions which may occur in the hydrophobic lipid region of the membrane (or lipid phases of foods), catalyzed by metal complexes, H_2O_2, reducing agents, or other reactants which diffuse or solubilize there. This may be partially attributed to a general expectation that metals are not soluble in hydrophobic media. Nevertheless, fatty acid complexation of metals has long been known to occur and is responsible for a major proportion of metal contamination of refined food oils. In recent studies determining lipid partitioning into lipid phases, fatty acids solubilized metals at concentrations of micromolar or higher, depending on the chelating or complexing agent; complexation of metals through the carboxylic acid groups was very likely involved (105). ADP and (des)ferrioxamine were surprisingly potent solubilizers, a characteristic which may partially explain why ADP as a chelator is unusually effective in enhancing lipid oxidation in membranes, and why desferrioxamine is not always protective against oxidative damage in tissues.

Iron complexes were 10-100× less soluble in the aprotic methyl esters of fatty acids, but even 10^{-8} M concentrations of iron were sufficient to drive oxidations detectable within minutes (seconds in some cases) by EPR spin trapping in these pure lipid phases (105).

Why should lipid phase reactions be critical? Reactions of metals occurring in the lipid phase, whether direct initiation, Fenton generation of $HO^{\cdot}$, or decomposition of LOOH, would be inaccessible to water-soluble interceptors. Thus, negative results in experiments using inhibition of lipid oxidation by water-soluble agents to derive mechanisms can be misinterpreted; rather than the causal connection being absent, the causal location may be wrong.

Perhaps an even more important reason is that Fenton and other iron reactions in aprotic solvents such as acetonitrile or lipid esters may not proceed as they would in water or protic organic solvents. Several factors may contribute to these reaction differences. First, the electrochemistry of iron (196) and the reactions of product oxyl radicals ($O_2^{\cdot-}/HO_2^{\cdot}$, $HO^{\cdot}$, $RO^{\cdot}$, and $ROO^{\cdot}$) in aprotic organic solvents differ from their aqueous counterparts. Ferric oxidation of hydroperoxides, which are relatively weak in aqueous solutions, are accelerated in aprotic solvents such as acetonitrile (107,108). Changes in the ligand structure and solvation state of the metal may shift electron transfer from an outer sphere (free radical) to an inner sphere (peroxide complex) process (39), thus altering oxidation mechanisms and products (108). Fe^{2+} has long been known to autoxidize much more rapidly (109), and hypervalent iron states form more readily and are more stable in aprotic solvents. Recent evidence that ferric iron is not easily reduced in aprotic fatty acid methyl esters (Schaich, K.M., unpublished data) is consistent with observed iron behavior in other aprotic organic solvents (110).

These observations may be extrapolated to membranes. Because the acyl chains forming the hydrophobic region of the lipid bilayer should be aprotic while the phosphate regions near the surface should be protic, the mechanisms of metal catalysis and the processes of lipid oxidation in these two regions are very likely to be different.

In research with Fenton and metal reactions in lipid phases, reactions in protic fatty acids have been shown to clearly differ from those in aprotic esters (105). EPR spin trapping studies of Fenton reactions (Fe^{3+}, H_2O_2, and hydroxylamine as a reducing agent) in lipid phases showed rapid Fenton reactions generating $HO^{\cdot}$ which could be trapped in fatty acids, but in fatty acid esters Fenton reactions were slower and $HO^{\cdot}$ were never trapped; instead, complex multiple-species spectra were produced.

Initial EPR spectra in lipid esters showed mixtures of radicals (105). Because Fe^{3+} reduction was inhibited and its oxidations were accelerated in the lipid phases, three oxidation reactions competed to produce the initial radicals: i) oxidation of H_2O_2 to $HO_2^{\cdot}$; ii) direct oxidation of unsaturated lipids to yield lipid peroxyl radicals (oxygen was present); and iii) oxidation of traces of preformed lipid hydroperoxides to peroxyl radicals. At later reaction stages, an unusual spectrum appeared; this was assigned to an epoxyallylic radical species.

Additional evidence that metal reactions in lipid or other non-aqueous aprotic phases do not follow classical aqueous reaction pathways has been provided in new reports showing production of ketones at the alkoxyl radical carbon (111,112) or a variety of epoxide or other rearrangement products rather than scission products when lipid hydroperoxides are reduced by iron complexes (113) or by ultraviolet light (114) in aprotic solvents. Similarly, in a reaction system containing $FeCl_3$, H_2O_2, and olefins in acetonitrile, epoxide and dioxetane products were formed rather than peroxy radicals, hydroxylation or scission products (115). An Fe^{III}-oxene [$Fe^{III}(O)$] species was proposed as the active catalyst in the latter study.

Another possible explanation for differences in oxidation mechanism in different media is offered by observations that linoleic acid and linoleate-containing phospholipids autoxidized neat, in chlorobenzene, or in buffered

vesicular systems yield hydroperoxides with very different *cis,trans* to *trans,trans* ratios (116–118). The *cis,trans/trans,trans* isomer ratios decreased as lipid concentrations increased, as hydrogen donating ability of the solvent decreased, and as intermolecular lipid associations increased. Analogous hydroperoxide patterns have been observed following *in vivo* lipid peroxidation (119). Thus, molecular conformations of hydroperoxides in lipid phases may facilitate isomerization and intramolecular rearrangements over scission reactions. A third factor which may favor intramolecular rearrangement is a reduction in the rate of intermolecular hydrogen abstraction by LOO$^{\bullet}$ due to the viscosity of lipid phases (120).

Additional research will be needed to determine definitively which effects of solvent have the dominant influence on metal catalyses of lipid oxidation: alteration of the electrochemistry of iron complexes, proton transfer mediation, changes in lipid conformations and molecular associations, or still other factors as yet unrecognized.

EFFECTS OF METALS ON PRODUCT MIXES

Numerous studies have documented the classical breakdown of oxidizing lipids into mixtures of hydroperoxides, aldehydes, ketones and other carbonyl compounds, alkanes and carboxylic acids *via* scission reactions of the lipid alkoxyl radicals (121,122, and references therein). Less attention has been given to internal rearrangements of hydroperoxides to epoxides and related product (113, 114,123,124), and very little research has focussed on determining what factors influence scission reactions and whether metal catalysis alters reaction pathways and product distribution. How metals and metal-solvent interactions affect the overall course of lipid oxidation and the scission reactions which produce malonaldehyde and similar reactive aldehydes, and how metals influence the dominance of scission reactions yielding carbonyls *versus* rearrangement reactions yielding epoxides in different environments are specific issues which have received little attention.

Why is this important? Metals affect not only the rate of initiation and total extent of lipid oxidation, but also the degree of chain branching and secondary reaction, and the nature of termination reactions, *i.e.* metals determine which final products are formed.

The thiobarbituric acid (TBA) test is a measure of lipid oxidation commonly used as a measure of the effectiveness of metals in catalyzing lipid oxidation. This test provides very convenient analyses because the products are water-soluble, eliminating the need for tedious lipid extractions. However, the TBA test depends on the production of "expected" scission products, predominantly malonaldehyde, although cyclic peroxides and dihydroperoxides of 18:3 and higher fatty acids are also known to react (125,126). Malonaldehyde is only produced in secondary scissions of fatty acids with three or more double bonds, and it is a relatively minor breakdown product. Media or reactants may alter the oxidation pathways, increasing or even eliminating cyclization reactions and malonaldehyde production.

For example, in the presence of water, metals increase both the breakdown of LOOH products to alkoxyl radicals and α- and β-scissions of alkoxyl radicals to aldehydes, other carbonyl compounds, and alkanes. Indeed, some versions of the TBA test include an iron reagent to accelerate these scission reactions and the subsequent formation of malonaldehyde from a secondary scission reaction.

In contrast, dominant products of metal-catalyzed oxidations in neat lipid phases or in aprotic solvents appear to be rearrangement products rather than scission products (113). As discussed above, whether this results predominantly from different catalytic species (*e.g.*, iron^{III}-oxenes or hypervalent iron) or rather from direct solvent influences on proton transfers (114) and molecular associations or conformations remains to be determined.

Internal rearrangements to epoxides, hydroxyepoxides, and hydroperoxyepoxides are facilitated by close alignment and association of the lipid chains, such as would be found in oriented monolayers in dry foods and in the bilayers of membranes (127,128). Solvent also plays a critical role. As discussed above, reactions of metals in lipid phases or in lipids dissolved in aprotic solvents do not produce classical distributions of lipid scission products. The epoxide and other rearrangement products observed with the cited metal-lipid reactions are consistent with reports that olefin hydroperoxide-metal complexes yield scission products in coordinating solvents but shift increasingly to epoxide production as the hydrogen donating power of the solvent decreases (129) and the epoxides are stabilized (124).

Also, heme catalyses do not yield classical distributions of hydroxy, aldehyde, and alkane products. Multiple reaction mechanisms are recognized for many heme compounds (130) producing keto, epoxyhydroxy, trihydroxy, and other non-scission compounds as dominant products.

The practical implications of these differences are enormous for both food chemistry and toxicology. A few obvious ones can be mentioned. First, given the considerations outlined above, serious questions must be raised regarding the validity of using malonaldehyde production as an indicator of metal catalysis in a lipid phase or in compartmentalized multiphase systems such as cells. Another implication: the secondary carbonyl and alkane products resulting from scission reactions are important sources of flavors and odors, some characteristic and most undesirable, from oxidized lipids (121). If a way could be found to block this pathway, shelf-life could be extended.

Reconsideration of mechanisms by which membrane lipid oxidation is involved in biological damage processes also seems to be warranted. Lipid free radical species (20, 131–138), hydroperoxides (139–141), and carbonyl oxidation products (135,136,142–147) have commonly been blamed for co-oxidation and crosslinking of critical cellular macromolecules (136) such as tocopherols (143,148), proteins, nucleic acids (149–153), and carcinogens (154,155). Nonetheless, many lipid rearrangement products, particularly the hydroperoxyepoxides, also have been found to be cytotoxic and perhaps carcinogenic. Whether the rearrangement products are also capable of crosslinking remains to be determined, but in light of Gardner's identification of methyl linoleate epoxy adducts with cysteine catalyzed by $FeCl_3$ (124,156,157) crosslinking capability would certainly be expected. Since lipid rearrangement products would be expected to form in membrane bilayers, where proteins and nucleic acids are also bound, epoxy-mediated lipid complexation to these critical macromolecules may provide an important mechanism for the cytotoxicity and cancer promoting activity of oxidized lipids.

FACTORS AFFECTING METAL CATALYSIS

In my own studies of metal reactions in lipid phases, oxidation kinetics have proven to be complex and dependent on a number of factors, including the specific metal complex or chelator used, the valence state of the metal, the concentration of the metal, the oxygen tension of the system, the types of lipid (protic or aprotic), and the presence of contaminating preformed hydroperoxides. These are all factors well known to affect the overall kinetics of lipid oxidation, but it is less understood how these and other system variables affect the mechanism and course of metal catalysis.

Solvents, especially water, affect metal catalysis in a variety of ways. As noted above, water and other hydrophilic solvents facilitate LO$^{\bullet}$ scission reactions in solution (158). However, it is known from research of Karel and others (8,9,159) that there are also conditions in which water inhibits LOOH decompositions. Two hypotheses previously advanced to explain these actions are i) hydration of metals, thus inhibiting electron transfers to and from the metals, and ii) hydrogen bonding between water and hydroperoxides, providing stabilization. Recent nuclear magnetic resonance (NMR) studies of water-lipid hydroperoxide-metal complexes and their effects on hydroperoxide decomposition kinetics have corroborated these mechanisms (160).

Chelators and complexing agents are critical determinants of the catalytic mode and effectiveness of metals. Chelators vary in their metal affinities, their charge and solubility in lipids, and thus how they partition between lipid and aqueous phases. They differ in the valence state of iron they stabilize, the metal coordination sites they occupy, and the type of electron transfer reactions they mediate (161). They also vary in the redox potentials of their complexes (Table 3) (162,163), and hence how they poise metals for redox cycling in the presence of cellular reducing agents. These should be obvious considerations, but they are often ignored when interpreting effects of different metal complexes, particularly in multiphase systems. The net effects of chelators may be complex and seemingly contradictory.

For example, EDTA complexation of iron removes "free" or weakly complexed iron from solution. It also lowers the Fe^{3+}/Fe^{2+} redox potential from 0.77 V to 0.12 V *vs* normal hydrogen electrode (NHE). Thus, by changing the localization of iron and by limiting the capability of Fe as an oxidizing agent, EDTA can markedly reduce *ab initio* (LH → L$^{\bullet}$) initiation of lipid oxidation. However, the lower redox potential makes EDTA-iron a better reducing agent, so EDTA-Fe^{2+} chelates reduce lipid hydroperoxides faster than uncomplexed iron. In the presence of reducing agents to recycle the iron, EDTA complexation may even result in a marked acceleration of chain propagation and branching reactions (LOOH → LO$^{\bullet}$ + $^{\bullet}$OH, and LO$^{\bullet}$ + L'H → L$^{\bullet}$ + LOH). The net effect of EDTA results from the balance between these actions in individual systems, and has led to apparently contradictory reports of EDTA effects on lipid oxidation (164).

Several other chelators deserve attention. Picolinic acid, a metabolic product of tryptophan, is a strong metal chelator and is potentially very important as a physiological chelator. Picolinates have received attention recently since the iron chelates are very effective at producing HO$^{\bullet}$, at least at low H_2O_2/chelate ratios (1:1) (165). At higher hydroperoxide ratios, a mechanism more like that with hypervalent iron dominates (111,112). With such behavior, it is imperative that we understand the redox chemistry of this molecule and its chelates. Reaction characteristics of picolinate complexes have been described by Sawyer (112).

ADP, histidine, bipyridyl, orthophenanthroline, and nitriloacetic acid are all chelators which have shown multiple, sometimes contradictory, effects on lipid oxidation in various systems. The first two are physiological chelators and the latter three are chelators known to be toxic to humans. Lipophilicity, singlet oxygen scavenging, hypervalent iron facilitation have been proposed as possible explanations for the actions of these chelators, but the mechanisms of their actions remain incompletely understood.

A characteristic of chelators which has received little general recognition is that HO$^{\bullet}$ and perhaps also lipid oxyl radicals, formed in the reaction cage of peroxide reduction, react with some chelators to form chelator free radicals which are themselves reactive. EDTA, for example, forms four different free radicals, most of which are strongly reducing and hence may be important determinants in the course of catalyzed oxidations (166). TRIS and phosphate (167) in buffers and desferrioxamine (168, 169; Schaich, K.M., manuscript in preparation) are known to form reactive radicals when attacked by HO$^{\bullet}$. These complexing agents are very likely to be both targets and initiators for lipid oxyl radicals.

Still another factor which may potentially have great importance for lipid oxidation in living systems is charge on chelators and on membrane surfaces. Surface charges

TABLE 3

Redox Potentials of Some Common Metal Complexes and Heme Compounds (refs. 162,163)

Complex	(V *vs* NHE)[a]	
	E^{o}	$E^{o'}$[b]
Bipyridyl	1.11	
1,10 (ortho)phenanthroline	1.06	
Fe^{3+}/Fe^{2+}	0.77	
Ferricyanide		0.36
Acetate, pH 5		0.34
Malonate, pH 4		0.26
Salicylate, pH 4		0.26
DTPA		0.126
EDTA		0.012
Oxalate		0.002
Pyrophosphate (PPi)		−0.14
8-Hydroxyquinoline		−0.15
Cyt c		0.254
Hemoglobin		0.144
Myoglobin		0.046
Cyt b_5 microsomes		0.02
Hematoporphyrin(pyridine)$_2$		0.004
Protoporphyrin IX(his)$_2$,		
pH 9.5		−0.138
pH 8.2 (borate)		−0.188
pH 7.0 (phosphate)		−0.226
Peroxidase (horseradish)		−0.271
Ferredoxin		−0.413

[a]Normal hydrogen electrode.

[b]Redox potential at pH 7 unless otherwise noted.

affect molecular access and binding as well as the dynamics of electron transfer reactions through lipid phases, and thus may be particularly important for porphyrin catalyses (170). Also, the electrostatic environment (the total effective charge) surrounding the binding site of a metal can markedly affect the redox potential and other thermodynamic properties of the metal (171). The net effect often results from a complex competition between several individual effects and thus may be difficult to predict. Nevertheless, these various effects of charge need to be understood and considered when interpreting mechanisms of metal action in complex systems.

Some very elegant studies have shown distinctly different behaviors in membranes or micelles incubated with Fenton reagents, depending on the surface charge of the vesicles (172–176). Negatively charged vesicles repelled metals, so direct initiation of lipids could not be effected. However, mutual repulsions of charged groups at the surface created more open molecular packing, so small molecules from the aqueous phase were able to penetrate the lipid layers and mediate reactions there. For example, $HO^{\cdot}$ generated near the surface was able to penetrate into the hydrophobic regions of the vesicles and initiate oxidation of lipids there, and this oxidation could be effectively inhibited by α-tocopherol and by water-soluble $HO^{\cdot}$ scavengers. In contrast, vesicles with negative surface charges bound metals, which reacted with traces of lipid hydroperoxides in the vesicles to initiate new oxidation chains. Water-soluble radical scavengers had little or no effect on this LOOH reaction, and tocopherol actually accelerated lipid oxidation in these vesicles because it recycled the Fe^{3+}, thus maintaining LOOH reductions. Effects of chelators in these systems could be explained by the changes in metal distribution and binding attributable to chelator charges (177).

SUMMARY

The five contemporary issues presented in this paper show clearly that metal catalyses of lipid oxidation in complex, often multiphasic or compartmentalized reaction systems are not straightforward. They often do not fit the classical electron transfer mechanisms for metal catalysis which have been accepted for decades. New understandings must be developed to more accurately explain the kinetics, mechanisms, and products of metal-catalyzed lipid oxidation in complex environments. Definitive evidence for intermediates in proposed mechanisms must be sought, and consideration must be given to all physical and chemical properties of reactants and reaction environments when interpreting mechanism from kinetic and product data. New integrative approaches and expertise from many different scientific disciplines will be necessary to elucidate the many factors affecting and controlling metal reactions in complex, multiphase systems.

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