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Emergence of Oxyl Radicals as Selective Oxidants

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Hydroxyl radicals ($HO^{\bullet}$) are derived in Fenton reaction with ferrous salt and H_2O_2 in acid medium, and at neutral pH, metal-oxyl radicals ($M\text{-}O^{\bullet}$) predominate. Evidence is accumulating that $M\text{-}O^{\bullet}$ radicals are also active in oxidation reactions, in addition to metal-oxo (M=O) now shown in many publications. Reactivity of these radicals gives selective oxidized products useful in cellular activities, in contrast to purported indiscriminate cell damage by hydroxyl radicals. Reactions with vanadium compounds, such as diperoxovanadate, peroxo-bridged mixed valency divanadate, vanadium-oxyl radical, tetravalent vanadyl and decavanadate illustrates selective gain in oxidative capacity of oxo- and oxyl- species. Occurrence of ESR signals typical of hydroxyl radicals is demonstrated in cell homogenates and tissue perfusates treated with spin trap agents. It is known for a long time lipid peroxides are formed in tissue microsomal systems exclusively in presence of salts of iron, among many metals tested. Oxygen and a reducing agent, ascorbate (non-enzymic) or NADPH (enzymic) are required to produce 'ferryl', the chelated Fe=O active form (possibly $Fe\text{-}O^{\bullet}$ and Fe-O-O-Fe ?) for the crucial step of H-atom abstraction. Yet literature is replete with unsupported affirmations that hydroxyl radicals initiate lipid peroxidation, an unexplained fixation of mindset. The best-known $^{\bullet}OH$ generator, a mixture of ferrous salt and H_2O_2, does not promote lipid peroxidation, nor do the many hydroxyl radical quenching agents stop it. The availability of oxo and oxyl-radical forms with transition metals, and also with non metals, P, S, N and V, calls for expansion of vision beyond superoxide and hydroxyl radicals and explore functions of multiple oxygen radicals for their biological relevance.

Keywords: Hydroxyl radicals, Metal oxyl radicals, Peroxo-vanadates, Lipid peroxidation,

Radicals, free radicals?

In the beginning of last millennium in 1900, Moses Gomberg[1] discovered $(C_6H_5)_3C^{\bullet}$, a carbon-centered triphenylmethyl radical present along with its dimer $(C_6H_5)_3C\text{-}C(C_6H_5)_3$. It is an unusual non-planar trivalent carbon compound with extensive electron delocalization that stabilizes the radical centre. He first used the name “free radical” and opened the concept of the short-lived radicals, serving as intermediates in chemical reactions. A simple and sufficient definition of a 'radical' is an atom having an unpaired electron. The unpaired electron of a radical has to be lost or has to acquire a partner. Thus, radicals can be powerful reductants or oxidants. During its fleeting existence, a radical rapidly exchanges an electron. Because of this radicals are highly reactive and are demonized as electron sucking, chain forming indiscriminate destroyers of all they encounter. An impression is, therefore, created that radicals are free to destroy any molecule. Moreover, chain reactions are proposed in radical reactivity. This very thought leads to fear of uncontrolled destruction of cells. Starting rightly with radiation damage, the current trendy apoptosis and many pathological conditions are attributed to the radicals that run free.

The field of reactive oxygen species (ROS) and antioxidants has witnessed a phenomenal growth in the last three decades essentially, due to suspected involvement of radical-mediated damages in many degenerative diseases, including arthritis, atherosclerosis, aging, Alzheimer, Parkinson, AIDS and carcinogenesis. Synthetic and natural antioxidants are found to scavenge free radicals in the experimental studies, raising expectation of their beneficial effects in what is popularly known as 'oxidative stress'. But this widespread belief was not supported by clinical studies carried out in some pathophysiological conditions[2]. Whither oxidative stress? The entrenched concept that radicals are always destructive does not seem to go away. Powerful phrases, such as oxidative stress and natural antioxidants ingrained into some dogmas overshadow the true role of redox regulation of superoxide and

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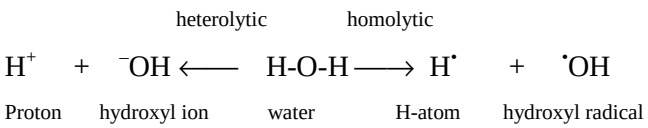
hydrogen peroxide. Awareness of reactivity of radicals in bio-systems as natural, essential process is increasing[3]. In the new millennium, let us look at radicals as useful agents in cells, which they are, without fear.

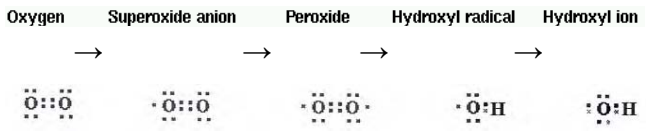
Oxygen radicals - Superoxide and hydroxyl

A covalent bond sharing a pair of electrons between two atoms can be split in two ways. Heterolytic split of water retains the electron pair on the oxygen atom, with both products having charges. Ionisation of water produces a hydrogen ion and a hydroxide ion, both charged. In homolytic split, each atom retains an electron, now unpaired and electrically neutral. Hydroxyl radicals are generated by homolytic split of H-O bond of water in a reaction that requires high-energy radiation, such as exposure to α-radiation or to a beam of accelerated electrons (pulse radiolysis). Covalent bonds, such as O-H, O-O, O-N, C-Hand S-H can undergo such reactions to produce radical species. Illustrated below is the example of split of H-O bond in water molecule (H-O-H) (conventionally a dot ($^{\bullet}$) in superscript indicates a radical with unpaired electron):



Molecular oxygen (dioxygen, O_2), the respiratory electron sink, is itself a diradical ($^{\bullet}O=O^{\bullet}$) with an unpaired electron on each oxygen atom in separate orbitals in its outer shell with parallel spin (triplet state). Yet oxygen is mercifully unreactive, thus saving organic cell material from unwanted destruction. Crucial for activation of dioxygen is its sequential reduction with each atom receiving the second electron from a metal center or another radical.

Sequential reduction of O_2 leads to other oxygen radical species (shown below): by one-electron gives the well known superoxide anion radical ($^{-}O\text{-}O^{\bullet}$) and by two-electrons gives the active oxidant, peroxide ($^{-}O\text{-}O^{-}$) (example, by the mitochondrial electron transport chain). The next reduction breaks the O-O bond homolytically to oxo-radical ($^{-}O^{\bullet}$), known as hydroxyl radical ($HO^{\bullet}$) in the protonated form in acid pH, which then is further reduced by another electron to a molecule of water.



Superoxide ($O_2^{\bullet -}$) is crucial first intermediate in the activation of dioxygen. The well-argued debate on superoxide as the crux of oxygen toxicity is now rested[4]. No controversy will ever resolve, as both parties are right to some extent. High hopes generated by the overrated name superoxide (remember superman!), given surprisingly by a chemist, Neumann[5] are now tempered because there is little "super" about superoxide, a "singularly unreactive" radical[6]. A one-electron reduction product of O_2 should really be called "semiperoxide", analogous to semiquinone. Its well-known reaction is reduction of Fe^{3+} in cytochrome *c*. It can also reduce Fe^{3+} in non-heme iron proteins such as ferritin and the free iron thus released can generate other radical species.

Hydroxyl radicals ($HO^{\bullet}$) can be detected by spin trap agents in cells or tissue homogenates on adding H_2O_2. The underlying reaction is the homolytic split H_2O_2 by a metal ion from an endogenous source of (Fe^{2+}). These adducts are typically sensitive to catalase and metal chelators, and a number of quenching compounds, such as mannitol, formate, ethanol, benzoate and possibly many other organic compounds which are destroyed by these radicals. When formed in cells the highly reactive hydroxyl radicals are destructive, and are indeed feared. Tight control mechanisms on generation of H_2O_2 on the one side, and release of free iron from sequestered forms on the other seem to have therefore evolved to protect the cells.

Electron spin resonance characteristics of oxyl radicals

Increasing number of investigations now include electron spin resonance (ESR) spectra to identify the presence of radicals species in the reactions. The unpaired electrons of radicals can be detected by their characteristic ESR spectra. ESR spectrum records the change in spin state of unpaired electrons, but not of paired electrons found in stable molecules. By using spin trap nitroxide compounds, commonly dimethylpyrroline N-oxide (DMPO), it is possible to obtain sufficiently stable adducts to record the ESR spectra of oxygen free radicals despite their fleeting existence.

Typical spectra obtained on pulse radiolysis of the aqueous reaction mixtures[7] in Fenton reaction mixture[8] and in cultured pancreatic cells[9] as DMPO-adducts and also in intact animals with PBN as the spin probe giving doublet of triplet lines[10,11] are given

in Fig. 1. The ESR signal consisting of a quartet with intensity ratio of 1:2:2:1 and hyperfine splitting of $_N$ = $_H$ = 14.9 Gauss obtained on scavenging of radiolytically produced HO• radical in aqueous solutions containing DMPO, is consistent with the DMPO-OH adduct. Similar signals have been obtained in many laboratories world over with a mixture of ferrous salt, H_2O_2 and DMPO, and this is accepted as evidence for formation of HO• radical. In the Fenton reaction mixture, wherein FeO• is also likely to be present, only the 1:2:2:1 quartet lines of HO• are found because the most abundant ferric iron (I = 0) has no influence. Hyperfine splitting characteristics of metal ions have been observed in the ESR lines, when their salts are treated with H_2O_2 representing metal-oxyl radicals. The example of the ESR spectrum of the Ru (O_2^-)-DMPO adduct[12] obtained on oxidation of Ru^{III}-hedta complex by H_2O_2 (seven-line pattern 1:2:2:2:2:2:1, A_N = 10.0 G, A_H = A_H = 5.5 G) is shown in Fig. 1.

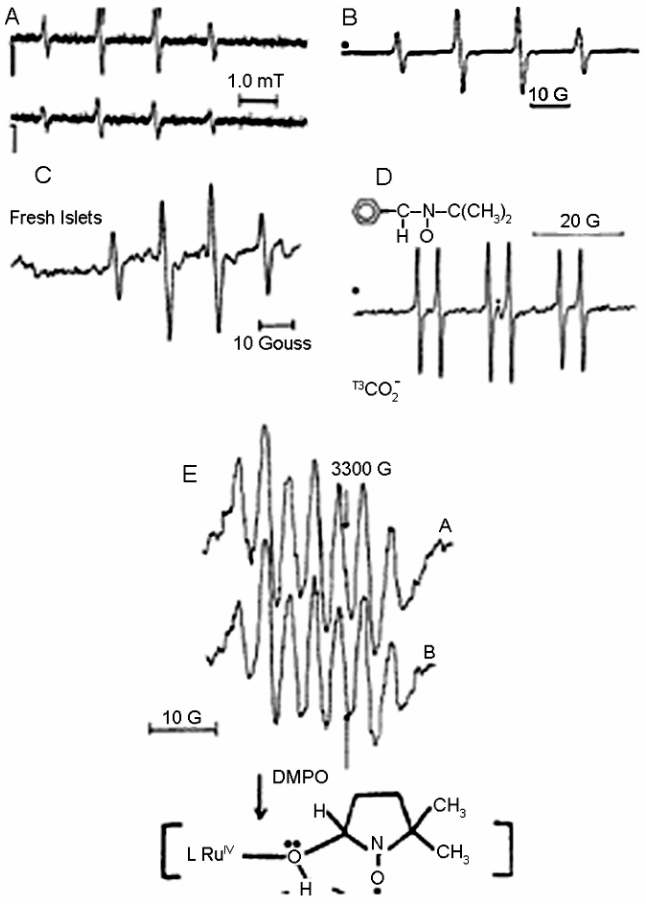


Fig. 1—ESR spectra of spin trap adducts of oxyl radicals. (A): Nitrogen-deoxygenated aqueous solution containing 15 mM DMPO after pulse radiolysis, ^{60}Co -irradiated, total dose about 5 Gy [adapted from Madden & Taniguchi[7]]; (B): Fenton reaction mixture (0.1 mM Fe^{II}, 0.2 mM H_2O_2, 40mM K-phosphate buffer pH 7.4) in presence of 40 mM DMPO [adapted from Yamazaki & Piette[8]]; (C): Homogenate of pancreatic islets of Langerhans in presence of 153 mM DMPO without any other addition, [adapted from Pieper *et al*[9]]. D. Effluent from liver perfused with CCl_4 (1 mM) and PBN (inset) (10 mM) [adapted from Connor *et al*[10]]; and (E): Radicals produced on oxidation of Ru^{III} (hedta complex) (about 3 mM) with H_20_2 (88 mM) in presence of, DMPO (30 mM) (at 0.80-G and 0.50-G). Ru-DMPO adduct is also shown [adapted from Zhang & Shepherd[12]].

Fenton reaction generates hydroxyl and also metal-oxyl radicals

The reduction of peroxide (O-O) bond requires a source of electron. This is supplied by a ferrous salt in the Fenton reaction**,** commonly used for generation of hydroxyl radical. In 1894 Fenton[13] observed a powerful oxidizing intermediate in the reaction between a ferrous salt and hydrogen peroxide in acidic solutions. This reaction became popular for oxidizing organic pollutants and in water treatment. Fenton neither stated HO• radical is formed in the reaction nor did he use it to explain the oxidation reaction. And he could not have, because free radicals were discovered later in 1900

Several proposals have been advanced on the active intermediates responsible for oxidations by Fenton reagents. In 1934, Haber and Weiss[14] first proposed that HO• radical, generated in Fenton reaction, is the oxidizing agent, popularly represented by the following equation: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$. This implies that one electron is donated by Fe^{2+} to reduce an oxygen atom to $^-$OH breaking the peroxo-bridge, leaving the other oxygen atom as $^{\bullet}$OH radical. Will not Fe^{3+} ions, abundantly present in such reaction mixtures, react with $^{\bullet}$OH radicals, like hydroxyl ions do to become insoluble ferric hydroxide at neutral pH? In such a reaction, Kremer[15] pointed out that 'the pair Fe^{3+} + $^{\bullet}$OH would not separate at all' and remain as the species $[Fe\ldots{}^{\bullet}OH]^{3+}$, or as ferryl $[Fe^{IV}{=}O]^{2+}$ or Fe-oxyl radical [Fe-O•].

Selected references that describe production of radicals on oxidation of metal ions by H_2O_2 are given in Table 1. The evidence given in these investigations indicated the presence of metal-oxyl intermediates. The extended work in the presence of chelating agents, emphasizes participation of metal-oxo (M=O) intermediates or metal-oxyl (M-O•) radicals, besides hydroxyl radicals in the biologically relevant oxidation reactions. Both forms of oxo species,

Table 1—Chronological findings and proposals on oxidant and radical species formed during the reaction between H_2O_2 and Fe^{2+} and other metal ions

Finding/proposal/comment	Authors (Year)	Ref.
Reaction between ferrous salt and H_2O_2 in acid solution yields powerful oxidizing intermediate	Fenton (1894)	13
Hydroxyl radical ($^{\bullet}OH$) is the principal oxidizing species in Fenton reaction under acidic conditions	Haber, Weiss (1932)	14
Metallo-oxo species is the predominant oxidant such as solvated ferryl ion $[Fe^{IV}=O]^{2+}$	Bray, Gorin (1932)	17
Experiments using ^{18}O-H_2O_2 supported a metallo-oxo species as the oxidant present under acidic conditions	Cahill, Taube (1952)	18
Oxidation of vanadyl cation, VO^{2+} by H_2O_2 in acid medium forms addition complex, $OVOOH^{+}$, and the peroxo-bridge breaks to yield OVO^{+} and $^{\bullet}OH$ radical	Brooks, Sicilio (1971)	19
Crypto hydroxyl radicals in paraquat toxicity	Youngman, Elstner (1981)	20
Both hydroxyl radical and Fe^{IV} species involved in oxidation of mandelic acid–iron complex by H_2O_2	Walling, Amarnath, (1982)	21
"Confusing pattern of inhibitor reactivity" led to inferences of ferryl or cupryl intermediation, named *crypto hydroxyl radicals*	Borg, Schaich, Forman (1983)	22
Co (II) ion in presence of H_2O_2 produces a 'reactive species' (***pseudo hydroxyl radical?***) that hydroxylates Aromatic compounds in a 'site-specific' manner 'not intercepted by $^{\bullet}OH$ scavengers'	Moorhouse, Halliwell Grootveld, Gutteridge, (1985)	23
Oxidation of Fe^{2+}-EDTA chelate generated an oxidant other than $^{\bullet}OH$.	Rahhal, Richter (1988)	24
Metal-oxo species with chelated iron in neutral solutions, hydroxyl radicals with free iron in acidic solutions, identified with stopped-flow spectrophotometry	Rush, Koppenol (1988)	25
Direct oxidant of DNA both *in vivo* and *in vitro E. coli* System, exhibits reactivity like ferryl radical, but not free $^{\bullet}OH$ radical	Imlay, Chin, Linn (1988)	26
Radical scavengers, formate and azide, but not ethanol, decrease DMPO-OH signals with H_2O_2+Cu,Zn-SOD	Yim, Chock, Stadtman (1990)	27
Three reaction paths produce hydroxyl radicals, ferryl species, and non-oxidizing species in Fenton reaction, depending on the nature of iron chelator	Yamazaki, Piette (1991)	28
Hydroxyl radical is the reactive intermediate in the reaction between Fe^{2+} and H_2O_2 in aqueous solution, but radicals formed from added alcohols kinetically differ	Smith, Whitwood Croft, Gilbert, Lindsay (1992)	29
Kinetic arguments against the intermediacy of the hydroxyl radical as oxidizing intermediate generated in the Fenton reagent	Wink, Wink, Nims, Ford (1994)	30
Reactive oxygen centred radicals hydroxyl and metal-bound oxyl radicals (M-$O^{\bullet}$) are also active intermediates in DNA-cleaving reactions	Breen, Murphy (1995)	31
Oxidant formed from H_2O_2 and Fe^{2+}.chelated with EDTA or ADP is different from that of hydroxyl radical	Henle, Linn (1997)	32
Hydroxyl radical 'remains bound at the iron center', ($[FeOOH]^{+}$?) with oxidizing properties distinguishable from free $^{\bullet}OH$', as pH increases	Lloyd, Hanna, Mason (1997)	33
Free $^{\bullet}OH$ radical is 'not the dominant reactant at all', a 'nucleophilic adduct reacts directly with substrates'	Sawyer, Kang, Llobet, Redman (1993)	34
Oxidation of Fe^{2+} or VO^{2+} by H_2O_2 yields $^{\bullet}OH$ and by diperoxovanandate[$OV(O_2)_2^{+}$] yields $^{\bullet}OV(O_2)^{2+}$ radical	Ramasarma, Ravishankar(2005)	35
Oxidants generated on homolytic cleavage of H_2O_2 by Ti-oxo radicals yield allylic, but not epoxy, oxidation products	Shetti, Srinivas, Ratnasamy (2005)	36

$HO^{\bullet}$ and M-$O^{\bullet}$, are oxidants and can accept electrons. Hydroxyl radical scavengers such as mannitol, formate and ethanol do not intercept actions of some metal-oxo species qualifying them as 'pseudo' and 'crypto' hydroxyl radicals. Coordination of iron with EDTA, ADP, porphyrin and other organic compounds gives raise to selective oxidants with the bound M=O possibly existing as metal-oxyl (M-$O^{\bullet}$) species with an electron delocalized in coordinated structures. Metal ions of biological relevance, Fe, V, Mo, Mn, Cr and Co can substitute H-atom to form metal-oxo species that can oxidize different substrates

selectively and yield variety of products. These studies reiterate that metal-oxyl radicals ($MO^{\bullet}$) predominate in physiological pH and $^{\bullet}OH$ radicals in acid medium in Fenton reaction. Yet it is baffling to note underplaying of the metal-oxyl radicals to the point of ignoring their due role. Continued undue veneration of hydroxyl radicals, as in lipid peroxidation, is more damaging. According to Crabtree[16], "the active form of the metal oxo is not always M=O as traditionally shown in textbooks, but the newly recognized oxyl radical form of M-$O^{\bullet}$".

Vanadium oxyl radical

Ability of vanadate (V^{V}) and vanadyl (V^{IV}) to form addition complexes, as well as peroxo-bridged divanadate complexes, with H_2O_2 gives them enhanced potential as oxidants. Experience with the versatile oxidation reactions of these vanadium peroxides enlightened us[37] on the reactivity of oxyl-radicals.

Brooks and Sicilio[19] first described in a classical paper in 1971 the multiple reactions that occur on mixing vanadyl sulfate and H_2O_2. A vanadium-peroxo radical ($OV^{2+}OO^{\bullet}$) was identified in this reaction mixture by its eight-line (4.5 G) ESR spectrum in acid medium. In phosphate buffer (pH 7.0) on similar oxidation of vanadyl by H_2O_2 or by diperoxovanadate (DPV) a radical species with quartet 1:2:2:1 ESR signals of DMPO adduct ($_N$ = $_H$ = 14.9 G), characteristic of $^{\bullet}OH$ radical was obtained[35]. On inclusion of ethanol in these reaction mixtures before adding vanadyl these signals change to triplet of doublet spectrum typical of DMPO-hydroxyethyl adduct ($_N$ = 15.9 G, $_H$ = 23.3 G) only with H_2O_2, but not with DPV, as the oxidant. Similar results were obtained when ferrous was used instead of vanadyl (Fig. 2). This gave the clue that the ESR signals represent oxyl radicals albeit with different properties.

The oxidant species derived from DPV was not strong enough to abstract H-atom from methyl group of ethanol but is capable of selectively oxidizing organic compounds (eg., ethanol → acetaldehyde, benzoate → *p*-hydroxybenzoate) (see ref. 37 for a summary). During this process, the -peroxo-bridge of divanadate complex [$OV^{IV}OOV^{V}(O_2)$] appears to split to vanadate [OVO] and a vanadium-oxo radical [$^{\bullet}OV(O_2)$], similar to HO^{-} and $^{\bullet}OH$ in Fenton reaction. The mixed valency divanadate is the active intermediate in the oxidation of NADH, bromide and others. The following reactions fit with the observations (vanadate V^{V}; vanadyl V^{IV}; all O and H atoms and charges not shown).

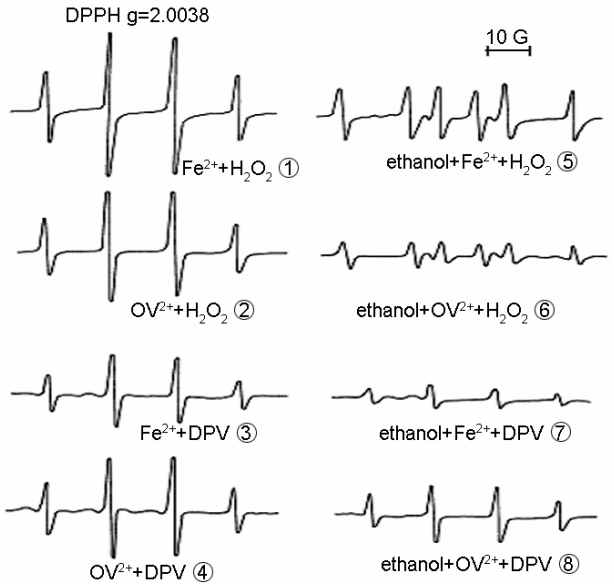


Fig. 2—ESR spectra of DMPO-adducts of radical species formed during oxidation of ferrous and vanadyl sulfates by H_2O_2 and diperxovanadate (DPV) [The reaction mixtures contained phosphate buffer (20 mM, pH 7.0) and DMPO (10 mM) in *1-8*, and ethanol (1.7 M) in *5-8* and the following where mentioned: H_2O_2 (1 mM in *1,5;* 0.4 mM in *2,6*), DPV (1 mM in *3,4,7,8*), ferrous ammonium sulfate (1 mM in *1,5* and 4 mM in *3,7*); vanadyl sulfate (0.4 mM in 2,6 and 4 mM in *4,8*). Note with added ethanol hydroxyethyl-radical is obtained with H_2O_2, but not DPV, as the oxidant [adapted from Ramasarma & Ravishankar[35]].

1 $V^{V} + 2\ HOOH \longrightarrow HOOV^{V}(O_2) + 3H^{+}$
(H_2O_2 addition reaction)

2 $V^{IV} + HOOV^{V}(O_2) \longrightarrow V^{IV}OOV^{V}(O_2) + H^{+}$
(peroxo-V addition reaction)

3 $V^{IV}OOV^{V} \longrightarrow V^{V}O^{-} + {}^{\bullet}OV^{V}(O_2)$
(electron transfer from V^{IV} breaks OO bridge)

Note reaction 3 is a homolytic split forced by transfer of the electron from V^{IV} to the first oxygen that becomes O^{-} ion and the second with unpaired electron remains $^{\bullet}O$ radical. Only the dinuclear vanadate produces the $^{\bullet}OV$-type radical.

H_2O_2 is a good oxidant in protonated form (HOOH) in acid medium, but not at neutral pH (e.g. bromide oxidation). Peroxidation reactions of H_2O_2 can be retained at physiological pH in cells by forming stable diperoxovanadate [$HOOV^{V}(O_2)$] in presence of ubiquitous trace amounts of cellular vanadium. The vanadium complex has the added advantage of resisting degradation by catalase.

Lipid peroxidation is not initiated by $HO^{\bullet}$ radical and is not a destructive chain reaction

It is known for a long time that vegetable oils get rancid on storage or during heating in iron pans.

Spoilage of the oil coincides with oxidation of unsaturated fatty acids to lipid-peroxides which then degrade into many products. One of these, malondialdehyde, on heating with thiobarbituric acid reagent yields a pink product enabling its quantitation. This is the well-known thiobarbituric acid reacting substances (TBARS) assay widely employed as an indicator of lipid peroxidation. Some sugars and including sucrose, commonly used for preparation of cell fractions, respond to this test. Lack of awareness of this non-specificity leads to wrong conclusions.

Interpretations and representations currently in vogue lead to wrong perspective of lipid peroxidation phenomenon. It is not general oxidation of lipids or fatty acids. It is selective addition of peroxide group only to polyunsaturated fatty acids (PUFA) with two or more double bonds. The name 'PUFA oxidation' is a better fit. The first observed product of oxidation is fatty acid hydroperoxide and this naturally led to naming the process peroxidation. But use of the word 'peroxidation' wrongly connects it to H_2O_2 as a causative agent and led to hydroxyl radicals unwittingly trumpeted as inducers of lipid peroxidation. Why look elsewhere when you have the infamous $HO^{\bullet}$ radical with its unpaired electron best suited for this initial 'electron sucking act'? It is not uncommon to see published articles in scientific journals, in books and in Internet files by peers, full of statements and illustrations haranguing that $HO^{\bullet}$ radicals initiate the chain reaction of lipid peroxidation. By their strong visual impact and authoritative peer assertions, these mislead, just like howlers in some textbooks, and leave behind damaging impressions. The sample illustration below, selected from many such in the Internet, colorfully shows hydroxyl radical as the electron-withdrawing agent (Fig. 3).

'The best-known $^{\bullet}OH$ generator, a mixture of ferrous salt and H_2O_2, does not promote lipid peroxidation, nor do the many hydroxyl radical quenching agents stop it' is a clear statement made by Devasagayam *et al*[38] in 2003 in a review on lipid peroxidation. Yet an emphatic assertion "The ability of the hydroxyl radical (generated *via* Fenton chemistry) to initiate lipid peroxidation is unquestionable" is made in a 2005 review[39], never mind all the overwhelming experimental evidence against it revealed since early 1980's (Table 2). What is unquestionable, on the other hand, is the *inability* of hydroxyl radicals generated in Fenton reaction to initiate lipid peroxidation**.** The present evaluation,

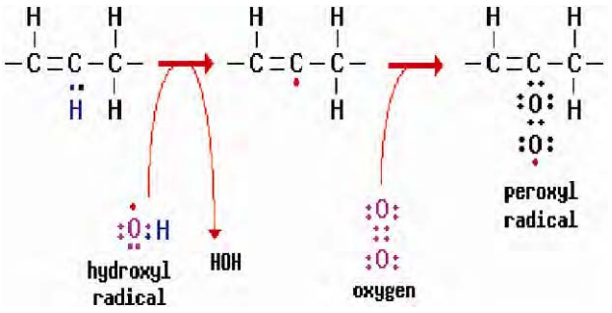


Fig. 3—Hydroxyl radical is assumed to withdraw electron from an unsaturated fatty acid to initiate lipid peroxidation in this appealing colorful scheme, adapted from 'biomedical hypertexts' under Free Radicals in Internet. This is not supported by experimental evidence (see section 6): $^{\bullet}OH$ generated by a mixture of ferrous salt and H_2O_2 (Fenton reaction) does not promote and hydroxyl radical quenching agents do not stop lipid peroxidation[37].

hopefully, will emancipate the entrapped mindset on hydroxyl radicals and lead to deciphering the true initiator of lipid peroxidation.

Unsaturated fatty acids with at least two double bonds, but not saturated fatty acids, undergo lipid peroxidation at good rates. Typically the process of lipid peroxidation oxidizes a PUFA molecule to hydroperoxy-PUFA (Fig. 4). Notice the initiation (reaction *A*) consists of removal of H-atom (electron + proton) from a carbon atom forming lipidC-radical by an "active Fe", popularly known as perferryl. Ubiquitous oxygen instantly reacts with the radical ($R^{\bullet}$) (reaction *B*) yielding the lipidC-peroxo radical ($ROO^{\bullet}$). This breaks into peroxidized products (reaction *C*). Loss of unsaturated fatty acid can proceed via *A* + *B* + *C* without a chain reaction. This depends on "active Fe" maintained by continuous supply of a reducing agent (NADPH or ascorbate) exhaustion of which terminates the reaction. The $R^{\bullet}$ radical is surmised to regenerate through interaction of $ROO^{\bullet}$ with second molecule of RH in the proposed propagation with ROOH as the product (reaction *D*) that may also give other peroxidized products (reaction *E*). By forming the chain *D*+*B*+*C*+*E*, the reaction becomes independent of reaction A and the reducing source until the fatty acid is exhausted. This can no doubt result in uncontrolled, extensive loss of polyunsaturated fatty acids (PUFA). Several papers refer, with respect and fear, to the concept of chain reaction in lipid peroxidation and the consequent damage to cellular membranes as the basis of pathophysiology of several diseases. Indeed, it is a dogma, like faith established, beyond review or inquiry. Once started, the Fe^{2+}-triggered reaction

Table 2—Developments on reactivity and mechanisms of lipid peroxidation

Reactivity/comment	Authors (Year)	Ref.
Ascorbate stimulates peroxide formation in tissue homogenates	Wolfson, Wilbur, Bernheim (1956)	43
Incubation of tissue homogenates produces lipid peroxides,	Bieri, Anderson (1960;	44
measured as TBARS	Zalkin, Tappel (1960)	45
Lipid peroxides damage erythrocyte membranes	Tsen, Collier (1960)	46
Microsomal lipid peroxidation is stimulated by ascorbate or NADPH	Hochstein, Ernster (1963)	47
Lipid peroxidation, stimulated by iron salts, damage membranes in mitochondria	Hunter et al (1964)	48
Lipid peroxides implicated in damage of membranes endoplasmic reticulum of liver (CCl_4 poisoning)	Recknagel, Ghoshal (1965)	49
Added unsaturated fatty acids linoleic acid, linolenic acid also peroxidized by homogenates and subcellular fractions	Wills (1966)	50
Microsomes of liver subcellular fractions show high rate of lipid peroxide formation and damage membranes	Wills, Wilkinson (1967)	51
PUFA from the membrane phospholipids disappear on lipid peroxidation	May, McCay (1968)	52
Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} can not substitute Fe^{2+} in lipid peroxide formation in microsomes	Wills (1969)	53
Lipid peroxyl radicals predominate in peroxidizing microsomes	Rosen, Rauckman (1981)	54
Microsomal lipid peroxidation was not sensitive to hydroxyl radical traps; exogenous hydrogen peroxide, expected to produce $^{\bullet}OH$, actually inhibited	Morehouse, Tien, Bucher, Aust. (1983)	55
Lipid peroxidation promoted by iron occurs *via* a free radical mechanism involving formation of a Fe(II):Fe(III)complex (Fe(II)-O_2-Fe(III) ?), and ADP-Fe(II)-dependent peroxidation of liposomes was not inhibited by catalase, SOD and $^{\bullet}OH$ scavengers	Bucher, Tien, Aust (1983)	56
Traps of $^{\bullet}OH$ do not inhibit, and $^{\bullet}OH$ radicals are not detected during lipid peroxidation caused by mixtures of Fe^{3+} and Fe^{2+}, data argue against the involvement of $OH^{\bullet}$	Braughler, Duncan, Chase (1986)	57
Fe^{2+} + H_2O_2 mixture generated $^{\bullet}OH$ radicals sensitive to catalase, mannitol and benzoate, but not lipid peroxidation	Minotti, Aust (1987)	58
Membranal lipid peroxidation by H_2O_2 + ADP-Fe^{3+} complex only in the presence of ascorbic acid	Harel, Kanner (1988)	59
Lipid peroxidation in brain microsomes needs small amount of vanadate in addition to Fe^{2+} and NADH, but not NADPH, with a stoichiometry of 1:4 for NADH:O_2	Patole, Ramasarma (1988)	42
Fe^{2+}-dependent peroxidation in liposomes, made from ox-brain phospholipids, is stimulated largely by ascorbic acid	Aruoma et al (1989)	60
Lipid peroxidation increased in thyroid homogenates with increasing concentration of $FeSO_4$ and constant H_2O_2 (*absence of* H_2O_2 *not tested, is it required at all?; homogenate could have provided ascorbate or ADP*)	Karbownik, Lewinski (2003)	61

should go on and on. In fact it stops on exhaustion of the reducing agent NADPH in the enzymic system with microsomes, sparing the residual PUFA. Often considered altruistic saviors, "antioxidants" are credited to neutralize the radical species (reaction *F* and possibly *A*) and terminate the presumed chain reaction. Lipid peroxidation reaction in the cell is under tight control, and is not always wasteful or destructive, and. The constraints are release of small amounts of free iron from the cellular stores required to trigger the process, and metabolic diversion to provide large amounts of NADPH. I proposed that it forms the basis of thermogenesis with its high rates of oxidation of NADPH accompanied by consumption of oxygen that release energy as heat[40].

A convincing "good experiment on the chain reaction is yet to appear"[38], and the purported propagation by chain reaction is hard to prove. The observed stoichiometry for NAD(P)H : O_2 of 1:4 exceeds the expected value of 0.5:1 for NADPH : Fe^{3+} for reduction of Fe in "active Fe" with liver microsomes[41], and of 1:1 for NADH:O_2 for reduction

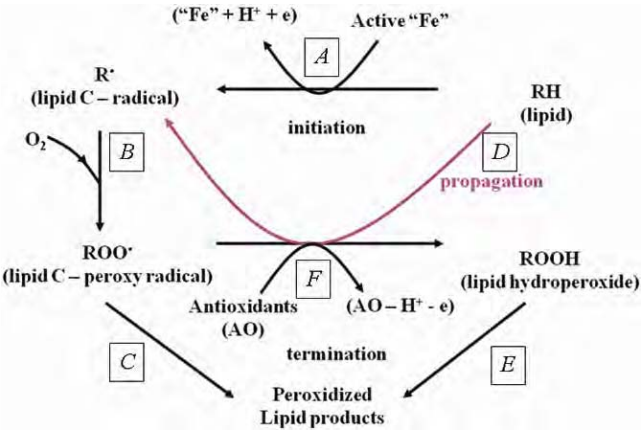


Fig. 4—Lipid peroxidation: a suggested scheme of reactions that fit some experimental observations. Lipid peroxidation reaction is initiated by withdrawing an electron from a polyunsaturated fatty acid (RH) (step A). Regeneration of "active Fe" requires a reducing source, NADPH (enzymic) or ascorbate (non-enzymic), for continuous activity. Ubiquitous dioxygen will react readily with $R^{\bullet}$ to form $ROO^{\bullet}$ (step B.). The implication of chain reaction is based on step D that can generate $R^{\bullet}$ using RH bypassing the "active Fe". "Antioxidants" are expected to terminate the reaction (step F) by neutralizing the radical, $ROO^{\bullet}$, but may also stop step A if a radical species is involved. Using another reductant (glutathione?), step F will produce ROOH, useful as a lipid-based peroxide-oxidant reducing itself to ROH, one of the observed products. Other minor products, such as malondialdehyde and isoprostanes, are likely to derive from $ROO^{\bullet}$ and ROOH (steps C and E).

of O_2 to H_2O_2 found during the polyvanadate-mediated lipid peroxidation in brain microsomes[42]. Consumption of large excess of O_2 over that of NAD(P)H points to lipid as the source of extra electrons and implies its oxidation. This offers indirect support to the occurrence of the chain reaction (*D* + *B* + *C*), operating for a limited number of cycles, but not after the reducing agent exhausts. Quantitation of the lipid-hydroperoxide and its products is needed to account for the extra oxygen consumed.

The extensive evidence embedded in the literature, partly cited in Table 2, supports a Fe-oxo species, but *not* H_2O_2 or $^{\bullet}OH$ radical, as the initiating oxidant for lipid peroxidation at physiological pH. The nature of this active form is yet to be deciphered but probably includes chelation, mixed valence Fe dimers and their O_2-addition complexes amenable for redox changes. The primary oxidant is able to abstract H-atom from C-H bond of the PUFA molecule yielding a $C^{\bullet}$-radical. Two examples of known H-atom abstracting reactions are selected here to showcase the active form of the oxidant for such activity.

First example is the formation of a $C^{\bullet}$-radical crucial in the conversion of ribose (2'-OH) to deoxyribose (2'-H) of nucleotides by the oxygen-dependent class I enzyme, ribonucleotide reductase. The proposed mechanism begins with O-O of dioxygen binding to Fe^{2+}-O-Fe^{2+} dimer in the protein to form Fe^{3+}-O-O-Fe^{3+} whose peroxo-bridge breaks to possibly Fe^{4+}-$O^{\bullet}$ + HO-Fe^{3+} (see reference[62] for an overview). The primary H-atom abstraction with these active iron forms is followed by sequential transfer of the electron through radical species of amino acids, tyrosine and cysteines, linked by hydrogen bridges over a long distance within the protein. Ultimately a cysteine-$S^{\bullet}$ withdraws H-atom from ribose-3' forming $C^{\bullet}$-radical, and returns it to ribose-2' $C^{\bullet}$-radical after its OH group is lost as water. In the overall process an oxidized cystine (S-S) had to be reduced back to cysteines (–SH) utilizing thioredoxin and NADH.

Second example is the initial step in the vanadate-dependent non-enzymic oxidation of NADH is the abstraction of H–atom from NADH to form $NAD^{\bullet}$ radical[63]. Both the reduced form vanadyl (OV^{IV}) a radical, and oxidized form diperoxovanadate [$V^{V}(O_2)_2$], required for activity, form peroxo-bridged divanadium complex [$OV^{IV}OOV^{V}(O_2)$] that can withdraw in steps two electrons from NADH. Also, the oxidizing capacity of H_2O_2 in bromoperoxidation, limited to acid conditions, is enhanced at physiological pH by forming this complex[64]. The radical, $^{\bullet}O$-$V^{V}(O_2)$, derived on breaking in the peroxo-bridge of this complex, can possibly abstract H-atom of C-H from a variety of compounds, eg., benzoate, ethanol (see reference 37 for an overview).

Drawing analogy from these examples, the potential candidates for abstracting an electron from RH (fatty acid) are likely to be Fe-$O^{\bullet}$ and Fe-O-O-Fe, with Fe being the preferred metal in lipid peroxidation. This proposal also validates of metal-oxyl, rather than hydroxyl radical, and peroxo-bridged Fe-dimer, instead of hydrogen peroxide to carry out such functions under physiological conditions.

Extended range of oxo-radicals

Possible intermediates of sequential reduction of dioxygen in association with protons, metals and carbon atoms are shown in Table 3. The protonated forms exist in acid conditions. The metal-associated forms are more likely to be active at physiological pH. Many metals do form some of these intermediates

Table 3—Possible intermediates of sequential reduction of dioxygen in association with protons, metals and carbon atoms

Addition	Dioxygen	Superoxide	Peroxide	Oxo-radical
	$^{\bullet}O=O^{\bullet}$	$^{-}O\text{-}O^{\bullet}$	$^{-}O\text{-}O^{-}$	$O^{\bullet}$
Proton-H^{+}		$HO\text{-}O^{\bullet}$	HO-OH	$HO^{\bullet}$
Metal-M		$MO\text{-}O^{\bullet}$	MO-OH	$MO^{\bullet}$
Carbon-C		$R\text{-}CO\text{-}O^{\bullet}$	R-CO-OH	$R\text{-}CO^{\bullet}$

M = V, Cr, Mn, Fe, Co, Cu, Mo, Ti, Ni, Ru, Re, Os; carbon-C: many phenolic compounds

with H_2O_2. Of these, Fe, Cu, Mn, Mo, V, Cr are physiologically relevant. Some of these metal-oxo radicals can act as oxidants similar to hydroxyl radicals but differ in oxidation properties and inhibitor responses, and are referred as '*crypto*' or '*pseudo*' hydroxyl radicals.

Oxo-radical ($R\text{-}CO^{\bullet}$), akin to hydroxyl ($HO^{\bullet}$), is also obtained on oxidation of quinols, tocols and phenolates. Also a large number of natural phenolic antioxidants act as radical-quenching agents and in the process do form such radicals that are neutralized by forming dimmers or by electron donors.

Some non-metallic elements, N, P, S, can also form oxo-radical species. Schwarz and coworkers[65] found by DFT calculation that oxyl species of oligomeric phosphate ($P_4O_{10}^{\bullet+}$), with one $P\text{-}O^{\bullet}$ bond (1.57 Å) longer than normal P=O (1.46 Å), (Fig. 5) is a good oxidant that can abstract H-atom from C-H group of methane. These will be cheap, safe and non-polluting for industrial use. Thus a variety of peroxo- and oxo- radical species can act as selective oxidants.

Vanadium (V^{V}) in the form of decavanadate ($V_{10}O_{28}^{6-}$), but not in ortho- or meta- forms (mixture of monomer and V_4), is a good acceptor of electrons from ascorbate[66], and enzymically from microsomal NADH-cytochrome c reductase[67]. Decavanadate has two segments of V_5 with unique pair of vanadium atoms loosely associated with unusual triply shared oxygen atoms at the core and also exposed six V-O groups at the periphery (Fig. 5). Notice the structure is similar to that of phosphate-oxyl ($P_4O_{10}^{\bullet+}$) compound described above. The radical species, $V\text{-}O^{\bullet}$ and $P\text{-}O^{\bullet}$, are possibly formed in these interesting cage-like condensed structures by internal electron delocalization.

Closing remarks

Many workers in this field of study, and also the common public, freely talk of free radical damage.

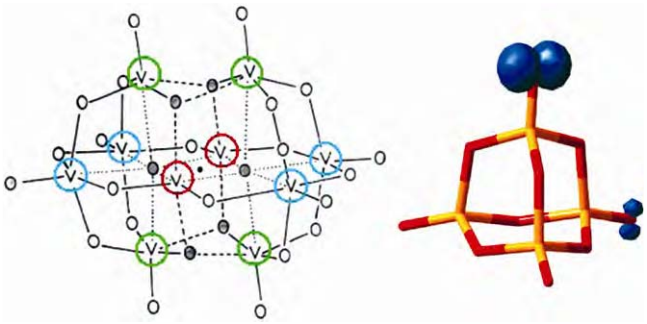


Fig. 5—Oligomeric forms of vanadate and phosphate. On the left is the computer graphic model of decavanadate ($V_{10}O_{28}^{6-}$) based on crystal structure [adapted from Ramasarma [37]]. The two most likely reducible vanadium atoms, circled in red, are linked to unusual triply shared oxygen atoms (shaded) and internal electron delocalization can form the basis of forming $V\text{-}O^{\bullet}$ species from the periferal V-O circled in green. On the right is the lowest energy structure of oligomeric tetraphosphate ($P_4O_{10}^{\bullet+}$) [adapted from Schwarz and coworkers [65]]. The phosphorus and oxygen atoms are shown in yellow and red, respectively, and the 'blue indicates the spin density'.

For example, statements like 'the reaction is inhibited by SOD and, therefore, it involves superoxide', appear patently obvious and, therefore, uncontested, oblivious of many other actions the SOD protein can do[68]. They infer the presence of radicals and their involvement in the reactions mostly based reaction mechanism and on the inhibitor studies, and rarely by ESR studies.

Also, it is common to see emphatic statements such as reactive oxygen species can damage protein, DNA and cell components, free radical chain reactions and lipid peroxidation lead to destruction of membranes, cells, tissues and organs, and the dangerous hydroxyl radicals and oxidative stress cause degenerative diseases and pathophysiological conditions. Such expressions are loosely introduced in 'introduction' and 'discussion' of articles as established facts. Bewilderingly these unsupported articles of faith are promoted by the peers.

Superoxide and hydroxyl are the two oxygen radicals predominantly cited for cellular toxicity or damage. Only these two and no other forms are present and functional? Enchanting radicals and chain reactions always charm their way through barriers of referees and editors into print. Everything about oxygen radicals seem so obvious that questioning about their role in oxidative stress is not tolerated. Other likely candidates of oxygen radical species bound to metals, carbon, and inorganic compounds are waiting to be recognized for their selective actions

in biologically relevant processes. It is time to expand vision beyond superoxide and hydroxyl radicals and explore functions of multiple naturally-occurring oxygen radicals.

Cells seem to promote generation of a variety of useful radicals to accomplish selective tasks rather than being their helpless victims. Intrinsically any chemical reaction involves breaking and making of bonds between atoms of molecules and the transient radical species cannot be normally detected. A number of relatively stable radicals, bound to protein-amino acid residues and cofactors, such as glycyl, tyrosyl, adenosyl, porphyrin cation, formyl, cysteine-thiyl, are now found to function as intermediates in enzyme-catalyzed reactions. Ribonucleotide reductase, pyruvate-formate lyase and methylmalonylCoA mutase are some examples in this new field of "Radical Enzymology" (see reference 62). The original 'Moses Gomberg concept' of a radical as an intermediate in one-electron transfer reaction needs to be revived, a renaissance, as it were.

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References

1 Gomberg M (1900) *Ber Chemische Gesselschaft* 33, 3150
2 Ceconi C, Boraso A, Caragnoni A & Ferrari R (2003) *Arch Biochem Biophys* 420, 217-221
3 Ramasarma T (2000) *Toxicology* 148, 85-91
4 Baum R (1984) *Chem Engg News*, 20-26
5 Neumann E W (1934) *J Chem Phy* 2, 31-33
6 Fee J (1980) In: *Metal Ion Activation of Dioxygen*, pp 209-237, Wiley & Sons, USA
7 Madden K P & Taniguchi H (2001) *Free Radic BiolMed* 30, 1374-1380
8 Yamazaki I & Piette L H (1991) *J Am Chem Soc* 113, 7588-7593
9 Pieper G M, Felix CC, Kalyanaraman B, Turk M & Roza A M (1995) *Free Radic Biol Med* 19, 219-225
10 Connor H D, Thurman R G, Gallizi M D & Mason R P (1986*) J Biol Chem* 261, 4542-4548
11 Bolli R, Jeroudi M O, Patel B S, Dubose C M, Lai E K, Roberts R & McCay P B (1989) *Proc Natl Acad Sci* (USA) 86, 4695-4699
12 Zhang S & Shepherd R E (1988) *Inorg Chem* 27, 4712-4719
13 Fenton H J H (1894) *J Chem Soc* 65, 899
14 Haber F & Weiss J (1934) *Proc Roy Soc (London) A* 147, 332
15 Kremer M L (1999) *Phys Chem Chem Phys* 1, 3595-3605
16 Crabtree R H (2009) *Nature Chem* 1, 348-349
17 Bray W C & Gorin M H (1932) *J Am Chem Soc* 54, 2124-2125
18 Cahill A E & Taube H (1952) *J Am Chem Soc* 74, 2312-2318
19 Brooks H B & Sicilio F (1971) *J Am Chem Soc* 10, 2530-2534
20 Youngman R J & Elstner E F (1981) *FEBS Lett* 129, 264-268
21 Walling C & Amarnath K (1982) *J Am Chem Soc* 104, 1185–1189
22 Borg D C, Schaich K M & Forman A (1984) In: *Oxygen Radicals in Chemistry and Biology*, pp 983-987, publ. Walter de Gruyter, Berlin
23 Moorhouse C P, Halliwell B, Grootveld M & Gutteridge J M C (1985) *Biochim Biophys Acta* 843, 261-268
24 Rahhal S & Richter H W (1988) *J Am Chem Soc* 110, 3126-3133
25 Rush J D & Koppenol W H (1988) *J Am Chem Soc* 110, 4957-4963
26 Imlay J A, Chin S M & Linn S (1988) Science 240. 640-.642.
27 Yim M B, Chock P B & Stadtman E R (1990) *Proc Natl Acad Sci* (USA) 87, 5006-5010
28 Yamazaki I & Piette L H (1991) *J Am Chem Soc* 113, 7588-7593
29 Croft S, Gilbert BC, Smith L & Whitwood J R (1992) *Free Rad Res Comms* 17, 21–39
30 Wink D A, Wink C B, Nims R W & Ford P C (1994) *Environ Hlth Perspect* 102 (Suppl 3), 11-15
31 Breen A P & Murphy JA (1995) *Free Radic Biol Med* 18, 1033-1077
32 Henle E S & Linn S (1997) *J Biol Chem* 272, 19095-19098
33 Lloyd RV, Hanna PM & Mason PM*)* (1997) *Free Radic Biol Med* 22, *885-888*
34 Sawyer D T, Kang C, Llobet A & Redman C (1993) *J Am Chem Soc* 115, 5817–5818
35 Ramasarma T & Ravishankar H N (2005) *Biochim Biophys Acta* 1722, 30-35
36 Shetti VN, Srinivas D & Ratnasamy P (2005) *Zeitschrift Physikalische Chemie, Oldenbourg Wissenschaftsverlag*, 219, 905–920
37 Ramasarma T (2003) *Proc Indian Natn Acad Sci* B69, 649-672; and in *Vanadium Biochemistry* (Alves M A, ed), Publisher, Research Signpost, Trivandrum, India, pp 45-74 (2008)
38 Devasagayam T P A, Bloor K K & Ramasarma T (2003) *Indian J Biochem Biophys* 40, 300-308
39 Valko M, Morris H & Cronin D (2005) *Curr Med Chem* 12, 1161-1208
40 Ramasarma T (1998) *Adv Organ Biol* 6, 219-239
41 Ernster L & Nordenbrand K (1961) *Methods Enzymol* 10, 5764-580
42 Patole M S & Ramasarma T (1988) *J Neurochem* 51, 491-496
43 Wolfson N, Wilbur K M & Bernheim F (1956) *Exp Cell Res* 10, 556-558
44 Bieri J G & Anderson A A (1960) *Arch Biochem Biophys* 90, 105-110
45 Zalkin H & Tappel AL (1960) *Arch Biochem Biophys* 88, 113-117
46 Tsen C C & Collier H B (1960) *Canad J Biochem Physiol* 88, 957-964

47 Hochstein P & Ernster L (1963) *Biochem Biophys Res Commun* 12, 338-394
48 Hunter E E, Scott A. Hoffsten P E., Gebicki J M, Weinstein J & Schneider A (1964) *J Biol Chem* 289, 61
49 Recknagel R O & Ghoshal A K (1965) *Fed Proc* (USA) 24, 299
50 Wills E D (1966) *Biochem J* 99, 667-676
51 Wills E D & Wilkinson A E (1967) *Radiation Res* 31, 732
52 May H E & McCay P B (1968) *J Biol Chem* 243, 2288
53 Wills E D (1969) *Biochem J* (1969) 118, 315-324
54 Rosen G M & Rauckman E J (1981) *Proc Nat Acad Sci* (USA) 78, 7346-7349
55 Morehouse L A, Tien M, Bucher J R & Aust S D (1983) *Biochem Pharmacol* 32, 123-127
56 Bucher J R, Tien M & Aust S D (1983) *Biochem Biophys Res Communs* 111, 777–784
57 Braughler J M, Duncan L A & Chase R L (1986) *J Biol Chem* 261, 10282-10289
58 Minotti G & Aust S D (1987) *J Biol Chem* 262, 1098-1104
59 Harel S & Kanner J (1988) *Free Radic Res Commun* 5, 21-33
60 Aruoma O I, Halliwell B, Laughton M J, Quinlan G J & Gutteridge M (1989) *Biochem J* 258, 617-620
61 Karbownik M & Lewinski A (2003) *J Cell Biochem* 90, 806–811
62 Himo F & Siegbahn P E M (2003) *Chem Rev* 103, 2421-2456
63 Ravishankar H N & Ramasarma T (1995) *Arch Biochem Biophys* 316, 319-326
64 Rao A V S, Islam N & Ramasarma T (1997) *Arch Biochem Biophys* 342, 289-297
65 Dietel N, Engeser M & Schwarz H (2009) *Angew Chem Int ed* 48, 4861-4863
66 Rao A V S & Ramasarma T (2000) *Biochim Biophys Acta* 1474, 321-330
67 Ramasarma T & Rao A V S (2006) *Mol Cell Biochem* 281, 139-144
68 Ramasarma T (2007) *Curr Sci* 92, 184-191