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Oxidants and Antioxidants

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Free Radicals and Lipid Peroxidation: What They Are and How They Got That Way

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I. Introduction

The aim of this review is to outline what radicals are and how they react. We will start with a discussion of the lifetimes and reactivities of different types of radicals. Then we will examine the mechanistic types of reactions that radicals undergo. The peroxidation of lipids, one of the oldest examples of a radical chain reaction that plays a role in biological changes, will be discussed. Finally, we will consider some "side reactions" that very often accompany the main processes that occur in lipid peroxidation and that have biological consequences.

The term "free radical" was first used in the debates that took place among chemists in the period 1750–1890 about the possibility that parts of molecules could have an independent existence. In that period, analytical methods in general and molecular weight methods in particular were so crude that it was not clear whether a part or a whole molecule was under observation. For example, if butane were to split to form two ethyl radicals, as shown in reaction (1), the methods of the time could not distinguish between the two "free" ethyl radicals on the right and the two "bound" ethyl radicals in the molecule butane, shown on the left.

$$CH_3–CH_2–CH_2–CH_3 \rightarrow 2\ CH_3–CH_2^{\cdot} \qquad (1)$$

Some of the problems that chemists of the 1800s had with radicals can be seen from the quite acrimonious quotes from leading chemists of the era, taken from the

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review by Pryor (1968). For example, Laurent in 1842 stated that "chemistry has now become the science of [radicals] that do not exist!" In contrast, Frankland in 1850 argued that "the isolation of these four radicals [methyl, ethyl, valeryl, and amyl] eliminates any doubt of their actual existence and is a complete proof of the correctness of the theories of Berzelius and Liebig." The murky status of radicals becomes clear from the statement of Wurtz in 1856: "Gerhardt's assumptions that free radicals do not exist . . . cannot be maintained. Ethylene, carbon monoxide and sulfur dioxide may be regarded as free radicals since they form compounds by direct addition of two atoms of chlorine." Clearly, Wurtz did not use the term "radical" in the same way that we do now, because neither carbon monoxide nor sulfur dioxide is a radical (although nitric oxide, another common gas, is). Mechanisms as well as atomic structures were unknown in those days, and chemists did not recognize that compounds that contain a carbon–carbon double bond (olefins) can add "two atoms of chlorine," i.e., Cl_2, either in a radical chain process or in an ionic process that does not involve radicals, depending on conditions (Pryor, 1966).

Thanks to pioneering work by Gomberg in 1900, Nernst in 1918, Herzfeld and Polanyi in the 1920s, and Paneth in 1929, the existence of "free" radicals both in the gas phase and in solution was unequivocally established in the period 1900–1930 (Pryor, 1968). Today, we use the terms "radical" and "free radical" interchangeably.

II. Putting the Proper Spin on Radicals

We should start by defining the equivalent terms "radical" and "free radical"; the formal definition states that a radical is a chemical species with an unpaired electron (Pryor, 1966; Walling, 1957). Radicals can be neutral or negatively or positively charged. Most of the organic radicals we will be discussing are neutral ($CH_3^{\cdot}$, $RO^{\cdot}$, etc.), but many radical anions and radical cations are known and are important in biology. For example, superoxide, $O_2^{\cdot -}$, is a radical anion. The oxidation of polycyclic aromatic hydrocarbons (PAHs) by a one-electron step is relatively easy, because the odd electron can be delocalized over the (generally) large number of benzene rings in these "bathroom tile" types of molecules; when a PAH loses an electron, it becomes a PAH cation radical, $PAH^{\cdot +}$, and these species can be involved in the oxidative processes that convert PAHs to carcinogens (Todorovic *et al.*, 1993; RamaKrishna *et al.*, 1993; Cavalieri and Rogan, 1984; Rogan *et al.*, 1993; Devanesan *et al.*, 1993).

Chemical bonds are made up of a pair of electrons that have opposed spins, symbolized in this way: $\uparrow\downarrow$. In a two-electron bond, these two electrons occupy the same orbital (space), located between the two atomic nuclei that are bonded together by this electron pair. When a bond breaks, either both electrons can attach to one partner, as shown in reaction (2), or one electron can attach to each partner, as shown in reaction (3). In the former case, called heterolysis, ions are formed. In the latter case, called homolysis or homolytic bond scission, two fragments, each with one unpaired electron, are formed; these species with an odd number of electrons are radicals.

$$X{-}Y \rightarrow X^{+} + :Y^{-} \tag{2}$$

$$X{-}Y \rightarrow X\cdot + \cdot Y \tag{3}$$

Because virtually all stable molecules have an even number of electrons in "closed" (complete) electronic orbitals, the scission shown in reaction (3) gives species with an odd number of electrons. These free radicals are called "open shell" species, because the orbital holding the single electron could hold another electron. If another electron were to be placed in this orbital, it must have a spin opposed in direction to the electron already there. This gives two electrons with opposed, or "paired," spins, as shown in the representation $\uparrow\downarrow$. The spins of the two electrons must be different because the Pauli exclusion principle states that each electron in a molecule must have at least one quantum number (of the four total) that differs from those of all the other electrons. These quantum numbers can be thought of as the "address" of the electrons in a molecule. If the two electrons are in the same orbital, then the three quantum numbers that specify the location in space of the electrons are the same; therefore, to satisfy the Pauli principle, the spin quantum numbers of the two electrons must differ. One must be polarized "up" and one must be "down." Incidently, it is this small energy difference that is utilized in electron spin resonance (ESR), a spectrographic technique that measures only those species with an odd electron. [ESR can also be referred to as electron paramagnetic resonance (EPR).] When the molecule is put in a magnetic field (so the electrons have an external reference to allow them to distinguish up from down, i.e., with or against the field), and the energy is scanned, species with pairs of electrons undergo transitions and no net energy is absorbed. However, radicals have one odd electron that "flips" its orientation from being with to against the field; the absorption of the small energy required by this electron flip is detected in ESR.

This business of spin can produce the unusual situation of a diradical, a species with two unpaired electrons that have the same spin. Because the two electrons have the same spin, they cannot go into the same orbital (Pauli principle) and are forced to stay apart. (Of course electrostatics also tells us that the two electrons would repel each other.) Dioxygen has two electrons with equal energy that occupy similarly shaped orbitals with identical energies (called "degenerate"), one on each oxygen atom. Hund's rule states that when two electrons fill degenerate orbitals, they have the same spin, which forces them to stay apart. Thus, ground-state dioxygen (O_2) is a diradical. If this ground-state "triplet" dioxygen is excited by 23 kcal/mol, it can pair the spins of these two electrons and put them both in the same orbital, forming the reactive species called singlet oxygen. (Singlet and triplet are terms that describe the number of equivalent spectroscopic states the species has.) The conversion of ground-state dioxygen (O_2) to the excited singlet state (represented as $^1O_2$) is shown in reaction (4).

$$\underset{\text{triplet}}{\cdot O{-}O\cdot} \rightarrow \underset{\text{singlet}}{O{=}O} \tag{4}$$

III. Radical Lifetimes

The biological lifetimes of a number of types of radicals can be approximated by calculations in which their rate constants for reaction with their principal targets are combined with the estimated concentration of the targets in the vicinity where the radical is formed (Pryor, 1986). These calculated lifetimes are shown in Table I. Note that radical lifetimes vary from extremely short to infinitely long. The hydroxyl radical is so short-lived that it can only diffuse about 50 molecular diameters before it reacts. Thus, it is extremely reactive and can pull off a hydrogen atom from even the least likely molecules. However, this very reactivity makes the hydroxyl

Table I
An Estimate of the Half-Lives of Different Types of Radicals and Oxidizing Species that Can Play a Role in Biological Processes[a]

Radical	Name	Substrate[b]	Half-life (37°C)
HO·	Hydroxyl radical	LH[c]	10^{-9} sec
LO·	Lipid alkoxyl radical	LH	10^{-6} sec
LOO·	Lipid peroxyl radical	LH	7 sec
L·	Lipid carbon-centered radical	O_2	10^{-8} sec
H_2O_2	Hydrogen peroxide	—[d]	Minutes
$O_2^{\cdot-}$	Superoxide anion	—[d]	10^{-5} sec[e]
$^1O_2$	Singlet oxygen	H_2O	10^{-6} sec
HQ·[f]	Semiquinone radical	O_2	Days
NO	Nitric oxide	—[g]	~1 sec

[a]From Pryor (1986).

[b]This is the species that is assumed to be the primary target that reacts with the given radical.

[c]LH is a polyunsaturated fatty acid (PUFA) or a PUFA molecule in a lipid. The hydrogen atom shown (H) is one of the doubly allylic hydrogens that are readily abstracted by radicals.

[d]Both hydrogen peroxide and superoxide probably have their concentrations limited by reaction with enzymes: catalase/glutathione peroxidase and superoxide dismutase (SOD), respectively. Superoxide occurs in systems in which it is produced as well as destroyed and probably reaches steady-state levels of 0.1–1.0 n*M*. Similarly, H_2O_2 occurs at steady-state levels, but probably can occur at up to 1–100 n*M* (Fridovich, 1976; Koppenol, 1989; Gardner and Fridovich, 1992; Imlay and Fridovich, 1991).

[e]Unpublished calculation by Fridovich for the liver, with normal SOD concentrations (see also Gardner and Fridovich, 1992; Van Overveld *et al.* 1992).

[f]HQ· represents a semiquinone radical such as that in cigarette tar. These radicals are stable indefinitely (Pryor *et al.*, 1983a,b; Pryor and Stone, 1993).

[g]Nitric oxide occurs in polluted air and cigarette smoke and is inhaled; it also is produced by nitric oxide synthase in a number of cell types (e.g., macrophages, endothelial cells). It reacts with heme compounds, with superoxide (to form peroxynitrite), and with many other targets (Pryor *et al.* 1985; Beckman *et al.*, 1990; Snyder and Bredt, 1992; Kubes *et al.*, 1991; Radi *et al.*, 1991; Marletta, 1989; McCall *et al.*, 1989).

radical quite unselective in the damage it produces. For example, in an attack on the protein α-1-proteinase inhibitor, hydrogen peroxide specifically oxidizes a methionine residue and produces an inactive protein. In contrast, hydroxyl radicals appear to do a random, nonselective damage to the periphery of the protein, changing its electrophoretic mobility but *not* inactivating it (Evans and Pryor, 1992, 1994).

Semiquinone radicals can have essentially infinite lifetimes, and thus would be thought to be relatively benign. However, semiquinone radicals can reduce dioxygen, producing superoxide. Superoxide can reduce ferric ions (Fe^{3+}), producing ferrous ions (Fe^{2+}) that are more reactive in lipid peroxidation systems. Thus, the relatively stable, nonoxidizing semiquinone radical can lead to biological damage.

These remarks about the reducing properties of semiquinones lead us to recite this poem:

All radicals are not oxidants.
And all oxidants are not radicals.

For example, superoxide quite generally functions as a reducing agent, not an oxidizing agent, thus illustrating that all radicals are not oxidants. Also, as discussed above, hydrogen peroxide (H_2O_2, or HO–OH) oxidizes sulfur atoms to the sulfoxide by an S_N2 reaction of the sulfur atom with the O–O bond of HO–OH, illustrating that all oxidants are not radicals. This S_N2 reaction is shown below for a sulfide such as methionine:

$$R_2S + HO{-}OH \rightarrow R_2\overset{+}{S}{-}OH + HO^- \qquad (5a)$$

$$R_2\overset{+}{S}{-}OH \rightarrow R_2S{=}O + H^+ \qquad (5b)$$

As we will see in the discussion below of lipid peroxidation of the low-density lipoprotein (LDL) particle, the lifetime of a radical can be influenced by having it physically isolated in a particle so it cannot react with other radicals. Isolating radicals by some technique can lead to long lifetimes, and consequently can make even inherently short-lived and reactive radicals directly observable by ESR. The particles formed when polyfluoroethylene-type polymers are heated to high temperatures contain reactive, short-lived oxy radicals that ordinarily could only be studied by ESR spin trap techniques. However, these radicals have infinite lifetimes when they are trapped in the polymer particles (Seidel *et al.*, 1991; Pryor *et al.*, 1990). Therefore, in this unusual case, these radicals can be observed by direct ESR (without adding a spin trap) because the radicals are trapped in particles and cannot undergo termination reactions, and consequently have long lifetimes despite their high reactivity. This same technique of immobilizing a radical to lengthen its lifetime is used to examine biological samples; samples are frozen to retard diffusion of radicals, lengthen lifetimes, and allow observation of otherwise short-lived radicals (Borg, 1976).

Table I shows the effects on lifetimes of small changes in the structure of radicals. The hydroxyl radical, HO·, virtually reacts at every collision. However,

the substitution of an alkyl group for the hydrogen atom, to give an alkoxyl radical RO·, gives a species that exists 1000-fold longer than does HO· before it reacts. Similarly, the peroxyl radical, ROO·, exists 10^6 times longer than does the alkoxyl radical. In fact, peroxyl radicals can have lifetimes of seconds, as long as many biological messenger molecules, such as some eicosanoids or nitric oxide (which is itself a radical). The lifetime of a carbon-centered radical, such as the lipid L· shown in Table I, is very short in oxygenated tissues; this is true because the rate of the reaction of dioxygen with L· is very fast. This reaction should be fast, because dioxygen is a diradical and, of course, L· is a radical, so the reaction of L· with O_2 is a type of radical–radical recombination reaction. Such reactions are always very fast, with bimolecular rate constants near the diffusion limit, about $10^{10}\ M^{-1}\ \text{sec}^{-1}$. Another example of a biologically important radical–radical recombination is the reaction of nitric oxide with superoxide to give peroxynitrite, reaction (6a). The rate constant for reaction (6a) has recently been measured and is about $10^9\ M^{-1}\ \text{sec}^{-1}$ (Huie and Padmaja, 1993), as would be expected for a radical recombination. The peroxynitrite anion is in equilibrium with peroxynitrous acid [reaction (6b)], which is a potent oxidant (Moreno and Pryor, 1992; Koppenol *et al.*, 1992; Jin *et al.*, 1994).

$$O_2^{\cdot -} + {\cdot}NO \rightarrow {}^{-}O{-}O{-}N{=}O \tag{6a}$$

$$H^+ + {}^{-}O{-}O{-}N{=}O \rightleftharpoons HO{-}O{-}N{=}O \tag{6b}$$

IV. Radical Chain Reactions and Lipid Autoxidation

Let us now consider the effect of introducing a radical into a system that contains a polyunsaturated fatty acid (PUFA), and the autoxidation of the PUFA begins to occur. (Autoxidation is the slow, flameless oxidation of a material, generally by a radical-mediated chain reaction. Because the autoxidation of lipids generally produces lipid hydroperoxides, LOOH, as the principal product, lipid autoxidation often is given the name "lipid peroxidation.")

Free radicals invariably are involved in chain reactions. When a free radical reacts with a nonradical species, another free radical must be produced. (A free radical contains an odd number of electrons, and the nonradical species it reacts with contains an even number of electrons; the reaction of an odd species with an even species must produce at least one species that contains an odd number of electrons.) Reactions in which the number of free radicals is conserved are called "propagation reactions."

When one radical is introduced into a system, a chain reaction generally is started and the primordial radical introduced will cause many molecules to undergo a chemical change. Thus, the effect of introducing a single radical into a system containing PUFAs will be to cause the autoxidation of many of the PUFA molecules. The number of molecules converted to some type of product per primordial radical introduced is called the kinetic chain length (KCL). Radical reactions can

have a very large KCL *in vitro*; the useful industrial radical reactions (such as chlorinations, polymerizations, and oxidations) have KCL values as high as 10^6. However, *in vivo*, where defense mechanisms are effective, a typical KCL lies between 5 and 100.

Lipid peroxidation is one of the oldest studied free radical chain reactions (Ingold, 1961; Mead, 1976; Pryor, 1976; Witting, 1974; Aust and Svingen, 1982; Yagi *et al.*, 1992; Burton and Ingold, 1981; Schultz *et al.*, 1962). Lipid peroxidation occurs whenever fats are exposed to air and can be observed in many *in vitro* systems, including homogeneous organic solutions of olefins (including PUFAs), solutions of olefins (and PUFAs) emulsified in aqueous solvents, and synthetic and natural bilayer membranes. In addition, numerous lines of evidence indicate that lipid autoxidation occurs *in vivo* (Halliwell and Gutteridge, 1989; Morrow and Roberts, 1991; Morrow *et al.*, 1990, 1992; Laudicina and Marnett, 1990; Porter *et al.*, 1980; Marnett, 1987, 1990; Frei *et al.*, 1988, 1990, 1992; Frei, 1991; Stocker and Frei, 1991; Bowry *et al.*, 1992b).

The equations that describe lipid autoxidation are shown in Scheme I. Radicals are produced in a process called "*initiation*." Radicals formed in the initiation step then react with a PUFA molecule by abstracting a hydrogen atom in a step called

	Eq. number
Initiation	
$\text{Initiation} \longrightarrow 2R\cdot$	I-1
$R\cdot + O_2 \longrightarrow ROO\cdot$	I-2
$ROO\cdot + LH \longrightarrow ROOH + L\cdot$	I-3
Propagation	
$L\cdot + O_2 \xrightarrow{k_o} LOO\cdot$	I-4
$LOO\cdot + LH \xrightarrow{k_p} LOOH + L\cdot$	I-5
Termination	
(a) No inhibitor; high oxygen tension	
$2\ LOO\cdot \xrightarrow{k_t}$ Non-radical products (NRP)	I-6
(b) No inhibitor; low oxygen tension	
$L\cdot + LOO\cdot \longrightarrow NRP$	I-7
$2L\cdot \longrightarrow NRP$	I-8
(c) Inhibitor present (ArOH)	
$LOO\cdot + ArOH \xrightarrow{k_{inh}} LOOH + ArO\cdot$	I-9
$ArO\cdot + LOO\cdot \longrightarrow NRP$	I-10

Scheme I. The chemical equations that are involved in lipid autoxidation. The rate constants shown over the arrows are referred to in the text.

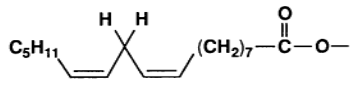
"*propagation*." The reactions in the propagation make up a chain reaction; that is, reactions (I-4) and (I-5) occur again and again until a *termination* reaction occurs.

In Scheme I, the PUFA molecule is represented as LH. In this notation, L is the skeleton of the polyunsaturated fatty acid molecule and the H is one of the hydrogen atoms between two double bonds; that is, it is a doubly allylic hydrogen. Unsaturated fatty acids that have just a single double bond (such as oleic) do not undergo autoxidation, although they may be cooxidized if they are present during the autoxidation of a PUFA. The structure of typical PUFA molecules with doubly allylic hydrogens is shown in Scheme II.

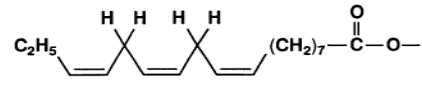
As remarked above, only fatty acids with more than one double bond undergo rapid autoxidation. That is true because doubly allylic hydrogen atoms are abstracted by peroxyl radicals in reaction (I-5) much more rapidly than are singly allylic hydrogens. The kinetic expression for the rate of oxidization of an olefin is given by Eq. (7). It can be seen that the ability of an olefin to undergo autoxidation is proportional to the concentration of the olefin LH, the square root of the rate at which radicals are formed in an initiation process, R_i, and the expression $k_p/(2k_t)^{0.5}$, where the rate constants for propagation, k_p, and termination, k_t, are defined in Scheme I. The term $k_p/(2k_t)^{0.5}$ is called the "oxidizability" of the olefin (because the rate of oxidation of an olefin is proportional to this term) and is symbolized by Δ (Pryor, 1984).

$$\frac{-[dO_2]}{dt} = \frac{-d[\text{PUFA}]}{dt} = \frac{k_p[\text{LH}]R_i^{0.5}}{(2k_t)^{0.5}} = \Delta[\text{LH}]R_i^{0.5} \tag{7}$$

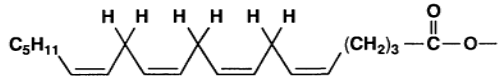
Using Eq. (7), the oxidizability factor, Δ, of different olefins can be obtained from the rate at which they absorb oxygen when primordial radicals are introduced.



Linoleate



Linolenate



Arachidonate

Scheme II. The structure of polyunsaturated fatty acid molecules, showing their doubly allylic hydrogen atoms (abbreviated as LH in Scheme I).

Table II
The Oxidizabilities of Olefins [$\Delta = k_p/(2k_t)^{0.5}$] and the Relative Propagation Rate Constants for Autoxidation, k_p, per Abstractable Hydrogen Atom[a]

Olefin	$\Delta = k_p/(2k_t)^{0.5}$	$k_p(M^{-1}\ sec^{-1})$
1-Octene	0.06	1
3-Heptene	0.5	1.4
Methyl oleate	0.9	1.1
Methyl linoleate	21	62
Methyl linolenate	39	234

[a]From Howard (1972), Korcek *et al.* (1972), and Pryor (1984).

(Generally, in studies of this type, a standard azo initiator is used as the source of radicals and a desired value of R_i can be obtained.) The oxidizability of several monoolefins and PUFAs are given in Table II, using data summarized by Howard (1972).

If oxidizability depends on the number of abstractable hydrogen atoms, then the rate of autoxidation for PUFAs should be proportional to the number of doubly allylic hydrogen atoms that a given PUFA molecule possesses. Older data had not shown that to be true, but a reexamination has shown that this prediction is followed quite closely (Cosgrove *et al.*, 1987), as shown by the data in Table III. Thus, on a scale wherein oleate (18:1) undergoes autoxidation too slowly to measure, linoleate (18:2), with two allylic hydrogens undergoes autoxidation half as fast as does linolenate (18:3) with four allylic hydrogens. The other PUFAs that were studied also undergo autoxidation in proportion to the number of doubly allylic hydrogens they possess.

Table III
Values of the Oxidizability of PUFA with Different Numbers of Doubly Allylic Hydrogens[a]

PUFA	Symbol	H[b]	$\Delta = k_p/(2k_t)^{0.5}$
Linoleate	18:2	2	20 ± 0.2
Linolenate	18:3	4	41 ± 2
Arachidonate	20:4	6	55 ± 2
Docosahexaenoate	22:6	10	102 ± 2

[a]From Cosgrove *et al.* (1987).
[b]The number of doubly allylic hydrogen atoms. The oxidizability of oleate, 18:1, is too small to observe in this system.

V. Autoxidation and Kinetic Chain Length

Expressions can be derived for the effect of various parameters on the rate of autoxidation of a PUFA using the reactions shown in Scheme I. The expressions are very different if an antioxidant such as vitamin E is absent or present, and the two cases will be discussed separately.

If the rate at which primordial radicals are formed is called R_i, then a steady-state condition states that the rate of initiation must equal the rate of termination, $R_i = R_t$. (If this relation were not true, either the reaction would stop or the entire system would become free radicals!) Thus, considering termination reaction (I-6), we can write Eq. (8a). From Eq. (8a), we can derive Eq. (8b), which shows that the steady-state concentration of peroxyl radicals increases with the square root of the rate of initiation. (This is the so-called square root law, and applies to chain reactions in which radical termination is second order.)

$$R_i = R_t = 2k_t\,[\mathrm{LOO}\cdot]^2 \tag{8a}$$

$$[\mathrm{LOO}\cdot] = (R_i/2k_t)^{0.5} \tag{8b}$$

As a model, consider the oxidation of linoleic acid (concentration = 0.63 *M*) in sodium dodecyl sulfate (SDS) micelles in a buffer at pH 7.0 and 37°C (Pryor, 1984; Pryor *et al.*, 1993). For this typical *in vitro* system, R_i is set at the typical value of 3×10^{-7} *M* sec^{-1}, and $k_t = 4 \times 10^6\ M^{-1}\ \mathrm{sec}^{-1}$; therefore, the steady-state concentration of lipid peroxyl radicals [from Eq. (8b)] is given by Eq. (9):

$$[\mathrm{LOO}\cdot] = [3 \times 10^{-7}\ M\ \mathrm{sec}^{-1}/2(4 \times 10^6\ \mathrm{M}^{-1}\ \mathrm{sec}^{-1})]^{0.5} = 2 \times 10^{-7}\ M \tag{9}$$

The relationship between the steady-state concentration of lipid carbon-centered radicals, L·, and the concentration of peroxyl radicals can be obtained from the steady-state relationship shown in Eq. (10a). This relationship follows from the fact that both steps in the chain sequence, reactions (I-4) and (I-5), must occur at the same rate because they alternate and one cannot go faster than the other. An alternative way to say this is that if Eq. (10a) were not true, one of the two propagating radicals (L· and LOO·) would soon disappear.

$$k_p[\mathrm{LOO}\cdot][\mathrm{LH}] = k_o[\mathrm{L}\cdot][\mathrm{O_2}] \tag{10a}$$

$$[\mathrm{L}\cdot] = k_p[\mathrm{LOO}\cdot][\mathrm{LH}]/k_o[\mathrm{O_2}] \tag{10b}$$

From the concentrations of peroxyl radicals, linoleic acid, oxygen, and the known values for k_p (Table II) and k_o, therefore, the steady-state concentration of L· is calculated as shown in Eq. (11), where numerical values are given for each of the quantities listed in Eq. (10b):

$$[\mathrm{L}\cdot] = \frac{(62)(2 \times 10^{-7})(0.63)}{(2 \times 10^9)(10^{-3})} = 4 \times 10^{-12}\ M \tag{11}$$

Thus, comparing Eqs. (9) and (11), the concentration of carbon-centered radicals, L·, is seen to be five orders of magnitude lower than peroxyl radicals. This is true because reaction (I-4) is so fast.

The kinetic chain length can be calculated for a system in which no antioxidant is present and termination occurs through reaction (I-7). [All of the termination occurs by reaction (I-7) as long as the tension of dioxygen is sufficiently high so that lipid radicals are rapidly converted to peroxyl radicals (Pryor, 1966).] The KCL is equal to the rate of the propagation reaction (I-5), divided by the rate at which primordial radicals are formed. That is, the KCL is given by Eq. (12), which can be seen to be a self-evident and quite reasonable definition.

$$\text{KCL} = \frac{\text{(rate of product formation)}}{\text{(rate of primordial radical formation)}} \tag{12}$$

Thus, the KCL is given by Eq. (13):

$$\text{KCL} = \frac{k_p[\text{LOO}\cdot][\text{LH}]}{R_i} = \frac{(62)(2 \times 10^{-7})(0.63)}{3 \times 10^{-7}} = 26 \tag{13}$$

Thus, about 26 molecules of linoleic acid are caused to be oxidized in this aqueous micellar solution by the introduction of a single radical into the system. Translating this into a biological context, introduction or production of a single radical in a lipid bilayer would lead to the destruction of about 26 PUFA molecules. Whether that is enough to alter significantly, and perhaps rupture, a bilayer is difficult to say, but it certainly is a large multiplying factor for radical damage.

VI. The Effects of Antioxidants

What is the effect of adding antioxidants to a PUFA system (Niki *et al.*, 1985; Takahashi *et al.*, 1992; Barclay, 1993; Burton and Ingold, 1981, 1989; Burton *et al.*, 1983)? In the SDS micellar system, if one α-tocopherol molecule is added for each 1000 PUFA molecules present (i.e., 0.1 mol%), then the radical concentrations and KCL are dramatically affected (Pryor, 1984). This is the level of antioxidants that has been used in commerce to protect products (such as plastics and rubber) from autoxidation. In biological systems, antioxidants are present in concentrations from 0.1 to more than 1 mol%, depending on the oxidative stress to which the tissue is subjected. When an antioxidant inhibitor (Inh) is present, a new steady-state relationship can be written, because termination now occurs primarily by reaction of peroxyl radicals with the antioxidant. This new equation is given by Eq. (14a):

$$R_i = R_{inh} = nk_{inh}[\text{LOO}\cdot][\text{Inh}] \tag{14a}$$

$$[\text{LOO}\cdot] = R_i/nk_{inh}[\text{Inh}] \tag{14b}$$

In Eq. (14a), k_{inh} is defined in Scheme I by reaction (I-9), and n is the number of radicals that react with a single molecule of inhibitor; in general, for a good inhibitor such as α-tocopherol (vitamin E), n is 2. This is because, just as would be expected, a good inhibitor reacts with radicals to form a very stable radical [reaction (I-9)] that just waits around until it can react with a second radical [reaction (I-10)], forming stable nonradical products and stopping the reaction of *two* of the chain-carrying peroxyl radicals in the process. The new steady-state concentration of

peroxyl radicals can be calculated by putting numerical values into Eq. (14b), as shown in Eq. (15):

$$[\mathrm{LOO}\cdot] = \frac{3 \times 10^{-7}}{(2)(2 \times 10^{5})(6 \times 10^{-4})} = 1 \times 10^{-9}\, M \tag{15}$$

(Because the linoleic acid concentration is 0.63 M, 0.1% as much vitamin E is equivalent to it being 6×10^{-4} M.) It can be seen that the addition of just one α-tocopherol molecule for every 1000 PUFA molecules reduces the steady-state concentration of peroxyl radicals by a factor of about 200 [compare Eqs. (15) and (9)]. This may not seem very great, but the kinetic chain length is then reduced from 26 to less than 1, as shown in Eq. (16):

$$\mathrm{KCL} = \frac{(62)(1 \times 10^{-9})(0.63)}{3 \times 10^{-7}} = 0.1 \tag{16}$$

Therefore, instead of one free radical causing the destruction of 26 lipid molecules, it takes roughly 10 free radicals to damage a single PUFA molecule. Clearly, the system is very highly protected by even a small amount of α-tocopherol.

These data were obtained by studies of the change in oxygen concentration when free radicals from a known initiator are generated in a system consisting of linoleic acid emulsified in SDS micelles in a phosphate buffer at pH 7.0 and 37°C. Figure 1 shows the trace. Interesting parameters can be calculated from a trace such as that in Fig. 1 (Pryor, 1984). For example, the stoichiometric factor n can be calculated from τ, the delay period, caused by a given concentration of initiator at a particular rate of initiation. (The variable, τ, can be thought of as the time it takes to "use up" the radicals made by a given amount of initiator, and thus is the ratio of the number of radicals scavenged divided by the rate at which they are produced.)

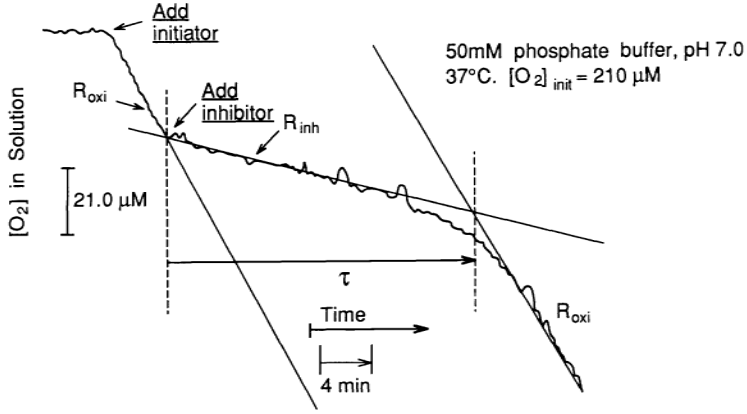


Figure 1. An oxygen uptake curve for the autoxidation of linoleic acid in sodium dodecyl sulfate (SDS) micelles at 37°C in aqueous buffer at pH 7.0. The parameters shown on the trace are R_{oxi}, the rate of use of oxygen; R_{inh}, the rate of use of oxygen during the inhibition period when inhibitor (α-tocopherol) is present; and τ, the time of the delay period caused by the added inhibitor (Pryor, 1984).

$$\tau = n[\text{Inh}]/R_i \tag{16}$$

With a value of τ and a measured value of R_{inh}, the ratio of k_p/k_{inh} can be obtained. The ratio k_p/k_{inh} gives the competition of peroxyl radicals to either carry on the chain reaction or be terminated by reaction with an inhibitor molecule [reaction (I-5) versus reaction (I-9)].

$$R_{inh} = k_p[\text{LH}]/k_{inh}\tau \tag{17}$$

Obviously, very good inhibitors, such as α-tocopherol, have values of k_{inh}/k_p that are very large (Pryor *et al.*, 1993). Unfortunately, however, these values seem to be somewhat dependent on the nature of the system undergoing autoxidation and there is not a single, unique "dictionary" of inhibitor constants (Pryor *et al.*, 1993).

VII. Sources of Radicals in Living Systems

The propagation reactions that are shown in Scheme I are the same as those that would occur during lipid peroxidation *in vivo*. However, in studies of lipid peroxidation *in vitro*, azo initiators are used that allow radicals to be produced at precisely determined rates from thermolysis (homolysis induced by heat). However, bond thermolysis probably is not responsible for radical production *in vivo* because nature does not generally make compounds that undergo a *simple unimolecular bond thermolysis* at temperatures near 37°C. For example, hydrogen peroxide and lipid hydroperoxides are reasonably stable at these temperatures (Pryor, 1966, 1976). (This statement refers to *unimolecular* bond homolysis; hydrogen peroxide and hydroperoxides can form radicals rapidly in the presence or iron and other transition metal ions.) Superoxide can react with nitric oxide to produce peroxynitrite and its conjugate acid, peroxynitrous acid (HOONO), as was shown in reactions (6a) and (6b). At first, it was suggested that HOONO could undergo homolysis to form hydroxyl radicals and nitrogen dioxide; however, that homolysis also is unlikely (Koppenol *et al.*, 1992; Jin *et al.*, 1994). It is probable, in fact, that none of the species that are normally observed in living systems undergo simple unimolecular homolysis at appreciable rates. Then how are radicals that can cause pathological changes formed in living systems?

It seems likely that most radicals derive from two main sources: the direct or indirect effects of toxins, or the leakage of superoxide from normal cellular processes. Toxins can exert a variety of effects. Some toxins, such as nitrogen dioxide, are themselves radicals and react with PUFAs to form carbon- and oxygen-centered radicals that can initiate lipid peroxidation (Pryor and Lightsey, 1981; Gallon and Pryor, 1993). Some toxins, although not radicals themselves, react with PUFAs to form radicals and initiate lipid autoxidation; ozone is an example of such a toxin (Pryor *et al.*, 1981). Some toxins release components that can reduce oxygen and produce superoxide; an example is cigarette tar (Pryor, 1987; Cosgrove *et al.*, 1985; Church and Pryor, 1985; Pryor and Stone, 1993). Other nonradical toxins can induce higher levels of enzyme systems that either convert substrates to radicals (Marnett and Eling, 1983; Markey *et al.*, 1987) or leak radicals to the cellular

milieu; one example of such an enzyme system is *P*-450 (Cavalieri and Rogan, 1984; Cavalieri *et al.*, 1987; Rogan *et al.*, 1993).

Probably the most important source of initiating radicals *in vivo,* however, is the release of superoxide produced in normal biochemical processes (Fridovich, 1976; Kellogg and Fridovich, 1975; McCord and Fridovich, 1969, 1978; Fridovich, 1978). Superoxide is a reducing radical (vide supra) and cannot initiate lipid autoxidation under normal circumstances. However, there are at least two pathways by which superoxide can be converted to initiating radicals. The first is called iron-dependent Fenton chemistry. In this process, superoxide dismutates to form hydrogen peroxide, which then is reduced by ferrous iron (or similar transition metals such as cuprous copper) to form the hydroxyl radical.

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{19}$$

$$H_2O_2 + Fe(II) \rightarrow HO\cdot + HO^- + Fe(III) \tag{20}$$

It is not clear what the function of superoxide dismutase (SOD) would be in protecting the cell against radicals from this process, because SOD would merely speed up reaction (19). However, it often is suggested that superoxide functions to rereduce ferric iron to ferrous, thus allowing a redox cycle in which an iron atom is used many times.

$$O_2^{\cdot -} + Fe(III) \rightarrow O_2 + Fe(II) \tag{21}$$

SOD, of course, would block reaction (21) from occurring, and thus protect the cell against reactions (19), (20), and (21) becoming a chain process.

An alternative way in which superoxide can form potent oxidants is by reaction with nitric oxide to give peroxynitrous acid, as discussed above.

$$O_2^{\cdot -} + \cdot NO \rightarrow {}^-O{-}O{-}N{=}O \tag{22}$$

$$H^+ + {}^-O{-}O{-}N{=}O \rightarrow HO{-}O{-}N{=}O \tag{23}$$

The importance of peroxynitrous acid, HOONO, was first pointed out by Beckman *et al.* (1990), who suggested that HOONO could be a source of the hydroxyl radical or hydroxyl-like species:

$$HO{-}O{-}N{=}O \rightarrow HO\cdot + \cdot NO_2 \tag{24}$$

Subsequently, Koppenol *et al.* (1992) pointed out that homolysis of HOONO is unlikely (vide supra). Rather, HOONO itself is a potent oxidant (Moreno and Pryor, 1992; Jin *et al.*, 1994). It appears that peroxynitrous acid can accomplish two types of oxidations. First, it can transfer an oxygen atom, as in the S_N2 reaction with methionine (Mets) to give methionine sulfoxide (MetS=O).

$$HOONO + MetS \rightarrow HONO + MetS{=}O \tag{25}$$

Second, it can oxidize substrates (S) by one electron:

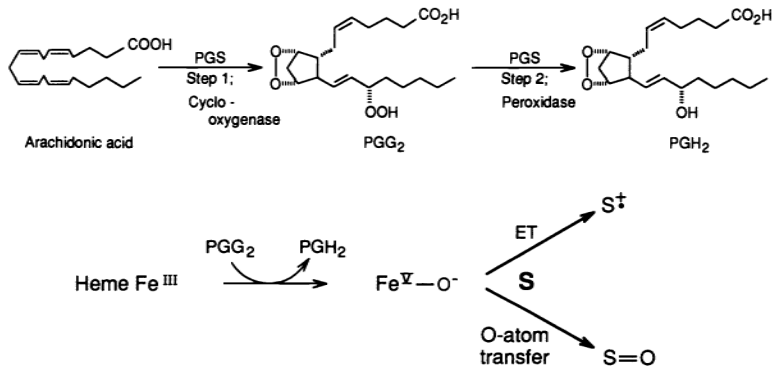
$$HOONO + S \rightarrow HO^- + \cdot NO_2 + S^{\cdot +} \tag{26}$$

VIII. Cooxidations and Related Troublemakers

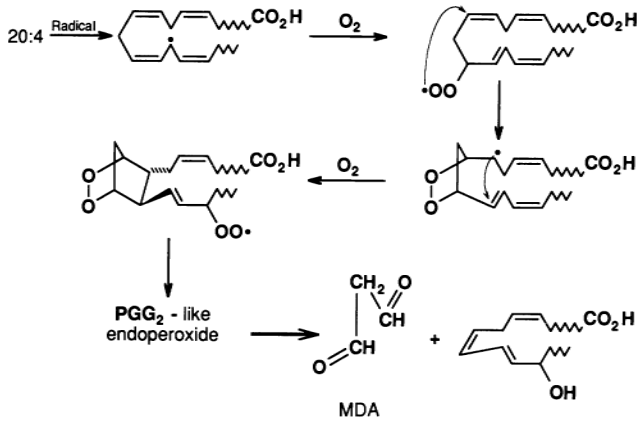
Polyunsatured fatty acids are particularly susceptible to autoxidation reactions. However, when they undergo oxidation, less susceptible molecules in the system can be carried along and cooxidized. One of the reasons for this is that peroxyl radicals can be converted to more reactive alkoxyl radicals by several processes that can occur during autoxidations. As can be seen from Table I, alkoxyl radicals are far more reactive (and short-lived) than are peroxyl radicals, and therefore are relatively unselective. Thus, alkoxyl radicals may attack a less susceptible molecule in the vicinity (for example, a protein) rather than another PUFA molecule.

There are more subtle ways in which cooxidations can occur. For example, arachidonic acid is oxidized to eicosanoids (prostaglandins and related compounds) by an enzyme system called prostaglandin synthase (PGS), as shown in the upper half of Scheme III. The PGS enzyme system has two activities. The first produces a bicyclic compound called PGG_2, an endoperoxide with a hydroperoxide function (Scheme III). The peroxidase activity of PGS that converts PGG_2 to PGH_2 uses electron donors that become cooxidized, as shown in the bottom half of Scheme III. Marnett and his group have studied these reactions in detail and found that the peroxidase function of PGS can either transfer an oxygen atom, as in the conversion of the 7,8-diol of benz[*a*]pyrene to the 7,8-diol-9,10-epoxide, or oxidize a substrate (such as pyrene) to the cation radical, as shown in outline in the bottom half of Scheme III (Markey *et al.*, 1987; Marnett and Eling, 1983).

When PUFAs with three or more double bonds undergo autoxidation, prostaglandin G_2-like compounds are formed by nonenzymatic pathways, as shown in Scheme IV (Pryor and Stanley, 1975). Recently, Morrow and Roberts have shown that these PG isomers are formed *in vivo*, and have used these compounds as a



Scheme III. (Top) The conversion of arachidonate to prostaglandin PGG_2 and then to PGH_2. (Bottom) The cooxidation of a substrate S either by oxygen-atom donation or one-electron transfer (ET) during the conversion of PGG_2 to PGH_2 by prostaglandin synthase (PGS) (Markey *et al.* 1987; Marnett and Eling, 1983).



Scheme IV. The cyclization of polyunsaturated fatty acids with three or more double bonds to give prostaglandin (PG) isomers (Pryor and Stanley, 1975). MDA, malondialdehyde.

method of following the oxidative stress status (OSS) of individuals (Morrow *et al.*, 1990; Morrow and Roberts, 1991; Pryor and Godber, 1991). Interestingly, these compounds are formed in the membrane, and are then cleaved from the membrane by lipases (Morrow *et al.*, 1992). This is the first proof that autoxidation of PUFAs *in membrane lipids* occurs *in vivo*.

IX. Generation of Radicals in the Lipid or Water Phase

When the autoxidation of lipids is initiated, the primordial radicals can be produced either in the aqueous phase or in the lipid bilayer, and it is conceivable that inhibitors could have different effects in these two situations. For example, if radicals are formed in the water phase, a water-soluble inhibitor such as vitamin C (ascorbate) can trap the radicals before they can diffuse into the lipid phase and initiate autoxidation (Frei *et al.*, 1988, 1989, 1990; Frei, 1991). In many biological systems, the generation of superoxide probably is the event that produces primordial radicals, and the radicals are produced in the aqueous phase outside the membrane.

A number of groups have studied questions regarding the effects of the site of radical generation, and the following generalities appear to apply (Frei *et al.*, 1990; Pryor *et al.*, 1993; Tsuchiya *et al.*, 1992; Sato *et al.*, 1990; Retsky *et al.*, 1993; Yoshida and Niki, 1992; Palozza *et al.*, 1992). If the inhibitor is *lipid-soluble* (e.g., α-tocopherol), then similar effects can be expected, regardless of whether the radicals are produced in the lipid or water phase. This makes sense, because the radicals must diffuse to the lipid phase before lipid peroxidation can be initiated, and the inhibitors are not sensitive to whether radicals were formed in the lipid phase or diffused into the lipid phase from the aqueous phase. If the inhibitor is *water-soluble*, then two effects may be observed. First, radicals from water-soluble initiators may be trapped before they can diffuse into the lipid phase (Frei *et al.*, 1988;

Frei, 1991; Retsky *et al.*, 1993). Second, water-soluble inhibitors may have a mechanism by which they can interact with radicals in the lipid phase and trap them. For example, when plasma is incubated with a lipid-soluble radical initiator, ascorbate can inhibit lipid autoxidation (Frei *et al.*, 1990).

X. Autoxidation of PUFAs in LDL

In the example we considered above, linoleic acid emulsified in SDS micelles underwent autoxidation. Micelles are very "leaky" and allow a rapid exchange of lipid-soluble materials from one micelle to another and from the aqueous solution into and out of the micelle (Barclay *et al.*, 1985). Bilayer membranes and the LDL particle behave somewhat differently (Barclay, 1993).

Perhaps one of the most important systems in which lipid peroxidation can have vitally important effects on human health is the oxidation of the LDL particle (Steinberg *et al.*, 1989, 1992; Palinski *et al.*, 1989; Steinbrecher *et al.*, 1984; Parthasarathy *et al.*, 1990; Steinberg, 1993; Frei, 1991; Retsky *et al.*, 1993; Lynch and Frei, 1993; Frei and Gaziano, 1993). Surprisingly, there are some reports that α-tocopherol is a rather ineffective inhibitor in that system (Frei *et al.*, 1988, 1990; Bowry *et al.*, 1992a; Stocker *et al.*, 1991; Frei and Gaziano, 1993; Frei and Ames, 1993). There are also studies that find α-tocopherol to be the critical inhibitor of LDL oxidation, more important than other LDL-associated antioxidants, e.g., β-carotene (Esterbauer *et al.*, 1989; Reaven *et al.*, 1993).

Bowry *et al.* (1992a) have compared the autoxidation of LDL lipids when they are extracted into a homogeneous solution in *tert*-butyl alcohol with lipid oxidation in the LDL particles suspended in aqueous buffer. Their results are shown in Figure 2. As can be seen from the right panel in Figure 2, when LDL is homogeneously dispersed in *tert*-butyl alcohol, all of the α-tocopherol disappears before much phosphatidylcholine hydroperoxide (PCOOH) or cholesteryl ester hydroperoxide

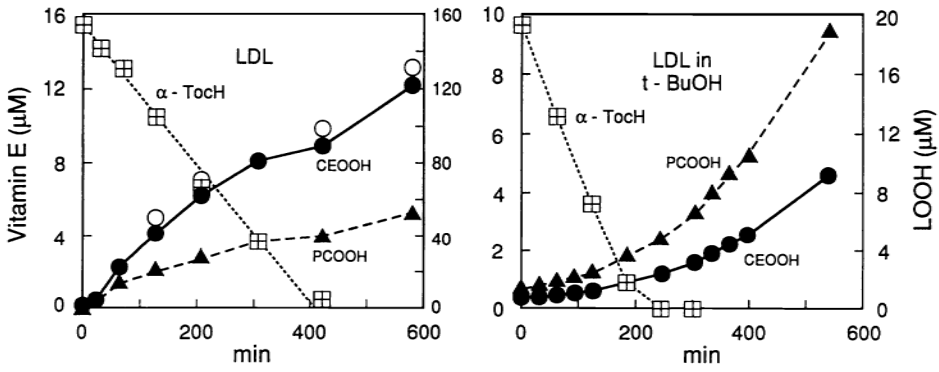


Figure 2. The autoxidation of low-density lipoprotein (LDL) in homogeneous solution in *tert*-butyl alcohol (*t*-BuOH) versus in the LDL particles dispersed in aqueous buffer (Bowry *et al.*, 1992a).

(CEOOH) is formed. However, when the LDL particles are present in an inhomogeneous phase that is dispersed in a buffer, lipid hydroperoxides begin appearing long before the α-tocopherol has been completely used. How could this be, when tocopherol normally stops all autoxidation chains dead?

This system has been studied in detail by a large group of investigators (Frei *et al.*, 1988; Bowry *et al.*, 1992a; Ingold *et al.*, 1993; Frei and Ames, 1993; Mohr *et al.*, 1992; Stocker *et al.*, 1991). Ingold *et al.* (1993) have explained the strange behavior of α-tocopherol in the LDL particle by comparison with the behavior of radicals in the emulsion polymerization of mixtures of styrene and butadiene in buffers to make rubber for automobile tires. In this system, radicals become isolated in a single emulsified oil droplet and have much longer lifetimes than normal. When radicals are isolated from each other, the rates of termination reactions are controlled by diffusion of one radical from one phase (the water) into another (the oil droplet) rather than by the chemical rate constants. Normal radical lifetimes are of the order of microseconds, whereas radicals have lifetimes of about 10 sec in the rubber polymerization emulsion system (Walling, 1957).

In the LDL particle, lipid-soluble components only slowly exchange from one LDL particle to another. In this system, Bowry *et al.* (1992a) find that α-tocopherol (ArOH) tends to *cooxidize* with PUFAs.

$$\mathrm{ArOH} + \mathrm{LOO}\cdot \rightarrow \mathrm{ArO}\cdot + \mathrm{LOOH} \tag{27}$$

$$\mathrm{ArO}\cdot + \mathrm{LH} \rightarrow \mathrm{ArOH} + \mathrm{L}\cdot \tag{28}$$

[Note that reaction (28) is a propagation rather than termination reaction.] The only way termination can occur in a system like this is either for a second radical to enter the LDL particle and react with the first, or to form a radical in the LDL particle that is water soluble and can be "exported" into the buffer. The following suggestions have been made by Bowry, Stocker, Ingold and their colleagues of ways that allow export of radicals to the buffer (Bowry *et al.*, 1992a; Ingold *et al.*, 1993). One involves the reaction of coenzyme Q with the tocopheroxyl radical to produce the coenzyme Q semiquinone radical. That radical can react with oxygen, as discussed above for semiquinones in general. As shown in reaction (29), this produces superoxide, which is water soluble and can be exported to the aqueous medium.

$$\mathrm{Q}^{\cdot-} + \mathrm{O}_2 \rightarrow \mathrm{Q} + \mathrm{O}_2^{\cdot-} \tag{29}$$

Another possibility is for the lipid peroxyl radical to react with a thiol of the protein (apolipoprotein B) in the LDL particle. This is shown in reactions (30)–(32).

$$\mathrm{LOO}\cdot + \mathrm{PSH} \rightarrow \mathrm{LOOH} + \mathrm{PS}\cdot \tag{30}$$

$$\mathrm{PS}\cdot + \mathrm{XS}^- \rightarrow \mathrm{PSSX}^{\cdot-} \tag{31}$$

$$\mathrm{PSSX}^{\cdot-} + \mathrm{O}_2 \rightarrow \mathrm{PSSX} + \mathrm{O}_2^{\cdot-} \tag{32}$$

The radical formed from the protein-bound thiol, shown as PS·, can react with a mercaptide ion (XS^-) from another thiol to form a disulfide radical ion, as shown in

reaction (31). The disulfide radical ion can then reduce oxygen to generate superoxide, as shown in reaction (32) (Ingold *et al.*, 1993).

XI. Epilogue

We have seen the variety of ways in which radicals can react and the enormous range in their lifetimes and reactivities. For many years radicals were thought of as some sort of exotic, "bad boys" of biology. Clearly, however, radicals are just another reactive species through which nature chooses to do her work.

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References

Aust, S. D., and Svingen, B. A. (1982). The role of iron in enzymatic lipid peroxidation. *In* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. 5, pp. 1–28. Academic Press, New York.

Barclay, L.R.C. (1993). Model biomembranes: Quantitative studies of peroxidation, antioxidant action, partitioning, and oxidative stress. *Can. J. Chem.* **71**, 1–16.

Barclay, L.R.C., Bailey, A.M.H., and Kong, D. (1985). The antioxidant activity of α-tocopherol-bovine serum albumin complex in micellar and liposome autoxidations. *J. Biol. Chem.* **260**, 15809–15814.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1620–1624.

Borg, D. C. (1976). Applications of electron spin resonance in biology. *In* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. 1, pp. 69–147. Academic Press, New York.

Bowry, V. W., Ingold, K. U., and Stocker, R. (1992a). Vitamin E in human low-density lipoprotein. When and how this antioxidant becomes a pro-oxidant. *Biochem. J.* **288**, 341–344.

Bowry, V. W., Stanley, K. K., and Stocker, R. (1992b). High density lipoprotein is the major carrier of lipid hydroperoxides in human blood plasma from fasting donors. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10316–10320. (Abstr.)

Burton, G. W., and Ingold, K. U. (1981). Autoxidation of biological molecules. *J. Am. Chem. Soc.* **103**, 6472–6477.

Burton, G. W., and Ingold, K. U. (1989). Vitamin E as an *in vitro* and *in vivo* antioxidant. *Ann. N.Y. Acad. Sci.* **570**, 7–22.

Burton, G. W., Joyce, A., and Ingold, K. U. (1983). Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch. Biochem. Biophys.* **221**, 281–290.

Cavalieri, E. L., and Rogan, E. G. (1984). One-electron and two-electron oxidation in aromatic hydrocarbon carcinogenesis. *In* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. 6, pp. 323–369. Academic Press, New York.

Cavalieri, E. L., Wong, A., and Rogan, E. G. (1987). Evidence for distinct binding sites in the cumene hydroperoxide-dependent metabolism of benzo[*a*]pyrene catalyzed by cytochrome. *Biochem. Pharmacol.* **36**, 435–440.

Church, D. F., and Pryor, W. A. (1985). Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ. Health Perspect.* **64**, 111–126.

Cosgrove, J. P., Borish, E. T., Church, D. F., and Pryor, W. A. (1985). The metal-mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. *Biochem. Biophys. Res. Commun.* **132**, 390–396.

Cosgrove, J. P., Church, D. F., and Pryor, W. A. (1987). The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids* **22**, 299–304.

Devanesan, P. D., RamaKrishna, N.V.S., Padmavathi, N. S., Higginbotham, S., Rogan, E. G., Cavalieri, E. L., Marsch, G. A., Jankowiak, R., and Small, G. J. (1993). Identification and quantitation of 7,12-dimenthylbenz[*a*]anthracene-DNA adducts formed in mouse skin. *Chem. Res. Toxicol.* **6**, 364–371.

Esterbauer, H., Striegl, G., Puhl, H., Oberreither, S., Rotheneder, M., El-Saadani, M., and Jürgens, G. (1989). The role of vitamin E and carotenoids in preventing oxidation of low density lipoprotein. *Ann. N.Y. Acad. Sci.* **570**, 254–267.

Evans, M. D., and Pryor, W. A. (1992). Damage to human α-1-proteinase inhibitor by aqueous cigarette tar extracts and the formation of methionine sulfoxide. *Chem. Res. Toxicol.* **5**, 654–660.

Evans, M. D., and Pryor, W. A. (1994). Cigarette smoking, emphysema and damage to alpha-1-proteinase inhibitor. *Am. J. Physiol: Lung Cell. Mol. Physiol.* (in press).

Frei, B. (1991). Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage. *Am. J . Clin. Nutr.* **54**, 1113S–1118S.

Frei, B., and Ames, B. N. (1993). Relative importance of vitamin E in antiperoxidative defenses in human blood plasma and low-density lipoprotein (LDL). *In* "Vitamin E in Health and Disease" (L. Packer and J. Fuchs, eds.), pp. 131–139. Dekker, New York.

Frei, B., and Gaziano, J. M. (1993). Content of antioxidants, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibilitiy of human LDL to metal ion-dependent and -independent oxidation. *J. Lipid Res.* **34**, 2135–2145.

Frei, B., Stocker, R., and Ames, B. N. (1988). Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9748–9752.

Frei, B., England, L., and Ames, B. N. (1989). Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6377–6381.

Frei, B., Stocker, R., England, L., and Ames, B. N. (1990). Ascorbate: the most effective antioxidant in human blood plasma. *In* "Antioxidants in Therapy and Preventive Medicine" (I. Emerit and L. Packer, eds.), pp. 155–163. Plenum, New York.

Frei, B., Stocker, R., and Ames, B. N. (1992). Small molecular antioxidant defenses in human extracellular fluids. *In* "Molecular Biology of Free Radical Scavenging Systems" (J. G. Scandalios, ed.), pp. 23–45. Cold Spring Harbor Lab. Press, Cold Spring Harbor, New York.

Fridovich, I. (1976). Oxygen radicals, hydrogen peroxide, and oxygen toxicity. *In* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. 1, pp. 239–277. Academic Press, New York.

Fridovich, I. (1978). The biology of oxygen radicals. The superoxide radical is an agent of oxygen toxicity; superoxide dismutases provide an important defense. *Science* **201**, 875–880.

Gallon, A. A., and Pryor, W. A. (1993). The identification of the allylic nitrite and nitro derivatives of methyl linoleate and methyl linolenate by negative chemical ionization mass spectroscopy. *Lipids* **28**, 125–133.

Gardner, P. R., and Fridovich, I. (1992). Inactivation–reactivation of aconitase in *Escherichia coli*. *J. Biol. Chem.* **267**, 8757–8763.

Halliwell, B., and Gutteridge, J.M.C. (1989). "Free Radicals in Biology and Medicine." Oxford Univ. Press, New York.

Howard, J. A. (1972). Absolute rate constants for reactions of oxyl radicals. *Adv. Free-Radical Chem.* **4**, 49–173.

Huie, R. E., and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Radical Res. Commun.* **18**, 195–199.

Imlay, J. A., and Fridovich, I. (1991). Assay of metabolic superoxide production in *Escherichia coli*. *J. Biol. Chem.* **266**, 6957–6965.

Ingold, K. U. (1961). Inhibition of the autoxidation of organic substances in the liquid phase. *Chem. Rev.* **61**, 563–589.

Ingold, K. U., Bowry, V. W., Stocker, R., and Walling, C. (1993). Autoxidation of lipids and antioxidation by α-tocopherol and ubiquinol in homogeneous solution and in aqueous dispersions of lipids: Unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 45–49.

Jin, X., Squadrito, G. L., and Pryor, W. A. (1994). Competing electron transfer and neucleophilic displacement reactions of methionine with peroxynitrite. *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Kellogg, E. W., and Fridovich, I. (1975). Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J. Biol. Chem.* **250**, 8812–8817.

Koppenol, W. H. (1989). Hormesis. *Science* **246**, 311.

Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulous, H., and Beckman, J. S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem. Res. Toxicol.* **5**, 834–842.

Korcek, S., Chenier, J.H.B., Howard, J. A., and Ingold, K. U. (1972). Absolute rate constants for hydrocarbon autoxidation. XXI. Activation energies for propagation and the correlation of propagation rate constants with carbon-hydrogen bond strengths. *Can. J. Chem.* **50**, 2285–2297.

Kubes, P., Suzuki, M., and Granger, D. N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4651–4655.

Laudicina, D. C., and Marnett, L. J. (1990). Enhancement of hydroperoxide-dependent lipid peroxidation in rat liver microsomes by ascorbic acid. *Arch. Biochem. Biophys.* **278**, 73–80.

Lynch, S. M., and Frei, B. (1993). Mechanisms of copper- and iron-dependent oxidative modification of human low-density lipoprotein. *J. Lipid Res.* **34**, 1745–1753.

Markey, C. M., Alward, A., Weller, P. E., and Marnett, L. J. (1987). Quantitative studies of hydroperoxide reduction by prostaglandin H synthase: Reducing substrate specificity and the relationship of peroxidase to cyclooxygenase activities. *J. Biol. Chem.* **262**, 6266–6279.

Marletta, M. A. (1989). Nitric oxide: Biosynthesis and biological significance. *Trends Biochem. Sci.* **14**, 488–492.

Marnett, L. J. (1987). Peroxyl free radicals: Potential mediators of tumor initiation and promotion. *Carcinogenesis* **8**, 1365–1373.

Marnett, L. J. (1990). Prostaglandin synthase-mediated metabolism of carcinogens and a potential role for peroxyl radicals as reactive intermediates. *Environ. Health Perspect.* **88**, 5–12.

Marnett, L. J., and Eling, T. E. (1983). Cooxidation during prostaglandin biosynthesis: A pathway for the metabolic activation of xenobiotics. *Rev. Biochem. Toxicol.* **5**, 135–172.

McCall, T. B., Boughton-Smith, N. K., Palmer, R.M.J., Whittle, B.J.R., and Moncada, S. (1989). Synthesis of nitric oxide from L-arginine by neutrophils. release and interaction with superoxide anion. *Biochem. J.* **261**, 293–296.

McCord, J. M., and Fridovich, I. (1969). Superoxide dismutase, an enzymic function for erythrocuproin (hemocuproin). *J. Biol. Chem.* **244**, 6049–6055.

McCord, J. M., and Fridovich, I. (1978). The biology and pathology of oxygen radicals. *Ann. Intern. Med.* **89**, 122–127.

Mead, J. F. (1976). Free radical mechanisms of lipid damage and consequences for cellular membranes. *In* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. 1, pp. 51–68. Academic Press, New York.

Mohr, D., Bowry, V. W., and Stocker, R. (1992). Dietary supplementation with coenzyme Q_{10} results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation. *Biochim. Biophys. Acta* **1126**, 247–254.

Moreno, J. J., and Pryor, W. A. (1992). Inactivation of α-1-proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol.* **5**, 425–431.

Morrow, J. D., and Roberts, L. J., II (1991). Quantification of noncyclooxygenase derived prostanoids as a marker of oxidative stress. *Free Radicals Biol. Med.* **10**, 195–200.

Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. M., Badr, K. F., and Roberts, L. J., II (1990). A series of prostaglandin F_2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9383–9387.

Morrow, J. D., Awad, J. A., Boss, H. J., Blair, I. A., and Roberts, L. J., II (1992). Noncyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed *in situ* on phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10721–10725.

Niki, E., Kawakami, A., Saito, M., Yamamoto, Y., Tsuchiya, J., and Kamiya, Y. (1985). Effect of phytyl side chain of vitamin E on its antioxidant activity. *J. Biol. Chem.* **260**, 2191–2196.

Palinski, W., Rosenfeld, M. E., Ylä-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., Steinberg, D., and Witztum, J. L. (1989). Low density lipoprotein undergoes oxidative modification *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1372–1376.

Palozza, P., Moualla, S., and Krinsky, N. I. (1992). Effects of β-carotene and α-tocopherol on radical-initiated peroxidation of microsomes. *Free Radical Biol. Med.* **13**, 127–136.

Parthasarathy, S., Khoo, J. C., Miller, E., Barnett, J., Witztum, J. L., and Steinberg, D. (1990). Low density lipoprotein rich in oleic acid is protected against oxidative modification: Implications for dietary prevention of atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3894–3898.

Porter, N. A., Wolf, R. A., Pagels, W. R., and Marnett, L. J. (1980). A test for the intermediacy of 11-hydroperoxyeicosa-5,8,12,14-tetraenoic acid [11-HPETE] in prostaglandin biosynthesis. *Biochem. Biophys. Res. Commun.* **92**, 349–355.

Pryor, W. A. (1966). "Free Radicals." McGraw-Hill, New York.

Pryor, W. A. (1968). Organic free radicals. *Chem. Eng. News* **46**, 70–89.

Pryor, W. A. (1976). The role of free radical reactions in biological systems. *In* "Free Radicals in Biology" (W. A. Pryor, ed.), Vol. I, pp. 1–50. Academic Press, New York.

Pryor, W. A. (1984). Free radicals in autoxidation and in aging. Part I. Kinetics of the autoxidation of linoleic acid in SDS micelles: Calculations of radical concentrations, kinetic chain lengths, and the effects of vitamin E. Part II. The role of radicals in chronic human diseases and in aging. *In* "Free Radicals in Molecular Biology, Aging and Disease" (D. Armstrong, R. S. Sohal, R. G. Cutler, and T. F. Slater, eds.), pp. 13–41. Raven, New York.

Pryor, W. A. (1986). Oxy-radicals and related species: their formation, lifetimes, and reactions. *Annu. Rev. Physiol.* **48**, 657–667.

Pryor, W. A. (1987). Cigarette smoke and the involvement of free radical reactions in chemical carcinogenesis. *Br. J. Cancer* **55**, Suppl. VIII, 19–23.

Pryor, W. A., and Godber, S. S. (1991). Oxidative stress status: an introduction. *Free Radical Biol. Med.* **10**, 173.

Pryor, W. A., and Lightsey, J. W. (1981). Mechanisms of nitrogen dioxide reactions: Initiation of lipid peroxidation and the production of nitrous acid. *Science* **214**, 435–437.

Pryor, W. A., and Stanley, J. P. (1975). A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. *J. Org. Chem.* **40,** 3615–3617.

Pryor, W. A., and Stone, K. (1993). Oxidants in cigarette smoke: Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. Tobacco smoking and nutrition: Influence of nutrition on tobacco associated health risks. *Ann. N.Y. Acad. Sci.* **686**, 12–28.

Pryor, W. A., Prier, D. G., and Church, D. F. (1981). Radical production from the interaction of ozone and PUFA as demonstrated by electron spin resonance spin-trapping techniques. *Environ. Res.* **24**, 42–52.

Pryor, W. A., Hales, B. J., Premovic, P. I., and Church, D. F. (1983a). The radicals in cigarette tar: Their nature and suggested physiological implications. *Science* **220**, 425–427.

Pryor, W. A., Prier, D. G., and Church, D. F. (1983b). Electron-spin resonance study of mainstream and sidestream cigarette smoke: Nature of the free radicals in gas-phase smoke and in cigarette tar. *Environ. Health Perspect.* **47**, 345–355.

Pryor, W. A., Dooley, M. M., and Church, D. F. (1985). Mechanisms of cigarette smoke toxicity: The inactivation of human alpha-1-proteinase inhibitor by nitric oxide/isoprene mixtures in air. *Chem.-Biol. Interact.* **54**, 171–183.

Pryor, W. A., Nuggehalli, S. K., Scherer, K. V., and Church, D. F. (1990). An electron spin resonance study of the particles produced in the pyrolysis of perfluorpolymers. *Chem. Res. Toxicol.* **3,** 2–7.

Pryor, W. A., Cornicelli, J. A., Devall, L. J., Tait, B., Trivedi, B. K., Witiak, D. T., and Wu, M.

(1993). A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem.* **58**, 3521–3532.

Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288**, 481–487.

RamaKrishna, N.V.S., Padmavathi, N. S., Cavalieri, E. L., Rogan, E. G., Cerny, R. L., and Gross, M. L. (1993). Synthesis and structure determination of the adducts formed by electrochemical oxidation of the potent carcinogen dibenzo[*al*]pyrene in the presence of nucleosides. *Chem. Res. Toxicol.* **6**, 554–560.

Reaven, P. D., Khouw, A., Beltz, W. F., Parthasarathy, S., and Witztum, J. L. (1993). Effect of dietary antioxidant combinations in humans: Protection of LDL by vitamin E but not by β-carotene. *Arterioscler. Thromb.* **13**, 590–600.

Retsky, K. L., Freeman, M. W., and Frei, B. (1993). Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification: Anti- rather than prooxidant activity of vitamin C in the presence of transition metal ions. *J. Biol. Chem.* **268**, 1304–1309.

Rogan, E. G., Devanesan, P. D., RamaKrishna, N.V.S., Higginbotham, S., Padmavathi, N. S., Chapman, K., Cavalieri, E. L., Jeong, H., Jankowiak, R., and Small, G. J. (1993). Identification and quantitation of benzo[*a*[pyrene-DNA adducts formed in mouse skins. *Chem. Res. Toxicol.* **6**, 356–363.

Sato, K., Niki, E., and Shimasaki, H. (1990). Free radical-mediated chain oxidation of low density lipoprotein and its synergistic inhibition by vitamin E and vitamin C. *Arch. Biochem. Biophys.* **279**, 402–405.

Schultz, H. W., Day, E. A., and Sinnhuber, R. O., eds. (1962). "Symposium on Foods: Lipids and Their Oxidation." Avi, Westport, Connecticut.

Seidel, W. C., Scherer, K. V., Jr., Cline, D., Jr., Olson, A. H., Bonesteel, J. K., Church, D. F., Nuggenhalli, S., and Pryor, W. A. (1991). Chemical, physical, and toxicological characterization of fumes produced by heating tetrafluoroethene homopolymer and its copolymers with hexafluoropropene and perfluoro(propyl vinyl ether). *Chem. Res. Toxicol.* **4**, 229–236.

Snyder, S. H., and Bredt, D. S. (1992). Biological roles of nitric oxide. *Sci. Am.* May, 68–877.

Steinberg, D. (1993). Modified forms of low-density lipoprotein and atherosclerosis. *J. Intern. Med.* **233**, 227–232.

Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320**, 915–924.

Steinberg, D., Berliner, J. A., Burton, G. W., Carew, T. E., Chait, A., Chisolm, G. M., III, Esterbauer, H., Fogelman, A. M., Fox, P. L., Furberg, C. D., Gaziano, J. M., Gey, K. F., Grundy, S. M., Harlan, W. R., Havel, R. J., Hennekens, C. H., Hoff, H. F., Jackson, R. L., Kayden, H. J., Keech, A., Krinsky, N. I., Manson, J., Parthasarathy, S., Probstfield, J., Pryor, W. A., Rifkind, B., Stadtman, E. R., Wallace, R. B., Witztum, J. L., Hlä-Herttuala, S., and Yusuf, S. (1992). Antioxidants in the prevention of human atherosclerosis. Summary of the Proceedings of a National Heart, Lung, and Blood Institute Workshop: September 5–6, 1991. *circulation* **85**, 2337–2344.

Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., and Steinberg, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3883–3887.

Stocker, R., and Frei, B. (1991). Endogenous antioxidant defenses in human blood plasma. *In* "Oxidative Stress: Oxidants and Antioxidants" (H. Sies, ed.), 2nd Ed., pp. 213–243. Academic Press, San Diego.

Stocker, R., Bowry, V. W., and Frei, B. (1991). Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation that does α-tocopherol. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1646–1650.

Takahashi, M., Komuro, E., Niki, E., and Tanaka, K. (1992). Action of fatty acid esters of L-ascorbic acids as antioxidants in phosphatidylcholine liposomal membranes. *Bull. Chem. Soc. Jpn.* **65**, 679–684.

Todorovic, R., Devanesan, P. D., Rogan, E. G., and Cavalieri, E. L. (1993). ^{32}P-postlabeling analysis of the DNA adducts of 6-fluorobenzo[*a*]pyrene and 6-methylbenzo[*a*]pyrene formed *in vitro*. *Chem. Res. Toxicol.* **6**, 530–534.

Tsuchiya, M., Scita, G., Freisleben, H., Kagan, V. E., and Packer, L. (1992). Antioxidant radical-scavenging activity of carotenoids and retinoids compared to α-tocopherol. *In* "Carotinoids. Part A: Chemistry, Separation Quantitation and Antioxidation" (L. Packer, ed.), Methods in Enzymology, Vol. 213, pp. 460–472. Academic Press, San Diego.

Van Overveld, F. J., Jorens, P. G., DeBacker, W. A., Rampart, M., Bossaert, L., and Vermeire, P. A. (1992). Release of arachidonic acid metabolites from isolated human alveolar type II cells. *Prostaglandins* **44**, 101–110.

Walling, C. (1957). "Free Radicals in Solution." Wiley, New York.

Witting, L. A. (1974). Vitamin E-polyunsaturated lipid relationship in diet and tissues. *Am. J. Clin. Nutr.* **27**, 952–959.

Yagi, K., Ishida, N., Komura, S., Ohishi, N., Kusai, M., and Kohno, M. (1992). Formation of hydroxyl radicals from lipid hydroperoxides. *In* "Oxygen Radicals" (K. Yagi, M. Kondo, E. Niki, and T. Yoshikawa, eds.), pp. 223–226. Elsevier, New York.

Yoshida, Y., and Niki, E. (1992). Oxidation of methyl linoleate in aqueous dispersions induced by copper and iron. *Arch. Biochem. Biophys.* **295**, 107–114.