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COMMENTARY

ANTIOXIDANT CHARACTERIZATION

METHODOLOGY AND MECHANISM

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WHAT IS AN ANTIOXIDANT?

The word "antioxidant" means different things to different people. Often (e.g. by food scientists) the term is implicitly restricted to chain-breaking inhibitors of lipid peroxidation, such as α-tocopherol. However, free radicals generated *in vivo* damage many other targets, including proteins, DNA and small molecules [1]. Hence, a broader definition of an antioxidant [1, 2] is *any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate*. The term "oxidizable substrate" includes every type of molecule found *in vivo*. This definition emphasizes the importance of the damage target studied and the source of ROS† used when antioxidant action is examined *in vitro*.

When ROS are generated *in vivo*, many antioxidants come into play [1–6]. Their relative importance depends upon which ROS is generated, how, where, and what target of damage is measured [2]. For example, when plasma is exposed to NO_2 or O_3, uric acid is protective [7]. By contrast, urate provides little protection against damage to plasma constituents by hypochlorous acid (HOCl) [8]. If the oxidative stress is the same but a different damage *target* is measured, different answers result. For example, gas-phase cigarette smoke causes lipid peroxidation in plasma, and ascorbate protects [9]. By contrast, ascorbate does not protect against damage to plasma proteins by cigarette smoke, as measured by the carbonyl assay [10].

Antioxidants are of interest to radiation [11] and polymer [12] chemists, food scientists and museum curators [13], but in this commentary I confine my discussion to the antioxidants thought to be important in living organisms. The antioxidant roles of vitamin E, ascorbic acid, superoxide dismutase, glutathione peroxidase, catalase and caeruloplasmin have been well reviewed [1–6] and will not be discussed further. However, many other substances have been proposed to act as antioxidants *in vivo*, ranging from β-carotene and metallothionein to carnosine, phytate, bilirubin, oestrogens, dihydrolipoic acid, polyamines and melatonin. How can such claims be evaluated? First, one must ask how the putative antioxidant is supposed to act. Does it act directly, e.g. by scavenging ROS or inhibiting their generation? This is the most common proposal. Does it act indirectly, e.g. by up-regulating endogenous antioxidant defences?

In evaluating the likelihood of direct antioxidant action *in vivo*, it is important to ask certain questions (Table 1). Simple experiments can answer some of these questions, and the results often allow one to dismiss the proposed antioxidant: a compound that is a poor antioxidant *in vitro* is unlikely to be any better *in vivo*. The present article outlines some approaches to characterization of direct antioxidant activity. Two obvious (but often forgotten) additional points are:

(i) A compound should be tested at concentrations achievable *in vivo*.

(ii) In assaying putative antioxidants, one should use biologically relevant ROS.

BIOLOGICALLY RELEVANT ROS

Superoxide and hydrogen peroxide

Superoxide formed *in vivo* is largely converted by superoxide dismutase (SOD)-catalysed or non-enzymic dismutation into H_2O_2 [3]. Some enzymes, e.g. glycollate and xanthine oxidases, produce H_2O_2 directly [1, 4]. Unlike $O_2^{\cdot -}$, H_2O_2 is thought to cross all cell and organelle membranes [1]. Both $O_2^{\cdot -}$ and H_2O_2 can damage a few cellular targets [3, 14, 15], but, in general, their reactivity is limited [1, 16]. Hence, few compounds react fast with $O_2^{\cdot -}$ or H_2O_2 (exceptions for $O_2^{\cdot -}$ are some iron–sulphur proteins, SOD, NO$^{\cdot}$ and, to a lesser extent, ascorbate [14, 16, 17]). For example, many thiols can react with

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† The term ROS (reactive oxygen species) is used in preference to oxygen radicals (since H_2O_2, singlet $O_2{}^1\Delta g$ and HOCl are non-radicals) or oxidants (since $O_2^{\cdot -}$ is also a reducing agent). "Reactive" is a relative term, e.g. $O_2^{\cdot -}$ is more reactive than O_2 but much less reactive than OH$^{\cdot}$ or HOCl.

Table 1. Questions to ask when evaluating "antioxidants" *in vivo*

1. What biomolecule is the antioxidant supposed to protect? Does enough antioxidant reach that target *in vivo*?
2. How does it protect—by scavenging ROS, preventing their formation, or repairing damage?
3. If the antioxidant acts by scavenging, can the resulting antioxidant-derived radicals themselves cause damage?
4. Can the antioxidant cause damage in other biological systems?

H_2O_2 and $O_2^{\cdot -}$, but the reaction rate constants are low, often $< 10^3\,M^{-1}\,sec^{-1}$ [16]. Hence, high thiol concentrations would be needed to scavenge $O_2^{\cdot -}$ or H_2O_2 *in vivo*.

Measuring superoxide scavenging. Superoxide is easily produced by radiolysis of water in the presence of O_2 and formate, and these techniques allow examination of the spectrum of products formed when $O_2^{\cdot -}$ reacts with putative antioxidants [18]. However, they are unsuitable for measuring slow (rate constants $< 10^5\,M^{-1}\,sec^{-1}$) reactions of $O_2^{\cdot -}$ in aqueous solution. Unfortunately, the rate constants for $O_2^{\cdot -}$ reaction with most biological molecules fall below this range. Stopped-flow methods can be used to study slower reactions [19]. However, approximate rate constants may be obtained using "test-tube" methods. Xanthine oxidase plus hypoxanthine (or xanthine) at pH 7.4 generates $O_2^{\cdot -}$ [3], which reacts with cytochrome *c* and nitro blue tetrazolium (NBT) with known rate constants, 2.6×10^5 and $6 \times 10^4\,M^{-1}\,sec^{-1}$, respectively [16]. If an $O_2^{\cdot -}$-reactive molecule is added, it decreases the rates of cytochrome *c* or NBT reduction, and competition plots allow calculation of approximate rate constants [20]. This approach can also be used with other sources of $O_2^{\cdot -}$, but controls are always *essential*:
(i) Check that the "antioxidant" does not directly inhibit $O_2^{\cdot -}$ generation. To assess xanthine oxidase activity, O_2 uptake or urate formation can be measured [20]. Many "antioxidants" absorb strongly at 290 nm, making spectrophotometric assessment of urate production inaccurate. HPLC analysis or O_2 uptake measurements avoid this problem.
(ii) Check that the "antioxidant" does not directly reduce cytochrome *c* or NBT (this is a particular problem with cytochrome *c*).
(iii) Consider the possibility that a radical formed by attack of $O_2^{\cdot -}$ on an "antioxidant" interacts with cytochrome *c* or NBT. This will be revealed as deviations from linear competition kinetics at high "antioxidant" concentrations.

Measuring H_2O_2 scavenging. H_2O_2 can be sensitively measured by peroxidase-based assay systems, e.g. horseradish peroxidase uses H_2O_2 to oxidize scopoletin into a non-fluorescent product [21]. If an "antioxidant" is incubated with H_2O_2 and the reaction mixture sampled at various times, rates of H_2O_2 disappearance can be used to calculate rate constants. Some points to consider are:
(i) Check that the "antioxidant" is not a substrate for peroxidase, which could decrease the fluorescence changes by competing with scopoletin rather than by really scavenging H_2O_2. An example is ascorbate, a substrate for most peroxidases. To check if a substance is oxidized by peroxidase, look for loss of the compound and changes in the absorbance spectrum upon incubation with peroxidase and H_2O_2: radicals derived by peroxidase-dependent oxidations often have spectra very different from those of the parent compounds, and there will be spectral changes in the peroxidase itself if it oxidizes the "antioxidant."
(ii) $O_2^{\cdot -}$ inhibits peroxidase (forming compound III) and may compromise measurement of H_2O_2 in systems generating $O_2^{\cdot -}$ [22].

If an "antioxidant" interferes with peroxidase-based systems, other assays for H_2O_2 can be used, including titration with acidified $KMnO_4$, measuring the O_2 release (1 mole per 2 moles of H_2O_2) when a sample of the reaction mixture is injected into an O_2 electrode containing a catalase solution, or measuring release of $^{14}CO_2$ from ^{14}C-labelled 2-oxoglutarate [1, 23].

Hydroxyl radical

Much of the damage done by $O_2^{\cdot -}$ and H_2O_2 *in vivo* is thought to be due to their conversion into more reactive species, including hydroxyl radical ($OH^{\cdot}$) [1, 24]. Formation of $OH^{\cdot}$ *in vivo* occurs by at least four mechanisms:
(i) Transition metal ion catalysis, especially by iron and copper [24].
(ii) Background exposure to radiation [11].
(iii) Reaction of $O_2^{\cdot -}$ with $NO^{\cdot}$ to give peroxynitrite [17],

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^- \qquad (1)$$

which is directly cytotoxic and can also decompose at physiological pH to several noxious products, apparently including nitronium ion (NO_2^+), nitrogen dioxide ($NO_2^{\cdot}$), and some $OH^{\cdot}$ [25, 26].
(iv) Reaction of HOCl with $O_2^{\cdot -}$ (rate constant about $10^7\,M^{-1}\,sec^{-1}$ [27]

$$HOCl + O_2^{\cdot -} \rightarrow OH^{\cdot} + O_2 + Cl^- \qquad (2)$$

Reactions of hydroxyl radical. Hydroxyl radical attacks most molecules found *in vivo*, with rate constants of $\geq 10^9\,M^{-1}\,sec^{-1}$ [11]. *Thus, almost everything in a cell can be regarded as an $OH^{\cdot}$* scavenger. Hence, suggestions that "antioxidants" act by scavenging $OH^{\cdot}$ *in vivo* are chemically unlikely. Their rate constants for $OH^{\cdot}$ scavenging may be high (often $> 10^{10}\,M^{-1}\,sec^{-1}$), but their molar concentrations *in vivo* are usually far less than that of endogenous molecules capable of rapidly reacting with $OH^{\cdot}$, including albumin (rate constant $> 10^{10}\,M^{-1}\,sec^{-1}$ [28]) and glucose (rate constant $\sim 10^9\,M^{-1}\,sec^{-1}$, but present at *millimolar* concentrations in body fluids).

Antioxidants affecting hydroxyl radical formation. An "antioxidant" that affects $OH^{\cdot}$-dependent damage *in vivo* is more likely to act by blocking $OH^{\cdot}$ formation, e.g. by removing its precursors ($O_2^{\cdot -}$,

H_2O_2, $ONOO^-$, HOCl, transition metal ions). Removal of transition metal ions can involve the following:
(i) Their binding to the "antioxidant" alters their redox potential and/or accessibility so as to stop them catalysing OH• production, e.g. binding of iron ions to desferrioxamine, transferrin or lactoferrin [1, 24].
(ii) Their binding to the "antioxidant" does not stop OH• formation, but the OH• is formed at the binding site, so that the "antioxidant" absorbs it and "spares" a more important target. For example, copper ions bound to albumin can still form OH•, and as a result the protein is damaged [29]. Such damage to albumin may matter much less *in vivo* than damage to other targets, such as plasma lipoproteins and cell membranes. Hence, the albumin, by binding copper and targeting damage to itself, is acting as a "sacrificial antioxidant" [30].

Measuring hydroxyl radical scavenging. The definitive technique for investigating reactions of "antioxidants" with OH• is pulse radiolysis [16, 18], but approximate rate constants can be obtained using simpler equipment. For example, the spin-trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) reacts with OH• (rate constant $> 10^9\ M^{-1}\ sec^{-1}$) to give a specific adduct. An added OH• scavenger will compete for OH• and decrease the DMPO-OH ESR signal, and its reaction rate constant can be obtained from a competition plot [31]. DMPO also reacts with $O_2^{\bullet -}$, although much more slowly (rate constant $\sim 10\ M^{-1}\ sec^{-1}$ [31]) to give a different adduct, and $O_2^{\bullet -}$ scavenging can similarly be measured by examining effects on the ESR signal of the DMPO-OOH adduct.

Another example of a detector for OH• is the sugar 2-deoxy-D-ribose [32]. In the "deoxyribose method" for studying reactions with OH•, the OH• is generated by a mixture of ascorbic acid, H_2O_2 and Fe^{3+}-EDTA. It attacks deoxyribose, degrading it into fragments that give a chromogen upon heating with thiobarbituric acid at low pH. If an OH• scavenger is added, it competes with deoxyribose for OH• and inhibits chromogen formation. Competition plots allow calculation of rate constants; the rate constant for the deoxyribose/OH• reaction is $3.1 \times 10^9\ M^{-1}\ sec^{-1}$ [32].

To gain valid rate constants with any competition method, one must show that:
(i) The "antioxidant" does not react with OH• precursors, e.g. H_2O_2.
(ii) If metal ion-dependent systems are used to make OH•, the "antioxidant" does not chelate them (the deoxyribose assay avoids this by using iron ions already chelated to EDTA).
(iii) The "antioxidant" does not interfere with product measurement. It should not inhibit when added to the reaction mixture *at the end of the incubation*, e.g. with the thiobarbituric acid (TBA) and acid during the deoxyribose assay, or after the DMPO-OH ESR spectrum has developed. Many compounds, e.g. ascorbate, reduce DMPO-OH to an ESR silent species.* Other pitfalls with DMPO are reviewed in Ref. 33.

(iv) In the deoxyribose assay, attack of OH• upon the "antioxidant" does not generate a false chromogen; omitting deoxyribose from the reaction mixture should eliminate colour development.

Inhibition of metal ion-dependent hydroxyl radical formation. Some "antioxidants" may block OH• *formation* by chelating metal ions. The deoxyribose method can also be used to test this possibility. When iron ions are added to the reaction mixture as $FeCl_3$ (not EDTA-chelated), some of them bind to deoxyribose. They still appear to catalyse OH• formation, but the OH• immediately attacks deoxyribose, so that OH• scavengers (at moderate concentrations) cannot inhibit chromogen formation [34, 35]. However, an "antioxidant" can inhibit in this version of the deoxyribose assay if it chelates iron ions away from the deoxyribose and renders them inactive or poorly active in generating OH• [35]. Hence, this version of the deoxyribose assay [subject to controls (i), (iii) and (iv) above] indicates the potential ability of a compound to interfere with "site-specific" generation of OH• catalysed by iron ions bound to a target.

If an "antioxidant" binds metal ions and decreases the amount of OH• detected, two possibilities exist:
(i) The "antioxidant"–metal ion complex cannot catalyse OH• formation.
(ii) OH• is still made but largely intercepted by the antioxidant. To distinguish between these mechanisms, one can examine the fate of the antioxidant in the reaction mixture; it will be chemically modified if it is reacting with OH•.

Peroxyl radicals

Formation of peroxyl radicals ($RO_2^{\bullet}$) is a key step in lipid peroxidation [1], but they can also be formed from DNA and proteins [36, 37] and when thiyl ($RS^{\bullet}$) radicals combine with oxygen [37–39].

Peroxyl radical scavenging. Peroxyl radical scavengers may be water soluble (e.g. dealing with radicals from DNA, thiols, proteins) or lipid soluble (e.g. the chain-breaking antioxidant inhibitors of lipid peroxidation). Glutathione (GSH) reacts rapidly (rate constants $\sim 10^7$–$10^8\ M^{-1}\ sec^{-1}$) with radicals resulting from attack of OH• upon DNA [11, 38, 39].

Many chain-breaking antioxidants exert *pro-oxidant* properties under certain circumstances *in vitro*, often because they bind and reduce Fe(III) or Cu(II). Even α-tocopherol can do this [40–42]. The α-tocopherol radical can abstract H atoms from polyunsaturated fatty acids (PUFAs) [43] although by orders of magnitude more slowly than do peroxyl radicals. Propyl gallate, a food antioxidant, has limited solubility in water, but this is enough to allow it to accelerate both OH• formation from H_2O_2 and DNA damage by the antibiotic bleomycin, by reducing Fe(III) to Fe(II) [44]. Many plant phenolics (especially flavonoids) inhibit lipid peroxidation, but some can *accelerate* oxidative damage to non-lipid biomolecules *in vitro* by reducing metal ions and/or by oxidizing to produce $O_2^{\bullet -}$ and H_2O_2 [45, 46], further illustrating the point that an antioxidant in one system is *not* an antioxidant in all.

Measuring peroxyl radical scavenging. Peroxyl radicals can be generated by pulse radiolysis [47]

* Gutteridge JMC, personal communication. Cited with permission.

and their reactions with "antioxidants" studied. ESR studies of peroxyl radical reactions can also be carried out [48].

Another approach is the total (peroxyl) radical-trapping antioxidant parameter (TRAP) assay [49], much used (in various versions) to study antioxidants in biological fluids. Peroxyl radicals are generated at a controlled rate by the thermal decomposition of a water-soluble "azo initiator," such as 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). Decomposition produces carbon-centred radicals, which react fast with O_2 to give peroxyl radicals that then attack a lipid to cause peroxidation. By analysing the effect of an "antioxidant" on the lag time to onset, and the rate, of peroxidation, information about its mechanism of action [49] and a relative rate for its reaction with $RO_2^{\bullet}$ [50] can be obtained. Lipid "targets" can be endogenous lipids in biological fluids, or added lipids, often linoleic acid/ester. Studies of the ability to protect against AAPH-induced peroxidation have been used to show, for example, that ascorbate is an excellent scavenger of water-soluble $RO_2^{\bullet}$ [49, 50], whereas desferrioxamine is not [50]. AAPH-derived radicals also inactivate lysozyme, providing a protein target for studies of protection by "antioxidants" [51, 52].

The carbon-centred radicals produced by AAPH decomposition can do direct damage (e.g. to DNA [53]) and can deplete antioxidants [54]. Thus, one must ensure that reaction mixtures contain enough O_2 to convert them completely into peroxyl radicals.

Lipid-soluble peroxyl radicals. It is difficult to generate "clean" lipophilic peroxyl radicals *in vitro*. One exception is trichloromethylperoxyl, formed by exposing a mixture of CCl_4, propan-2-ol and buffer to ionizing radiation [55]. Rate constants for reaction of several "antioxidants" with $CCl_3O_2^{\bullet}$ have been determined [56]. However, $CCl_3O_2^{\bullet}$ is more reactive than non-halogenated peroxyl radicals, and so the results should be taken only as approximations of relative reactivity with the peroxyl radicals formed *in vivo*.

Studies of lipid peroxidation

To test lipid antioxidant activity directly, one can simply examine the ability of an "antioxidant" to inhibit peroxidation of lipoproteins, tissue homogenates, fatty acid/ester emulsions, liposomes or membranes (e.g. erythrocytes, liposomes, microsomes). Such studies are popular, but several points must be noted:

(i) The lipid systems are usually kept under ambient pO_2, but some antioxidants (e.g. β-carotene [57]) work better at lower pO_2. Variable results can arise if rapid peroxidation depletes O_2 during the reaction.

(ii) Accurate measurement of peroxidation is not easy (reviewed in Ref. 58). The TBA test is widely used, but one *must* ensure that an apparent "antioxidant" action is not caused by interference with the assay. For example, much of the "lipid-peroxidation inhibitory effect" of carnosine and anserine in microsomes is due to interference with the TBA test [59]; these compounds still inhibit when added with the TBA reagents.

(iii) How lipid peroxidation is started. If azo initiators (e.g. AAPH) are used, it can be difficult to distinguish whether an antioxidant is acting by direct scavenging of the initiator-derived peroxyl radicals, or by scavenging the chain-propagating peroxyl radicals generated from the lipid substrate. Lipophilic antioxidants added to reaction mixtures seldom partition completely into membranes.

Lipid peroxidation is often started by adding metal ions, e.g. as Fe^{2+}, $FeCl_3$ plus ascorbate or $FeCl_3$-ADP plus NADPH (for microsomes). In these cases, an "antioxidant" effect could occur not only by peroxyl radical scavenging but also by metal ion chelation. However, these two possibilities can be distinguished. If the antioxidant is acting only by chelation, it will not be consumed during the reaction, as shown by, for example, HPLC analysis. A chain-breaking antioxidant *is* consumed as it scavenges peroxyl radicals. Chain-breaking antioxidants at low concentrations often (not always) introduce a lag period into the peroxidation process, corresponding to the time taken for antioxidant consumption, whereas metal-binding antioxidants usually give a constant inhibition throughout the reaction.

Microsomal peroxidation assays. "Microsomes" prepared by differential centrifugation of tissue homogenates are a complex mixture of vesicles from endoplasmic reticulum, plasma membrane and other cell membranes. They contain variable amounts of endogenous antioxidants, such as α-tocopherol. Hence an "antioxidant" inhibiting microsomal peroxidation could (in addition to the mechanisms discussed above) be acting by "recycling" endogenous antioxidants. For example, dihydrolipoate does not inhibit iron/ascorbate-dependent peroxidation in liposomes [60], but it recycles vitamin E radical in microsomes to inhibit peroxidation [61]. If microsomal lipid peroxidation is started by adding NADPH plus Fe^{3+}-ADP [62], a control (usually measuring NADPH consumption) is needed to check that the "antioxidant" does not inhibit enzymic reduction of Fe^{3+}-ADP. Addition of NADPH to microsomes activates cytochromes P450, so added "antioxidants" could be metabolized to products more (or less) active in inhibiting peroxidation.

Lipid peroxidation is often started by adding Fe^{3+}/ascorbate. For microsomes, this avoids problems with P450. However, ascorbate may be capable of reducing "antioxidant" radicals (generated as they scavenge peroxyl radicals) back to the antioxidant, thus enhancing their action. Such a reaction will only occur if the antioxidant-derived radicals become accessible for reduction at the membrane surface, as α-tocopheryl radicals appear to do [6]. Hence, the antioxidant activity of some lipid-soluble chain-breaking antioxidants may appear to be greater if ascorbate is present.

Overall, it is wise to compare antioxidant ability using peroxidation of different lipids, started by different mechanisms.

Phagocyte-derived ROS

Activated neutrophils, macrophages, eosinophils and monocytes produce $O_2^{\bullet -}$ and H_2O_2. Most, if not all, of the H_2O_2 arises by dismutation of $O_2^{\bullet -}$, the initial product of the NADPH oxidase enzyme complex [63].

If phagocyte-derived ROS are causing tissue damage, protection could be achieved not only by scavenging ROS, but also by blocking their formation. Many drugs have been claimed to block the respiratory burst, but few claims meet the criterion that *the drug at the concentrations achieved* in vivo *must slow the respiratory burst that is triggered using physiologically relevant stimuli.*

Methods for isolating phagocytes and measuring their production of $O_2^{\cdot -}$ and H_2O_2 are well described [63, 64]. In using them, one must ensure that "antioxidants" do not interfere with the methods used to measure ROS production, e.g. by directly reducing cytochrome *c* (many thiols do this and have been falsely claimed to inhibit neutrophil $O_2^{\cdot -}$ generation) or by interfering with peroxidase-based measurement of H_2O_2.

Hypochlorous acid. Neutrophils contain myeloperoxidase, which uses H_2O_2 to oxidize Cl^- into HOCl [64]. Eosinophils contain a similar enzyme, which prefers to oxidize bromide (Br^-) ions and presumably produces HOBr [65]. Hypohalous acids contribute not only to phagocyte killing of foreign organisms, but also to tissue damage. For example, HOCl inactivates α_1-antiproteinase, an important inhibitor of serpins, such as elastase [64].

Hypochlorous acid scavenging assays. "Antioxidants" that prevent HOCl-mediated damage could scavenge HOCl directly and/or inhibit its production by myeloperoxidase. Myeloperoxidase can be assayed by standard tests of peroxidase activity [1] (e.g. oxidation of guaiacol to a chromogen in the presence of H_2O_2) or by measuring its production of HOCl [66]. Often the former type of assay is easier when looking for inhibitors, since the latter type can give confusing results if compounds that also scavenge HOCl are tested. If an apparent inhibition of myeloperoxidase is found, it should be checked whether the "antioxidant" is really inhibiting myeloperoxidase or is simply acting as a competing substrate. Thus, several thiols are not only HOCl scavengers [15, 67, 68], but also substrates for myeloperoxidase, presumably slowing HOCl formation [69]. Ascorbate also acts in both ways and has complex effects on myeloperoxidase [70, 71]. The plant phenol 4-hydroxy-3-methoxyacetophenone (apocynin) inhibits neutrophil $O_2^{\cdot -}$ release *in vitro*, apparently because it is oxidized by myeloperoxidase to generate the "real" inhibitor [72].

Once established that an "antioxidant" does not inhibit myeloperoxidase, HOCl scavenging can then be examined using myeloperoxidase/H_2O_2/Cl^- to generate it. More simply, HOCl can be made by acidifying sodium hypochlorite (Na^+OCl^-) and using A_{235} measurements to calculate HOCl concentration [73]. If a physiologically relevant concentration of an "antioxidant" is mixed with α_1-antiproteinase, a good HOCl scavenger should protect the α_1-antiproteinase against inactivation when HOCl is added [68]. Controls are needed to show that the "antioxidant" does not interfere with the assay by:
(i) inactivating elastase directly,
(ii) stopping α_1-antiproteinase from inhibiting elastase,
(iii) re-activating α_1-antiproteinase after inactivation by HOCl.

If a substance fails to protect α_1-antiproteinase against HOCl, it may be that:
(i) It reacts too slowly (if at all) with HOCl.
(ii) It reacts with HOCl to form a "long-lived" oxidant that is itself capable of inactivating α_1-antiproteinase [64]. Taurine reacts with HOCl to give chloramines, which also inactivate α_1-antiproteinase. The spectra of chloramines have been published [64], and it is also of interest to see if putative antioxidants can scavenge such products. An alternative assay for HOCl scavenging involves testing to see if the "antioxidant" can prevent oxidation of 5-thio-2-nitrobenzoic acid by HOCl [74].

The abilities of several drugs to scavenge HOCl have been examined [67, 68, 74]. Almost all react with HOCl, but few react fast enough for HOCl scavenging to be feasible *in vivo*. Even if these drugs did react with HOCl *in vivo*, the possibility of forming toxic reaction products should be considered [75].

Haem proteins/peroxides

Mixtures of H_2O_2 with haem proteins (including cytochrome *c*, haemoglobin, myoglobin) oxidize many substrates and catalyse lipid peroxidation and protein damage [76–78], apparently by the action of both amino acid radicals and haem-associated oxo-iron species [79, 80]. Such reactions may contribute to ischaemia–reperfusion injury, atherosclerosis, neurodegenerative disease, muscle injury and chronic inflammation. The ability of a substance to react with activated haem proteins can be examined spectrophotometrically by looking for loss of the ferryl myoglobin (or haemoglobin) spectrum as the compound reduces it to the ferrous or ferric state [81]. "Antioxidants" can also be tested for ability to inhibit oxidative protein damage or lipid peroxidation by haem protein/peroxide mixtures [78, 81].

Exposure of haem proteins to excess H_2O_2 causes haem breakdown and iron ion release [82]. Some antioxidants, such as ascorbate, prevent iron release [81] providing an additional assay method for testing the effects of "antioxidants."

A recent application of myoglobin/H_2O_2 systems is the development of a colorimetric assay for "total antioxidant status." Myoglobin/H_2O_2 is used to oxidize 2,2′-azinobis(3-ethylbenzo-thiazoline 6-sulphonate) (ABTS) into a coloured radical cation, $ABTS^{\cdot +}$, which reacts with several antioxidants [83].

Peroxynitrite

Peroxynitrite (equation 1) is easily prepared [25], allowing its reactions with "antioxidants" to be investigated [25, 26]. Careful control of pH is essential; peroxynitrite solutions are highly alkaline and can be contaminated with H_2O_2. The bicarbonate content of the reaction mixture affects peroxynitrite reactivity [25, 84].

Singlet oxygen

Oxygen has two singlet states, but the $^1\Delta g$ state is probably the most important. Singlet $O_2{}^1\Delta g$, although not a free radical, is a powerful oxidizing

agent, able to attack rapidly several molecules, including PUFAs. Singlet oxygen can be produced by photosensitization reactions [1]. It is also formed when O_3 reacts with human body fluids [85], when $ONOO^-$ reacts with H_2O_2 [86], and by self-reaction of peroxyl radicals during lipid peroxidation [87].

Assessing singlet oxygen quenching. Singlet O_2 can be generated by photosensitization reactions, but one must ensure that damage to a target molecule is due to $^1O_2$ rather than to direct interactions with the excited state of the sensitizer or by reactions involving other ROS, such as $O_2^{\cdot -}$ and $OH^{\cdot}$, that are often generated in light/pigment systems [1]. A technique has been described [88] in which $^1O_2$ generated by an immobilized sensitizer is allowed to diffuse a short distance to reach the target molecule. Singlet O_2 can also be generated by the thermal decomposition of endoperoxides, such as 3,3′-(1,4-naphthylidene)dipropanoate [89].

CONCLUDING COMMENTS

The tests outlined above enable one to examine the *possibility* that a given compound acts directly as an antioxidant *in vivo*. *The tests may clearly show that a direct antioxidant role is unlikely*. Alternatively, they could show that an antioxidant action is *feasible*, in that the compound shows protective action at concentrations within the range present *in vivo*. How then does one prove that the compound actually *does* act as an antioxidant *in vivo*?

For some naturally occurring antioxidants, it has been possible to remove them and observe increased oxidative damage, e.g. mutants of *Escherichia coli* lacking both MnSOD and FeSOD show severe damage when grown aerobically [90]. For dietary antioxidants, the effect of depletion can be studied, e.g. prolonged vitamin E deficiency in patients with disorders of intestinal fat absorption produces neurodegeneration [91].

These approaches are not feasible for most putative "antioxidants." Evidence supporting their antioxidant role *in vivo* can be provided by at least two approaches:

(i) Is the compound depleted under conditions of oxidative stress (e.g. ascorbate is rapidly lost at sites of oxidative stress [92])? However, antioxidant action *in vivo* need not result in antioxidant depletion; vitamin E and perhaps certain flavonoids can be "recycled" by ascorbate.

(ii) If an "antioxidant" scavenges radicals, is it degraded into products whose concentrations can be measured and shown to increase during oxidative stress? Thus, ascorbate produces ascorbate radical [92], and attack of ROS upon urate gives allantoin, cyanuric acid and parabanic acid [93].

As far as the ability of nutrients and drugs to act as antioxidants *in vivo* is concerned, specific assays are being developed to measure rates of oxidative damage to protein, DNA and lipid (reviewed in Ref. 94). Steady-state and total body oxidative damage to these targets can now be approximated, providing a tool to examine the effects of "antioxidants" *in vivo*.

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