Free Rad. Res. Comms., Vol. 9, No. 1, pp. 1-32
Reprints available directly from the publisher
Photocopying permitted by license only

REVIEW ARTICLE

HOW TO CHARACTERIZE A BIOLOGICAL ANTIOXIDANT

BARRY HALLIWELL

Department of Biochemistry, University of London King's College, Strand Campus, London WC2R 2LS, UK

(Received October 20th, 1989; in revised form November 27th, 1989)

An antioxidant is a substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. Many substances have been suggested to act as antioxidants *in vivo*, but few have been proved to do so. The present review addresses the criteria necessary to evaluate a proposed antioxidant activity. Simple methods for assessing the possibility of physiologically-feasible scavenging of important biological oxidants (superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, haem-associated ferryl species, radicals derived from activated phagocytes, and peroxyl radicals, both lipid-soluble and water-soluble) are presented, and the appropriate control experiments are described. Methods that may be used to gain evidence that a compound actually does function as an antioxidant *in vivo* are discussed. A review of the pro-oxidant and anti-oxidant properties of ascorbic acid that have been reported in the literature leads to the conclusion that this compound acts as an antioxidant *in vivo* under most circumstances.

KEY WORDS: Antioxidant, free radical, lipid peroxidation, ascorbic acid, oxygen radicals, DNA damage, peroxyl radicals, neutrophils, hypochlorous acid.

INTRODUCTION

The word "antioxidant" can be defined in various ways. Often, the term is implicitly restricted to chain-breaking antioxidant inhibitors of lipid peroxidation, such as vitamin E. However, the author prefers a broader definition – an antioxidant is "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate."[1] The term "oxidizable substrate" includes almost everything found in living cells, including proteins, lipids, carbohydrates and DNA. Antioxidants are of interest to radiation chemists, food scientists, polymer chemists and even to curators of museums,[1-4] but I shall confine discussion here to the antioxidants known, or proposed, to be important in aerobic organisms. Recent reviews have covered the well-established physiological antioxidant roles of such proteins as superoxide dismutase,[5] glutathione peroxidase,[6] catalase[1,6,7] and caeruloplasmin.[8] The role of the lipid-soluble chain-breaking antioxidant vitamin E has also been extensively discussed.[9,10]

*The term ROS 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^- is also a reducing agent). "Reactive" is a relative term, e.g. O_2^- is more reactive than O_2 but much less so than ·OH or HOCl.

TABLE I
Questions to ask when evaluating the proposed role of an "antioxidant" *in vivo*

1. What biomolecule is the compound supposed to protect? Is the compound present *in vivo* at or near that biomolecule at sufficient concentration?

2. How does it protect – by scavenging ROS, by preventing their formation or by repairing damage?

3. Is antioxidant protection the primary biological role of the molecule or a secondary one? For example, SOD has probably evolved as an antioxidant enzyme.[2] By contrast, transferrin has probably evolved as an iron transport protein, although the binding of iron ions to transferrin stops them accelerating radical reactions,[38] giving this protein an important secondary role in extracellular antioxidant defence.[39]

4. If the antioxidant acts by scavenging a ROS, can the antioxidant-derived radicals themselves do biological damage?

5. Can the antioxidant cause damage in biological systems different from those in which it exerts protection?

Many other substances have been proposed to act as antioxidants *in vivo*. They include β-carotene,[11] albumin,[12] metallothionein,[13] carnosine and related compounds,[14,15] mucus,[16] phytic acid,[17] taurine and its precursors,[18-20] bilirubin,[21] uric acid,[22] oestrogens,[23] creatinine,[24] ergothioneine (reviewed in[25]), dihydrolipoic acid,[26] ovothiols,[27] coenzyme Q,[28] polyamines,[29] retinol,[30] flavonoids and other phenolic compounds of plant origin[31] and ascorbic acid, a compound that has been reported to have both antioxidant and pro-oxidant properties, depending on the reaction conditions.[32,33] Some drugs administered to humans, such as non-steroidal anti-inflammatory drugs (reviewed in[34]), desferrioxamine[35,36] and N-acetylcysteine ([reviewed in[37]]) might have antioxidant properties *in vivo*.

In evaluating the likelihood of a proposal that a given compound acts as an antioxidant *in vivo*, it is important to ask the right questions, as summarized in Table 1. Some quite simple experiments can be performed *in vitro* to answer certain of these questions, and the results can allow one to rule out the proposed antioxidant ability in several cases. The purpose of the present review is to outline a battery of fairly-simple experiments that may be used to approach this problem. Let us first remind ourselves what reactive oxygen species (ROS)* are actually formed *in vivo*.

BIOLOGICALLY-IMPORTANT REACTIVE OXYGEN SPECIES

In testing putative antioxidant activity it is important to use biologically-relevant reactive oxygen species (ROS)*. One of these is superoxide, O_2^-, which is known to be formed *in vivo*.[1,5,40] Some of this O_2^- production is accidental, e.g. by leakage onto O_2 of electrons from the electron-transport chains of mitochondria and endoplasmic reticulum.[1,5,40] Some O_2^- production is deliberate, e.g. that by activated phagocytic cells.[41] It has been suggested that vascular endothelium may also constantly produce small amounts of O_2^- as part of a vaso-regulatory mechanism.[42]

Superoxide formed *in vivo* is converted by superoxide dismutase (SOD) or by non-enzymic dismutation into H_2O_2. Some enzymes, such as glycollate oxidase, also produce H_2O_2 directly.[1,43] Unlike O_2^-, H_2O_2 is able to cross all biological membranes. Both O_2^- and H_2O_2 can find some targets within cells at which they can do direct damage,[5,40] but on the whole their reactivity is limited. Thus only a few compounds, other than specific enzymes such as SOD and catalase, are able to remove O_2^- and

H_2O_2 at rapid rates. For example, many thiols react with H_2O_2 and with O_2^-, but the rate constants for these reactions are low, usually $< 10^3\,M^{-1}\,s^{-1}$.[37,44] Thus very high thiol concentrations (often > 1 mM) would be required to achieve significant scavenging. It is therefore unlikely that most thiol compounds administered to humans as drugs (such as N-acetylcysteine or penicillamine) could act *in vivo* by scavenging O_2^- or H_2O_2, simply because such high drug concentrations are not achieved in body fluids. GSH is present at intracellular concentrations in the high millimolar range, but the reaction of GSH with O_2^- or H_2O_2 can produce reactive sulphur-containing radicals that might be capable of doing more damage than the O_2^- and H_2O_2 would by themselves.[45,46] It is perhaps therefore fortunate that reactions of O_2^- and H_2O_2 with thiols are slow.

Ascorbic acid reacts with O_2^- with a rate constant of approximately $2.7 \times 10^5\,M^{-1}\,s^{-1}$ at pH 7.4.[47] Many animal tissues (e.g. eye lens, lung, brain) and the chloroplasts of green plants contain ascorbate at millimolar concentrations, and so scavenging of O_2^- *in vivo* might be feasible.[47–49] Thus although SOD reacts with O_2^- about four orders of magnitude faster than does ascorbate, the concentration of ascorbate *in vivo* is often more than four orders of magnitude greater than that of SOD.[1,48,49] Such arguments based on rate laws assume, of course, completely homogeneous distribution of substances within the cell, which is rarely the case. For example, most O_2^- formed in chloroplasts is produced by the thylakoids. Much SOD is bound to the thylakoid surface, whereas ascorbic acid is in the stroma, giving the SOD an advantage.[1,48] Reaction of ascorbic acid with O_2^- produces the semidehydroascorbate radical, which appears to be an unreactive species, incapable of doing significant biological damage.[50,51] Indeed, the stability of this radical means that it can easily be observed by ESR in biological material.[50]

Several keto-acids, such as glyoxylate, pyruvate and 2-oxoglutarate[52–56] react with H_2O_2. Rate-constants for the reactions have not been determined but are likely to be low, since millimolar concentrations of keto-acid have to be added to achieve high rates of H_2O_2 removal[53–56] and it is not clear if such concentrations are usually achieved *in vivo*. Pyruvate at high concentrations in cell-culture media can protect cells against damage by H_2O_2 [53,56] and Varma *et al.*[55] have argued that pyruvate concentrations in the mammalian lens might be sufficiently high to offer protection against H_2O_2 *in vivo*.

Hydroxyl radical

Much of the damage done by O_2^- and H_2O_2 *in vivo* is thought to be due to their conversion into highly-reactive oxidants.[1,5,35,39,57,58] After a long debate, it seems to be firmly established that one of these oxidants is hydroxyl radical, $\cdot OH$,[57–59] confirming a view held by the author[60] and others[61–63] for over ten years now.

Formation of $\cdot OH$ from O_2^- requires traces of catalytic transition metal ions, of which iron seems likely to be the most important *in vivo*[57,64] although copper ions might also play a role.[58,65] In systems containing O_2^-, H_2O_2 and iron ions, reactive species additional to $\cdot OH$ are probably also formed, including perferryl and ferryl (Figure 1). With copper ion/H_2O_2 systems the chemistry is even less clear – debate continues as to whether $\cdot OH$ is formed at all, or if it is formed *in addition to* a reactive copper(III) species (e.g. $Cu(OH)_2^+$).[58,65,66] However, the ability of copper ion/H_2O_2 systems to do severe damage to proteins[65,67–69] and to DNA[70,71] is well established, so that whatever species are formed are highly reactive. Even in systems containing iron

$$O_2^- + Fe^{3+}\text{-chelate} \rightleftharpoons [O_2^-\text{-}Fe^{3+}\text{-chelate} \xleftarrow{\text{PERFERRYL}} O_2\text{-}Fe^{2+}\text{-chelate}] \rightleftharpoons O_2 + Fe^{2+}\text{-chelate}$$

$$Fe^{2+}\text{-chelate} + H_2O_2 \longrightarrow \left[\begin{array}{c}\text{Ferryl?}\\ \text{Fe(IV)} = \text{O}\end{array}\right] \longrightarrow \cdot OH + OH^- + Fe^{3+}\text{-chelate}$$

Initial reaction of O_2^- with Fe^{3+} -chelates produces *perferryl* as an intermediate. Reaction of Fe^{2+} -chelates with H_2O_2 eventually produces $\cdot OH$, possibly via a ferryl intermediate.[57]

$$O_2^- + Cu^{2+}\text{-chelate} \longrightarrow \underset{[Cu^{2+}\text{-}O_2^-\ ?]}{\text{intermediate}} \longrightarrow Cu^+\text{-chelate} + O_2$$

$$Cu^+\text{-chelate} + H_2O_2 \longrightarrow \text{intermediate?} \xrightarrow{??} \cdot OH + OH^- + Cu^{2+}\text{ species}$$

$$O_2^- + Cu^{2+}\text{-chelate} + 2H^+ \xrightarrow{H_2O_2}$$

With copper ions, even less is understood about the reaction mechanism. Thus O_2^- might reduce Cu^{2+} to Cu^+, or oxidize it to a reactive copper(III) complex. Reaction of H_2O_2 with copper ions might produce copper(III) and/or $\cdot OH$.

FIGURE 1 *Intermediates in superoxide-driven Fenton-type Chemistry.*

ions, debate continues as to which biological iron chelators might actually mediate $\cdot OH$ formation *in vivo* and where they might be located in the body.[57,58,64,64a] Nevertheless, evidence for the biological importance of superoxide-driven Fenton chemistry is now substantial, as recently discussed in several articles.[57,59,72-75] Background exposure to ionizing radiation will also cause a steady low rate of $\cdot OH$ formation within cells, by splitting of water.

Hydroxyl radical is fearsomely reactive: it combines with almost all molecules found in living cells, with rate constants of $10^9 - 10^{10}\,M^{-1}s^{-1}$.[76] Thus almost everything in a cell is an $\cdot OH$ scavenger: no specific molecule has evolved for this role. Hence a suggestion that, for example, plasma metallothionein[13] acts to scavenge $\cdot OH$ *in vivo* seems chemically unlikely. The rate constant for scavenging (at $> 10^{10}\,M^{-1}s^{-1}$) may be high, but the molar concentration of metallothionein in plasma is far less than that of substances that are also capable of rapidly scavenging $\cdot OH$ such as albumin (rate constant also $> 10^{10}\,M^{-1}s^{-1}$ [76]). Glucose is a poorer scavenger (rate constant[76] about $10^9\,M^{-1}s^{-1}$) but its presence at 4.5 *millimolar* concentration would allow it to compete favourably with metallothionein. In the same way, suggestions that several anti-inflammatory drugs act *in vivo* by scavenging $\cdot OH$ formed at sites of inflammation are unlikely to be true: in almost all cases the drug does not accumulate to a high enough concentration *in vivo*.[34,77] One *possible* exception may be salicylate.[78]

It seems much more likely that an antioxidant acting to interfere with damage caused by $\cdot OH$ *in vivo* will act not by scavenging, but by binding the transition metal ions needed for $\cdot OH$ formation from O_2^- and H_2O_2 (Figure 1). Indeed, it has been argued that iron and copper ion transport and storage proteins have evolved so as to safely sequester the metal ions that they transport or store into forms that are incapable of stimulating free radical reactions.[1,8,39,57,64] This may be achieved by two mechanisms. First, binding may so alter the redox potential and/or accessibility of the

metal ion that it cannot participate in ·OH formation: this appears to be true of iron ions bound to transferrin or lactoferrin[38] and of copper ions bound to caeruloplasmin.[8] Metallothionein could also help to sequester copper and other transition metal ions in "safe" forms. In the same way, the iron chelator desferrioxamine, at the concentrations actually achieved *in vivo* during therapeutic administration, is far more likely to protect against damage mediated by ·OH by binding iron ions and preventing ·OH formation, rather than by scavenging this radical directly.[36]

A second possibility is that binding of a "catalytic" transition metal ion to an "antioxidant" does not prevent the redox reactions, but that they are directed on to the antioxidant, so sparing a more important target. Because ·OH is highly reactive, it will combine with the biological molecules that are present at or very close to its site of formation, and so the location of "catalytic" transition metal ions *in vivo* is an important determinant of the actual cellular damage that is done by oxidative stress.[12,39,64,65,75] Hence the presence of copper or iron ions bound to DNA *in vivo* can lead to DNA damage by site-specific Fenton chemistry[59,70,71,73,79] whereas metal ion binding to membranes can lead to lipid peroxidation.[39,57,80,81] When copper ions bind to plasma albumin, Fenton-type reactions can still occur on the binding sites and the protein is damaged.[67–69] However, albumin is much less significant as a target of damage than are the membranes of erythrocytes[81] or vascular endothelial cells,[82,83] so that the binding of copper ions to albumin may represent a protective mechanism overall,[12] since the damaged albumin can be quickly replaced. Binding of copper ions to the amino acid histidine in plasma might also be a protective mechanism: formation of ·OH radicals detectable in free solution is suppressed,[84] but the histidine is destroyed.[85] Both albumin and histidine might thus represent safe temporary transport forms for copper ions in plasma, until they can be cleared from the circulation by the liver.

Peroxyl radicals

Attack of ·OH upon biological molecules can proceed by addition, hydrogen atom abstraction or electron transfer reactions (reviewed in[1]). In all cases, a radical is formed from the biomolecule attacked. Detailed studies have been carried out on the radicals that can result from attack of ·OH upon lipids,[1,86] DNA[87,88] and some proteins.[87,89,90] In many cases, carbon-centred radicals are produced, that can then react with O_2 to give peroxyl radicals, e.g.

$$R\text{–}H \xrightarrow{\cdot OH} H_2O + R^{\cdot} \xrightarrow{O_2} RO_2^{\cdot} \qquad (1)$$

Formation of peroxyl radicals is the major chain-propagating step in lipid peroxidation,[1,86] but they can also be formed in non-lipid systems. For example, radicals resulting from the attack of ·OH upon uric acid can inactivate yeast alcohol dehydrogenase[91] or human α_1-antiproteinase.[92]

Reaction of ·OH with thiols can produce thiyl radicals (RS·) that then react with oxygen to give reactive oxysulphur radicals such as RSO· and $RSO_2^{\cdot}$ (thiyl peroxyl): the exact chemistry of these reactions is still uncertain.[87,93,94] Oxysulphur radicals resulting from attack of ·OH upon penicillamine appear to be capable of inactivating α_1-antiproteinase,[95] and probably of damaging other proteins. Whether or not ·OH radicals ever react with uric acid and penicillamine *in vivo* is uncertain: the concentrations of uric acid present in normal body fluids, and the concentrations of penicilla-

mine present in the body fluids of patients treated with this drug are less than 1 mM, and so they may not compete effectively for any ·OH generated. On the other hand, both uric acid and penicillamine readily bind iron and copper ions[96,97] and so they might direct ·OH formation onto themselves, resulting in production of reactive radicals.

Scavengers able to remove peroxyl radicals might be effective in the aqueous phase (e.g. dealing with radicals from DNA, thiols, uric acid etc) or in the hydrophobic (membrane interior) phase. Glutathione (GSH) reacts rapidly (rate constants $\sim 10^7 - 10^{-8}\,M^{-1}s^{-1}$) with radicals resulting from attack of ·OH upon DNA.[87,98] Indeed, "repair" of DNA-derived radicals in irrdiated cells is thought to be the major mechanism accounting for the radioprotective action of GSH,[87,98] although damage potentially resulting from the GS· radical and its derivatives must not be ignored.[93,94] Ascorbic acid also reacts very quickly (rate constants $\sim 10^8\,M^{-1}s^{-1}$)[93,99,100] with both peroxyl radicals and with sulphur-containing radicals and so might serve an important repair function. Thus, at physiological concentrations, ascorbate was able to protect α_1-antiproteinase against radicals resulting from the attack of ·OH upon uric acid.[92]

The scavengers of peroxyl radicals that operate in the hydrophobic interior of biological membranes, are, of course, the chain-breaking anti oxidant inhibitors of lipid peroxidation. By far the most important of these *in vivo* is α-tocopherol.[9,10,101] The author believes that the *main* biological function of α-tocopherol in Man is to act as an antioxidant, although it may have other functions as well.[9] Some other compounds present in membranes may be able to act as chain-breaking antioxidants, but I believe that this is *not* their major physiological function: perhaps they are oxidized simply because they happen to be there. Thus peroxidation of mitochondrial membranes

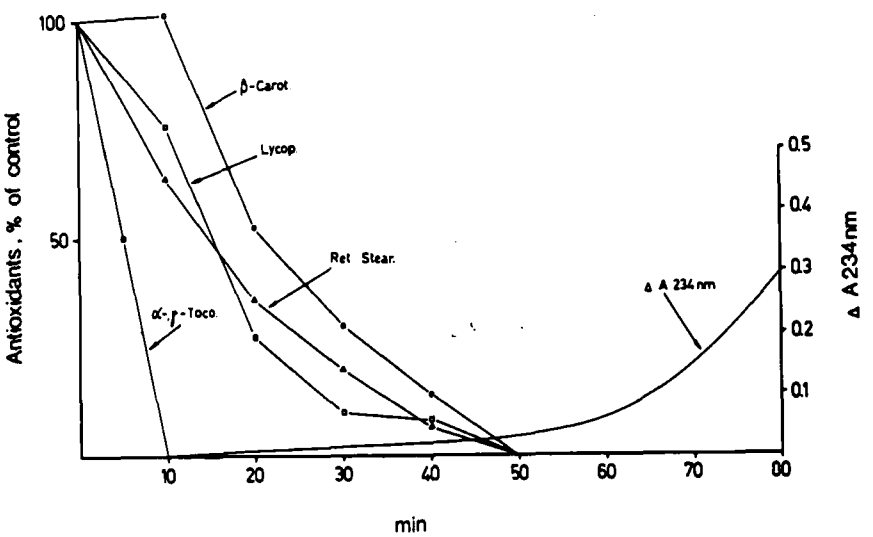


FIGURE 2 Time-course of the consumption of antioxidants and formation of conjugated dienes during oxidation of human plasma low-density lipoproteins. The diene increase was measured by continuous monitoring of the increase in absorbance at 234 nm. Reproduced with permission from Esterbauer *et al.* (1989) *Free Radical Res. Commun.*, **6**, 67–75.

TABLE 2
Polyunsaturated fatty acids and antioxidants in human low-density lipoproteins. The values given are the mean and the highest and lowest values of 16 LDL preparations from different donors. The relative molecular mass of the LDL particle was assumed to be 2.5×10^6. Reproduced with permission from Esterbauer *et al.* (1989) in *Medical, Biochemical and Chemical Aspects of Free Radicals* (eds Hayaishi, O *et al.*) pp. 1203–1209.

	Number of molecules per LDL particle	
	mean	range
linoleic acid ester	957	680–1247
arachidonic acid ester	87	48–172
α-tocopherol	6.5	4.0–9.9
γ-tocopherol	0.5	0.3–0.7
β-carotene	0.3	0.1–0.7
lycopene	0.2	0.05–0.3

results in loss of ubiquinone, and this loss appears to protect the lipid. Hence ubiquinone has been called an antioxidant.[28,102] It may well be, however, that the damage to ubiquinone is more significant in terms of loss of mitochondrial function than is the damage to lipid. Indeed, when peroxyl radicals in membranes react with membrane proteins, the lipid is spared, but the attack upon the proteins can sometimes be much more physiologically-damaging in interrupting membrane function.[103] Thus one would not call the membrane proteins "antioxidants".

Retinol[104,105] and lycopene[104,105a] in membranes might also be attacked by free radicals during membrane lipid peroxidation simply because of their location, as might β-carotene within membranes. β-carotene inhibits peroxidation of lipids only at low O_2 concentrations,[11,105] but this is not without biological relevance since many human tissues have low intracellular O_2 concentrations *in vivo*. When low density lipoproteins isolated from plasma undergo peroxidation, oxidation of lipids does not reach its maximum rate until tocopherols, lycopene, retinol and β-carotene have been oxidized (Figure 2). Does this mean that retinol, β-carotene and lycopene are selectively accumulated in LDL in order to act as antioxidants? Probably not, since there is less than 1 molecule of β-carotene per LDL particle *in vivo* (Table 2). By contrast, about 7 α-tocopherol molecules are present per LDL particle (Table 2), consistent with its important physiological role as a chain-breaking antioxidant.[101,106]

Many lipid-soluble chain-breaking antioxidants can have *pro-oxidant* properties under certain circumstances, often because they can bind Fe(III) ions and reduce them to Fe^{2+}. Even α-tocopherol can be made to exert pro-oxidant effects *in vitro*,[107,108] although these have no physiological relevance. Propyl gallate, an antioxidant sometimes used in the food industry, has limited solubility in water, but this is enough to allow it to accelerate both $\cdot OH$ formation from H_2O_2 by Fenton chemistry and DNA damage by the antibiotic bleomycin, in both cases by its ability to reduce Fe(III) to Fe^{2+}.[108,109] Many plant phenolics (especially flavonoids) have been styled as "antioxidants" because they inhibit lipid peroxidation – hence the appearance of "bioflavonoids" (plus or minus vitamin C) on the shelves of health-food stores. However, several plant phenolics can *accelerate* oxidative damage to non-lipid biomolecules such as DNA.[110–12] They do this by reducing Fe(III) ions to Fe^{2+} [112] and/or by oxidizing to produce O_2^- and H_2O_2.[110,111] Thus an antioxidant in one system is *not* an antioxidant in all systems, and this must be borne in mind.

Phagocyte-derived oxidants

Activated neutrophils, macrophages, eosinophils and monocytes produce O_2^- and H_2O_2. Most, if not all, of the H_2O_2 arises by dismutation of O_2^-, the first product of the NADPH oxidase enzyme complex.[41,113,114] After a long debate, it now seems clear that neutrophils (and presumably other kinds of phagocytic cell) do not themselves secrete or contain an iron catalyst capable of forming ·OH from O_2^- and H_2O_2.[115,116] Indeed, neutrophils may secrete the iron ion-binding protein lactoferrin in order to *minimize* iron-dependent radical reactions in their surroundings.[39,115,117,118] This is *not* to say that ·OH does not contribute to bacterial killing or digestion in the phagocytic vacuole. Thus H_2O_2 might enter the bacteria and kill them by generating ·OH[119] using intra-bacterial iron ions.[120] Alternatively, attack of hypochlorous acid upon bacteria may cause iron ion liberation into the vacuole, so allowing ·OH formation.[121]

If neutrophil-derived O_2^- and H_2O_2 are involved in producing oxidative damage to tissues, antioxidant protection could be achieved not only by scavenging these species, but also by agents that block the respiratory burst and stop their formation. Thus several anti-inflammatory drugs have been suggested to interfere with phagocyte functioning (reviewed in[34]). However, very few of these claims meet the criterion that the drug *at the concentrations actually achieved in vivo during normal therapeutic regimes* must slow the respiratory burst that is triggered by using physiological stimuli such as opsonized bacteria. Drugs for which interference with the respiratory burst is a feasible contributor to their action *in vivo* include piroxicam,[122,123] and antimalarials.[124]

Activated neutrophils contain and secrete the enzyme myeloperoxidase, which uses H_2O_2 to oxidize chloride ions into the powerful oxidant hypochlorous acid, HOCl (reviewed in[113,114,125]). Human eosinophils contain a similar enzyme, which prefers bromide (Br^-) ions as a substrate and presumably produces HOBr.[126] Hypohalous acids contribute something to the mechanisms by which neutrophils and eosinophils attack ingested bacteria or parasites respectively, but the extent of the contribution is uncertain.[113,114,125,127] HOCl produced outside the phagocyte might also contribute to tissue damage. For example, it is a powerful oxidant of critical –SH groups on cell surfaces, so interfering with membrane transport systems.[128–130] HOCl can also oxidize an essential methionine residue in α_1-antiproteinase (formerly called α_1-antitrypsin). α_1-Antiproteinase is the major inhibitor of serine proteases, such as elastase, in human body fluids. Oxidation of the methionine causes this protein to lose its protease inhibitory capacity, so facilitating proteolytic injury in the surrounding area, e.g. damage to elastic fibres in lung by elastase.[125,131] The rate of inactivation of α-antiproteinase by HOCl is extremely fast, being complete within minutes.[131]

However, because HOCl is highly reactive, it can also combine with many of the other molecules in its immediate vicinity. Thus when HOCl is added to human plasma, α_1-antiproteinase is not inactivated because the HOCl preferentially reacts with other plasma components present at greater molar concentrations than those of α_1-antiproteinase. These components include albumin[132] and ascorbic acid.[133] Indeed, ascorbic acid at physiological concentrations is a powerful scavenger of HOCl, being able to protect α_1-antiproteinase against inactivation.[133] Scavenging of HOCl by albumin may well be due to rapid reaction with –SH groups on this protein.[132] The bilirubin bound to circulating albumin does not contribute significantly to the HOCl-scavenging ability,[134] although bilirubin may help to prevent unsaturated fatty acids, transported in an albumin-bound form in the blood, from becoming peroxidized.[21] Oxidized albumin may be rapidly cleared from the circulation and degraded.[12,135]

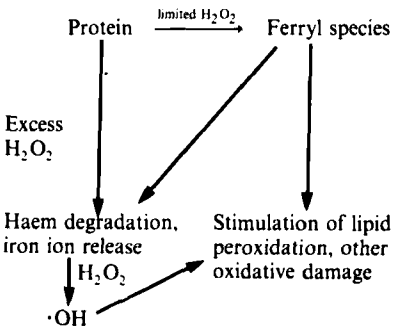
Recent surveys have examined the ability of several anti-inflammatory drugs to scavenge HOCl.[136,137] It was concluded that almost all of the drugs examined could react, but only a few drugs would, at the concentrations present *in vivo*, be capable of reacting fast enough to protect biological targets such as α_1-antiproteinase from attack by HOCl. Thus HOCl scavenging *in vivo* in patients treated with these drugs is only feasible in the case of thiol compounds (penicillamine, N-acetylcysteine), phenylbutazone and aminosalicylates. Again, even if these drugs are oxidized *in vivo* by HOCl, the possibility of forming toxic products as a result of oxidation must not be ignored.[37,95,136–141]

An antioxidant protecting against damage by HOCl might also act by inhibiting myeloperoxidase. Thus several thiols are not only good scavengers of HOCl, but also act as competing substrates for myeloperoxidase and therefore slow down HOCl formation.[140–142] Aminosalicylates and dapsone may also inhibit myeloperoxidase.[143] Ascorbic acid probably also acts as an alternative substrate for myeloperoxidase and might slow HOCl production.

Ferryl and perferryl species

Interaction of O_2^- with iron ions can form perferryl species (Figure 1), which have been suggested to be capable of initiating lipid peroxidation. However, a perferryl species is known to be present at the active site of peroxidase compound III, a poorly-active form of the enzyme. Hence the author feels that perferryl is not a likely candidate for a reactive damaging species *in vivo* (discussed in[57,64]).

Ferryl, an oxo-iron species in which the iron has an oxidation number of 4, may also be formed in Fenton systems (possibly as an intermediate in ·OH formation: Figure 1), and would be expected to be powerfully oxidizing. Haem-associated ferryl species are well-known to be generated by reaction of certain haem proteins (horseradish peroxidase, myoglobin and probably haemoglobin) with H_2O_2.[144–147] Mixtures of myoglobin or haemoglobin with H_2O_2 are capable of accelerating lipid peroxidation,[148–150] a property that has been attributed by some groups to initiation of peroxidation by the ferryl proteins (reviewed in[148]). In addition, incubation of



Myoglobin, and probably haemoglobin, react with H_2O_2 to form ferryl species. Excess H_2O_2 leads to haem degradation and iron ion release, perhaps via a ferryl intermediate.

FIGURE 3 *Interaction of myoglobin and haemoglobin with H_2O_2.*

myoglobin and haemoglobin with an excess of H_2O_2 can cause haem breakdown to release iron ions,[151-153] as shown in Figure 3. Both ferryl formation, and the release of iron ions able to accelerate free radical reactions, from haemoglobin might account for the observation that bleeding into a site of inflammation exacerbates tissue injury.[34,154] Formation of ferrylmyoglobin as a result of production of H_2O_2 upon reoxygenation of ischaemic heart tissues has also been suggested to contribute to myocardial reoxygenation injury.[155,156] Thus agents that scavenge ferryl species might prevent lipid peroxidation, stimulated by ferryl haem proteins. They might also prevent decomposition of the proteins to release catalytic iron ions.[148,155-159] Ascorbic acid seems especially effective in this respect, presumably by being preferentially oxidized and so reducing the iron in the haem ring back to an Fe(III) state.[157,159] Thus ascorbic acid inhibits lipid peroxidation stimulated by mixtures of haem proteins and H_2O_2, and also slows or prevents haem degradation.[157,159]

Other species capable of reacting with ferryl haem include thiols and uric acid,[155,157] although the potential pro-oxidant effects of radicals resulting from one-electron oxidation of uric acid or of thiols must be considered. Hence, tests of the ability of a substance to scavenge haem-associated ferryl species may be relevant in the evaluation of antioxidant activity, particularly if antioxidants are being developed for use against myocardial reoxygenation injury.

Singlet oxygen

Oxygen has two singlet states, but the $^1\Delta g$ state is probably the most important in biological systems (reviewed in[1]). Singlet O_2 $^1\Delta g$ has no unpaired electrons and is therefore not classified as a radical, but it is known to be a powerful oxidizing agent, able to combine directly with many molecules that are unreactive with ground-state O_2, such as polyunsaturated fatty acids.[1] The term "singlet O_2" is used in the rest of this review to refer to both $^1\Delta g$ and $^1\Sigma g^+$ states collectively.

Singlet oxygen can be produced on or in the skin as a result of photosensitization reactions triggered by certain drugs,[160-163] cosmetics,[161] plant toxins[164] or by the porphyrins that accumulate in some forms of porphyria.[165] Singlet O_2 is also important in the "phototherapy" of certain diseases, e.g. the use of psoralens to treat psoriasis[160] and of haematoporphyrin derivatives in lung cancer.[166] Singlet O_2 may also be generated in the lens of the eye and contribute to the development of cataract.[167]

However, whether singlet O_2 is produced as a major tissue-damaging species in the deeper tissues of the body is uncertain. Suggestions that singlet O_2 is produced by non-enzymic dismutation of O_2^- or during the respiratory burst of phagocytic cells have not received much experimental support.[168-170] During peroxidation of membrane lipids, singlet O_2 (or at least a species closely resembling it) is produced, probably largely by the self-reaction of lipid peroxyl radicals (reviewed in[171]).

$$>CHO_2\cdot + >CHO_2\cdot \rightarrow {}^1O_2 + >C=O + ->C\text{-}OH \qquad (3)$$

This singlet O_2 could then react with further polyunsaturated fatty acid side-chains and contribute to the overall peroxidation process.[171] However, singlet O_2 seems only to make a minor contribution in most peroxidation systems, possibly because $LO_2^\cdot$ radicals do not accumulate to high concentrations in membranes and so their self-reaction is kinetically disfavoured.

Ascorbic acid is not only a chemical scavenger of singlet O_2, but also quenches this molecule very effectively in aqueous solution.[172-174] Similarly, α-tocopherol can both

quench and scavenge singlet O_2 within membranes.[1,175] In addition, carotenoids can both quench and scavenge singlet O_2: this role is of the greatest importance in protecting illuminated chloroplasts against photodestruction reviewed in,[48] but whether it is of relevance to normal mammalian metabolism is uncertain. Administered carotenoids have been observed to have beneficial effects in patients exposed to photosensitizers[165,176] and there is a weak inverse correlation between a high dietary intake of carotenoids and the incidence of cataract[177] or of cancer.[178] Lycopene is especially effective in quenching singlet O_2.[105a]

METHODOLOGY – HOW TO TEST FOR ANTIOXIDANT ACTIVITY

Now that the multiple ways in which a substance might exert antioxidant activity have been reviewed, let us see how simple tests to evaluate putative antioxidant properties may be carried out *in vitro*. It is essential to examine the action of a compound over a physiologically-relevant concentration range. For example, if compound X is present *in vivo* at concentrations < 1 mM, its ability to inhibit lipid peroxidation only at concentrations > 20 mM is irrelevant. One must also bear in mind that, if acting as a scavenger, an "antioxidant" may itself give rise to damaging radical species.

Scavenging of superoxide

Superoxide is easily produced by radiolysis of water in the presence of O_2 and formate ions. Pulse radiolysis allows examination of the spectrum of any products formed when O_2^- reacts with a putative antioxidant.[179] However, pulse radiolysis is unsuitable for measuring reactions of O_2^- in aqueous solution where the rate is much lower than the overall rate of non-enzymic dismutation of O_2^-. This limits measurements of rate constants to those of $10^5\,M^{-1}s^{-1}$ or greater. Unfortunately, the rate constants for the reaction of O_2^- with most biological molecules, except ascorbate and SOD, are less than this.[44] Stopped-flow methods can be used to study these slower reactions (e.g.[180]). However, provided that suitable control experiments are done, good approximations to rate constants may be achieved using simple test-tube systems. Thus a mixture of hypoxanthine and xanthine oxidase at pH 7.4 generates O_2^- [181] which reacts with cytochrome c and nitro-blue tetrazolium (NBT) with defined rate constants,[44] namely 2.6×10^5 and $6 \times 10^4\,M^{-1}s^{-1}$ respectively. Any added molecule capable of reacting with O_2^- will decrease the rates of cytochrome c or NBT reduction, and analysis of the inhibition produced allows calculation of an approximate rate constant.[182] This simple method has been used in attempts to establish rate constants for the reactions of desferrioxamine,[182] taurine,[183] carnosine[184] and N-acetylcysteine[37] with O_2^-. It should be noted that the method cannot distinguish between a reaction of the added compound with O_2^- or a much faster reaction with the small amount of $HO_2^\cdot$ in equilibrium with O_2^- at pH 7.4.[36] The potassium salt of superoxide ($K^+O_2^-$) is available commercially and may be used as an alternative source of O_2^- (e.g. references[180,184a]).

Some controls are essential in using these simple "test-tube" methods to investigate scavenging of O_2^-, particularly in systems using xanthine oxidase

i) it must be checked that the substance under test does not inhibit O_2^- generation, e.g. by inhibiting xanthine oxidase. This can be checked in the case of the enzyme by measuring uric acid formation[182] (using HPLC, or spectrophotometrically).

ii) it must be checked that the substance does not itself reduce cytochrome c or NBT. This is a particular problem with cytochrome c, which is easily reduced (e.g. by ascorbic acid and by thiols at high concentration), especially at high pH. The author's experience is that it is less of a problem with NBT, provided that pH values of 7.4 or less are used.

iii) One must consider the possibility that a radical formed by attack of O_2^- on a substance could itself reduce cytochrome c or NBT. This will be revealed as deviations from linear competition kinetics at high scavenger concentrations. Thus the nitroxide radical formed when O_2^- reacts with desferrioxamine can probably reduce cytochrome c, since it reduces many other molecules.[185]

Hydrogen peroxide

H_2O_2 is easily and sensitively measured by using peroxidase-based assay systems. The most common employs horseradish peroxidase, which uses H_2O_2 to oxidize scopoletin into a non-fluorescent product.[186,186a] Thus if a putative scavenger is incubated with H_2O_2 and the reaction mixture sampled for analysis of H_2O_2 at various times, rates of loss of H_2O_2 can be measured to allow calculation of rate constants. The *essential* control is to check that the substance being tested is not itself a substrate for peroxidase, which could compete with scopoletin and cause an artefactual inhibition. Thus ascorbic acid and thiol compounds can be oxidized by horseradish peroxidase; hence they seriously interfere with peroxidase-based assay systems.[1,37] In order to check if a substance might be oxidized by peroxidase, one should look for changes in the absorption spectrum when the compound is added to a peroxidase-H_2O_2 mixture: 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 is oxidizing the compound under test.

If the compound does interfere with peroxidase-based systems, other assays for H_2O_2 can be used. Thus H_2O_2 can be estimated by simple titration with acidified potassium permangante or by measuring the O_2 release (1 mole of O_2 per 2 moles of H_2O_2) when a sample of the reaction mixture is injected into an O_2 electrode containing buffer and a large amount of catalase.[1] Varma[54] has described a sensitive radiochemical assay for H_2O_2, based on its ability to decarboxylate [^{14}C]-labelled 2-oxoglutarate. Measurement of $^{14}CO_2$ is performed by scintillation counting. Electrodes that detect H_2O_2 have also been described (e.g.[187,187a]), although they are subject to interference.

Hydroxyl radical

The definitive technique for measuring the rate constant for reaction of a substance with ·OH, and for studying the products of that reaction, is pulse radiolysis.[179] Most compounds react with ·OH with rate constants of $10^9 - 10^{10}\,M^{-1}\,s^{-1}$.[1,76]

If pulse radiolysis facilities are not available, approximate rate constants can often be calculated using the "deoxyribose method". Hydroxyl radicals are generated by a mixture of ascorbic acid, H_2O_2 and Fe^{3+}-EDTA. Those radicals not scavenged by other components of the reaction mixture attack the sugar deoxyribose, degrading it into a series of fragments,[87] some or all of which react on heating with thiobarbituric acid (TBA) at low pH to give a pink chromogen[188,189] which is an adduct of TBA with

malondialdehyde.[189,190] If a scavenger of ·OH is added to the reaction mixture, it will compete with deoxyribose for the ·OH radicals and inhibit deoxyribose degradation, to an extent depending on the scavenger's concentration and on its rate constant for reaction with ·OH. Competition plots allow this rate constant to be calculated, assuming that deoxyribose reacts with ·OH with a rate constant of $3.1 \times 10^9 M^{-1} s^{-1}$.[191]

The deoxyribose assay works well with most substances.[183,184,191,192] Essential controls include checking that

i) the substance does not react rapidly with H_2O_2, which could block ·OH formation. This has rarely been a problem because H_2O_2 is poorly reactive.

ii) the substance is not a powerful iron chelator, capable of removing iron ions from EDTA. This is again rarely a problem.

iii) attack of ·OH upon the substance does not produce TBA-reactive material. A control should be performed in which deoxyribose is omitted from the reaction mixture.

iv) the substance does not interfere with measurement of deoxyribose degradation products. This can be checked by showing that it does not inhibit when added to the reaction mixture at the end of the incubation, with the TBA and acid.[184]

As argued previously, direct scavenging of ·OH is a generally-unlikely mode of antioxidant action *in vivo*, simply because very high concentrations of scavenger are required to compete with biological molecules for any ·OH generated. It is therefore of interest to examine the ability of a putative antioxidant to chelate metal ions in such a way that it interferes with metal ion-dependent *generation* of ·OH, particularly that involving iron ions bound to a target ("site-specific" Fenton chemistry). The deoxyribose method affords a way of doing this also (Figure 4). When iron ions are added to the reaction mixture as $FeCl_3$ (not chelated to EDTA), some of them bind to deoxyribose.[193,194] The bound iron ions still participate in Fenton chemistry, but any ·OH radicals formed immediately attack the sugar and are not released into free solution.[195] Hydroxyl radical scavengers, at moderate concentrations, do not inhibit this deoxyribose degradation because they cannot compete with the deoxyribose for ·OH generated by bound iron ions.[195] The only substances that do inhibit in this assay are those that bind iron ions strongly enough to remove them from the deoxyribose.[195,77] Hence this assay indicates the potential ability of a compound to interfere with "site-specific" generation of ·OH radicals catalyzed by bound iron ions. Indeed, the assay has been used to evaluate the abilities of anti-inflammatory drugs,[77] taurine and its precursors[183] and carnosine, homocarnosine or anserine[184] to act in this way. The essential controls are numbers (i), (iii) and (iv) above, i.e. the compound must not scavenge H_2O_2 rapidly, be degraded to TBA-reactive material or interfere with measurement of the deoxyribose degradation products.

It must be emphasized that this version of the deoxyribose assay (Figure 4) measures a combination of two factors: the ability to remove iron ions from deoxyribose *and* to render those iron ions inactive or poorly active in generating ·OH. Thus EDTA removes iron ions from deoxyribose, but iron-EDTA chelates are very effective in generating ·OH (for reasons discussed in[196]) so that the deoxyribose is still degraded – this time by ·OH in "free solution", rather than by ·OH formed upon the deoxyribose molecule.

If a compound inhibits site-specific radical damage to deoxyribose by chelating iron

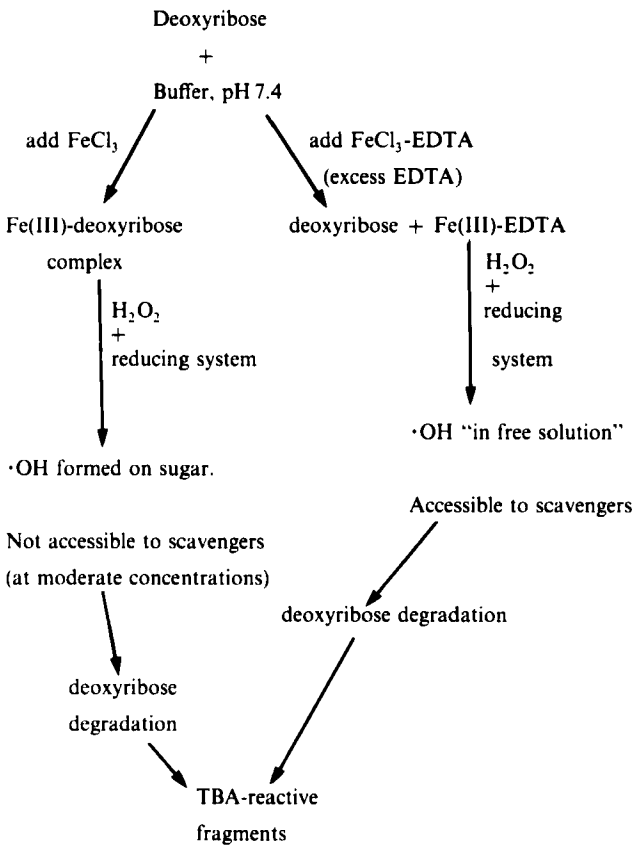


FIGURE 4 Principles of the use of the deoxyribose assay to measure scavenging of hydroxyl radicals, and the ability to inhibit site-specific ·OH generation.

ions and rendering them less active in producing ·OH, two possibilities can account for the latter property. First, the inhibitor-metal ion complex may be *incapable* of reacting with O_2^- or H_2O_2, so blocking ·OH formation. Second, it may be that the inhibitor-metal ion complex still undergoes redox reactions, but that the ·OH is largely intercepted by the inhibitor and is not allowed to escape into free solution, i.e. damage is directed onto the inhibitor and away from the deoxyribose. To distinguish between these mechanisms, one can examine the fate of the inhibitor in the reaction mixture. For example, HPLC analysis should show whether or not the inhibitor is undergoing degradation as the reaction proceeds.

To summarize, the deoxyribose method performed in the absence of EDTA may represent an approximate, but simple, test of the ability of a compound to interfere with iron ion-dependent site-specific Fenton chemistry.

Water-soluble peroxyl radicals

To what extent water-soluble peroxyl radicals contribute to oxidative damage *in vivo* is uncertain, since they are usually easily "repairable" by ascorbic acid.[92,93,99] Indeed,

a recent study of the reactivity of peroxyl radicals produced as a result of attack of ·OH on several anti-inflammatory drugs in the presence of O_2 suggested that such radicals are, in general, of limited reactivity.[95] However, damage to biological targets by several such radicals has been demonstrated[91,92,99,197] and a complete "screen" of antioxidant ability should therefore include an assessment of the capacity of a putative antioxidant to scavenge peroxyl radicals. How can this be done? Peroxyl radicals derived from anti-inflammatory drugs can easily be generated by pulse radiolysis under appropriate conditions, and their scavenging by antioxidants studied.[99,197] Attack of ·OH upon uric acid produces radicals that may be observed by ESR[198] and that can attack certain proteins.[91,92] The ability of antioxidants to scavenge the ESR-detectable species and/or to protect the proteins against inactivation may be examined, as has been done for ascorbic acid.[92,198]

Another method of examining peroxyl radical scavenging is the TRAP assay, introduced by Ingold *et al.*[32,199] and much used in studies of antioxidants in body fluids.[10,199-203] Peroxyl radicals are generated at a controlled rate by the thermal decomposition of a water-soluble "azo initiator", such as ABAP (Figure 5). They are allowed to react with a lipid, whereupon they cause peroxidation. Thus by analyzing the effect of a water-soluble antioxidant on the rate of peroxidation, a relative rate for its reaction with peroxyl radicals can be measured.[32,199,204] A suspension of linoleic acid or an ester of it is frequently used as lipid substrate.[10,32,199-202] Studies of the ability to protect linoleic acid or other lipids against peroxidation by ABAP-derived radicals have been used to show, for example, that ascorbic acid is an excellent scavenger of water-soluble peroxyl radicals[32,199-201] whereas desferrioxamine is a much-poorer scavenger.[204]

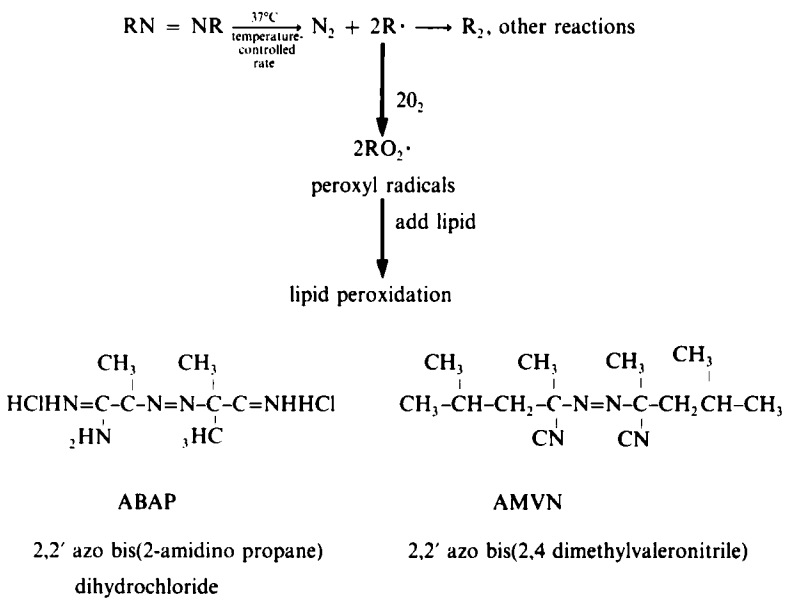


FIGURE 5 Decomposition of ABAP and AMVN leading to peroxyl radical generation.

Lipid-soluble peroxyl radicals

Peroxyl radicals are the major chain-propagating species in the process of lipid peroxidation in membranes.[1] Thus a standard test of antioxidant ability is the action of a substance in inhibiting peroxidation of membranes such as erythrocytes, tissue homogenates, liposomes or microsomes.[10,205–208] Although such studies are widely performed, several points must be considered in interpreting them. Firstly, the lipid systems are usually maintained under ambient pO_2, although some antioxidants work best at lower O_2 concentrations.[11,105] Variable results may arise because rapid peroxidation often depletes O_2 in the reaction mixture.

Secondly, how should peroxidation be measured? O_2 uptake is one possibility [reviewed in.[1]] The thiobarbituric acid (TBA) test is widely used and works well in liposomal and microsomal systems[205–210] but it is *essential* to ensure that the apparent antioxidant effect of an added compound is not due to interference with the TBA test itself. Table 3 shows why this control is necessary: much of the apparent antioxidant effect of carnosine and anserine in inhibiting microsomal lipid peroxidation is still seen when these compounds are added with the TBA and acid, i.e. they are interfering with the assay.[184] Detailed reviews of methods for measuring lipid peroxidation in biological material have recently been published,[1,209–212] so I will not dwell on these points here.

The third major point to consider is how peroxidation of the lipid substrate can be started. If a water-soluble azo initiator such as ABAP (Figure 5) is used, it will be difficult to distinguish whether an antioxidant acts by direct scavenging of the ABAP-derived peroxyl radicals, or by scavenging of the lipid peroxyl radicals within the lipid

TABLE 3

Action of the putative antioxidants anserine and carnosine on lipid peroxidation: interference with the thiobarbituric acid assay. Peroxidation of rat-liver microsomes was studied, using a standard TBA test as described in.[184] Desferrioxamine exerted its inhibitory effect during the incubation: when added with the TBA reagents it had little effect. By contrast, much of the apparent inhibition of lipid peroxidation by carnosine and anserine is due to interference with the TBA test. Results abstracted from.[184]

Peroxidation stimulated by adding	Extent of peroxidation A_{532}	% Inhibition by antioxidant added					
		12 mM carnosine		12 mM anserine		0.2 mM desferrioxamine	
		at start of expt	during TBA assay only	at start of expt	during TBA assay only	at start of expt	during TBA assay only
Fe^{3+}/ascorbate	1.18 (100%)	75	61	80	53	84	7
Fe^{3+}-ADP/NADPH	1.22 (100%)	79	54	81	40	80	0
Fe^{2+}	1.05 (100%)	66	58	54	50	75	5
Fe^{2+}-ADP	1.13 (100%)	69	56	62	48	75	13

subtrate. This problem is not overcome by treating the membrane with a lipid-soluble azo initiator such as AMVN (Figure 5). Although the hydrophobic AMVN decomposes within the membrane interior to form peroxyl radicals, it is still difficult to distinguish whether an antioxidant that has entered the membrane is acting by scavenging the AMVN-derived radicals, or the (more biologically-important) peroxyl radicals arising from the fatty acid side-chains within the membrane.

Peroxidation can also be accelerated by adding iron salts to membranes[205–207] e.g. as Fe^{2+}, $FeCl_3$ plus ascorbate or $FeCl_3$-ADP plus NADPH (in the case of microsomes). In metal ion-dependent systems, an added antioxidant might act not only by scavenging peroxyl radicals but also by binding iron ions and stopping them from accelerating peroxidation. However, these two possibilities can be distinguished easily. If the antioxidant is acting by metal binding, it will not be consumed during the reaction, as shown by direct analysis (e.g. by HPLC). A chain-breaking antioxidant is consumed by reaction with peroxyl radicals in the membrane. Chain-breaking antioxidants at low concentrations often introduce a lag period into the peroxidation process, corresponding to the time taken for the antioxidant to be consumed, whereas metal-binding antioxidants will give a constant inhibition throughout the reaction.

Most lipid peroxidation *in vivo* is metal ion-dependent, probably involving iron[1,39,64,207] The author therefore feels that the most biologically-relevant evaluation of ability to inhibit lipid peroxidation is achieved by testing a putative antioxidant against iron-stimulated lipid peroxidation in liposomes, microsomes or other biological membranes, paying particular attention to possible artefacts in the assay methods used[1,210–212] and to checking what is actually happening to the antioxidant in the reaction mixture. It should, of course, be noted that microsomal fractions will already contain variable amounts of endogenous antioxidants, such as α-tocopherol.[205] If peroxidation is started by adding NADPH plus Fe^{3+}-ADP to the microsomes, a control should also be performed to check that an added compound does not inhibit enzymic reduction of the iron complex.[207] This is usually done by measuring consumption of NADPH. Addition of NADPH to microsomes also activates the cytochrome P-450 system, which is capable of metabolizing certain antioxidants. Thus the observed inhibitory effect of chlorpromazine on NADPH-dependent peroxidation of microsomes was claimed to be due to its conversion into antioxidant hydroxylated derivatives by microsomal mixed function oxidases.[213]

Peroxidation may also be started by adding Fe^{3+}/ascorbate mixtures. This avoids problems with the mixed function oxidase system, but it should be noted that ascorbate may well be capable of chemically reducing lipid-soluble radicals (derived by reaction of the antioxidant with peroxyl radicals), back to the antioxidant molecule, so facilitating antioxidant action. Such a reaction will only occur, of course, if the antioxidant-derived radicals become accessible for reduction at the membrane surface, as has been shown to be the case for α-tocopheryl radicals in membranes, and in low-density lipoproteins isolated from human plasma.[10,104,214–216] Thus it is possible that the antioxidant activity of some lipid-soluble chain-breaking antioxidants might appear to be greater in systems containing ascorbic acid, as has been observed with some plant phenolics.[112] Hence it is wise to compare antioxidant ability using peroxidation started by several different mechanisms (e.g. Table 3). The importance of checking that an antioxidant in lipid systems does not exert pro-oxidant effects in non-lipid systems[108,112] has already been stressed.

Phagocyte-derived oxidants

Methods for isolating phagocytic cells and measuring their production of O_2^- and H_2O_2 are well-described in the literature (e.g.[114]) and in a recent book.[113,127] Standard methods[41,127] can be used to check the ability of compounds to interfere with phagocyte oxidant production after triggering the respiratory burst. It is essential to ensure that compounds do not interfere with the methods used to measure oxidant production, e.g. by directly reducing cytochrome c or by interfering with peroxidase-based measurement of H_2O_2 (see previous sections). The concentrations of compounds tested must also be comparable to those present *in vivo*.

In recent studies, Ames *et al.*[201,217] have used neutrophils, activated by exposure to phorbol myristate acetate, as a *physiological* source of oxidants, in order to examine the antioxidant defences in human plasma. Ascorbic acid was the first product to be oxidized: its complete consumption was associated with the detection of lipid peroxidation products in plasma lipids. Depletion of ascorbic acid has already been noted to occur at sites of inflammation,[218,219a] such as in synovial fluid from the knee-joints of patients with active rheumatoid arthritis.[218,219] Ascorbic acid oxidation might occur by its reaction with O_2^- [219] and HOCl,[133] and by its ability to reduce α-tocopheryl radicals at the surface of membranes.[214,216]

Compounds can also be tested for their potential to interfere with tissue damage by HOCl, which they could do by scavenging HOCl and/or by inhibiting its production by myeloperoxidase. Myeloperoxidase can be assayed in several ways including standard tests of peroxidase activity[1] (e.g. its ability to oxidize guaiacol to a chromogen in the presence of H_2O_2 [220]) or by specific measurements of HOCl production using monochlorodimedon [discussed in[221]]. Often the former type of assay is easier to use in looking for inhibition, since the latter type of assay is prone to interference if compounds that can also act as HOCl scavengers are tested. If an apparent inhibition of myeloperoxidase is found, it should be checked whether the compound is really inhibiting myeloperoxidase or is simply acting as a competing substrate (perhaps being oxidized to damaging products).[138,139,142]

If it has been established that a compound does not inhibit myeloperoxidase directly, then scavenging of HOCl can be examined using myeloperoxidase/H_2O_2/Cl^- as a source of this substance.[141] More simply, HOCl can be made as required by acidifying commercial sodium hypochlorite (Na^+OCl^-) to pH 6.2 and using a molar absorption coefficient of 100 at 235 nm to calculate its concentration.[222] Thus a concentration of the putative antioxidant that is achievable *in vivo* can be mixed with α_1-antiproteinase, a protein that is highly susceptible to attack by HOCl. A good scavenger of HOCl should protect α_1-antiproteinase against inactivation when HOCl is added subsequently. Such a method has been used to show that 5-aminosalicylate,[136] N-acetylcysteine,[37] ascorbic acid,[133] albumin,[12] some anti-inflammatory drugs,[137] bile pigments,[134] hypotaurine,[183] mercaptopropionylglycine[223] and tetracycline[224] could conceivably act as scavengers of HOCl *in vivo*.

If a substance fails to protect α_1-antiproteinase against inactivation by HOCl in this assay system, there are two possible explanations. Firstly, its reaction with HOCl may be too slow, or nonexistent. Secondly, its reaction with HOCl may form a "long-lived" oxidant that is also capable of inactivating α_1-antiproteinase. Taurine has been shown to do this[125] and the possibility must always be considered for other compounds.

Singlet oxygen

Singlet O_2 can be easily generated by photosensitization reactions, but it is important to ensure that any damage caused to a biological molecule in such systems is due to singlet O_2 rather than by direct interaction with the excited state of the sensitizer or by reactions involving other oxidants, such as O_2^- and ·OH, that can sometimes be generated in illuminated pigment-containing systems.[1] A simple technique has been described[225] in which singlet O_2 is generated by an immobilized sensitizer and allowed to diffuse a short distance to react with the target molecule. This system has proved useful for studying biological damage produced by singlet O_2 [226] and it should be easily applicable to studies of quenching and scavenging activity. Both ascorbic acid[172-174] and some sulphur-containing compounds (e.g.[227]) might be efficient protectors against singlet O_2 generated in aqueous media, whereas carotenoids may play a similar role in membranes under certain circumstances.[48,165,176-178,228,105a] Singlet O_2 can also be generated by the thermal decomposition of endoperoxides, such as 3,3′-(1,4-naphthylidene)dipropanoate.[105a]

Ferryl species

Mixtures of H_2O_2 and haemoglobin or myoglobin stimulate peroxidation of fatty acids and of microsomes, apparently by the action of haem-associated ferryl radicals.[148-151,155] The ability of a substance to react with ferryl species can be examined spectrophotometrically by looking for loss of the ferryl myoglobin (or haemoglobin) spectrum. Such studies have been carried out with ascorbic acid, desferrioxamine, thiol compounds and salicylic acid.[155-159,223,229,230] A good "quencher" of ferryl, such as ascorbate, will inhibit ferryl-dependent peroxidation of fatty acids or membrane lipids. In using this assay it is essential to check that the compound does not act as a chain-breaking antioxidant as well.[155-159,223]

Exposure of haem proteins to a large molar excess of H_2O_2 causes haem breakdown and iron ion release (Figure 3). Some antioxidants, such as ascorbic acid, prevent this process by reducing the ferryl species. The ability to inhibit release of iron ions provides an additional assay method for testing the effect of putative antioxidants on haem protein-H_2O_2 systems.[223]

PROVING THAT A PUTATIVE ANTIOXIDANT IS IMPORTANT *IN VIVO*

The battery of tests outlined above enables one to examine the *possibility* that a given compound could act as an antioxidant in one or more ways *in vivo*. The tests may show that an antioxidant role is unlikely, as has been concluded for taurine.[183] 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*. In the latter case, how then can one prove that the compound actually does act as an antioxidant *in vivo*?

In some cases involving naturally-occurring antioxidants, it has been possible to remove the compound and look for increased oxidant damage. Thus mutants of *E. coli* genetically-engineered to lack both MnSOD and FeSOD show severe damage when grown aerobically,[231,232] and damage can be minimized by introducing a gene coding for SOD, even mammalian CuZnSOD.[233] This and other[234,235] experiments clearly illustrate the physiological antioxidant role of SOD. For antioxidants of dietary origin, the effect of removing them from the diet can sometimes be studied.

Thus examination of patients with disorders of intestinal fat absorption has shown that severe deficiency of vitamin E in humans produces neurological symptoms that are consistent with, although certainly not yet proved to be caused by, increased oxidative damage *in vivo*.[9,236]

For most putative antioxidants, such as uric acid, this approach has not been feasible. Evidence consistent with an antioxidant role *in vivo* can then be provided by at least two types of method. Firstly, is the compound depleted under conditions of oxidant stress? Thus ascorbic acid is rapidly oxidized in synovial fluid in the knee-joints of patients with rheumatoid arthritis.[218,219] Presumably ascorbate is acting to scavenge oxidants derived from the many activated phagocytes present.[133,219] Ascorbate is also rapidly oxidized in the plasma of patients with adult respiratory distress syndrome, in which there is often massive infiltration of neutrophils into the lung, where they become activated.[219a] However, antioxidant action *in vivo* need not necessarily result in depletion of the antioxidant. Thus the α-tocopheryl radical can be "repaired" by ascorbic acid, as discussed previously.

Secondly, if a compound acts as a radical scavenger, it might be degraded to form specific products whose concentrations increase during oxidant stress, and can be measured. Thus oxidation of uric acid by ·OH, HOCl or ferryl species produces a range of products, including allantoin.[237] Increased allantoin concentrations have been detected in the plasma of patients with rheumatoid arthritis,[238] a disease in which oxidant formation *in vivo* is known to be increased.[1,34] This perhaps suggests that uric acid is exerting at least some scavenging activity *in vivo*.[237]

As far as the ability of administered "foreign" compounds (such as drugs) to act as antioxidants *in vivo* is concerned, specific assays are now becoming available to measure rates of DNA damage and lipid peroxidation due to oxidant attack *in vivo* (reviewed in[239]). For example, when the putative antioxidant probucol is administered to humans, low-density lipoprotein subsequently isolated from the plasma is more resistant to peroxidation *in vitro*, showing that suggestions that probucol acts as an inhibitor of peroxidation *in vivo*[240] are at least feasible. Methods for measuring rates of formation of H_2O_2,[1,43,241-245] and possibly ·OH[116,239,246,247] *in vivo* are now available and may be of use in assessments of antioxidant ability.

TABLE 4
Antioxidant roles of ascorbic acid

Scavenges O_2^- and HO_2· (overall rate constant about $2.7 \times 10^5 M^{-1}s^{-1}$ at pH 7.4).
Scavenges water-soluble peroxyl (RO_2·) radicals.
Scavenges thiyl and sulphenyl radicals.
"Repairs", and so prevents damage by, radicals arising by attack of ·OH upon uric acid.
Can reduce carcinogenic nitrosamines to inactive products.
Powerful scavenger of HOCl and an alternative substrate for myeloperoxidase (slowing HOCl formation).
Quencher and scavenger of singlet oxygen.
Inhibits lipid peroxidation by haemoglobin- or myoglobin-H_2O_2 mixtures, and prevents haem breakdown to release iron ions, by being preferentially oxidized by ferryl proteins.
Powerful scavenger and quencher of singlet O_2 in aqueous solution.
Regenerates α-tocopheryl radicals in membranes, so generating a renewable lipid-soluble chain-breaking antioxidant (α-tocopherol).
Scavenges nitroxide radicals, e.g. the radicals formed by attack of O_2^- or ·OH upon desferrioxamine.[185]
Scavenges ·OH radicals (rate constant $> 10^9 M^{-1}s^{-1}$).
Protects plasma lipids against peroxidation induced by activated neutrophils.[201,217]
May protect against oxidants present in cigarette smoke.[249]

CAN ANTIOXIDANTS BE PRO-OXIDANT? THE CASE OF ASCORBIC ACID

The importance of testing putative antioxidants for pro-oxidant properties has already been stressed. Ascorbic acid has a multiplicity of antioxidant properties, as reviewed previously in this article and in[248]: these properties are summarized in Table 4. Ascorbate's ability to show antioxidant properties is related to the fact that the semidehydroascorbate radical is poorly reactive.[50,51] Enzymic systems exist *in vivo* to reduce this radical back to ascorbate at the expense of NADH (the NADH-semidehydroascorbate reductase enzyme) or of GSH (the dehydroascorbate reductase enzyme). However these enzymes seem to be largely intracellular[1] and so ascorbic acid is rapidly depleted in human extracellular fluids under conditions of oxidant stress, probably by the reactions

$$\text{ascorbate} \xrightarrow{\text{radical attack}} \text{semidehydroascorbate} \quad (3)$$

$$2\ \text{semidehydroascorbate} \xrightarrow{\text{disproportionation}} \text{ascorbate} + \text{dehydroascorbate} \quad (4)$$

$$\text{dehydroascorbate} \xrightarrow[\text{breakdown}]{\text{rapid non-enzymic}} \text{oxalate, threonate, other oxidation products} \quad (5)$$

Ascorbic acid has also been reported to show pro-oxidant properties (e.g.[33,250–253]). Thus H_2O_2 produced in the eye has been suggested to arise from the non-enzymic oxidation of ascorbate.[253] Mixtures of copper or iron salts and ascorbic acid are well-known to stimulate lipid peroxidation and formation of reactive oxygen species such as $\cdot OH$.[1,205] Indeed, all of the reported pro-oxidant properties of ascorbate probably involve its interaction with transition metal ions.[33,250,254,255] Thus ascorbate has sometimes been described as mutagenic when to cells in culture[251] but metal ions added to, or contaminating, the culture media could easily account for this.[1]

One of the major antioxidant defences in mammals is the sequestration of transition metal ions in forms incapable of stimulating free radical reactions.[1,12,38,39,57] Thus iron and copper complexes capable of accelerating such reactions are not generally available in the extracellular fluids of the human body, except in some "metal overload" disease states such as iron overload[1,12,38,39,57,256–259] and after severe injury, when metal ions can be released as a result of cell destruction.[39,208,260,261] It therefore follows that the antioxidant properties of ascorbic acid in the human body will normally predominate, a conclusion supported by the available clinical evidence.[177,261,262] However, administration of ascorbic acid to iron-overloaded patients is contra-indicated unless desferrioxamine is being simultaneously given.[33]

Acknowledgements

I am very grateful to Dr. John Gutteridge for his helpful comments on this review article.

References

1. Halliwell, B. and Gutteridge, J.M.C. *Free Radicals in Biology and Medicine*, second edition, Clarendon Press, Oxford, UK, (1989).
2. Scott, G. Potential toxicological problems associated with antioxidants in plastic and rubber consumables. *Free Rad. Res. Commun.* **5**, 141–147, (1988).

3. Grootveld, M. and Jain, R. Recent advances in the development of a diagnostic test for irradiated foodstuffs. *Free Rad. Res. Commun.*, **6,** 271–292, (1989).
4. Daniels, V. Oxidative damage and the preservation of organic artefacts. *Free Rad. Res. Commun.*, **5,** 213–220, (1988).
5. Fridovich, I. Superoxide dismutases. An adaptation to a paramagnetic gas. *J. Biol. Chem.* **264,** 7761–7764, (1989).
6. Sies, H. (ed) *Oxidative Stress*, Academic Press, New York and London, (1985).
7. Beyer, W.F. Jr. and Fridovich I. Catalases – with and without heme. In *Oxygen Radicals in Biology and Medicine* (eds. M.G. Simic, K.A. Taylor, J.F. Ward and C. von Sonntag), Plenum Press, New York, pp. 651–661, (1988).
8. Gutteridge, J.M.C. and Stocks, J. Caeruloplasmin: physiological and pathological perspectives. *CRC Crit. Rev. Clin. Lab. Sci.* **14,** 257–329, (1981).
9. Diplock, A.T. Vitamin E. In *Fat – Soluble Vitamins* (ed. A.T. Diplock). Heinemann, London, pp. 154–224, (1985).
10. Niki, E. Antioxidants in relation to lipid peroxidation. *Chem. Phys. Lipids*, **44,** 227–253, (1987).
11. Burton, G.W. and Ingold, K.U. β-carotene, an unusual type of lipid antioxidant. *Science*, **224,** 569–573, (1984).
12. Halliwell, B. Albumin – an important extracellular antioxidant? *Biochem. Pharmacol.*, **37,** 569–571, (1988).
13. Thornalley, P.J. and Vasak, M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim. Biophys. Acta*, **827,** 36–44, (1985).
14. Boldyrev, A.A., Dupin, A.M., Bunin, A.Y., Babizhaev, M.A. Severin, S.E. The antioxidative properties of carnosine, a natural histidine containing dipeptide. *Biochem. Int.*, **15,** 1105–1113, (1987).
15. Kohn, R. Yamamoto, Y., Cundy, K.C. and Ames, B.N. Antioxidant activity of carnosine, homocarnosine and anserine present in muscle and brain. *Proc. Nat. Acad. Sci. USA*, **85,** 3175–3179, (1988).
16. Cross, C.E., Halliwell, B. and Allen, A. Antioxidant protection: a function of tracheobronchial and gastrointestinal mucus. *Lancet*, **i,** 1328–1330, (1984).
17. Graf, E. Applications of phytic acid. *J. Am. Oil Chem. Soc.*, **60,** 1861–1867, (1983).
18. Banks, M.A., Martin, W.G., Pailes, W.H. and Castranova, V. Taurine uptake by isolated alveolar macrophages and type II cells. *J. Appl. Physiol.*, **66,** 1079–1086, (1989).
19. Wright, C.E., Tallan, H.H., Lin, Y.Y. and Gaull, G.E. Taurine: biological update. *Ann. Rev. Biochem.*, **55,** 427–453, (1986).
20. Schurr, A. and Rigor, B.M. The mechanism of neuronal resistance and adaptation to hypoxia. *FEBS Lett.*, **224,** 4–8, (1987).
21. Stocker, R., Glazer, A.N. and Ames, B.N. Antioxidant activity of albumin-bound bilirubin. *Proc. Nat. Acad. Sci. USA*, **84,** 5918–5922, (1987).
22. Ames, B.N., Cathcart, R., Schwiers, E. and Hochstein, P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. *Proc. Nat. Acad. Sci. USA*, **78,** 6858–6862, (1981).
23. Sugioka, K., Shimosegawa, Y. and Nakano, M. Estrogens as natural antioxidants of membrane phospholipid peroxidation. *FEBS Lett.*, **210,** 37–39, (1987).
24. Glazer, A.N. Fluorescence-based assay for reactive oxygen species: a protective role for creatinine. *FASEB J.*, **2,** 2487–2491, (1988).
25. Hartman, P.E. Ergothioneine as an antioxidant. *Meth. Enzymol.*, in press.
26. Scholich, H., Murphy, M.E. and Sies, H. Antioxidant activity of dihydrolipoate against microsomal lipid peroxidation and its dependence on α-tocopherol. *Biochim. Biophys. Acta*, **1001,** 256–261, (1989).
27. Holler, T.P. and Hopkins, P.B. Ovothiols as biological antioxidants. The thiol groups of ovothiol and glutathione are chemically distinct. *J. Am. Chem. Soc.*, **110,** 4837–4838, (1988).
28. Landi, L., Cabrini, L., Sechi, A.M. and Pasquali, P. Anti-oxidative effect of ubiquinones on mitochondrial membranes. *Biochem. J.*, **222,** 463–466, (1984).
29. Tadolini, B. Polyamine inhibition of lipid peroxidation. *Biochem. J.*, **249,** 33–36, (1988).
30. D'Aquino, M., Dunster, C. and Willson, R.L. Vitamin A and glutathione-mediated free radical damage: competing reactions with polyunsaturated fatty acids and vitamin C. *Biochem. Biophys. Res. Commun.*, **161,** 1199–1203, (1989).
31. Ratty, A.K. and Das, N.P. Effects of flavonoids on non-enzymatic lipid peroxidation: structure-activity relationship. *Biochem. Med. Metabol. Biol.*, **39,** 69–79, (1988).

32. Wayner, D.D.M., Burton, G.W. and Ingold, K.U. The antioxidant efficiency of vitamin C is concentration-dependent. *Biochim. Biophys. Acta*, **884**, 119–123, (1986).
33. Halliwell, B. Ascorbic acid, iron overload and desferrioxamine. *Br. Med. J.*, **285**, 296, (1983).
34. Halliwell, B., Hoult, J.R.S. and Blake, D.R. Oxidants, inflammation and anti-inflammatory drugs. *FASEB J.*, **2**, 2867–2873, (1988).
35. Gutteridge, J.M.C., Richmond, R. and Halliwell, B. Inhibition of the iron-catalyzed formation of hydroxyl radicals from superoxide and lipid peroxidation by desferrioxamine. *Biochem. J.* **184**, 469–472, (1979).
36. Halliwell, B. Protection against tissue damage *in vivo* by desferrioxamine. What is its mechanism of action? *Free Rad. Biol. Med.*, in press.
37. Aruoma, O.I., Halliwell, B., Hoey, B.M. and Butler, J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid. *Free Rad. Biol. Med.*, **6**, 593–597, (1989).
38. Aruoma, O.I. and Halliwell, B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl radical generation? *Biochem. J.*, **241**, 273–278, (1987).
39. Halliwell, B. and Gutteridge, J.M.C. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.*, **246**, 501–514, (1986).
40. Fridovich, I. Superoxide radical: an endogeneous toxicant. *Ann. Rev. Pharmacol. Toxicol.*, **23**, 239–257, (1983).
41. Curnutte, J.T. and Babior, B.M. Chronic granulomatous disease. *Adv. Human Genet.*, **6**, 229–297, (1987).
42. Halliwell, B. Superoxide, iron, vascular endothelium and reperfusion injury. *Free Rad. Res. Commun.*, **5**, 315–318, (1989).
43. Chance, B., Sies, H. and Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.*, **59**, 527–605, (1979).
44. Bielski, B.H.J. Reactivity of HO_2/O_2^- radicals in aqueous solution. *J. Phys. Chem. Ref. Data*, **14**, 1041–1100, (1985).
45. Wefers, H. and Sies, H. Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen. *Eur. J. Biochem.*, **137**, 29–36, (1983).
46. Schoneich, C., Asmus, K.D., Dillinger, U. and Bruchhausen, Fv. Thiyl radical attack on polyunsaturated fatty acids: a possible route to lipid peroxidation. *Biochem. Biophys. Res. Common.*, **161**, 113–120, (1989).
47. Nishikimi, M. Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. *Biochem. Biophys. Res. Commun.*, **63**, 463–468, (1975).
48. Halliwell, B. Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chem. Phys. Lipids*, **44**, 327–340, (1987).
49. Varma, S.D. and Richards, R.D. Ascorbic acid and the eye lens. *Ophthal. Res.*, **20**, 164–173, (1988).
50. Cabelli, D.E. and Bielski, B.H.J. Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO_2/O_2^- radicals. A pulse radiolysis and stopped-flow photolysis study. *J. Phys. Chem.*, **87**, 1809–1812, (1983).
51. Bielski, B.H.J., Richter, H.W. and Chan, P.C. Some properties of the ascorbate free radical. *Ann. N. Y. Acad. Sci.*, **258**, 231–237, (1975).
52. Halliwell, B. and Butt, V.S. Flavin mononucleotide-sensitized photooxidation of glyoxylate in Good's buffers. *Biochem. J.*, **129**, 1157–1158, (1972).
53. Andrae, U., Singh, J. and Ziegler-Skylakakis, K. Pyruvate and related α-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. *Toxicol. Lett.*, **28**, 93–98, (1985).
54. Varma, S.D. Radio-isotopic determination of subnanomolar amounts of peroxide. *Free Rad. Res. Commun.*, **5**, 359–368, (1989).
55. Varma, S.D. and Morris, S.M. Peroxide damage to the eye lens *in vitro*. Prevention by pyruvate. *Free Rad. Res. Commun.*, **4**, 283–290, (1988).
56. O'Donnell-Tormey, J., Nathan, C.F., Lanks, K., De Boer, C.J. and de La Harpe, J. Secretion of pyruvate. An antioxidant defense of mammalian cells. *J. Exp. Med.*, **165**, 500–514, (1987).
57. Halliwell, B. and Gutteridge, J.M.C. Iron as a biological prooxidant. *ISI Atlas Sci., Biochem.*, **1**, 48–52, (1988).
58. Sutton, H.C. and Winterbourn, C.C. On the participation of higher oxidation states of iron and copper in Fenton reactions. *Free Rad. Biol. Med.*, **6**, 53–60, (1989).
59. Aruoma, O.I., Halliwell, B. and Dizdaroglu, M. Iron ion-dependent modification of bases in DNA

by the superoxide radical generating system hypoxanthine/xanthine oxidase. *J. Biol. Chem.*, **264,** 13024–13028, (1989).
60. Halliwell, B. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates. Is it a mechanism for hydroxyl radical production in biochemical systems? *FEBS Letts.*, **92,** 321–326, (1978).
61. McCord, J.M. and Day, E.D. Superoxide-dependent production of hydroxyl radical catalysed by an iron-EDTA complex. *FEBS Letts.*, **86,** 139–142, (1978).
62. Beauchamp, C. and Fridovich, I. A mechanism for the production of ethylene from methional. *J. Biol. Chem.*, **245,** 4641–4646, (1970).
63. Fong, K.L., McCay, P.B., Poyer, J.L., Misra, H.P. and Keele, B.B. Evidence for superoxide-dependent reduction of iron(III) and its role in enzyme-generated hydroxyl radical formation. *Chemico-Biol. Int.*, **15,** 77–89, (1976).
64. Halliwell, B. and Gutteridge, J.M.C. The importance of free radicals and catalytic metal ions in human disease. *Mol. Asp. Med.*, **8,** 89–193, (1985).
64a. Aruoma, O.I., Halliwell, B., Gajewski, E. and Dizdaroglu, M. Damage to the bases in DNA induced by hydrogen peroxide and ferric ion chelates. *J. Biol. Chem.*, **264,** 20509–20512, (1989).
65. Czapski, G., Goldstein, S. and Meyerstein, D. What is unique about superoxide toxicity as compared to other biological reductants? A hypthesis. *Free Rad. Res. Commun.*, **4,** 231–236, (1985).
66. Masarwa, M., Cohen, H., Meyerstein, D., Hickman, D.L. Bakac, A. and Espenson, J.H. Reactions of low-valent transition metal complexes with hydrogen peroxide. Are they "Fenton-like" or not? 1. The case of Cu^{+}_{aq} and Cr^{2+}_{aq}. *J. Am. Chem. Soc.*, **110,** 4293–4297, (1988).
67. Gutteridge, J.M.C. and Wilkins, S. Copper salt-dependent hydroxyl radical formation. Damage to proteins acting as anti-oxidants. *Biochim. Biophys. Acta*, **759,** 38–41, (1983).
68. Wolff, S.P. and Dean, R.T. Fragmentation of proteins by free radicals and its effect on their susceptibility to enzymic hydrolysis. *Biochem. J.*, **234,** 399–403, (1986).
69. Marx, G. and Chevion, M. Site-specific modification of albumin by free radicals. Reaction with copper(II) and ascorbate. *Biochem. J.*, **236,** 397–400, (1986).
70. Chiou, S.H., Chang, W.C., Jou, Y.S., Chung, H.M.M. and Lo, T.B. Specific cleavages of DNA by acorbate in the presence of copper ions or copper chelates. *J. Biochem. (Tokyo)*, **98,** 1723–17126, (1985).
71. Stoewe, R. and Prutz, W.A. Copper-catalyzed DNA damage by ascorbate and hydrogen peroxide: kinetics and yield. *Free Rad. Biol. Med.*, **3,** 97–105, (1987).
72. Brandi, G., Cattabeni, F., Albano, A. and Cantoni, O. Role of hydroxyl radicals in *Escherichia coli* killing by hydrogen peroxide. *Free Rad. Res. Commun.*, **6,** 47–55, (1989).
73. Imlay, J.A. and Linn, S. DNA damage and oxygen radical toxicity. *Science*, **240,** 1302–1309, (1988).
74. Kyle, M.E., Nakae, D., Sakaida, I., Miccadei, S. and Farber, J.L. Endocytosis of superoxide dismutase is required in order for the enzyme to protect hepatocytes from the cytotoxicity of hydrogen peroxide. *J. Biol. Chem.*, **263,** 3784–3789, (1988).
75. Halliwell, B. Oxidants and human disease – some new concepts. *FASEB J.*, **1,** 358–364, (1987).
76. Anbar, M. and Neta, P. A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqueous solution. *Int. J. Appl. Rad. Isotopes*, **18,** 495–523, (1967).
77. Aruoma, O.I. and Halliwell, B. The iron-binding and hydroxyl radical scavenging actions of anti-inflammatory drugs. *Xenobiotica*, **18,** 459–470, (1988).
78. Grootveld, M. and Halliwell, B. Aromatic hydroxylation as a potential measure of hydroxyl-radical formation *in vivo*. Identification of hydroxylated derivatives of salicylate in human body fluids. *Biochem. J.*, **237,** 499–504, (1988).
79. Larramendy, M., Mello-Filho, A.C., Leme Martins, E.A. and Meneghini, R. Iron-mediated induction of sister-chromatid exchanges by hydrogen peroxide and superoxide anion. *Mut. Res.*, **178,** 57–63, (1987).
80. Hebbel, R.P. Auto-oxidation and a membrane-associated "Fenton reagent": a possible explanation for development of membrane lesions in sickle erythrocytes. *Clin. Haematol.*, **14,** 129–140, (1988).
81. Hochstein, P., Sree Kumar K. and Forman, S.J. Lipid peroxidation and the cytoxicity of copper. *Ann. N. Y. Acad. Sci.*, **355,** 240–248, (1980).
82. Stam, H., Hulsmann, W.C., Jongkind, J.F., van der Kraaij, A.M.M. and Koster, J.F. Endothelial lesions, dietary composition and lipid peroxidation. *Eicosanoids*, **2,** 1–14, (1989).
83. Matsubara, T., Saura, Y., Hirohata, K. and Ziff, M. Inhibition of human endothelial cell proliferation in vitro and neo-vascularization in vivo by D-penicillamine. *J. Clin. Invest.*, **83,** 158–167,

(1989).
84. Rowley, D.A. and Halliwell, B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: a physiologically-significant reaction? *Arch. Biochem. Biophys.*, **225**, 279–284, (1983).
85. Uchida, K. and Kawakishi, S. Selective oxidation of imidazole ring in histidine residues by the ascorbic acid-copper ion system. *Biochem. Biophys. Res. Commun.*, **138**, 659–665, (1986).
86. Gardner, H.W. Oxygen radical chemistry of polyunsaturated fatty acids. *Free Rad. Biol. Med.*, **7**, 65–86, (1989).
87. Von Sonntag, C. *The Chemical Basis of Radiation Biology*, Taylor and Francis, London, (1988).
88. Steenken, S. Purine bases, nucleosides and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical cations and e^- and OH adducts. *Chem. Rev.*, **89**, 503–520, (1989).
89. Whitburn, K.D., Shieh, J.J., Sellers, R.M. Hoffman, M.Z. and Taub, I.A. Redox transformations in ferrimyoglobin induced by radiation generated free radicals in aqueous solution. *J. Biol. Chem.*, **257**, 1860–1869, (1982).
90. Nagy, I.Z. and Floyd, R.A. Hydroxyl free radical reactions with amino acids and proteins studied by electron spin resonance spectroscopy and spin-trapping. *Biochim. Biophys. Acta*, **790**, 238–250, (1984).
91. Kittridge, K.J. and Willson, R.L. Uric acid substantially enhances the free radical-induced inactivation of alcohol dehydrogenase. *FEBS Letts*, **170**, 162–164, (1984).
92. Aruoma, O.I. and Halliwell, B. Inactivation of α_1-antiproteinase by hydroxyl radicals. The effect of uric acid. *FEBS Letts*, **244**, 76–80, (1989).
93. Asmus, K.D. Sulfur-centered free radicals. in *Radioprotectors and Anticarcinogens* (ed. T.F. Slater) Academic Press, London, pp. 23–42, (1987).
94. Sevilla, M.D., Yan, M., Becker, D. and Gillich, S. ESR investigations of the reactions of radiation-produced thiyl and DNA peroxyl radicals: formation of sulfoxyl radicals. *Free Rad. Res. Commun.*, **6**, 21–24, (1989).
95. Aruoma, O.I., Halliwell, B., Butler, J. and Hoey, B.M. Apparent inactivation of α_1-antiproteinase by sulphur-containing radicals derived from penicillamine. *Biochem. Pharmacol.*, **38**, 4353–4357, (1989).
96. Davies, K.J.A. Sevanian, A., Muakkassah-Kelly, S.F. and Hochstein, P. Uric acid-iron complexes. *Biochem. J.*, **235**, 747–754, (1986).
97. Rowley, D.A. and Halliwell, B. Formation of hydroxyl radicals from NADH and NADPH in the presence of copper salts. *J. Inorg. Biochem.*, **23**, 103–108, (1985).
98. Fahey, R.C. Protection of DNA by thiols. *Pharmacol. Therapeut.*, **39**, 101–108, (1988).
99. Willson, R.L. Organic peroxy free radicals as ultimate agents in oxygen toxicity. *In Oxidative Stress* (ed. H. Sies), Academic Press, London, pp. 41–72, (1985).
100. Tamba, M., Simone, G., Quintiliani, M. and Del Soldato, P. Model experiments to evaluate the protective role of thiols and other reductants against oxidative damage. *Pharmacol. Therapeut.* **39**, 155–156, (1988).
101. Burton, G.W. and Ingold, K.U. Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.*, **19**, 194–201, (1986).
102. Landi, L., Pasquali, P., Bassi, P. and Cabrini, L. Effect of oxygen free radicals on ubiquinone in aqueous solution and phospholipid vesicles. *Biochim. Biophys. Acta*, **902**, 202–206, (1987).
103. Richards, D.M.C., Dean, R.T. and Jessup, W. Membrane proteins are critical targets in free radical mediated cytolysis. *Biochim. Biophys. Acta*, **946**, 281–288, (1988).
104. Esterbauer, H., Striegl, G., Puhl, H. and Rotheneder, M. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Rad. Res. Commun.*, **6**, 67–75, (1989).
105. Vile, G. and Winterbourn, C.C. Inhibition of adriamycin-promoted microsomal lipid peroxidation by β-carotene, α-tocopherol and retinol at high and low oxygen partial pressures. *FEBS Letts.*, **238**, 356–357, (1988).
105a. Di Maschio, P., Kaiser, S. and Sies, H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys.*, **274**, 532–538, (1989).
106. Esterbauer, H., Jurgens, G., Puhl, H. and Quehenberger, O. Role of oxidatively modified LDL in atherogenesis. In *Medical, Biochemical and Chemical Aspects of Free Radicals* (eds. O. Hayaishi, E. Niki, M. Kondo and T. Yoshikawa), Elsevier, Amsterdam, pp. 1203–1209, (1988).
107. Yamamoto, K. and Niki, E. Interaction of α-tocopherol with iron: antioxidant and pro-oxidant effects of α-tocopherol in the oxidation of lipids in aqueous dispersions in the presence of iron. *Biochim. Biophys. Acta.*, **958**, 19–23, (1988).

108. Aruoma, O.I., Evans, P.J., Kaur, H., Sutcliffe, L. and Halliwell, B. An evaluation of the antioxidant and potential pro-oxidant properties of food additives and of Trolox C, Vitamin E and probucol *Free Rad. Res. Commun.*, in press.
109. Gutteridge, J.M.C. and Xaio-Chang, F. Enhancement of bleomycin-iron free radical damage to DNA by anti-oxidants and their inhibition of lipid peroxidation. *FEBS Letts.*, **123,** 71–74, (1981).
110. Ochiai, M., Nagao, M., Wakabayashi, K. and Sugimura, T. Superoxide dismutase acts as an enhancing factor for quercetin mutagenesis in rat-liver cytosol by preventing its decomposition. *Mut. Res.*, **129,** 19–24, (1984).
111. Srivastava, A.K. and Padmanaban, G. Gossypol-mediated DNA degradation. *Biochem. Biophys. Res. Commun.*, **118,** 1515–1522, (1987).
112. Laughton, M.J., Halliwell, B., Evans, P.J. and Hoult, J.R.S. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. *Biochem. Pharmacol.*, **38,** 2859–2865, (1989).
113. Borregaard, N. The respiratory burst: an overview. In *The Respiratory Burst and its Physiological Significance* (eds. A.J. Sharma and P.R. Strauss) Plenum Press, New York, pp. 1–31, (1989).
114. Hurst, J.K. and Barrette, W.C., Jr. Leukocyte oxygen activation and microbicidal oxidative toxins. *CRC Crit. Rev. Biochem. Mol. Biol.*, **24,** 271–328, (1989).
115. Britigan, B.E., Rosen, G.M., Thompson, B.Y., Chai, Y. and Cohen, M.S. Stimulated neutrophils limit iron-catalyzed hydroxyl radical formation as detected by spin-trapping. *J. Biol. Chem.*, **261,** 17026–17032, (1986).
116. Kaur, H., Fagerheim, I., Grootveld, M., Puppo, A. and Halliwell, B. Aromatic hydroxylation of phenylalanine as an assay for hydroxyl radicals: application to activated human neutrophils and to the heme protein leghemoglobin. *Anal. Biochem.*, **172,** 360–367, (1988).
117. Halliwell, B., Gutteridge, J.M.C. and Blake, D.R. Metal ions and oxygen radical reactions in human inflammatory joint disease. *Phil. Trans. Royal Soc. series B,* **311,** 659–671, (1985).
118. Gutteridge, J.M.C., Paterson, S.K., Segal, A.W. and Halliwell, B. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. *Biochem. J.*, **199,** 259–261, (1981).
119. Repine, J.E., Fox, R.B. and Berger, E.M. Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radical. *J. Biol. Chem.*, **256,** 7094–7096, (1981).
120. Gutteridge, J.M.C. and Wilkins, S. Non-protein-bound iron within bacterial cells and the action of bleomycin. *Biochem. Int.*, **8,** 89–93, (1984).
121. Rosen, H. and Klebanoff, S.J. Oxidation of microbial iron-sulfur centers by the myeloperoxidase-H_2O_2-halide antimicrobial system. *Infect. Immun.*, **47,** 613–618, (1985).
122. Biemond, P., Swaak, A.J.G., Penders, J.M.A., Beindorff, C.M. and Koster, J.F. Superoxide production by polymorphonuclear leucocytes in rheumatoid arthritis and osteoarthritis: in vivo inhibition by the antirheumatic drug piroxicam due to interference with the activation of the NADPH oxidase. *Ann. Rheum. Dis.*, **45,** 249–255, (1986).
123. Kaplan, H.B., Edelson, H.S., Korchak, H.M., Given, W.P., Abramson, S. and Weissmann, G. Effects of non-steroidal anti-inflammatory agents on human neutrophil functions *in vitro* and *in vivo*. *Biochem. Pharmacol.*, **33,** 371–378, (1984).
124. Hurst, N.P., French, J.K., Bell, A.L., Nuki, G., O'Donnell, M.L., Betts, W.H. and Cleland, L.G. Differential effects of mepacrine, chloroquine and hydroxychloroquine on superoxide anion generation, phospholipid methylation and arachidonic acid release by human blood monocytes. *Biochem. Pharmacol.*, **35,** 3083–3089, (1986).
125. Weiss, S.J. Tissue destruction by neutrophils. *N. Eng. J. Med.*, **320,** 365–376, (1989).
126. Mayeno, A.N., Curran, A.J., Roberts, R.L. and Foote, C.S. Eosinophils preferentially use bromide to generate halogenating agents. *J. Biol. Chem.*, **264,** 5660–5668, (1989).
127. Sbarra, A.J. and Strauss, R.R. (eds.) *The Respiratory Burst and its Physiological Significance*, Plenum Press, New York, (1989).
128. Fliss, H. Oxidation of proteins in rat heart and lungs by polymorphonuclear leukocyte antioxidants. *Mol. Cell. Biochem.*, **84,** 177–178, (1988).
129. Kukreja, R.C., Weaver, A.B. and Hess, M.L. Stimulated human neutrophils damage cardiac sarcoplasmic reticulum function by generation of oxidants. *Biochim. Biophys. Acta*, **990,** 198–205, (1989).
130. Albrich, J.M., Gilbaugh III, J.H., Callahan, K.B. and Hurst, J.K. Effect of the putative neutrophil-generated toxin, hypochlorous acid, on membrane permeability and transport systems of *Escherichia coli*. *J. Clin. Invest.*, **78,** 177–184, (1989).
131. Clark, R.A., Stone, P.J., El Hag, A., Calore, J.D. and Franzblau, C. Myeloperoxidase-catalyzed inactivation of α_1-protease inhibitor by human neutrophils. *J. Biol. Chem.*, **256,** 3348–3353, (1981).
132. Wasil, M., Halliwell, B., Hutchison, D.C.S. and Baum, H. The antioxidant action of extracellular

fluids. Effect of human serum and its protein components on the inactivation of α_1-antiproteinase by hypochlorous acid and by hydrogen peroxide. *Biochem. J.*, **243,** 219–223, (1987).
133. Halliwell, B., Wasil, M. and Grootveld, M. Biologically-significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by ascorbic acid. Implications for antioxidant protection in the inflamed rheumatoid joint. *FEBS Letts,* **213,** 15–18, (1987).
134. Stocker, R. and Peterhans, E. Antioxidant properties of conjugated bilirubin and biliverdin: biologically relevant scavenging of hypochlorous acid. *Free Rad. Res. Commun.*, **6,** 57–66, (1989).
135. Olszowska, E., Olszowski, S., Zgliczynski, J.M. and Stelmaszynska, T. Enhancement of proteinase-mediated degradation of proteins modified by chlorination. *Int. J. Biochem.*, **21,** 799–805, (1989).
136. Aruoma, O.I., Wasil, M., Halliwell, B., Hoey, B.M. and Butler, J. The scavenging of oxidants by sulphasalazine and its metabolites. *Biochem. Pharmacol.*, **36,** 3739–3742, (1987).
137. Wasil, M., Halliwell, B., Moorhouse, C.P., Hutchison, D.C.S. and Baum, H. Biologically-significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by some anti-inflammatory drugs. *Biochem. Pharmacol.*, **36,** 3847–3850, (1987).
138. Kalyanaraman, B. and Sohnle, P.G. Generation of free radical intermediates from foreign compounds by neutrophil-derived oxidants. *J. Clin. Invest.*, **75,** 1618–1622, (1985).
139. Uetrecht, J.P. Idiosyncratic drug reactions: possible role of reactive metabolites generated by leukocytes. *Pharmacol. Rev.*, **6,** 265–273, (1989).
140. Cuperus, R.A., Muijsers, A.O. and Wever, R. Antiarthritic drugs containing thiol groups scavenge hypochlorite and inhibit its formation by myeloperoxidase from human leukocytes. *Arth. Rheum.*, **28,** 1228–1233, (1985).
141. Matheson, N.R. The effect of antiarthritic drugs and related compounds on the human neutrophil myeloperoxidase system. *Biochem. Biophys. Res. Commun.*, **108,** 259–265, (1982).
142. Svensson, B.E. and Lindvall, S. Myeloperoxidase-oxidase oxidation of cysteamine. *Biochem. J.*, **249,** 521–530, (1988).
143. Von Ritter, C., Grisham, M.B. and Granger, D.N. Sulfasalazine metabolites and dapsone attenuate formyl-methionyl-leucyl-phenylalanine-induced mucosal injury in rat ileum. *Gastroenterol.*, **96,** 811–816, (1989).
144. Ortiz de Montellano, P.R. Control of the catalytic activity of prosthetic heme by the structure of hemoproteins. *Acc. Chem. Res.*, **20,** 289–294, (1987).
145. Whitburn, K.D. The interaction of oxymyoglobin with hydrogen peroxide: a kinetic anomaly at large excesses of hydrogen peroxide. *Arch. Biochem. Biophys.*, **267,** 614–622, (1988).
146. Aviram, I., Wittenberg, B.A. and Wittenberg, J.B. The reaction of ferrous leghemoglobin with hydrogen peroxide to form leghemoglobin(IV). *J. Biol. Chem.*, **253,** 5685–5689, (1978).
147. Petersen, R.L., Symons, M.C.R. and Taiwo, F.A. Application of radiation and electron spin resonance spectroscopy to the study of ferryl myoglobin. *J. Chem. Soc., Faraday Trans. I,* **85,** 2435–2443, (1989).
148. Kanner, J., German, J.B. and Kinsella, J.E. Initiation of lipid peroxidation in biological systems. *CRC Crit. Rev. Food Sci. Nutr.*, **25,** 317–364, (1987).
149. Grisham, M.B. Myoglobin-catalyzed hydrogen peroxide dependent arachidonic acid peroxidation. *J. Free Rad. Biol. Med.*, **1,** 227–232, (1985).
150. Gutteridge, J.M.C. The antioxidant activity of haptoglobin towards haemoglobin-stimulated lipid peroxidation. *Biochim. Biophys. Acta,* **917,** 219–223, (1987).
151. Gutteridge, J.M.C. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Letts.*, **201,** 291–295, (1986).
152. Puppo, A. and Halliwell, B. Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglobin a biological Fenton reagent? *Biochem. J.*, **249,** 185–190, (1988).
153. Puppo, A. and Halliwell, B. Formation of hydroxyl radicals in biological systems. Does myoglobin stimulate hydroxyl radical formation from hydrogen peroxide? *Free Rad. Res. Commun.*, **4,** 415–422, (1988).
154. Yoshino, S., Blake, D.R., Hewitt, S., Morris, C. and Bacon, P.A. Effect of blood on the activity and persistence of antigen-induced inflammation in the rat air pouch. *Ann. Rheum. Dis.*, **44,** 485–490, (1985).
155. Mitsos, S.E., Kim, D., Lucchesi, B.R. and Fantone, J.C. Modulation of myoglobin-H_2O_2-mediated peroxidation reactions by sulfhydryl compounds. *Lab. Invest.*, **59,** 824–830, (1988).
156. Galaris, D., Cadenas, E. and Hochstein, P. Glutathione-dependent reduction of peroxides during ferryl- and met-myoglobin interconversion: a potential protective mechanism in muscle.*Free Rad. Biol. Med.*, **6,** 473–478, (1989).
157. Kanner, J. and Harel, S. Initiation of membranal lipid peroxidation by activated metmyoglobin and methemoglobin. *Arch. Biochem. Biophys.*, **237,** 314–321, (1985).

158. Harel, S. and Kanner, J. Haemoglobin and myoglobin as inhibitors of hydroxyl radical generation in a model system of "iron redox" cycle. *Free Rad. Res. Commun.*, **6**, 1–10, (1989).
159. Rice-Evans, C., Okunade, G. and Khan, R. The suppression of iron release from activated myoglobin by physiological electron donors and by desferrioxamine. *Free Rad. Res. Commun.*, **7**, 45–54, (1989).
160. Pathak, M.A. Molecular aspects of drug photosensitivity with special emphasis on psoralen photosensitization reaction. *J. Nat. Cancer Inst.*, **69**, 163–170, (1982).
161. Epstein, J.H. Chemical phototoxicity in humans. *J. Nat. Cancer Inst.*, **69**, 265–268, (1982).
162. Hall, R.D., Buettner, G.R., Motten, A.G. and Chignell, C.F. Near-infrared detection of singlet molecular oxygen produced by photo-sensitization with promazine and chlorpromazine. *Photochem. Photobiol.*, **46**, 295–300, (1987).
163. Hasan, T. and Khan, A.U. Phototoxicity of the tetracyclines: photosensitized emission of singlet delta dioxygen. *Proc. Nat. Acad. Sci. USA*, **85**, 4604–4606, (1986).
164. Dodge, A.D. and Knox, J.P. Photosensitizers from plants. *Pest. Sci.*, **17**, 579–586, (1986).
165. Mathews-Roth, M.M. Photoprotection by carotenoids. *Fed. Proc.*, **46**, 1890–1893, (1987).
166. Cortese, D.A. and Kinsey, J.H. Hematoporphyrin derivaive phototherapy in the treatment of bronchogenic carcinoma. *Chest*, **86**, 8–13, (1984).
167. Goosey, J.D., Zigler, J.S. Jr., Matheson, I.B.C. and Kinoshita, J.H. Effects of singlet oxygen on human lens crystallins in vitro. *Invest. Ophthal. Vis. Sci.*, **20**, 679–683, (1981).
168. Arudi, R.L., Bielski, B.H.J. and Allen, A.O. Search for singlet oxygen luminescence in the disproportionation of HO_2/O_2^-. *Photochem. Photobiol.*, **39**, 703–706, (1984).
169. Nagano, T. and Fridovich, I. Does the aerobic xanthine oxidase reaction generate singlet oxygen? *Photochem. Photobiol.*, **41**, 33–37, (1985).
170. Kanofsky, J.R., Wright, J., Miles-Richardson, G.E. and Tauber, A.I. Biochemical requirements for singlet oxygen production from purified human myeloperoxidase. *J. Clin. Invest.*, **74**, 1489–1495, (1984).
171. Wefers, H. Singlet oxygen in biological systems. *Bioelectrochemistry and Bioenergetics*, **18**, 91–104, (1987).
172. Chou, P.T. and Khan, A.U. L-ascorbic acid quenching of singlet delta molecular oxygen in aqueous media: generalized anti-oxidant property of vitamin C. *Biochem. Biophys. Res. Commun.*, **115**, 932–937, (1983).
173. Bodannes, R.S. and Chan, P.C. Ascorbic acid as a scavenger of singlet oxygen. *FEBS Letts.*, **105**, 195–196, (1979).
174. Kwon, B.M. and Foote, C.S. Chemistry of singlet oxygen. 50. Hydroperoxide intermediates in the photooxygenation of ascorbic acid. *J. Am. Chem. Soc.*, **110**, 6852–6853, (1988).
175. Neely, W.C., Martin, J.M. and Barker, S.A. Products and reaction rates of the oxidation of tocopherols with singlet molecular oxygen. *Photochem. Photobiol.*, **48**, 423–428, (1988).
176. Giles, A., Jr., Warner, W. and Kornhauser, A. *In vivo* protective effect of β-carotene against psoralen phototoxicity. *Photochem. Photobiol.*, **41**, 661–666, (1985).
177. Machlin, L.J. Influences of antioxidant vitamins on cataract formation. In *Medical, Biochemical and Chemical Aspects of Free Radicals* (eds. O. Hayaishi, E. Niki, M. Kondo and T. Yoshikawa), Elsevier, Amsterdam, pp. 351–359, (1988).
178. Bendich, A. and Olson, J.A. Biological action of carotenoids. *FASEB J.*, **3**, 1927–1932, (1989).
179. Butler, J., Hoey, B.M. and Lea, J.S. The measurement of radicals by pulse radiolysis. In *Free Radicals, Methodology and Concepts* (eds. C. Rice-Evans and B. Halliwell), Richelieu Press, London, pp. 457–479, (1988).
180. Bull, C., McClune, G.J. and Fee, J.A. The mechanism of Fe-EDTA catalyzed superoxide dismutation. *J. Am. Chem. Soc.*, **105**, 5290–5300, (1983).
181. McCord, J.M. and Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.*, **244**, 6049–6055, (1969).
182. Halliwell, B. Use of desferrioxamine as a probe for iron-dependent formation of hydroxyl radicals. Evidence for a direct reaction between desferal and the superoxide radical. *Biochem. Pharmacol.*, **34**, 229–233, (1985).
183. Aruoma, O.I., Halliwell, B., Hoey, B.M. and Butler, J. The antioxidant action of taurine, hypotaurine and their metabolic precursors. *Biochem. J.*, **256**, 251–255, (1988).
184. Aruoma, O.I., Laughton, M.J., and Halliwell, B. Carnosine, homocarnosine and anserine. Could they act as antioxidants in vivo? *Biochem. J.*, **264**, 863–869 (1989).
184a. Henry, L.E.A., Halliwell, B. and Hall, D.O. The superoxide dismutase activity of various photosynthetic organisms measured by a new and rapid assay technique. *FEBS Letts.*, **66**, 303–306, (1976).

185. Davies, M.J., Donkor, R., Dunster, C.A., Gee, C.A., Jonas, S. and Willson, R.L. Desferrioxamine (Desferal) and superoxide free radicals. Formation of an enzyme-damaging nitroxide. *Biochem. J.*, **246,** 725–729, (1987).
186. Boveris, A. Martino, E. and Stoppani, A.O.M. Evaluation of the horseradish peroxidase scopoletin method for the measurement of hydrogen peroxide formation in biological systems. *Anal. Biochem.*, **80,** 145–158, (1977).
186a. Corbett, J.T. The scopoletin assay for hydrogen peroxide. A review and a better method. *J. Biochem. Biophys. Meth.*, **18,** 297–308, (1989).
187. Schroy, C.B. and Biaglow, J.E. Use of an oxidase electrode to determine factors affecting the *in vitro* production of hydrogen peroxide by Ehrlich cells and 1-chloro-2,4-dinitrobenzene. *Biochem. Pharmacol.*, **30,** 3201–3207, (1981).
187a. Tatsuma, T., Okawa, Y. and Watanabe, T. Enzyme monolayer-and bilayer-modified tin oxide electrodes for the determination of hydrogen peroxide and glucose. *Anal. Chem.*, **61**, 2352–2355, (1989).
188. Halliwell, B. and Gutteridge, J.M.C. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. *FEBS Letts.*, **128,** 347–352, (1981).
189. Gutteridge, J.M.C. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. *FEBS Letts.*, **128,** 343–346, (1981).
190. Cheeseman, K.H., Beavis, A. and Esterbauer, H. Hydroxyl-radical-induced iron-catalyzed degradation of 2-deoxyribose. Quantitative determination of malondialdehyde. *Biochem. J.*, **252,** 649–653, (1988).
191. Halliwell, B., Gutteridge, J.M.C. and Aruoma, O.I. The deoxyribose method: a simple "test tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.*, **165,** 215–219, (1987).
192. Hoey, B.M., Butler, J. and Halliwell, B. On the specificity of allopurinol and oxypurinol as inhibitors of xanthine oxidase. A pulse radiolysis determination of rate constants for reaction of allopurinol and oxypurinol with hydroxyl radicals. *Free Rad. Res. Commun.*, **4,** 259–263, (1988).
193. Aruoma, O.I., Grootveld, M. and Halliwell, B. The role of iron in ascorbate-dependent deoxyribose degradation. Evidence consistent with a site-specific hydroxyl radical generation caused by iron ions bound to the deoxyribose molecule. *J. Inorg. Biochem.*, **29,** 289–299, (1987).
194. Aruoma, O.I., Chaudhary, S.S., Grootveld, M. and Halliwell, B. Binding of iron(II) ions to the pentose sugar 2-deoxyribose. *J. Inorg. Biochem.*, **35,** 149–155, (1989).
195. Gutteridge, J.M.C. Reactivity of hydroxyl and hydroxyl-like radical discriminated by release of thiobarbituric-acid-reactive material from deoxyribose, nucleosides and benzoate. *Biochem. J.*, **224,** 761–767, (1984).
196. Grootveld, M. and Halliwell, B. An aromatic hydroxylation assay for hydroxyl radicals utilizing high-performance liquid chromatography. Use to investigate the effect of EDTA on the Fenton reaction. *Free Rad. Res. Commun.*, **1,** 243–250, (1986).
197. Hiller, K.O., Hodd, P.L. and Willson, R.L. Anti-inflammatory drugs: protection of a bacterial virus as an in vitro biological measure of free radical activity. *Chemico-Biol. Int.*, **47,** 293–305, (1983).
198. Maples, K.R. and Mason, R.M. Free radical metabolite of uric acid. *J. Biol. Chem.*, **263** 1709-1712, (1988).
199. Wayner, D.D.M., Burton, G.W., Ingold, K.U., Barclay, L.R.C. and Locke, S.J. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. *Biochim. Biophys. Acta*, **924,** 408–419, (1987).
200. Thurnham, D.I., Situnayake, R.D., Koottathep, S., McConkey, B. and Davis, M. Antioxidant status as measured by the "TRAP" assay in rheumatoid arthritis. In *Free Radicals, Oxidant Stress and Drug Action* (ed. C. Rice-Evans), Richelieu Press, London, pp. 169–192, (1987).
201. Frei, B. Stocker, R. and Ames, B.N. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Nat. Acad. Sci. USA*, **85,** 9748–9752, (1988).
202. Lindeman, J.N.H., Zoeeren-Grobben, D.V., Schriver, J., Speek, A.J., Poorthuis, B.H.J. and Berger, H.M. The total free radical trapping ability of cord blood plasma in preterm and term babies. *Pediat. Res.*, **26,** 20–24, (1989).
203. Halliwell, B. and Gutteridge, J.M.C. Antioxidants of extra-cellular fluids. *Arch. Biochem. Biophys.*, submitted.
204. Darley-Usmar, V.M., Hersey, A. and Garland, L.G. A method for the comparative assessment of antioxidants as peroxyl radical scavengers. *Biochem. Pharmacol.*, **38,** 1465–1469, (1989).
205. Wills, E.D. Lipid peroxide formation of microsomes. The role of non-haem iron. *Biochem. J.*, **113,** 325–332, (1969).

206. Gutteridge, J.M.C. The effect of calcium on phospholipid peroxidation. *Biochem. Biophys. Res. Commun.*, **77,** 379–386, (1979).
207. Minotti, G. and Aust, S.D. The role of iron in the initiation of lipid peroxidation. *Chem. Phys. Lipids*, **44,** 191–208, (1987).
208. Stocks, J., Gutteridge, J.M.C. Sharp, R.J. and Dormandy, T.L. Assay using brain homogenate for measuring the antioxidant activity of biological fluids. *Clin. Sci. Mol. Med.*, **47,** 215–222, (1974).
209. Albro, P.W. Corbett, J.T. and Schroeder, J.L. Application of the thiobarbiturate assay to the measurement of lipid peroxidation products in microsomes. *J. Biochem. Biophys. Meth.*, **13,** 185–194, (1986).
210. Gutteridge, J.M.C. Apects to consider when detecting and measuring lipid peroxidation. *Free Rad. Res. Commun.*, **1,** 173–184, (1986).
211. Gutteridge, J.M.C. and Halliwell, B. Lipid peroxidation in biological systems – measurement and mechanism. *Trends Biochem. Sci.*, in press.
212. Pryor, W.A. Forum on the detection of lipid hydroperoxides in biological samples. *Free Rad. Biol. Med.*, **7,** 177–21, (1989).
213. Eluashvili, I.A., Pashinova, T.P., Bogdanova, E.D., Kagan, V.E. and Prilipko, L.L. Effect of chlorpromazine on enzymic oxidation of lipids. *Byull. Eksp. Biol. Med.*, **84,** 323–326, (1977).
214. Wefers, H. and Sies, H. The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. *Eur. J. Biochem.*,**174,** 353–357, (1988).
215. Liebler, D.C., Kling, D.S. and Reed, D.J. Antioxidant protection of phospholipid bilayers by α-tocopherol. *J. Biol. Chem.*, **261,** 12114–12119, (1986).
216. Packer, J.E., Slater, T.F. and Willson, R.L. Direct observation of a free radical interaction between vitamin E and vitamin C. *Nature*, **278,** 737–738, (1979).
217. Frei, B., England, L. and Ames, B.N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Nat. Acad. Sci. USA*, **86,** 6377–6381.
218. Lunec, J. and Blake, D.R. The determination of dehydroascorbic acid and ascorbic acid in the serum and synovial fluid of patients with rheumatoid arthritis. *Free Rad. Res. Commun.*, **1,** 31–39, (1985).
219. Blake, D.R., Hall, N.D., Treby, D.A. Halliwell, B. and Gutteridge, J.M.C. Protection against superoxide and hydrogen peroxide in synovial fluid from rheumatoid patients. *Clin. Sci*, **61,** 483–486, (1981).
219a. Cross, C.E., Forte, T., Stocker, R., Louie, S., Yamamoto, Y., Ames, B.N. and Frei, B. Oxidative stress and abnormal cholesterol metabolism in patients with adult respiratory distress syndrome. *J. Lab. Clin. Med.*, in press.
220. Iwamoto, H., Kobayashi, T., Hasegawa, E. and Morita, Y. Reactions of human myeloperoxidase with hydrogen peroxide and its true catalase activity. *J. Biochem. (Tokyo)*, **101,** 1407–1412, (1987).
221. Kettle, A.J. and Winterbourn, C.C. The mechanism of myeloperoxidase-dependent chlorination of monochlorodimendon. *Biochim. Biophys. Acta*, **957,** 185–191, (1988).
222. Green T.R., Fellman, J.H. and Eicher, A.L. Myeloperoxidase oxidation of sulfur-centered and benzoic acid hydroxyl radical scavengers. *FEBS Letts.*, **192,** 33–36, (1985).
223. Puppo, A., Aruoma, O.I., Bolli, R. and Halliwell, B. Biochemical mechanisms accounting for the cardioprotective action of mercapto-propionylglycine. *Biochem. J.*, submitted.
224. Wasil, M., Halliwell, B. and Moorhouse, C.P. Scavenging of hypochlorous acid by tetracycline, rifampicin and some other antibiotics: a possible antioxidant action of rifampicin and tetracycline? *Biochem. Pharmacol.*, **37,** 775–778, (1988).
225. Midden, W.R. and Wang, S.Y. Singlet oxygen generation for solution kinetics: clean and simple. *J. Am. Chem. Soc.*, **105,** 4129–4135, (1983).
226. Dahl, T.A., Midden, W.R. and Hartman, P.E. Pure exogenous singlet oxygen: nonmutagenicity in bacteria. *Mut. Res.*, **201,** 127–136, (1988).
227. Rougee, M., Bensasson, R.V., Land, E.J. and Pariente, R. Deactivation of singlet molecular oxygen by thiols and related compounds, possible protectors against skin photosensitivity. *Photochem. Photobiol.*, **47,** 485–489, (1988).
228. Krinsky, N.I. and Deneke, S.M. Interaction of oxygen and oxy-radicals with carotenoids. *J. Nat. Cancer Inst.*, **69,** 205–210, (1982).
229. Puppo, A. and Halliwell, B. Generation of hydroxyl radicals by soybean nodule leghaemoglobin. *Planta*, **173,** 405–410, (1988).
230. Kanner, J. and Harel, S. Desferrioxamine as an electron donor. Inhibition of membranal lipid peroxidation initiated by H_2O_2-activated metmyoglobin and other peroxidizing systems. *Free Rad. Res. Commun.*, **3,** 309–317, (1987).
231. Touati, D. The molecular genetics of superoxide dismutase in *E. coli*. An approach to understand-

ing the biological role and regulation of SODs in relation to other elements of the defence system against oxygen toxicity. *Free Rad. Res. Commun.*, **8**, 1–9, (1989).
232. Schellhorn, H. and Hassan, H.M. Response of hydroperoxidase and superoxide dismutase deficient mutants of *Escherichia coli* K-12 to oxidative stress. *Can. J. Microbiol.*, **34**, 1171–1176, (1988).
233. Natvig, D.O., Imlay, K., Touati, D. and Hallewell, R.A. Human copper-zinc superoxide dismutase complements superoxide dismutase-deficient *Escherichia coli* mutants. *J. Biol. Chem.*, **262**, 14697–14701, (1989).
234. Van Loon, A.P.G.M., Pesold-Hurt, B. and Schatz, G. A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc. Nat. Acad. Sci. USA*, **83**, 3820–3824, (1986).
235. Phillips, J.P., Campbell, S.D., Michaud, D., Charbonneau, M. and Hilliker, A.J. Null mutation of copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat and reduced longevity. *Proc. Nat. Acad. Sci. USA*, **86**, 2761–2765, (1989).
236. Harding, A.E. Matthews, S., Jones, S., Ellis, C.J.K., Booth, I.W. and Muller, D.P.R. Spinocerebellar degeneration associated with a selective defect of vitamin E absorption. *N. Eng. J. Med.*, **313**, 32–35, (1985).
237. Kaur, H. and Halliwell, B. Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chemico-Biological Int.*, in press.
238. Grootveld, M. and Halliwell, B. Measurement of allantoin and uric acid in human body fluids. A potential index of free radical reactions in vivo? *Biochem. J.*, **243**, 803–808, (1987).
239. Rice-Evans, C. and Halliwell, B. (eds). *Free Radicals: Methodology Concepts*, Richelieu Press, London, (1988).
240. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Eng. J. Med.*, **320**, 915–924, (1989).
241. Curtis, W.E., Muldrow, M.E., Parker, N.B., Barkley, R. Linas, S.L. and Repine, J.E. N,N′-dimethylthiourea dioxide formation from N,N′-dimethylthiourea reflects hydrogen peroxide concentrations in simple biological systems. *Proc. Nat. Acad. Sci. USA*, **85**, 3422–3425, (1988).
242. Sasaki, F., Kinoshita, T., Takahama, H. and Watanabe, K. Cytochemical studies of hydrogen peroxide production in the tadpole tail of *Rana japonica* during metamorphic climax, *Histochem. J.*, **20**, 99–107, (1988).
243. Oshino, N., Jamieson, D. and Chance, B. The properties of hydrogen peroxide production under hyperoxic and hypoxic conditions of perfused rat liver. *Biochem. J*, **146**, 53–65, (1975).
244. Yusa, T., Beckman, J.S., Crapo, J.D. and Freeman, B.A. Hyperoxia increases H_2O_2 production by brain *in vivo*. *J. Appl. Physiol.*, **63**, 353–358, (1987).
245. Foerster, E.C., Fahrenkemper, T., Rube, U., Graf, P. and Sies, H. Peroxisomal fatty acid oxidation as detected by H_2O_2 production in intact perfused liver. *Biochem. J.*, **196**, 705–712, (1981).
246. Halliwell, B. and Grootveld, M. The measurement of free radical reactions in humans. Some thoughts for future experimentation. *FEBS Letts.*, **213**, 9–14, (1987).
247. Babbs, C.F. and Griffin, D.W. Scatchard analysis of methane sulfinic acid production from dimethyl sulfoxide: a method to quantify hydroxyl radical formation in physiologic systems. *Free Rad. Biol. Med.*, **6**, 493–503, (1989).
248. Bendich, A., Machlin, L.J., Scandurra, O., Burton, G.W. and Wayner, D.D.M. The antioxidant role of vitamin C. *Adv. Free Rad. Biol. Med.*, **2**, 419–444, (1986).
249. Chow, C.K., Thacker, R.R., Changchit, C., Bridges, R.B., Rehm, S.R., Humble, J. and Turbek, J. Lower levels of vitamin C and carotenes in plasma of cigarette smokers. *J. Am. Coll. Nut.*, **5**, 305–312, (1986).
250. Davison, A.J., Kettle, A.J. and Fatur, D.J. Mechanism of the inhibition of catalase by ascorbate. Roles of active oxygen species, copper and semidehydroascorbate. *J. Biol. Chem.*, **261**, 1193–1200, (1986).
251. Shamberger, R.J. Genetic toxicology of ascorbic acid *Mut. Res.*, **133**, 135–159, (1984).
252. Acedo, G.N. Redei, G.P. and Sandhu, S.S. Mutagenicity of ascorbic acid for the plant Arabidopsis. *Mut. Res.*, **129**, 57–62, (1984).
253. Giblin, F.J., McCready, J.P., Kodama, T. and Reddy, V.N. A direct correlation between the levels of ascorbic acid and H_2O_2 in aqueous humor. *Exp. Eye Res.*, **38**, 87–93, (1984).
254. Halliwell, B. and Foyer, C.H. Ascorbic acid, metal ions and the superoxide radical. *Biochem. J.*, **155**, 697–700, (1976).
255. Buettner, G.R. Ascorbate oxidation in the presence of iron and copper chelates. *Free Rad. Res. Commun.*, **1**, 349–353, (1986).

256. Gutteridge, J.M.C. Copper-phenanthroline induced site specific oxygen radical damage to DNA. Detection of loosely bound trace copper in biological fluids. *Biochem. J.*, **218,** 983–985, (1984).
257. Gutteridge, J.M.C., Rowley, D.A., Griffiths, E. and Halliwell, B. Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. *Clin. Sci.*, **68,** 463–467, (1985).
258. Grootveld, M., Bell, J.D., Halliwell, B., Aruoma, O.I., Bomford, A. and Sadler, P.J. Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. *J. Biol. Chem.*, **264,** 4417–4422, (1989).
259. Evans, P.J., Bomford, A. and Halliwell, B. Non-caeruloplasmin copper and ferroxidase activity in mammalian serum. Ferroxidase activity and phenanthroline-detectable copper in human serum in Wilson's disease. *Free Rad. Res. Commun.*, **7,** 55–62, (1989).
260. Halliwell, B., Aruoma, O.I., Mufti, G. and Bomford, A. Bleomycin-detectable iron in serum from leukaemic patients before and after chemotherapy. Therapeutic implications for treatment with oxidant-generating drugs. *FEBS Letts.*, **241,** 202–204, (1988).
261. Halliwell, B. Oxidants and the central nervous system: some fundamental questions. *Acta Neurol. Scand.*, in press
262. Gey, K.F., Stahelin, H.B. and Brubacher, G.B. Plasma levels of essential antioxidants inversely related to subsequent cancer. In *Medical, Biochemical and Chemical Aspects of Free Radicals* (eds. O. Hayaishi, E. Niki, M. Kondo and T. Yoshikawa), Elsevier, Amsterdam, pp. 377–384, (1988).

Accepted by Prof. H. Esterbauer