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Review Section

The Characterization of Antioxidants

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Abstract—The role of antioxidants in nutrition is an area of increasing interest. Antioxidants are used (1) to prolong the shelf life and maintain the nutritional quality of lipid-containing foods, and (2) to modulate the consequences of oxidative damage in the human body. This review discusses what an antioxidant is and how the properties of antioxidants may be characterized.

Introduction: what is an antioxidant?

Antioxidants are of interest to the food industry, because they prevent rancidity (reviewed by Löliger, 1991). As an example, Fig. 1 shows some of the active antioxidant components in herbs, spices and vegetables. Antioxidants are also of interest to biologists and clinicians, because they may help to protect the human body against damage by reactive oxygen species (ROS)‡.

However, the term 'antioxidant' is very loosely used. How can it be defined? Often, the term is implicitly restricted to chain-breaking antioxidant inhibitors of lipid peroxidation, such as α-tocopherol. Indeed, food scientists often simply equate antioxidants to inhibitors of lipid peroxidation. However, free radicals generated *in vivo* damage many targets other than lipids, including proteins, DNA and small molecules. Hence a broader definition of 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' (Halliwell, 1990; Halliwell and Gutteridge, 1989). The term 'oxidizable substrate' includes almost everything found in foods and in living tissues including proteins, lipids, carbohydrates and DNA. This definition emphasizes the importance of the target chosen and the source of oxidative damage in characterizing an antioxidant.

When ROS are generated in living systems, a wide variety of antioxidants comes into play (Aruoma, 1993; Diplock, 1985 and 1994; Fridovich, 1989; Gutteridge and Stocks, 1981; Halliwell, 1990; Halliwell and Gutteridge, 1989; Sies, 1985). Many reviews have covered the well-established physiological antioxidant roles of α-tocopherol, ascorbic acid and proteins such as superoxide dismutase (SOD), glutathione peroxidase, catalase and caeruloplasmin (Diplock, 1985; Fridovich, 1989; Halliwell, 1994a; Halliwell and Gutteridge, 1989; Kappus and Diplock, 1992; Liebler, 1993; Sies, 1985; Sies *et al.*, 1992). From the food perspective, the role of superoxide dismutase in foods has been extensively discussed (Donnelly and Robinson, 1991).

The relative importance of these various antioxidants *in vivo* depends on which ROS is generated, how it is generated, where it is generated, and what target of damage is measured. For example, if human blood plasma is tested for its ability to inhibit iron ion-dependent lipid peroxidation, transferrin and caeruloplasmin are found to be the most important protective agents (Gutteridge and Quinlan, 1992; Gutteridge and Stocks, 1981). When plasma is exposed to the toxic gas nitrogen dioxide (found in polluted air), uric acid seems to exert protection against oxidative damage by this gas (Halliwell *et al.*, 1992). In contrast, when hypochlorous acid (HOCl) is added to plasma, uric acid plays little protective role (Hu *et al.*, 1992).

Similarly, if the oxidative stress is kept the same but a different *target* of oxidative damage is measured, the results can be different. For example,

Abbreviations: AAPH = 2,2′-azo*bis* (2-amidinopropane hydrochloride); AMVN = 2,2′-azo*bis* (2,4-dimethylvaleronitrile); BHA = butylated hydroxyanisole; ESR = electron spin resonance; GSH = reduced glutathione; NBT = nitro-blue tetrazolium; PUFA = polyunsaturated fatty acids; ROS = reactive oxygen species; SOD = superoxide dismutase; TBA = thiobarbituric acid.

‡ROS is a collective term used to include oxygen radicals ($O_2^{\cdot-}$, $OH^{\cdot}$, $RO_2^{\cdot}$, $RO^{\cdot}$ etc) and several non-radical oxidizing agents, such as HOCl, H_2O_2, O_3 and $ONOO^-$. Reactive is a relative term (e.g. $O_2^{\cdot-}$ is more reactive than O_2 but much less so than hydroxyl radical $OH^{\cdot}$ or HOCl).

when plasma is exposed to gas-phase cigarette smoke, lipid peroxidation occurs and ascorbic acid inhibits this process (Frei *et al.*, 1991). In contrast, ascorbic acid has no effect on damage to plasma proteins caused by cigarette smoke, as measured by the carbonyl assay (Reznick *et al.*, 1992).

Hence it is perfectly possible for an antioxidant to protect in one system but to fail to protect, or even sometimes to cause damage, in others. Antioxidant inhibitors of lipid peroxidation may not protect other targets (such as DNA and protein) against damage, and sometimes can even aggravate such damage (see below). This may not matter much in the food matrix, since damage to DNA and proteins, unless very excessive, will not alter the taste or texture of food (unlike lipid peroxidation). However, it is of the greatest importance *in vivo*. For example, butylated hydroxyanisole (BHA) is a powerful inhibitor of lipid peroxidation, and yet huge dietary doses of it can induce cancer of the rat forestomach, and it has been suggested that oxidative DNA damage could be involved (Life Science Research Office, 1994; Schildermann *et al.*, 1995).

A wide range of compounds has been suggested to act as antioxidants *in vivo* and in foods—from β-carotene and metallothionein to histidine-containing dipeptides (carnosine, homocarnosine, anserine), mucus, phytic acid, taurine, bilirubin, oestrogens, creatinine, dihydrolipoic acid, polyamines and melatonin. Antioxidant compounds derived from plants, especially phenols such as quercetin, carnosol, thymol, carnosic acid, hydroxytyrosol, gallic acid derivatives, tannins, catechins, rutin, morin, ellagic acid, eugenol and rosemarinic acids, are of considerable interest from the viewpoint of dietary antioxidant

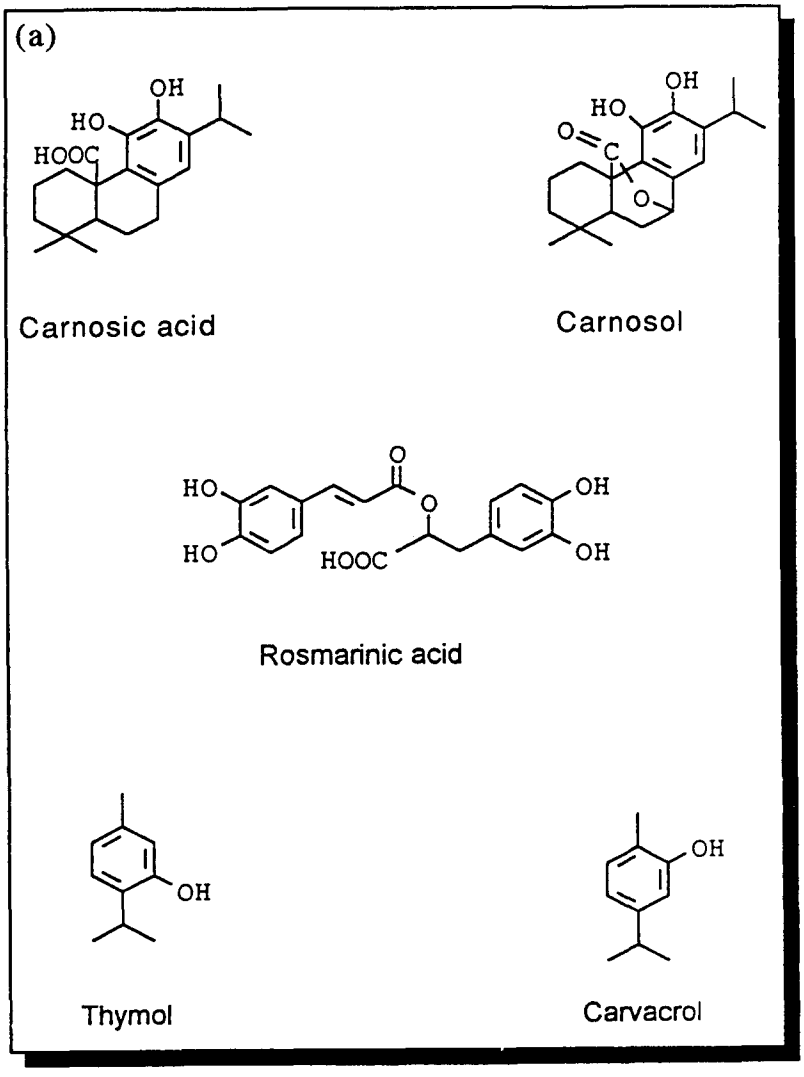

Fig. 1a—*Caption on p. 604*

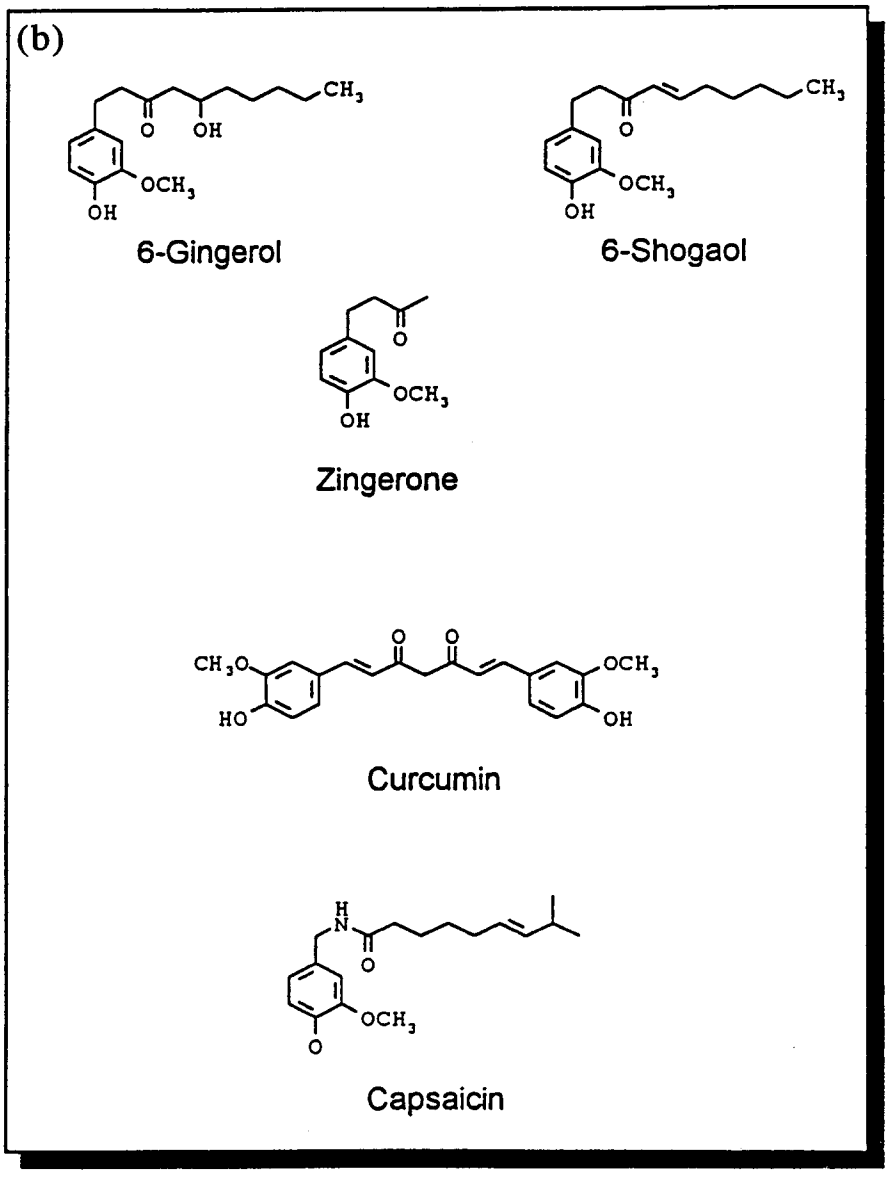

Fig. 1b—*Caption on p. 604*

supplementation and food preservation (Aeschbach *et al.*, 1994; Ahmed *et al.*, 1994; Anton, 1988; Aruoma, 1994a; Aruoma *et al.*, 1992; Bahorun *et al.*, 1994; Das, 1989; Ho *et al.*, 1994; Huang *et al.*, 1994; Hudson, 1990; Kahnau, 1976; Kinsella *et al.*, 1993; Larson, 1988; Laughton *et al.*, 1989; Löliger, 1991; Nagababu and Lakshmaiah, 1994; Page and Charbonneau, 1989; Ramanathan and Das, 1992 and 1993a; Regnault-Roger, 1988; Rice-Evans and Diplock, 1993; St Angelo, 1992; Wu *et al.*, 1994; Yoshida *et al.*, 1993). But how can they be evaluated as antioxidants? If they are absorbed into the body, will they be safe? This is an important question; the known carcinogen diethylstilboestrol is a powerful inhibitor of lipid peroxidation *in vitro* (Wiseman and Halliwell, 1993), while being a DNA-damaging agent *in vivo* (Roy and Liehr, 1991).

Basics of antioxidant characterization

A compound might exert antioxidant actions *in vivo* or in food by inhibiting generation of ROS, or by directly scavenging free radicals. Additionally, *in vivo* an antioxidant might act by raising the levels of endogenous antioxidant defences (e.g. by upregulating expression of the genes encoding SOD, catalase or glutathione peroxidase)—for reviews see Aruoma

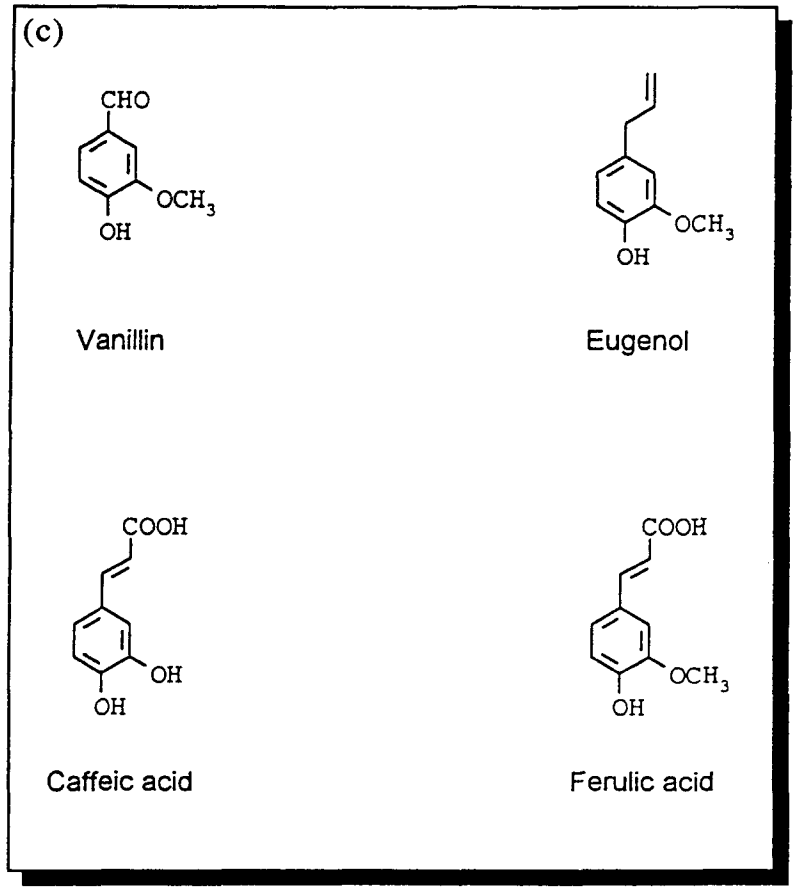

Fig. 1. Some of the many antioxidants found in plants.

(1994a) and Halliwell (1990). Some quite simple experiments can be performed to examine direct antioxidant ability *in vitro* and to test for possible pro-oxidant effects on different molecular targets. This 'screening' approach can be used to rule out direct antioxidant activity *in vivo*: a compound that is poorly effective *in vitro* will not be any better *in vivo*. It can also alert one to the possibility of damaging effects. During *in vitro* testing, it is essential to examine the action of a compound over a concentration range that is relevant to its intended use. For example, if compound X is present *in vivo* at concentrations less than 1 μM, its ability to inhibit lipid peroxidation only at concentrations greater than 20 mM is irrelevant unless there is good reason to suspect that it concentrates at a particular site *in vivo*. One must also bear in mind that, if the compound is acting as a scavenger, an 'antioxidant' may itself give rise to damaging radical species (radicals beget radicals). It is also important to use relevant reactive oxygen species and sources generating such species (Table 1); the choice will depend on whether effects *in vivo* or effects in the food matrix are being considered.

Important reactive oxygen species

Superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2)

Superoxide formed *in vivo* is largely—if not completely—converted by SOD-catalysed or non-enzymic dismutation, into H_2O_2 (Fridovich, 1989). SOD is also present in many uncooked foods (Donnelly and Robinson, 1991). Some enzymes, such as glycollate oxidase, also produce H_2O_2 directly *in vivo* (Chance *et al.*, 1979; Halliwell and Gutteridge, 1989). Unlike $O_2^{\cdot -}$, H_2O_2 is able to cross biological membranes (Halliwell and Gutteridge, 1989). Both $O_2^{\cdot -}$ and H_2O_2 can find some molecular targets to which they can do direct damage, 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 react with $O_2^{\cdot -}$ and H_2O_2 at rapid rates. For example, many thiols react with H_2O_2 and with $O_2^{\cdot -}$, but the rate constants for these reactions are low, usually less than 10^3 M^{-1} sec^{-1} (Bielski, 1985; Winterbourn and Metodiewa, 1994). Thus very high thiol concentrations (often greater than 1 mM) would be required to compete with non-enzymic dismutation

Table 1. Relevant ROS for examining potential antioxidants

In vivo	Food matrix
$O_2^{\cdot-}$ (superoxide radical	$O_2^{\cdot-}$
Singlet O_2	Singlet O_2
H_2O_2 (hydrogen peroxide)	H_2O_2
Lipid peroxides	Lipid peroxides
HOCl (hypochlorous acid)*	—
$RO^{\cdot}$ (alkoxyl radicals)†	$RO^{\cdot}$
$RO_2^{\cdot}$ (peroxyl radicals)†	$RO_2^{\cdot}$
$NO^{\cdot}$ (nitric oxide), $NO_2^{\cdot}$ (nitrogen dioxide), $ONOO^-$ (peroxynitrite), HNO_2 (nitrous acid)	NO_2^- and products derived from it (use of nitrite for preservation)
$OH^{\cdot}$ (hydroxyl radical)	$OH^{\cdot}$

*HOCl is produced by activated phagocytes in the human body. Many bleaches and disinfectants contain its sodium salt (NaOCl, sodium hypochlorite), so it is possible that food constituents might sometimes come into contact with this molecule. Irradiation of chloride ion-containing materials might also make HOCl under certain conditions (Czapski *et al.*, 1992).

†Radicals formed by the breakdown of lipid hydroperoxides, either thermally (as in heated oils or fats) or catalysed by transition metal ions (both in foods and *in vivo*).

of $O_2^{\cdot-}$ and achieve significant scavenging. It is therefore very unlikely that most thiol compounds administered to humans as drugs (such as *N*-acetylcysteine or penicillamine) could act *in vivo* by scavenging $O_2^{\cdot-}$ or H_2O_2, simply because the high drug concentrations needed for direct scavenging are not achieved in body fluids.

Assessment of superoxide scavenging

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 $O_2^{\cdot-}$ reacts with a putative antioxidant (Butler *et al.*, 1988; Willson, 1985). However, pulse radiolysis is unsuitable for measuring most reactions of $O_2^{\cdot-}$ in aqueous solution, since the reaction rates are usually lower than the overall rate of non-enzymic dismutation of $O_2^{\cdot-}$. This limits measurements of rate constants to those of $10^5\ M^{-1}\ sec^{-1}$ or greater. Unfortunately, the rate constants for the reaction of $O_2^{\cdot-}$ with most biological molecules, except ascorbate, $NO^{\cdot}$ and SOD, are less than this (Bielski, 1985; Butler *et al.*, 1988). Stopped-flow methods can be used to study these slower reactions (Bull *et al.*, 1983). 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 (or xanthine) and xanthine oxidase at pH 7.4 generates $O_2^{\cdot-}$ which reacts with cytochrome c and nitroblue tetrazolium (NBT) with defined rate constants, namely 2.6×10^5 and $6 \times 10^4\ M^{-1}\ sec^{-1}$, respectively (Bielski, 1985; Fridovich, 1989). Any added molecule capable of reacting with $O_2^{\cdot-}$ will decrease the rates of cytochrome c or NBT reduction, and analysis of the inhibition produced allows calculation of an approximate rate constant (Halliwell, 1985). This approach has been widely used by ourselves and others to establish rate constants for the reactions of $O_2^{\cdot-}$ with various molecules. However, some essential controls must not be forgotten.

(1) It must be checked that the substance under test does not inhibit $O_2^{\cdot-}$ generation (e.g. by inhibiting xanthine oxidase). This can be checked by measuring uric acid formation. One artefact that has confused some scientists is that many compounds being tested for $O_2^{\cdot-}$ scavenging absorb strongly at 290 nm, making spectrophotometric assessment of enzyme activity highly inaccurate. HPLC, or measurement of O_2 uptake, can be used instead.

(2) 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 certain thiols). The authors' experience is that it is less of a problem with NBT, provided that pH values of 7.4 or less are used.

(3) One must consider the possibility that a radical formed by attack of $O_2^{\cdot-}$ on a substance could itself reduce cytochrome c or NBT. This will be revealed as deviations from linear competition kinetics at high scavenger concentrations.

Assessment of hydrogen peroxide scavenging

H_2O_2 is easily and sensitively measured by using peroxidase-based assay systems. The most common system uses horseradish peroxidase, and follows the oxidation of scopoletin by H_2O_2 to form a non-fluorescent product (Boveris *et al.*, 1977; Corbett, 1989). Thus, if a putative scavenger is incubated with H_2O_2 and the reaction mixture is sampled for analysis of H_2O_2 at various times, rates of loss of H_2O_2 can be measured to allow the calculation of rate constants. It is essential to check that the substance being tested is not itself a substrate for peroxidase, and could compete with scopoletin to cause an artefactual inhibition. For example, ascorbic acid and thiol compounds can be oxidized by horseradish peroxidase, and hence they often interfere with peroxidase-based assay systems. 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. Superoxide radical can inactivate peroxidase (forming compound III) and may compromise measurement of H_2O_2 in systems generating $O_2^{\cdot-}$. This can be avoided by addition of SOD (Kettle *et al.*, 1994).

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 permanganate ($KMnO_4$) or by measuring the O_2 release (1 mol of O_2 per 2 mol 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 (Halliwell and Gutteridge,

1989). Varma (1989) has described a sensitive radiochemical assay for H_2O_2, based on its ability to decarboxylate ^{14}C-labelled 2-oxoglutarate to $^{14}CO_2$ (measured by scintillation counting).

Hydroxyl radical

Much of the molecular damage that can be done by $O_2^{\cdot-}$ and H_2O_2 is thought to be due to their conversion into much more reactive species (reviewed by Halliwell and Gutteridge, 1989 and 1990), the most important of which is the hydroxyl radical ($OH^{\cdot}$).

Formation of $OH^{\cdot}$ from $O_2^{\cdot-}$ can be achieved by at least four different mechanisms. One requires traces of catalytic transition metal ions, of which iron and copper seem likely to be the most important *in vivo* (Fig. 2). Iron ions, and possibly copper ions, are released during processing of foods, especially meats, and could also catalyse such reactions (Igene *et al.*, 1979; Kanner *et al.*, 1988; Miller *et al.*, 1994; Ramanathan and Das, 1993a,b).

A second mechanism is that background exposure to ionizing radiation causes a steady-state low rate of $OH^{\cdot}$ formation within cells and in food by splitting of water (von Sonntag, 1987). Food irradiation (Elias, 1994) for sterilization or prevention of germination will generate much more $OH^{\cdot}$. A third means of forming some $OH^{\cdot}$ is the reaction of $O_2^{\cdot-}$ with the free radical nitric oxide ($NO^{\cdot}$), a reaction that proceeds at a rate comparable to that of $O_2^{\cdot-}$ with SOD (Huie and Padmaja, 1993). Indeed, $NO^{\cdot}$ is one of the few molecules with which $O_2^{\cdot-}$ reacts quickly.

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^- \qquad [1]$$

The product, peroxynitrite, can itself cause damage (e.g. it oxidizes –SH groups and destroys methionine) (Beckman *et al.*, 1994). In addition, at physiological pH, peroxynitrite protonates and decomposes to a range of noxious products, which are identical with (or closely resemble) nitronium ion (NO_2^+), the free radical gas nitrogen dioxide ($NO_2^{\cdot}$) and $OH^{\cdot}$ (Beckman *et al.*, 1994; van der Vliet *et al.*, 1994a). Considerable chemical knowledge of nitrite/$NO^{\cdot}$ chemistry has come from studies by food scientists of the use of nitrite in meat preservation (Igene *et al.*, 1979; Kanner *et al.*, 1988; Ramanathan and Das, 1993a,b). Reaction of HOCl with $O_2^{\cdot-}$ also makes some $OH^{\cdot}$; the rate constant is close to $10^7\ M^{-1}\ sec^{-1}$ (Candeias *et al.*, 1993). It is possible that HOCl could be generated in irradiated foods (Czapski *et al.*, 1992).

$$HOCl + O_2^{\cdot-} \rightarrow OH^{\cdot} + O_2 + Cl^- \qquad [2]$$

Reactions of hydroxyl radical

Hydroxyl radical is fearsomely reactive: it combines with almost all molecules found in the food matrix or in living cells, with rate constants of 10^9–$10^{10}\ M^{-1}\ sec^{-1}$ (Anbar and Neta, 1967). *Thus almost everything in food or in vivo can be regarded as an $OH^{\cdot}$ scavenger: no specific molecule has evolved for this role.* Hence literature suggestions that, for example, flavonoids, carotenoids or administered drugs (unless they reach high levels) act to scavenge $OH^{\cdot}$ *in vivo* or in foods are chemically unlikely. The rate constants for scavenging (at $>10^{10}\ M^{-1}\ sec^{-1}$) may be high, but the molar concentrations of these substances are far less than that of endogenous molecules that are also capable of rapidly scavenging $OH^{\cdot}$. For example, blood plasma albumin is an excellent $OH^{\cdot}$ scavenger [rate constant $>10^{10}\ M^{-1}\ sec^{-1}$. (Smith *et al.*, 1992)]. Glucose is not quite as good an $OH^{\cdot}$ scavenger [rate constant (Anbar and Neta, 1967) about $10^9\ M^{-1}\ sec^{-1}$] but it is present at relatively high concentrations (4.5 mM) in body fluids. The very high concentrations of sugars in certain foods could represent a powerful potential for scavenging $OH^{\cdot}$.

Antioxidants affecting hydroxyl radical formation

It seems much more likely that an antioxidant that interferes with damage caused by $OH^{\cdot}$ will act not by direct $OH^{\cdot}$ scavenging, but by scavenging or blocking formation of its precursors ($O_2^{\cdot-}$, H_2O_2, HOCl, $ONOO^-$) and/or by binding the transition metal ions needed for $OH^{\cdot}$ formation from $O_2^{\cdot-}$ and H_2O_2

$$H_2O_2 + \underset{(Fe^{2+})}{Cu^+} \rightarrow OH^{\cdot} + OH^- + \underset{(Fe^{3+})}{Cu^{2+}}$$

$$\underset{(Fe^{3+})}{Cu^{2+}} + O_2^{\cdot-} \rightarrow \underset{(Fe^{2+})}{Cu^+} + O_2$$

$$\textbf{Net:}\ H_2O_2 + O_2^{\cdot-} \xrightarrow[\text{catalysis}]{Fe,\ Cu} OH^{\cdot} + OH^- + O_2$$

Fig. 2. Catalysis of hydroxyl radical formation from superoxide and hydrogen peroxide by iron and copper ions.

(Fig. 2). Inhibition of $OH^{\cdot}$ generation by binding metal ions can occur by two mechanisms. First, binding of the metal ions may so alter their redox potential and/or accessibility that they cannot participate in $OH^{\cdot}$ formation. For example, this appears to be true for iron ions bound to the physiological iron-binding proteins, transferrin and lactoferrin (Aruoma and Halliwell, 1987). In the same way, the iron-chelating drug desferrioxamine, at the concentrations actually achieved *in vivo* when it is administered to patients to remove unwanted iron, is far more likely to protect against damage mediated by $OH^{\cdot}$ by binding iron ions and preventing $OH^{\cdot}$ formation than by scavenging this radical directly (Halliwell, 1989).

A second possibility is that binding of a 'catalytic' transition metal ion to an 'antioxidant' does not prevent the redox reactions, but that these reactions are directed onto the antioxidant, so sparing a more important target (Aruoma *et al.*, 1987; Gutteridge, 1984). Because $OH^{\cdot}$ 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 exact location of 'catalytic' transition metal ions is an important determinant of the molecular nature of the damage that is caused *in vivo* or in foods (Halliwell and Gutteridge, 1989 and 1990; Marx and Chevion, 1986). For example, when copper ions bind to albumin in human blood plasma, Fenton-type reactions (Fig. 2) can still occur on the binding sites and the protein is damaged by $OH^{\cdot}$. However, albumin is much less significant as a target of damage than are plasma lipoproteins and the membranes of blood cells and vascular endothelial cells, and so the binding of copper ions to albumin may represent a protective mechanism, since the damaged albumin may be replaced quickly (Halliwell, 1988). Histidine-containing dipeptides, found in foods and in many mammalian tissues, might also act as copper ion chelators (Kohn *et al.*, 1988) and they could exert antioxidant effects in meats.

Assessment of hydroxyl radical scavenging

The definitive technique for measuring the rate constant for reaction of a substance with $OH^{\cdot}$, and for studying the products of that reaction, is pulse radiolysis (Anbar and Neta, 1967; Butler *et al.*, 1988). Most compounds react with $OH^{\cdot}$ with rate constants of 10^9–10^{10} M^{-1} sec^{-1}.

If pulse radiolysis facilities are not available, approximate rate constants can often be calculated by one of several simpler assays. In our laboratories we most often use the 'deoxyribose method' (Aruoma 1994b; Halliwell and Gutteridge, 1981; Halliwell *et al.*, 1987). Hydroxyl radicals are generated by a mixture of ascorbic acid, H_2O_2 and Fe^{3+}–EDTA. Those radicals that are not scavenged by other components of the reaction mixture attack the sugar deoxyribose, degrading it into a series of fragments, some or all of which react on heating with thiobarbituric acid (TBA) at low pH to give a pink chromogen (Halliwell *et al.*, 1987) which is an adduct of TBA with the three-carbon aldehyde malondialdehyde. If a scavenger of $OH^{\cdot}$ is added to the reaction mixture, it will compete with deoxyribose for the $OH^{\cdot}$ radicals and inhibit deoxyribose degradation. Competition plots allow this rate constant to be calculated, assuming that deoxyribose reacts with $OH^{\cdot}$ with a rate constant of 3.1×10^9 M^{-1} sec^{-1} (Halliwell *et al.*, 1987).

Control experiments are essential.

(1) Check that the substance does not react rapidly with H_2O_2, which could block $OH^{\cdot}$ formation.

(2) If metal ion-dependent systems are being used to make $OH^{\cdot}$, check that the substance is not a powerful metal ion chelator. The deoxyribose assay avoids this problem by using iron ions already chelated to EDTA.

(3) Check that attack of $OH^{\cdot}$ on the substance does not produce TBA-reactive material. A control should be performed in which deoxyribose is omitted from the reaction mixture.

(4) Ensure that the substance does not interfere with measurement of products. This can be checked by showing that it does not inhibit when added to the reaction mixture at the end of the incubation (e.g. with the TBA and acid during the deoxyribose assay).

Inhibition of metal ion-dependent hydroxyl radical formation

As argued previously, direct scavenging of $OH^{\cdot}$ will be an infrequent mechanism of antioxidant action, simply because very high concentrations of scavenger are required to compete with adjacent molecules *in vivo* or in the food matrix for any $OH^{\cdot}$ 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 $OH^{\cdot}$, particularly that involving iron ions bound to a target ('site-specific' Fenton chemistry). The deoxyribose method also affords a way of doing this. When iron ions are added to the reaction mixture as $FeCl_3$ (not chelated to EDTA), some of them bind to deoxyribose (Aruoma *et al.*, 1987; Gutteridge, 1984). The bound iron ions still participate in Fenton chemistry (Fig. 2), but any $OH^{\cdot}$ radicals formed immediately attack the sugar and are not released into free solution. Hydroxyl radical scavengers, at moderate concentrations, do not inhibit this deoxyribose degradation because they cannot compete with the deoxyribose for $OH^{\cdot}$ generated by bound iron ions (Aruoma and Halliwell, 1988). The only substances that do inhibit in this assay are those that bind iron ions strongly enough to remove them from the deoxyribose. A good example is citrate—a fairly poor $OH^{\cdot}$ scavenger but a good inhibitor in this system (Aruoma and Halliwell,

1988). Hence, this assay [subject to controls (1), (3) and (4) above] indicates the potential ability of a compound to interfere with 'site-specific' generation of $OH^{\cdot}$ radicals catalysed by bound iron ions. Indeed, the assay has been used to evaluate the abilities of citric acid (widely present in foods), anti-inflammatory drugs (Aruoma and Halliwell, 1988) and several plant phenolics (Aeschbach *et al.*, 1994; Aruoma, 1994a; Aruoma *et al.*, 1992; Laughton *et al.*, 1989; Scott *et al.*, 1993) to act in this way.

It must be emphasized that this version of the deoxyribose assay measures a combination of two factors: the ability both to remove iron ions from deoxyribose and to render those iron ions inactive or poorly active in generating $OH^{\cdot}$. Thus EDTA removes iron ions from deoxyribose, but iron–EDTA chelates are very effective in generating $OH^{\cdot}$ so that the deoxyribose is still degraded—this time by $OH^{\cdot}$ in 'free' solution, rather than by $OH^{\cdot}$ formed on the deoxyribose molecule.

If a compound inhibits site-specific radical damage to deoxyribose by chelating iron ions and rendering them less active in producing $OH^{\cdot}$, two possibilities can account for the latter property. First, the inhibitor–metal ion complex may be incapable of reacting with $O_2^{\cdot-}$ or H_2O_2, so blocking $OH^{\cdot}$ formation. Secondly, it may be that the inhibitor–metal ion complex still undergoes redox reactions, but that the $OH^{\cdot}$ 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 by chemical analysis (i.e. does the inhibitor undergo chemical modification as the reaction proceeds?) An example may be found in Gutteridge *et al.* (1990).

Peroxyl radicals

Formation of peroxyl radicals ($RO_2^{\cdot}$) is the major chain-propagating step in lipid peroxidation (Halliwell and Gutteridge, 1989) but $RO_2^{\cdot}$ can also be formed in non-lipid systems, such as proteins (Davies *et al.*, 1993; Dean *et al.*, 1993). Decomposition of peroxides by heating or by transition metal ion catalysis can generate both peroxyl and alkoxyl radicals (e.g. Fig. 3).

Reaction of $OH^{\cdot}$ with thiols in foods or *in vivo* can produce thiyl radicals ($RS^{\cdot}$), which can then combine with oxygen to give reactive oxysulfur radicals such as $RSO^{\cdot}$ and $RS_2^{\cdot}$ (thiyl peroxyl): the exact chemistry of these reactions is still uncertain (Asmus, 1987; Sevilla *et al.*, 1989; von Sonntag, 1987). An illustration of their potential importance *in vivo* is that oxysulfur radicals resulting from attack of $OH^{\cdot}$ on the drug penicillamine appear to be capable of inactivating α_1-antiproteinase (Aruoma *et al.*, 1989a). Free radical damage to sulfur-containing compounds in foods could conceivably lead to generation of abnormal smells and flavours.

Peroxyl radical scavenging

Scavengers able to remove peroxyl radicals might be effective in the aqueous phase (e.g. dealing with radicals from DNA, thiols and proteins). Thus glutathione (GSH) reacts rapidly (rate constants about 10^7–$10^8\ M^{-1}\ sec^{-1}$) with radicals resulting from attack of $OH^{\cdot}$ on DNA (Fahey, 1988; von Sonntag, 1987). Peroxyl radical scavengers could also act in hydrophobic (food lipid, membrane, lipoprotein interior) phases: these are, of course, the chain-breaking antioxidant inhibitors of lipid peroxidation.

Many lipid-soluble chain-breaking antioxidants can have pro-oxidant properties under certain circumstances *in vitro*, often because they can bind Fe(III) or Cu(II) ions and reduce them to Fe^{2+} or Cu^{+}. Even α-tocopherol can be made to exert pro-oxidant effects *in vitro* (Maiorino *et al.*, 1993; Yamamoto and Niki, 1988) and this has also been observed in food systems (Cillard *et al.*, 1980). α-Tocopherol can reduce metal ions; in addition, the α-tocopherol radical is capable of abstracting H atoms from polyunsaturated fatty acids (PUFAs; Mukai *et al.*, 1993), although it does so at a rate an order of magnitude slower than for peroxyl radicals. Propyl gallate, a food antioxidant, has limited solubility in water, but this is enough to allow it to accelerate both $OH^{\cdot}$ formation from H_2O_2 by Fenton chemistry and DNA damage by the antibiotic bleomycin, in both cases by its ability to reduce

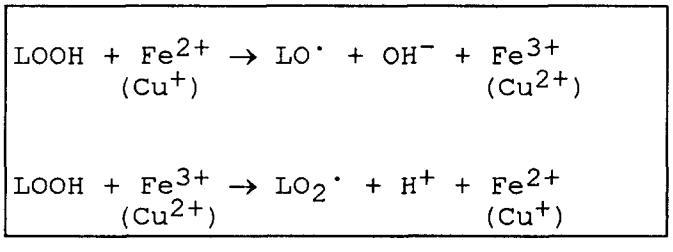

Fig. 3. Decomposition of lipid peroxides* (LOOH) by transition metal ions: $LO^{\cdot}$ = lipid alkoxyl radical; $LO_2^{\cdot}$ = lipid peroxyl radical.

*This is a gross oversimplification of the extremely complex chemistry that is taking place.

Fe(III) to Fe^{2+} (Aruoma *et al.*, 1990; Gutteridge and Xiao-Chang, 1981). Many plant phenolics (especially flavonoids) have been styled as 'antioxidants' because they inhibit lipid peroxidation. When propyl gallate, α-tocopherol and other plant phenolics are added to lipid systems, their chain-breaking antioxidant activity usually greatly outweighs their 'pro-oxidant' action in reducing metal ions, so that peroxidation is inhibited. However, several plant phenolics can accelerate oxidative damage to non-lipid biomolecules, such as DNA, *in vitro* (Aruoma *et al.*, 1992 and 1993; Laughton *et al.*, 1989; Ochiai *et al.*, 1984). This further illustrates the point that an antioxidant in one system is not an antioxidant in all systems.

Assessment of peroxyl radical scavenging

Peroxyl radicals derived from anti-inflammatory drugs can easily be generated by pulse radiolysis under appropriate conditions, and their scavenging by antioxidants studied (Willson, 1985). The ability of antioxidants to scavenge ESR-detectable peroxyl radicals is an additional approach (Greenley and Davies, 1992).

Another method of examining peroxyl radical scavenging is the TRAP assay (Wayner *et al.*, 1986 and 1987), which is much used (in its original or adapted versions) to study antioxidants in body fluids. Peroxyl radicals are generated at a controlled rate by the thermal decomposition of a water-soluble 'azo initiator', such as AAPH. This yields carbon-centered radicals, which react fast with O_2 to give peroxyl radicals. These are allowed to react with a lipid, whereupon they cause peroxidation. Thus by analysing the effect of a water-soluble antioxidant on the rate of peroxidation, a relative rate for its reaction with peroxyl radicals can be measured (Darley-Usmar *et al.*, 1989). A suspension of linoleic acid, or an ester of it, is frequently used as lipid substrate. Studies of the ability to protect linoleic acid or other lipids against peroxidation by AAPH-derived radicals have been used to show, for example, that ascorbic acid is an excellent scavenger of water-soluble peroxyl radicals (Darley-Usmar *et al.*, 1989; Wayner *et al.*, 1986 and 1987), whereas desferrioxamine is not. Radicals derived from AAPH also inactivate the enzyme lysozyme, which provides a protein target for studies of protection by 'antioxidants' (Lissi and Clavero, 1990; Paya *et al.*, 1992).

The carbon-centred radicals produced by AAPH decomposition can do direct damage (e.g. to DNA; Hiramoto *et al.*, 1993) and can deplete antioxidants (Soriani *et al.*, 1994). Thus one must ensure that reaction mixtures contain enough O_2 to convert them completely into peroxyl radicals.

Lipid-soluble peroxyl radicals

It has often proved difficult to generate 'clean' lipid-soluble peroxyl radicals *in vitro* for studies of scavenging ability. One exception is trichloromethylperoxyl (Alfassi *et al.*, 1993; Aruoma *et al.*, 1992; Lal *et al.*, 1988; Monig *et al.*, 1985; Packer *et al.*, 1978), which is formed by exposing a mixture of carbon tetrachloride (CCl_4), propan-2-ol and buffer to ionizing radiation, so producing hydrated electrons (e^-_{aq}) and $OH^{\cdot}$.

$$e^-_{aq} + CCl_4 \rightarrow {}^{\cdot}CCl_3 + Cl^- \quad [5]$$

$$OH^{\cdot} + CH_3CHOHCH_3 \rightarrow H_2O + CH_3\dot{C}OHCH_3 \quad [6]$$

$$CH_3\dot{C}OHCH_3 + CCl_4 \rightarrow CH_3COCH_3 + {}^{\cdot}CCl_3 + H^+ + Cl^- \quad [7]$$

$${}^{\cdot}CCl_3 + O_2 \rightarrow CCl_3O_2^{\cdot} \quad [8]$$

Rate constants for reaction of several known and putative antioxidants with $CCl_3O_2^{\cdot}$ have been published (Aruoma, 1994a; Aruoma *et al.*, 1995; Simic, 1991; Willson, 1985). However, $CCl_3O_2^{\cdot}$ is more reactive than non-halogenated peroxyl radicals, and so the results should be taken only as approximations of relative reactivity with the peroxyl radicals that are generated *in vivo* or during lipid peroxidation reactions in the food matrix.

Direct studies of lipid peroxidation

A direct test of antioxidant ability towards lipids is to examine whether a substance inhibits peroxidation of artificial lipid systems (e.g. fatty acid or fatty acid ester emulsions, liposomes), food systems, or biological systems such as erythrocytes, lipoproteins, tissue homogenates or microsomes. Although such studies are widely performed, several points must be considered in interpreting them. First, in biological assays the lipid systems are usually maintained under ambient pO_2, although some putative antioxidants (e.g. β-carotene) work better at the lower O_2 concentrations that often exist *in vivo* (Burton and Ingold, 1984). 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. The TBA test is widely used in food systems and by biological scientists, 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. For example, much of the apparent antioxidant effect of carnosine and anserine in inhibiting lipid peroxidation is still seen when these compounds are added with the TBA and acid (Table 2)—that is, they interfere with the assay (Aruoma *et al.*, 1989b). Detailed reviews of methods for measuring lipid peroxidation in biological materials (Packer, 1994) and in food systems (St. Angelo, 1992) have recently been published, so they will not be considered in detail here.

The third major point to consider is how peroxidation of the lipid substrate can be started. Lipoxygenase addition is one method. If a water-soluble azo initiator such as AAPH is used, it will be difficult to distinguish whether an antioxidant acts by direct scavenging of the AAPH-derived peroxyl radicals, or

Table 2. Effects of carnosine, anserine and desferrioxamine on lipid peroxidation: interference with the TBA assay system*

		Inhibition of peroxidation (%)					
Antioxidant added:		Carnosine (12 mM)		Anserine (12 mM)		Desferrioxamine (0.2 mM)	
Point at which antioxidant added:	Extent of peroxidation (A_{532})	Start of experiment	During TBA assay only	Start of experiment	During TBA assay only	Start of experiment	During TBA assay only
Addition							
Fe^{3+}/ascorbate	1.18	75	61	80	53	84	7
Fe^{3+}–ADP/NADPH	1.22	79	54	81	40	80	0
Fe^{2+}	1.05	66	58	54	50	75	5
Fe^{2+}–ADP	1.13	69	56	62	48	75	13

*Adapted from Aruoma *et al.* (1989b).

Peroxidation in rat liver microsomes was studied. Note that desferrioxamine has little effect when added only during the TBA assay, showing that it is really inhibiting lipid peroxidation. In contrast, much of the apparent inhibitory effect of carnosine and anserine is still seen when they are added with the TBA reagents, (i.e. they interfere with the assay).

by scavenging the lipid peroxyl radicals within the lipid substrate. This problem is not overcome by treating the lipid with a lipid-soluble azo initiator such as AMVN. Although the hydrophobic AMVN dissolves in lipids and decomposes to form peroxyl radicals, it is still difficult to establish whether an antioxidant is acting by scavenging the AMVN-derived radicals or the peroxyl radicals arising from the lipid substrate itself.

Peroxidation can also be accelerated by adding iron salts to lipids (Kanner *et al.*, 1988; Wills, 1969); for example 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. If the antioxidant is acting by metal binding, it will not be consumed during the reaction, as can be shown by direct analysis (e.g. by HPLC). In contrast, a chain-breaking antioxidant is consumed as it reacts with peroxyl radicals. Chain-breaking antioxidants at low concentrations often (not always) introduce a lag period into the peroxidation process, corresponding to the time taken for the antioxidant to be consumed, whereas metal-binding antioxidants would be expected to give a constant inhibition through the reaction. Table 3 illustrates this 'lag period' concept by showing one measurement of the lipid antioxidant action of plant extracts. Rosemary antioxidants showed the best antioxidant activity out of a series of plant extracts.

Microsomal peroxidation assays

Microsomal fractions from mammalian tissues (e.g. turkey muscle, rat liver) already contain variable amounts of endogenous antioxidants, such as α-tocopherol. Hence some antioxidants might not act directly, but by 'recycling' endogeneous antioxidants. For example, the dithiol compound dihydrolipoic acid had no inhibitory effect on iron/ascorbate-dependent peroxidation in liposomes made from pure phospholipids [in fact, it accelerated peroxidation somewhat (Scott *et al.*, 1994)], but it could recycle vitamin E radical in microsomes to inhibit peroxidation (Packer, 1992). This is a very important concept in the food industry: can added antioxidants help to preserve food levels of vitamin E, which is essential in the human diet? If microsomal lipid peroxidation is started by adding NADPH plus

Table 3. Antioxidant index of herb, spice and plant extracts (Rancimat test)

	Antioxidant index in:			
Extract*	Chicken fat	Lard	Soya oil	Sunflower oil
Rosemary	12.6	11.4	2.1	2.3
Sage	8.4	8.5	1.8	1.8
Thyme	5.7	4.8	1.2	1.3
Oregano	3.4	2.9	1.3	1.3
Cocktail P†	8.3	9.1	1.8	2.0
Ginger	2.4	2.9	1.1	1.1
Turmeric	1.8	1.6	1.1	1.1
Cayenne pepper	1.2	1.1	1.0	1.1
Cocktail O‡	1.8	2.0	1.0	1.1
Laurel	1.5	1.5‖		
Basil	1.1	1.5‖		
Marjoram	1.3	1.2‖		
Savory	1.1	1.1‖		
Cinnamon	1.1	1.0‖		
Allspice	1.2	1.1‖		
Cloves	2.3	2.0‖		
Zingerone§	1.5 (7.6¶)	3.0‖		
Green tea	1.0		—	
Vine leaves	1.6 (5.3¶)	2.3‖		

*Extracts were added at 1% (w/w) except where otherwise indicated.

†The mixture contained: rosemary, 40%, sage, 20%, thyme, 20%, oregano, 20%.

‡The mixture contained: ginger, 50%; turmeric, 25%; cayenne pepper, 25%.

§Prepared by retroaldol condensation from 200 g ginger: solvent extraction/alkaline hydrolysis/neutralization/liquid–liquid extraction/evaporation of solvent/taking-up in MCT.

‖Tested at 2% in lard.

¶In parentheses, tested at 10% in chicken fat.

The rancimat test is an accelerated oxidation test widely used in the food industry (Löliger 1991). The test consists of blowing air through a sample of oil at a given temperature (110°C) and monitoring the formation of ionic volatiles by a conductivity determination in a sample of water through which the air is passed after having passed through the oil. Values in the Table represent the ratio of the induction periods of an oil *with* added antioxidant and the induction period of the same oil *without* antioxidant. Thus the higher the number the greater the activity of the extract as a chain-breaking antioxidant.

Fe^{3+}–ADP to the microsomes (Wills, 1969) a control should also be performed to check that an added compound does not inhibit enzymic reduction of the iron complex. 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.

Peroxidation may also be started by adding Fe^{3+}/ascorbate mixtures to lipids. For microsomes, this avoids problems with the mixed function oxidase system, but it should be noted that ascorbate may well be capable of chemically reducing antioxidant radicals (derived by reaction of the antioxidant with peroxyl radicals), back to the antioxidant molecule, thus facilitating antioxidant action. Such a reaction will only occur, of course, if the antioxidant-derived radicals become accessible for reduction at the lipid surface, as has been shown to be the case for α-tocopheryl radicals in membranes and plasma lipoproteins (Diplock, 1985 and 1994). 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. These interactions of vitamin C, vitamin E and other antioxidants present in (or added to) foods are very important, not only in preventing rancidity but also in maintaining the nutritional quality of the food (keeping high levels of the antioxidant vitamins).

Hence, to gain a full understanding, it is wise to compare antioxidant ability using peroxidation started by several different mechanisms, in different lipid substrates, and to consider interactions between added and endogenous antioxidants.

Phagocyte-derived reactive oxygen species

Activated neutrophils, macrophages, eosinophils and monocytes produce $O_2^{\cdot -}$ and H_2O_2. Most, if not all, of the H_2O_2 arises by dismutation of $O_2^{\cdot -}$, the first product of the NADPH oxidase enzyme complex (Curnutte and Babior, 1987).

If neutrophil-derived $O_2^{\cdot -}$ 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 it has been suggested that several anti-inflammatory drugs interfere with phagocyte functioning. However, very few of these claims meet the criterion that the drug at the concentrations actually achieved *in vivo* during normal therapeutic regimens must slow the respiratory burst that is triggered by using physiologically-relevant stimuli, such as opsonized bacteria.

Methods for isolating phagocytic cells and measuring their production of $O_2^{\cdot -}$ and H_2O_2 are well documented in the literature. Standard methods (e.g. cytochrome c or NBT reduction for $O_2^{\cdot -}$, peroxidase-based assays for H_2O_2) can be used to check the ability of compounds to interfere with phagocyte ROS production after triggering the respiratory burst. It is essential to ensure that compounds do not interfere with the methods used to measure ROS production (e.g. by directly reducing cytochrome c or by interfering with peroxidase-based measurement of H_2O_2—see previous sections).

Hypochlorous acid. Activated neutrophils contain and secrete the enzyme myeloperoxidase, which uses H_2O_2 to oxidize chloride ions into the powerful oxidant hypochlorous acid (HOCl) (Weiss, 1989). Human eosinophils contain a similar enzyme, which prefers bromide (Br^-) ions as a substrate and presumably produces HOBr (Mayeno *et al.*, 1989). Hypohalous acids contribute to the mechanisms by which neutrophils and eosinophils attack ingested bacteria, and HOCl produced outside the phagocyte can cause tissue damage (Weiss, 1989). For example, HOCl oxidizes an essential methionine residue in α_1-antiproteinase (formerly called α_1-antitrypsin), which is an important inhibitor of serine proteases, such as elastase, in human body fluids (Clark *et al.*, 1981; Weiss, 1989). Hypochlorous acid may not have a great relevance to food materials, although it is widely used in bleaches (as the sodium salt, NaOCl) and could be generated during irradiation of foods that contain chloride ions under anoxic conditions (Czapski *et al.*, 1992).

Hypochlorous acid scavenging assays. Compounds can be tested for their potential to interfere with tissue damage by HOCl. This interference could be achieved by scavenging HOCl and/or by inhibiting HOCl production by myeloperoxidase (Wasil *et al.*, 1987). Myeloperoxidase can be assayed in several ways, including standard tests of peroxidase activity (Halliwell and Gutteridge, 1989)—for example its ability to oxidize guaiacol to a chromogen in the presence of H_2O_2 (Iwamoto *et al.*, 1987). It can also be assayed by specific measurements of HOCl production using monochlorodimedon [discussed by Cuperus *et al.* (1987) and Kettle and Winterbourn (1988)]. 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).

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. 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 (Green *et al.*, 1985). 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. [This inactivation is also

physiologically important (Weiss, 1989)]. A good scavenger of HOCl should protect α_1-antiproteinase against inactivation when HOCl is added subsequently. Controls are necessary, to show that the substance under test does not:

(1) itself inactivate elastase,

(2) interfere with the ability of α_1-antiproteinase to inactivate elastase, and

(3) re-activate α_1-antiproteinase that has been inactivated by HOCl.

If a substance fails to protect α_1-antiproteinase against inactivation by HOCl in this assay system, there are two possible explanations. First, its reaction with HOCl may be too slow, or non-existent. Secondly, its reaction with HOCl may form a 'long-lived' oxidant that is also capable of inactivating α_1-antiproteinase (Weiss *et al.*, 1983). Taurine has been shown to do this and the possibility must always be considered for other compounds. The spectra of various chloramines have been published (Weiss *et al.*, 1983) and it is also of interest to see whether putative antioxidants can scavenge such products (as demonstrated with captopril by Aruoma *et al.* (1991). HOCl and chloramines are particularly reactive with thiol compounds and methionine (Weiss, 1989).

Several papers have examined the ability of anti-inflammatory drugs to scavenge HOCl (Cuperus *et al.*, 1987; Wasil *et al.*, 1987). It was concluded that almost all of the drugs examined could react with HOCl *in vivo*, but only a few drugs would, at the concentrations present *in vivo*, be capable of reacting fast enough to protect important biological targets from attack by HOCl (this does not, of course, mean that they would actually do so). 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 (Green *et al.*, 1985; Kalyanaraman and Sohnle, 1985; Uetrecht, 1983).

An antioxidant protecting against damage by HOCl might do so not only by scavenging HOCl but also 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 (Cuperus *et al.*, 1985; Svensson and Lindvall, 1988). Ascorbic acid is a good HOCl scavenger and a substrate for myeloperoxidase, but its effects on HOCl production are complex (Marquez and Dunford, 1990). The plant phenol 4-hydroxy-3-methoxyacetophenone (apocynin) inhibits neutrophil $O_2^{\cdot-}$ release *in vitro*, apparently because it is oxidized by myeloperoxidase to generate the 'real' inhibitory agent (Hart *et al.*, 1990).

Several phenolic compounds (such as vanillin, ferulic acid, catechins, carnosic acid, carnosol and propyl gallate) react quickly with HOCl and can protect α_1-antiproteinase and other susceptible targets against damage *in vitro* (Aruoma *et al.*, 1992 and 1993; Scott *et al.*, 1993). This may have physiological significance, given the interest in the use of natural phenolic antioxidants as therapeutic agents (Aruoma, 1994a).

Haem proteins/peroxides

Mixtures of H_2O_2 and cytochrome c, haemoglobin or myoglobin oxidize many products and stimulate lipid peroxidation (Grisham, 1985; Kanner *et al.*, 1987; Yoshino *et al.*, 1985), apparently by the generation of both amino acid radicals on the protein and radicals associated with the haem group (Rao *et al.*, 1994; Kelman *et al.*, 1994). Such reactions may contribute to ischaemia-reperfusion injury, crash injury and chronic inflammation (Galaris *et al.*, 1989; Radi *et al.*, 1991). They can also occur in meat products (Igene *et al.*, 1979; Kanner *et al.*, 1987). The ability of a substance to react with activated haem proteins can be examined spectrophotometrically by looking for loss of the ferryl myoglobin (or haemoglobin) spectrum as the compound reduces it to the ferrous or ferric state (e.g. Rice-Evans *et al.*, 1989). A good 'quencher' of haem protein radicals, such as ascorbate, will also inhibit ferryl-dependent peroxidation of fatty acids or membrane lipids (Kanner *et al.*, 1987; Rice-Evans *et al.*, 1989) but it is essential to check in such experiments that the compound does not inhibit lipid peroxidation directly.

Exposure of haem proteins to a large molar excess of H_2O_2 causes haem breakdown and iron ion release (Gutteridge, 1986; Harel *et al.*, 1988). Some antioxidants, such as ascorbic acid, prevent this process by reducing the ferryl species (Rice-Evans *et al.*, 1989) and this provides an additional assay method for testing the effect of putative antioxidants on haem protein–H_2O_2 systems.

Peroxynitrite

Peroxynitrite (equation 1) can be simply prepared (Beckman *et al.*, 1994) and its reaction with biological molecules investigated. Careful control of pH is essential, and the effects produced depend on the bicarbonate content of the system (van der Vliet *et al.*, 1994b). The effects of addition of peroxynitrite to human plasma have been described (van der Vliet *et al.*, 1994b).

Singlet oxygen

Oxygen has two singlet states, but the $^1\Delta g$ state is probably the most important in biological and food systems. 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 PUFAs (see Halliwell and Gutteridge, 1989). The term 'singlet O_2' is used in the rest of this article to refer to both the $^1\Delta g$ and $^1\Sigma g^+$ states collectively.

Singlet oxygen can be produced in foods, and on or in the skin, as a result of photosensitization reactions (e.g. bright sunlight can damage milk by

reactions involving riboflavin). Singlet O_2 is also formed when ozone reacts with several molecules, including thiols and proteins (Kanofsky and Sima, 1993). Singlet O_2 is important in the 'phototherapy' of certain diseases, for example, using psoralens and haematoporphyrin derivatives (Cortese and Kinsey, 1984; Pathak, 1982). Singlet O_2 may also be generated in the lens of the eye and contribute to the development of cataract (Goosey *et al.*, 1981). During lipid peroxidation $^1O_2$ (or at least a species closely resembling it) is produced, probably largely by the self-reaction of lipid peroxyl radicals (reviewed in Wefers, 1987).

$$>CHO_2^{\cdot} + >CHO_2^{\cdot} \rightarrow {}^1O_2 + >C=O + -->C\text{-}OH \quad [9]$$

Singlet O_2 can be generated easily by photosensitization reactions. However, in studying $^1O_2$ quenching it is important to ensure that any damage caused to a target molecule in such systems is due to singlet O_2 and not caused by direct interaction with the excited state of the sensitizer, or by reactions involving other ROS such as $O_2^{\cdot -}$ and $OH^{\cdot}$, that are often generated in illuminated pigment-containing systems. A technique has been described in which singlet O_2 is generated by an immobilized sensitizer and allowed to diffuse a short distance to react with the target molecule (Midden and Wang, 1983). This system has proved to be useful for studying biological damage produced by singlet O_2 (Dahl *et al.*, 1988) and it should be easily applicable to studies of quenching and scavenging activity. Singlet O_2 can also be generated by the thermal decomposition of endoperoxides, such as 3,3′-(1,4-naphthylidene) dipropanoate, and such sources have been used to examine the reactions of singlet O_2 with human plasma (Di Maschio *et al.*, 1989).

What do we learn from these studies?

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* or in the food matrix. The tests may clearly show that an antioxidant role is unlikely. Alternatively, they could show that an antioxidant action is *feasible*, in that the compound shows protective action at concentrations within the range present in foods or *in vivo*. One can then select compounds for further investigation.

Concluding comments

Foods for human consumption—and the human body—contain many molecules (lipids, DNA, proteins, vitamins and carbohydrates) that are susceptible to attack by ROS. Efforts are being made to address the real biological significance to humans of the antioxidant ability of food nutrients (such as ascorbic acid, vitamin E and polyphenolic compounds including flavonoids) and the pro-oxidant properties of nutrient components such as plant phenols as demonstrated *in vitro* [e.g. see Aruoma (1994a) and Halliwell (1994b)].

The premise is that the antioxidant status of a biological system (including the human body) is important and that the biological system must tend towards avoidance of environments where there is excessive ROS formation. The authors believe that there is a need to determine the contribution of nutrients to the modulation of the pathological consequences of ROS in the human body, as well as to evaluate the potential use of 'natural' and 'synthetic' antioxidants during food processing. Are the antioxidant molecules really protective of all substrates susceptible to attack by ROS? The characterization of antioxidants, which we have reviewed here, may help scientists to provide an answer.

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