Free Rad. Res., Vol. 25, No. 1, pp. 57–74
Reprints available directly from the publisher
Photocopying permitted by license only

Commentary*
Oxidative Stress, Nutrition and Health. Experimental Strategies for Optimization of Nutritional Antioxidant Intake in Humans

BARRY HALLIWELL

Neurodegenerative Disease Research Centre, Pharmacology Group, King's College, Manresa Road, London SW3 6LX, UK. Fax +44 171 333 4949

Accepted by Professor C. Rice-Evans

(Received January 15th, 1996)

Reactive oxygen species and reactive nitrogen species are formed in the human body. Endogenous antioxidant defences are inadequate to scavenge them completely, so that ongoing oxidative damage to DNA, lipids, proteins and other molecules can be demonstrated and may contribute to the development of cancer, cardiovascular disease and possibly neurodegenerative disease. Hence diet-derived antioxidants may be particularly important in protecting against these diseases. Some antioxidants (e.g. ascorbate, certain flavonoids) can exert pro-oxidant actions *in vitro*, often by interaction with transition metal ions. The physiological relevance of these effects is uncertain, as is the optimal intake of most diet-derived antioxidants. In principle, these questions could be addressed by examining the effects of dietary composition and/or antioxidant supplementation upon parameters of oxidative damage *in vivo*. The methods available for measuring steady-state damage (i.e. the balance between damage and repair or replacement of damaged molecules) and the actual rate of damage to DNA, proteins and lipids are reviewed, highlighting areas in which further methodological development is urgently required.

INTRODUCTION

Free radicals and antioxidants are widely discussed in the clinical and nutritional literature and lay press. The assumption often is that free radicals are always bad and antioxidants always good, but recent clinical trials suggest that giving the 'antioxidant' β-carotene to smokers can accelerate the development of lung cancer.[1,1a] So how much dietary antioxidant do we need? The purpose of this article is to provide an overview of our current knowledge and to propose experimental strategies for the evaluation of optimal antioxidant intake in humans.

The need for antioxidants

Aerobes have evolved antioxidant defences essentially because O_2 is poisonous.[2–4] The evolution of

*Based on an overview lecture given at the October 1995 meeting of contractors in the Antioxidants Programme of the Ministry of Agriculture, Fisheries and Food (UK).

O_2 tolerance mechanisms has allowed aerobes to use O_2 for metabolic transformations (e.g. oxidase and hydroxylase enzymes, such as cytochromes P-450) and for efficient energy production using electron transport chains with O_2 as the terminal electron acceptor. The mitochondrial electron transport chain generates over 80% of the ATP needed by aerobic cells, and the lethal effects of such electron transport chain inhibitors as cyanide show how important this activity is to humans.

Antioxidants are needed to limit the formation of, and to scavenge, a range of molecules loosely classified as 'reactive oxygen species' and 'reactive nitrogen species' which are generated in the human body (Table 1). Some of these species arise by 'accidents of chemistry'. For example, superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) can arise by direct oxidation of several biomolecules by O_2. Examples are adrenaline, dopamine, tetrahydrofolates and some components of mitochondrial and P450 electron transport chains.[3] Such $O_2^{\bullet-}$ generation is the unavoidable consequence of having these 'autoxidizable' molecules in a body that needs oxygen.[3,4] Humans are exposed to radiation from the environment, both natural (e.g. radon gas, cosmic radiation) and from man-made sources. Low-wavelength electromagnetic radiation (e.g. gamma rays) can split water in the body to generate the viciously-reactive hydroxyl radical ($OH^{\bullet}$).[5] Ultraviolet light is insufficiently energetic to split H_2O, but it can cleave the O-O covalent bond in H_2O_2 to give $2OH^{\bullet}$.[4]

TABLE 1

REACTIVE OXYGEN SPECIES (ROS)	
Radicals	Non-Radicals
Superoxide, $O_2^{\bullet-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\bullet}$	Ozone, O_3
Alkoxyl, $RO^{\bullet}$	Singlet oxygen $^1\Delta g$
Hydroperoxyl, $HO_2^{\bullet}$	
REACTIVE NITROGEN SPECIES (RNS)	
Radicals	Non-radicals
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitrosyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium cation, NO_2^+
	Alkyl peroxynitrates, ROONO

ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals (HOCl, O_3, $ONOO^-$, $^1O_2$, H_2O_2). RNS is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as such non-radicals as HNO_2 and N_2O_4. $ONOO^-$ is often included in both categories. 'Reactive' is not always an appropriate term: H_2O_2, $NO^{\bullet}$ and $O_2^{\bullet-}$ react quickly with few molecules whereas $OH^{\bullet}$ reacts quickly with almost everything. $RO_2^{\bullet}$, $RO^{\bullet}$, HOCl, $NO_2^{\bullet}$, $ONOO^-$ and O_3 have intermediate reactivities. HOCl could also be regarded as a 'reactive chlorine species'.

In addition to this unavoidable generation of ROS, some are made *deliberately in vivo*. For example, the phagocytes (neutrophils, monocytes, macrophages, eosinophils) that defend against foreign organisms, generate $O_2^{\bullet-}$, H_2O_2 and (in the case of neutrophils) hypochlorous acid as one of their mechanisms for killing foreign organisms.[6] They can also generate RNS, for a similar reason.[7] This essential defence mechanism can go wrong: several diseases (such as rheumatoid arthritis and inflammatory bowel disease) are accompanied by excessive phagocyte activation and resulting tissue damage, to which ROS/RNS contribute.[7-9] It has been estimated[3] that 1–3% of the oxygen we breathe in is used to make $O_2^{\bullet-}$.[3] Since humans consume a lot of O_2, a simple calculation[4] shows that over 2 kg of $O_2^{\bullet-}$ is made in the human body every year – people with chronic inflammations may make much more. Measurement of levels of nitrate/nitrite in human body fluids such as urine and plasma suggest that the total body 'load' of RNS may also be large[200-203] for example, in one human study basal plasma NO_3^- was 29 ± 1 μM corresponding to a basal body production of 840 ± 146 μmol per day. Dietary NO_3^-/NO_2^- can have a confounding effect, however: plasma NO_3^- rose to 205 μM 2 h after intake of nitrate-rich food.[203]

Some RNS are, like $O_2^{\bullet-}$, also useful, but toxic in excess. The prime example is **nitric oxide** ($NO^{\bullet}$). It has many useful functions, such as regulation of

blood pressure,[7] but too much $NO^{\bullet}$ is toxic: excess $NO^{\bullet}$ production is thought to be an important tissue injury mechanism in such conditions as chronic inflammation, stroke and septic shock.[7–9] Other RNS, such as nitrogen dioxide ($NO_2^{\bullet}$) and peroxynitrite ($ONOO^-$), are probably always damaging. The *balance* between RNS/ROS is also important in determining tissue injury.[10,11] Thus $NO^{\bullet}$ reacts very fast with $O_2^{\bullet-}$ to generate $ONOO^-$.[12] Peroxynitrite may mediate several of the cytotoxic effects of excess $NO^{\bullet}$, such as the destruction of iron/sulphur clusters in certain enzymes.[13] Nitric oxide also reacts fast with lipophilic peroxyl radicals, important propagating species in the biological chain reaction of lipid peroxidation, to generate alkyl peroxynitrates (ROONO). These appear far more stable than $ONOO^-$.[10] If ROONO derivatives can be metabolised without the release of toxic free radicals then the reaction of $NO^{\bullet}$ with peroxyl radicals is potentially *beneficial* because it allows $NO^{\bullet}$ to inhibit lipid peroxidation. The ratio of $NO^{\bullet}$ to ROS may be all important since $ONOO^-$ can *cause* lipid peroxidation. Thus, a 1:1 ratio of $O_2^{\bullet-}$ to $NO^{\bullet}$ generates $ONOO^-$ and induces lipid peroxidation whereas an excess of $NO^{\bullet}$ can inhibit lipid peroxidation by scavenging peroxyl radicals.[10] Such interactions may be particularly important in cardiovascular disease, especially the development of atherosclerosis.[11]

Vascular endothelial cells generate $NO^{\bullet}$[7] for a multitude of physiological reasons and they may also secrete $O_2^{\bullet-}$ (reviewed in[14]). Hence $ONOO^-$ can form in the vasculature. Phagocytes adhering to vascular endothelium can also produce RNS and ROS and interactions between them may have regulatory significance, e.g. $NO^{\bullet}$ inhibits platelet and phagocyte adhesion to the endothelium.[7] In advanced atherosclerotic lesions thrombosis plays an important role and this normally beneficial biological process is controlled in part by the $NO^{\bullet}$-dependent inhibition of platelet aggregation.[7] However, in atherosclerotic lesions excess production of $O_2^{\bullet-}$ may cause loss of the modulatory action of $NO^{\bullet}$ and at the same time yield $ONOO^-$ which is pro-aggregatory and so could commit platelets in this environment to thrombus formation.[15]

At an early stage in the development of atherosclerosis, peroxidation of low density lipoproteins (LDL) occurs in the vessel wall.[16] We do not really know how oxidation of LDL begins in the artery wall. However, peroxynitrite could be involved since it oxidises LDL,[17] causes a rapid depletion of several antioxidants (ascorbate, urate, protein thiols and ubiquinol)[18] and releases copper ions from the plasma protein caeruloplasmin.[19] Copper ions are powerful catalysts of LDL oxidation[20] which have been detected in advanced human atherosclerotic lesions.[21] Preliminary immunological evidence consistent with formation of $ONOO^-$ in atherosclerotic lesions has been presented.[22] As emphasized earlier, the ratio of $NO^{\bullet}$ to $O_2^{\bullet-}$ may be all important, since an excess of $NO^{\bullet}$ could *inhibit* LDL peroxidation.[10,23]

HUMAN ANTIOXIDANTS: ENDOGENOUS

Some antioxidants are synthesized in the human body: they include enzymes, certain other proteins and low-molecular-mass species.[3,24–27] Examples are superoxide dismutases, catalases, glutathione peroxidases, thiol-specific antioxidants, metallothioneins, other metal ion-binding and storage proteins, urate, GSH and ubiquinol.[3,24–33]

Aerobes have developed antioxidant defences to protect against the toxic effects of current levels of atmospheric O_2 (21%), but no more than that. This is evidenced by the fact that all aerobes suffer injurious effects if exposed to O_2 at concentrations greater than 21%.[2] For example, exposure of adult humans to pure O_2 at 1 atm pressure for as little as 6 hours causes chest soreness, cough, and sore throat in some subjects, and longer periods of exposure lead to alveolar damage.[2] The incidence of retinopathy of prematurity increased abruptly in the early 1940s among infants born prematurely and often led to blindness. Not until 1954 was it realized that retrolental fibroplasia is associated with the use of high O_2 concentrations in incubators

TABLE 2 Evidence that Damage by RNS and ROS Occurs in the Human Body

Target of damage	Evidence
DNA	Low baseline levels of DNA base damage products are present in DNA isolated from human cells (reviewed in [36]). Urinary excretion of DNA base damage products, presumably resulting from repair of oxidative damage to DNA.[37,38]
Protein	Attack of free radicals upon proteins produces protein carbonyls and other modified amino acid residues.[39] Low levels of carbonyls and certain other products (e.g. *ortho* tyrosine) have been detected in human tissues and body fluids.[40–42] Nitrotyrosines, products of reaction of $ONOO^-$ with tyrosine residues in proteins, have been detected in atherosclerotic lesions[22] and nitrotyrosine is excreted in human urine.[43] Bityrosine can also be detected in body fluids (our unpublished data).
Lipid	Accumulation of 'age pigments' in tissues. Lipid peroxidation in atherosclerotic lesions.[16] Presence of end products of peroxidation in human body fluids (for a recent review that examines the methodological problems in measuring such products see ref [44]). The most specific products seem to be the isoprostanes.[45]
Uric acid	Attacked by several ROS to generate allantoin, cyanuric acid, parabanic acid, oxonic acid, and other products, which can be detected in human body fluids. Levels increase during oxidative stress.[46,47]

for premature babies. More careful control of O_2 concentrations (continuous transcutaneous O_2 monitoring, with supplementary O_2 given only where necessary) and administration of the lipid-soluble antioxidant α-tocopherol have decreased its incidence. However, the problem has not disappeared, since many premature infants need increased levels of O_2 in order to survive at all.[34,35]

Indeed, there is good evidence that endogenous antioxidants do *not* completely prevent damage by RNS/ROS in the human body (Table 2). Hence efficient repair systems are needed. Repair of the damage done to DNA by ROS/RNS is particularly important,[48] as the constant assault by these species on DNA throughout the long human lifespan may contribute to the age-related development of spontaneous human cancers.[38,49–51] Indeed, the ageing process itself may involve the cumulative effects of oxidative damage over a lifespan.[49,52]

HUMAN ANTIOXIDANTS: DIET-DERIVED

We also obtain certain antioxidants from the diet. The physiological role of some of these is well-established (e.g. vitamin E, ascorbate) whereas the role of others is uncertain as yet (Table 3). Some of these antioxidants can be made to exert *pro-oxidant* effects *in vitro*, often by interaction with transition metal ions. The physiological role (if any) of these effects is uncertain: it would presumably depend on the availability of 'catalytic' metal ions. This relates to another important nutritional question; what is the optimal intake of iron? Iron is essential for human health, especially in children and pregnant women, but could too much iron intake cause harm?[89,90] In the healthy human body, metal ions appear largely sequestered in forms unable to catalyse free radical reactions.[27] Hence the antioxidant properties of ascorbate (and any plant phenolics that are absorbed) are usually thought to predominate over pro-oxidant effects.[64,65]

There are two caveats. First, some apparently-healthy people are not. It has been suggested that twice as many adult men in the USA have the inborn disease idiopathic haemochromatosis (IH) as have real iron-deficiency anaemia.[90,91] IH can lead to iron overload, with iron 'catalytic' for free radical reactions being present in blood plasma.[92] Giving vitamin C to iron-overloaded patients without an iron chelating agent (such as desferal) can produce serious effects.[93,94] Similarly, there is considerable debate about the possible pro- oxidant effect of ascorbate in premature babies, who may have saturated transferrin and iron 'catalytic' for free radical reactions in their plasma.[95,96]

The second caveat is that injury to tissues can release iron and copper ions (reviewed in [97]). For example, metal ions catalytic for free radical

TABLE 3 Dietary Antioxidants. A diet rich in fruits, nuts, grains and vegetables is protective against several human diseases. This may be due to the antioxidants they contain and/or to the many other compounds present.[53–59]

PUTATIVE ANTIOXIDANT	STATUS
Vitamin E	Essential antioxidant in humans, protective against cardiovascular disease. Severe deficiency causes neurodegeneration and accelerates atherosclerosis.[58,60–63]
Vitamin C	Multiple metabolic roles, antioxidant action only one of its effects (reviewed in [64,65]). Can exert pro-oxidant actions *in vitro* by interaction with iron and copper ions.[65]
β-carotene, other carotenoids, related plant pigments	Epidemiological evidence that high body levels are associated with diminished risk of cancer and cardiovascular disease, particularly in smokers (e.g. [61,62]). Carotenoids are good singlet O_2 quenchers/scavengers.[66] Often simplistically grouped with vitamins E and C as 'antioxidant nutrients', but it is not yet rigorously proved that any protective effects these pigments exert against human disease are due to antioxidant action (discussed in [67–69]). For example, β-carotene supplementation of the diet did not decrease the elevated urinary excretion of 8-hydroxydeoxyguanosine, a putative index of oxidative DNA damage, in smokers.[69] Conversion to retinoids and/or effects on cell communication may be equally or more important explanations of the biological effects of these compounds.[70–73] Many of the apparent protective effects of carotenoids as well as reports of deleterious effects[1,1a] involve smokers, suggesting possible interactions between carotenoids and constituents of cigarette smoke, for which there is some *in vitro* evidence.[74]
Flavonoids, other plant	Many plant phenols inhibit lipid peroxidation and lipoxygenase enzymes *in vitro*[75–78] and 'wine phenolics' may be important dietary antioxidants.[81–83] It has been speculated that flavonoids in red wine could explain the'"French paradox'[81,82] although the identity of the phenolics responsible is uncertain.[83] Like ascorbate, some plant phenolics can be pro-oxidant *in vitro* if mixed with copper or iron ions.[77,84,85] More data are needed on absorption and bioavailability of phenolics, but evidence is growing that some wine and tea phenolics are absorbed.[79–81,86,87] Plant phenols might also scavenge RNS, e.g. preventing tyrosine nitration by $ONOO^-$, but the biological properties of any resulting nitroso/nitro-phenolics must be considered.[88]

reactions have been measured in advanced human atherosclerotic lesions.[21] There are repeated (although controversial) suggestions that high body iron and/or copper stores are associated with increased risk of cancer and cardiovascular disease (reviewed in [90]). Could this be because the more iron or copper is present within a tissue, the more could be liberated to catalyse free radical reactions after injury? If so, then the *in vitro* pro-oxidant effects of ascorbate and flavonoids might become physiologically (or pathologically) relevant.

EVALUATION OF THE CLAIMS AND COUNTER-CLAIMS: THE AUTHOR'S APPROACH

There are multiple suggestions in the biomedical and lay literature that various natural dietary constituents or dietary supplements act as 'antioxidants'. For some (e.g. α-tocopherol) there is good *in vivo* data (Table 3) whereas for others (e.g. wine flavonoids, melatonin, garlic, ginseng, *Ginkgo biloba*) data to support their effectiveness *in vivo* in humans are limited or absent. Even for 'established' antioxidants, it has rarely been possible to determine the level of intake that maximizes antioxidant effects whilst minimizing toxicity, e.g. by putative pro-oxidant effects. The approach of my laboratory to this problem has been two-pronged. First, we have developed a battery of antioxidant characterization methods to establish *in vitro* what putative 'antioxidants' are actually capable of doing.[98,99] The results may be used to evaluate the possibility (or impossibility) that a compound can exert *direct* antioxidant effects *in vivo* and the results enable one to reason from simple chemical principles and dismiss outright many of the crazier claims.

However, even an excellent *in vitro* antioxidant will not necessarily work as such *in vivo*. In addition, some compounds may exert antioxidant actions by upregulating endogenous antioxidant defences and/or inhibiting generation of ROS/RNS rather than by direct antioxidant action. Hence, we and others are developing methods for assessing both total and steady-state oxidative damage in the human body. It is important to assess all major molecular targets of damage by ROS/RNS (DNA, proteins, lipids) since an antioxidant that protects one target may fail to protect (or even exacerbate injury to) another. For example, flavonoids usually inhibit lipid peroxidation *in vitro*, but can sometimes stimulate metal ion-dependent damage to DNA and proteins (Table 3). In principle, one should be able to measure 'markers' of baseline oxidative damage in humans and examine how they are affected by changes in diet, such as alterations in fruit and vegetable intake, changes in consumption of saturated/ polyunsaturated fats or supplementation with antioxidants (e.g. pure compounds or complex herbal extracts). The optimal intake could then be determined, as could the biological relevance of putative pro-oxidant effects.

What 'markers' are available?

DNA

ROS- and RNS-mediated 'spontaneous' DNA damage is thought to contribute to cancer development e.g. via mutations in the tumour suppressor gene *p53*.[51] Several ROS/RNS have the potential to contribute to cancer development in that they can

(1) cause structural alterations in DNA e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification. ROS and RNS able to modify DNA directly include hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, ozone (O_3), nitrous acid (HNO_2), $ONOO^-$ and its decomposition products;

(2) activate[100] or inhibit[101] signal transduction pathways

(3) block cell-cell communication,[102,103] an event important in restricting cell proliferation

(4) modulate cell growth, differentiation and death by apoptosis or necrosis[104–106]

(5) damage proteins, such as DNA repair enzymes and DNA polymerases, perhaps thereby decreasing fidelity of replication.[105]

The chemistry of DNA damage by several ROS/RNS has been well characterised *in vitro*[5,107–112] although further studies are needed with $RO_2^{\bullet}$, $RO^{\bullet}$ and O_3. Nitric oxide ($NO^{\bullet}$) and products derived from it ($NO_2^{\bullet}$, HNO_2, $ONOO^-$, N_2O_3, etc.) can cause nitrosation and deamination of amino groups on DNA bases leading to point mutations. Deamination products of purine bases include xanthine (from guanine) and hypoxanthine (from adenine).[112] 8-Nitroguanine may be a useful 'marker' of attack on DNA by certain RNS such as $ONOO^-$.[113–115] Whereas $O_2^{\bullet-}$ and H_2O_2 do not react with DNA bases at all, $OH^{\bullet}$ generates a multiplicity of products from all four DNA bases.[108] By contrast, $^1O_2$ appears selective for attack upon guanine.[110,111] The most common base lesion, and the one most often measured as an index of oxidative DNA damage, is 8-hydroxyguanine.[116]

Oxidative damage to DNA appears to occur continuously *in vivo*, in that low levels (presumably a 'steady state' balance between DNA damage and repair) have been detected in DNA isolated from human cells and tissues.[36] The pattern of damage to the purine and pyrimidine bases bears the chemical fingerprint of $OH^{\bullet}$ attack, suggesting that $OH^{\bullet}$ formation occurs within the nucleus *in vivo*.[36,117] However, this raises the question of how $OH^{\bullet}$ could be produced in the nucleus. If $OH^{\bullet}$ is attacking DNA, it must be made very close to the DNA since $OH^{\bullet}$ is so reactive that it cannot diffuse from its site of formation. Background radiation may be one source but radiation-generated $OH^{\bullet}$ is formed over the whole cell and only a small fraction hits DNA.[117] Other potential sources of $OH^{\bullet}$ or $OH^{\bullet}$-like species include the decomposition of $ONOO^-$[118,119]

and the reaction of $O_2^{\bullet-}$ with HOCl.[120] By far the greatest interest has been, however, in reactions of transition metal ions with H_2O_2 as a source of $OH^{\bullet}$. The question of whether 'catalytic' transition metal ions (e.g. iron and copper ions) really are in close proximity to DNA *in vivo* is clearly an important one. Although iron and copper appear to be present in the nucleus (e.g. refs.[121,121a,210]), it remains to be established why and how 'catalytic' iron and copper ions reach the DNA, since these ions are normally carefully sequestered by the human body. However, oxidative stress can liberate metal ions from their normal sequestration sites[14,19,21,27,204] which might then bind to DNA, which is a powerful metal ion chelator. Low levels of xanthine and hypoxanthine are also detected in DNA from mammalian cells[112,122]: these presumably arise by deamination reactions, although their formation by other pathways is also feasible.

Measurement of oxidative DNA damage

Basic principles

There are two types of measurement of oxidative DNA damage. **Steady-state damage** can be measured when DNA is isolated from human cells and tissues and analyzed for base damage products: it presumably reflects the balance between damage and DNA repair. Hence a rise in steady state oxidative DNA damage (e.g. as has been reported in some human cancerous tumours[123,124]) could be due to increased damage and/or decreased repair. It is worth mentioning that the measurement of baseline levels of oxidatively modified DNA bases, although very important, does not provide information as to whether this damage is in active genes or quiescent DNA.

However, it is important also to have an index of total DNA damage in the human body, i.e. the 'input' side of the steady-state equation. The most common approach has been to assess the 'output' side, i.e. trying to estimate the rate of repair of oxidized DNA.

Studies upon urine

Several DNA base damage products are excreted in human urine, including the nucleoside 8-hydroxy-deoxyguanosine (8-OHdG), 8-hydroxy-adenine and 7-methyl-8-hydroxyguanine[37,38] but the one most exploited is 8-OHdG, usually measured by a method involving HPLC with electrochemical detection.[38,125–127] In a study of 169 humans, the average 8-OHdG excretion was 200–300 pmol/kg per 24 h, corresponding to 140–200 oxidative modifications of guanine per cell per day.[125,126] Furthermore, 32 smokers in this study excreted 50% more 8-OHdG than 53 non-smokers, suggestive of a 50% increased rate of oxidative DNA damage from smoking. GC-MS has also been used to measure 8-OHdG in urine and the limit of detection was 1.8 pmol corresponding to a level of 8-OHdG in urine of 35 nM.[128]

The validity of these urinary measurements of oxidative DNA damage must be considered. The level of 8-OHdG in urine is presumably unaffected by the diet since nucleosides are not absorbed from the gut. The question of whether any 8-OHdG is metabolized to other products in humans has not been rigorously addressed. Additionally, it is possible that some or all of the 8-OHdG excreted in urine may arise not from DNA, but from deoxyGTP (dGTP) in the DNA precursor pool of nucleotides. An enzyme has been described which hydrolyzes dGTP containing oxidized guanine to prevent its incorporation into DNA.[129,130] Because of these uncertainties, my research group is working hard to develop alternative urinary markers of total body oxidative damage.

The effects of dietary manipulation on the urinary excretion of 8-OHdG in humans have been reported in a few papers. Von Poppel *et al*[69] reported no inhibitory effect of β-carotene supplementation in male smokers. They also found no inhibitory effect of caloric restriction for 10 weeks.[131] However, consumption of 300 g of cooked Brussels sprouts per day decreased 8-OHdG generation by 28% in healthy male

non-smokers.[132] This could reflect less damage to DNA and/or its precursor pool (dGTP): it is also conceivable that constituents of this vegetable induce enzymes that metabolize 8-OHdG.

Measurement of guanine damage products in DNA by HPLC and GC-MS

8-Hydroxyguanine (8-OHG) and 8-OHdG are the products most frequently measured as indicators of oxidative DNA damage. Analysis of 8-OHdG using HPLC coupled to electrochemical detection (ECD)[116] is a highly sensitive technique that is frequently used after release of 8-OHdG from DNA, usually by enzymic hydrolysis. Gas Chromatography-Mass Spectrometry (GC-MS) with Selected Ion Monitoring (SIM) has also been used to characterize oxidative DNA base damage by the identification of a spectrum of products,[108] including 8-OHG, after formic acid hydrolysis of DNA and derivatization (often by trimethylsilylation) to generate volatile products. When GC-MS is used to measure modified DNA bases, a quantitative analysis of these bases in a DNA sample can be achieved by adding a suitable internal standard to the sample at an early stage of the analysis, such as before the hydrolysis of the DNA.[108] Stable isotope-labelled analogues of the modified bases can also be used as internal standards.[133]

One advantage of the GC-MS approach is that measurement of a wide range of base damage products allows more accurate quantitation of DNA damage and can help to identify the ROS/RNS species that caused the damage (e.g. $^1O_2$ selectively attacks guanine whereas $OH^\bullet$ attacks all four DNA bases). However, the levels of 8-OHdG measured in DNA by HPLC/ECD are often[36] (but not always[134,205]) less than the levels of 8-OHG measured by GC-MS/SIM (selected ion monitoring). HPLC could underestimate the real amount of 8-OHdG in DNA if the enzymic hydrolysis is incomplete; the action of the exonucleases and endonucleases used to hydrolyse the DNA may be affected by the modification of the bases,[36,135] and the acid pH often used for nuclease digestions could cause hydrolysis of 8-OHdG to 8-OHG, resulting in the loss of HPLC-detectable material (discussed in [36,136]). By contrast GC-MS might overestimate 8-OHG (and perhaps other base damage products) as a result of their artifactual formation during the heating-step involved in classical silylation-based derivatization procedures.[36,137] Hamberg and Zhang[138] have developed a 'cold' derivatization procedure that should avoid this problem. The important factor is that any necessary heating stages should be done anoxically: heating DNA bases in the presence of O_2 is bound to oxidize them! Hence some of the claimed artefacts[36,137] are due to failure to remove O_2. However, it is difficult to remove O_2 *completely*.

As an alternative means of avoiding possible problems with derivatization we have developed an HPLC-ECD method that allows measurement of 8-OHG and three of the other oxidized base products in acid-hydrolyzed DNA, thus avoiding the need for derivatization.[136] Liquid chromatography-mass spectrometry techniques are under development in several laboratories: this is another approach to avoiding derivatization problems if sufficient sensitivity can be achieved.

DNA isolation problems

A problem to be considered in all these techniques is the possibility that DNA is oxidatively damaged during its isolation from cells and tissues, particularly if phenol based methods are used, since oxidizing phenols generate ROS.[139,207] It has been shown that routine phenol-based DNA purification procedures can increase 8-OHdG levels 20-fold in samples that are exposed to air following removal of the phenol. Indeed, exposure to air alone results in a fourfold increase compared to DNA samples that have been solubilized in buffers purged with nitrogen.[139] Furthermore, the sensitization by phenol of DNA to subsequent exposure to air occurs despite extensive dialysis between phenol treatment and enzymatic DNA

digestion.[139] However, rigorous control of isolation procedures and avoidance of phenol in many laboratories (e.g. by studying isolated chromatin or by using different DNA isolation methods) does not abolish oxidative damage detected in isolated DNA,[36,108,127,140–142,207] strongly supporting the view that there is a low steady-state DNA damage *in vivo*. Indeed the presence of a DNA repair enzyme system and the excretion of base damage products support the view that oxidative damage really does occur *in vivo*.

There has been little work to date on effects of dietary manipulation on 'steady-state' levels of oxidative DNA damage in humans, but dietary vitamin E concentrations were reported not to affect 8-OHdG levels in rat liver DNA, whereas green tea in the drinking water decreased the rise in liver 80HdG levels produced by treatment of rats with 2-nitropropane.[143,206]

LIPIDS: LIPID PEROXIDATION

Lipid peroxidation is important *in vivo* for several reasons, in particular because it contributes to the development of atherosclerosis.[16,144,145] Hence a common test of the effectiveness of dietary antioxidants is to measure their effects on the 'peroxidizability' of LDL isolated from blood plasma. For example, Esterbauer *et al*[144] showed that dietary supplementation of humans with vitamin E increased the 'lag period' before peroxidation accelerated when LDL isolated from the plasma was subsequently incubated with copper ions *in vitro*. For example, 1200 IU of α-tocopherol increased the lag period by about 75%.

Lipid peroxidation is also important because end products of this process (particularly cytotoxic aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal, HNE) can cause damage to proteins and to DNA.[145–148] For example, an HPLC-ECD assay has been developed for the detection of the 1,N^2-propano adducts formed by the reaction of mutagenic α,β-unsaturated aldehydes with DNA.[147] Other biologically relevant aldehyde adducts to proteins and DNA that can be detected include those of MDA and HNE.[145,147–149] A deoxyguanosine-MDA adduct has been identified in human and rat urine,[150] but the levels excreted in rat urine were not affected by vitamin E status.

The more unsaturated a fatty acid side-chain, the greater its propensity to undergo lipid peroxidation. But do PUFAs really peroxidize at a faster rate *in vivo*? This question is particularly important in relation to proposals that increases in dietary polyunsaturate/saturate ratios are beneficial to health.[151] The brain and nervous system are particularly rich in highly polyunsaturated fatty acids, making them prone to undergo lipid peroxidation, at least *in vitro*.[152]

Steady-state measures of lipid peroxidation

Human atherosclerotic lesions have been shown to contain lipid hydroperoxides,[16] but the steady-state levels of peroxides in human body fluids, such as blood plasma, appear very low, usually <100 nM. These data come from assays that measure 'real' lipid peroxides[153–155] rather than notoriously-unspecific methods such as diene conjugation or the simple thiobarbituric acid (TBA) test.[44] HPLC-based TBA tests can, however, record comparably-low values, provided that BHT is added with the TBA reagents.[44,156] Human body fluids also contain low levels of F_2-isoprostanes, compounds isomeric to prostaglandins that appear to arise by free radical oxidation of phospholipids containing arachidonic acid.[45,157,158] However, it has been suggested that 8-epi$PGF_2\alpha$ can be generated by cyclooxygenase in human platelets, although this does not appear to be a significant contributor to total body production of this compound.[159,209] Isoprostanes appear to exist in human plasma largely esterified to phospholipids rather than 'free' and sensitive assays to measure them have been described.[45,157,158,160] Preliminary data (Gopaul, Angaard and Halliwell unpublished) suggest that PUFAs other than arachidonate (including

eicosapentaenoic and docosahexaenoic acids) can give rise to different families of isoprostane-like compounds upon peroxidation. This might be an approach to assessing the relative rates of peroxidation of different PUFAs in the human body.

Measuring 'total' lipid peroxidation in the human body

Peroxide levels in cells and tissues represent a balance between peroxide formation and peroxide metabolism or decomposition, i.e. they are essentially a 'steady-state' measurement. Can some measure of 'total' peroxidation (i.e. the input side of the equation) be obtained?

This has most-commonly been attempted by measuring hydrocarbon gases (ethane, pentane) in exhaled air[161] and urinary excretion of MDA (more properly called TBA-reactive material).[162] The latter assay is confounded by diet: most of the lipid-related TBARS appearing in urine seems to arise from lipid peroxides or aldehydes in ingested food, which are presumably largely generated during cooking.[163,164] For example, Brown *et al*[164] showed that a diet rich in cooked meat promoted urinary TBARS excretion, to an extent depending on the temperature at which the meat was cooked. Hence urinary TBARS is not a suitable assay to assess whole body lipid peroxidation in response to changes in dietary composition, although it could theoretically be used to look at effects of antioxidant supplementation of people on a 'fixed' diet.[163] In any case, HPLC must be used to separate the real $(TBA)_2MDA$ adduct; much TBARS in urine is not even lipid-derived[165] or arises from aldehydes other than MDA.[166] A recent study[167] showed that urinary TBARS (measured by HPLC) was unaffected by 50 days supplementation with 300 mg of d-α-tocopherol in 4 healthy Japanese subjects.

Breath excretion of ethane and pentane, minor end-products of lipid peroxidation, is very difficult (but not impossible[168]) to measure in humans because of the problem of contamination of the atmosphere by these gases, resulting in their partitioning into body fat stores.[169] Particular problems with pentane include the fact that it is metabolized by cytochromes P-450[168,170] and that GC columns frequently used to separate 'pentane' for measurement have in fact failed to separate it from isoprene, a hydrocarbon also excreted in exhaled air.[169,171,172] Indeed, the real levels of excreted pentane seem close to zero in most humans.[169,171,172] Perhaps further evaluation of the technique of hydrocarbon gas exhalation should focus upon ethane,[161] but in general the technique would be difficult to use reliably in human studies except where subjects are confined to controlled environments breathing air of minimal hydrocarbon content. The possible effect of dietary changes upon hydrocarbon gas production by gut flora is another potential confounding factor.

Isoprostanes and their metabolites can be measured in human urine,[45,158,209] and this may prove to be a valuable assay of whole body lipid peroxidation if a confounding effect of diet can be ruled out.

PROTEINS: DAMAGE BY ROS AND RNS

Oxidative damage to proteins may be of particular importance *in vivo* both in its own right (affecting the function of receptors, enzymes, transport proteins etc and perhaps generating new antigens that provoke immune responses), and because it can contribute to secondary damage to other biomolecules, e.g. inactivation of DNA repair enzymes and loss of fidelity of DNA polymerases in replicating DNA.[173] Attack of various RNS ($ONOO^-$, $NO_2^\bullet$ and possibly some other species) upon tyrosine (both free and in proteins) leads to production of 3-nitrotyrosine, which can be measured immunologically or by HPLC or GC/MS techniques (reviewed in [174]). Reduction of nitrotyrosine to aminotyrosine increases the sensitivity of measurement, since the latter compound can be measured using highly-sensitive

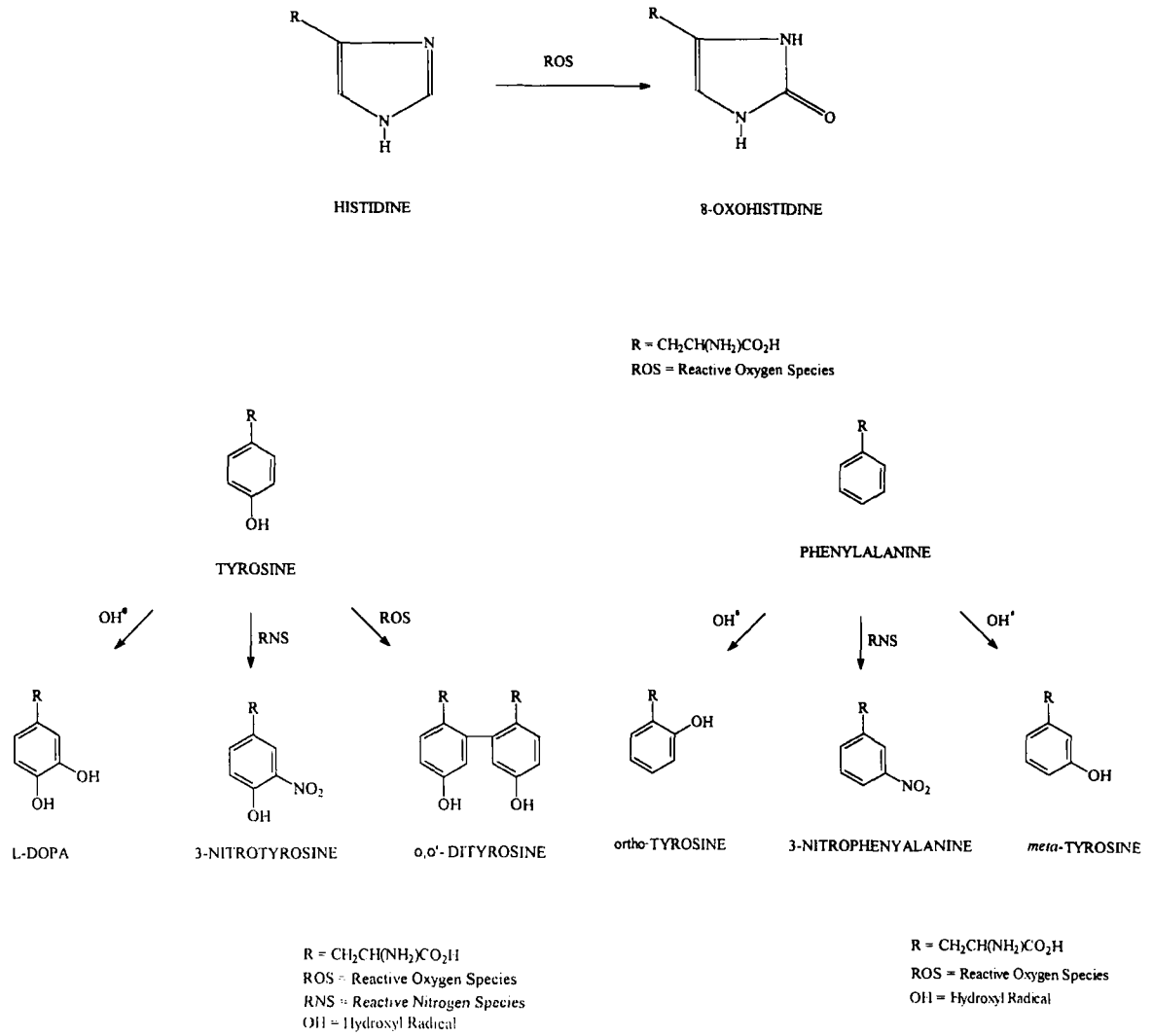

FIGURE 1 Some of the products of attack by reactive oxygen and nitrogen species upon amino acid residues in proteins.

electrochemical detection. Nitrotyrosine is also excreted in human urine,[43] although the possible confounding effect of dietary nitrotyrosine (if any) and of dietary nitrate/nitrite requires evaluation. For example, NO_2^- can interact with HOCl to generate a nitrating species that can convert tyrosine to 3-nitrotyrosine.[175]

The chemical reactions resulting from attack of ROS/RNS upon proteins are complex. Free radical attack can generate protein peroxides, which can decompose in complex ways.[176,177] Assays of human tissues and body fluids by simple 'peroxide determinations' such as those involving xylenol orange[178,179] or iodometric methods[180] could measure such peroxides; this could conceivably explain why levels of alleged 'lipid peroxides' measured by such techniques in human body fluids tend to be higher (often in the μM range) than those revealed by the more-specific techniques for measuring lipid peroxides that were discussed earlier.

Steady-state protein damage

Several assays for damage to specific amino acid residues in proteins have been developed (Figure 1) including assays of L-DOPA (produced by

tyrosine hydroxylation),[181] valine hydroxides (produced from valine hydroperoxides[177]), tryptophan hydroxylation and ring-opening products,[182,183] 8-oxohistidine,[184,185] dityrosine[174,186–188] and *ortho*- and *meta*-tyrosines, products of attack of $OH^{\bullet}$ upon phenylalanine.[42,189–190] The levels of any one (or, preferably, of more than one) of these products in proteins could in principle be used to assess the balance between oxidative protein damage and the repair or (more likely) hydrolytic removal of damaged proteins. The only products exploited to date have been the hydroxylated phenylalanines. For example, levels of *ortho*-tyrosine and dityrosine in human lens proteins have been reported in relation to age.[42] These products were also measured in hair from 'Alpine Man' *Homo tirolensis*.[191]

More use has been made of the *carbonyl assay*, a 'general' assay of oxidative protein damage,[40] to assess steady-state protein damage in human tissues and body fluids. The carbonyl assay is based on the fact that several ROS can attack amino acid residues in proteins (particularly histidine, arginine, lysine and proline) to produce carbonyl functions that can be measured after reaction with 2,4-dinitrophenylhydrazine.[40,192] The carbonyl assay has become widely used and many laboratories have developed individual protocols for it (e.g. [40,41,193]). Sometimes the assay procedures used in a particular laboratory are not precisely specified in published papers and even when they are, they often differ from those used originally by the group of Stadtman *et al.* (e.g. refs [40,192–196]). This point is important because there is a considerable variation in the 'baseline' levels of protein carbonyls in certain tissues, depending on how the assay is performed.[197,198] For example, levels reported for human brain cortex range from 1.5 to 6.4 nmol/mg protein.[198] By contrast, most groups seem to obtain broadly-comparable values for protein carbonyls in human plasma, of <1 nmol/mg protein, so plasma protein carbonyls should be a useful marker of oxidative protein damage for nutritional studies. More work needs to be done to identify the molecular nature of the carbonyls, i.e. which amino acid residues have been damaged and on what proteins. Western-blotting assays based on the use of anti-DNPH antibodies have been developed in an attempt to identify oxidatively-damaged proteins in tissues and body fluids.[193,199] Covalent binding of certain aldehyde end-products of lipid peroxidation to proteins can generate 'carbonyls' and this must be borne in mind.[145]

Measures of total ongoing protein damage

As mentioned above, urinary nitrotyrosine[43] might be useful as a generalized index of attack by reactive nitrogen species. Very little research has been carried out on the presence of oxidized amino acids and their metabolites in urine, except that bityrosine has been detected and can be measured by HPLC with fluorescence detection (our unpublished data). Alysine-MDA adduct was found in rat and human urine.[208] More work needs to be done in this area, and the possible confounding effects of oxidized proteins/amino acids in the diet (e.g. in irradiated foods[189]) must be considered.

CONCLUSION

The proposal[4] that 'state of the art' biomarkers of oxidative damage could be used to answer questions about dietary antioxidant effects in humans is a fairly novel one. Some of the assays currently available need further development, particularly those of 'total ongoing' DNA and protein damage. However, even at the present stage of development, useful information can be (and has been) gained. We can at last begin to put the speculations of recent years about optimal antioxidant nutrition onto a firm scientific basis.

Acknowledgements

I am particularly grateful to the Ministry of Agriculture, Fisheries and Food (MAFF, UK) for research support.

References

1. α-Tocopherol, β-Carotene Prevention Study Group (1994) The effect of vitamin E and beta carotene on the

incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine*, **330**, 1029–35.
1a. P.M. Rowe (1996) Beta-carotene takes a collective beating. *Lancet*, **347**, 249.
2. J. Balentine (1982) *Pathology of oxygen toxicity*. Academic Press, New York.
3. I. Fridovich (1986) Superoxide dismutases. *Methods in Enzymology*, **58**, 61–97.
4. B. Halliwell (1994) Free radicals and antioxidants: a personal view. *Nutrition Reviews*, **52**, 253–265.
5. C. von Sonntag (1987) *The chemical basis of radiation biology*. Taylor and Francis, London.
6. B.M. Babior and R.C. Woodman (1990) Chronic granulomatous disease. *Seminars in Hematology*, **27**, 247–59.
7. S. Moncada and A. Higgs (1993) The L-arginine-nitric oxide pathway. *New England Journal of Medicine*, **329**, 2002–2011.
8. M.B. Grisham (1994) Oxidants and free radicals in inflammatory bowel disease. *Lancet*, **344**, 859–861.
9. B. Halliwell (1995) Oxygen radicals, nitric oxide and human inflammatory joint disease. *Annals of Rheumatic Disease*, **54**, 505–510.
10. H. Rubbo, R. Radi, M. Trujillo *et al.* (1994) Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. *Journal of Biological Chemistry*, **269**, 26066–26075.
11. V. Darley-Usmar, H. Wiseman and B. Halliwell (1995) Nitric oxide and oxygen radicals: a question of balance. *FEBS Letters*, **369**, 131–135.
12. R. E. Huie and S. Padmaja (1993) The reaction of NO with superoxide. *Free Radical Research Communications*, **18**, 195–199.
13. L. Castro, M. Rodriguez and R. Radi (1994) Aconitase is readily inactivated by peroxynitrite but not by its precursor nitric oxide. *Journal of Biological Chemistry*, **269**, 29409–29415.
14. V. Darley-Usmar and B. Halliwell (1996) Blood radicals. *Pharmaceutical Research*, **13**, 649–662.
15. M.A. Moro, V.M. Darley-Usmar, D.A. Goodwin *et al.* (1994) Paradoxical fate and biological action of peroxynitrite in human platelets. *Proceedings of the National Academy of Sciences of the USA*, **91**, 6702–6706.
16. D. Steinberg, S. Parthasarathy, T.E. Carew *et al.* (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine*, **320**, 915–924.
17. A. Graham, N. Hogg, B. Kalyanaraman *et al.* (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by macrophage scavenger receptors. *FEBS Letters*, **330**, 181–185.
18. A. Van der Vliet, D. Smith, C.A. O'Neill *et al.* (1994) Interactions of peroxynitrite with human plasma and its constituents. *Biochemical Journal*, **303**, 295–301.
19. J.A. Swain, V. Darley-Usmar and J.M.C. Gutteridge (1994) Peroxynitrite releases copper from caeruloplasmin: implications for atherosclerosis. *FEBS Letters*, **342**, 49–52.
20. H. Esterbauer, G. Striegl, H. Puhl and M. Rotheneder (1989) Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Research Communications*, **6**, 67–74.
21. C. Smith, M.J. Mitchinson, O.I. Aruoma and B. Halliwell (1992) Stimulation of lipid peroxidation and hydroxyl radical generation by the contents of human atherosclerotic lesions. *Biochemical Journal*, **286**, 901–905.
22. J.S. Beckman, Y.Z. Ye, P.G. Anderson *et al.* (1994) Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biological Chemistry Hoppe-Seyler*, **375**, 81–88.
23. N. Hogg, B. Kalyanaraman, J. Joseph, A. Struck and S. Parthasarathy (1993) Inhibition of low-density lipoprotein oxidation by nitric oxide. *FEBS Letters*, **334**, 170–174.
24. B. Chance, H. Sies and A. Boveris (1979) Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, **59**, 527–605.
25. H. Sies (ed) (1991) *Oxidative stress, oxidants and antioxidants*. Academic Press, London and New York.
26. B. Frei (ed) (1994) *Natural Antioxidants in Human Health and Disease*, Academic Press, San Diego.
27. B. Halliwell and J.M.C. Gutteridge (1990) The antioxidants of human extracellular fluids. *Archives of Biochemistry and Biophysics*, **280**, 1–8.
28. F. Ursini and A. Bindoli (1987) The role of selenium peroxidases in the protection against oxidative damage of membranes. *Chemistry and Physics of Lipids*, **44**, 255–276.
29. L.S. Chubatsu and R. Meneghini (1993) Metallothionein protects DNA from oxidative damage. *Biochemical Journal*, **291**, 193–198.
30. J.S. Lazo, Y. Kondo, D. Dellapiazza *et al.* (1995) Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. *Journal of Biological Chemistry*, **270**, 5506–5510.
31. K. Kim, I.H. Kim, K.Y. Lee, S.G. Rhee and E.R. Stadtman (1988) The isolation and purification of a specific 'protector' protein which inhibits enzyme inactivation by a thiol/Fe(III)/O_2 mixed-function oxidation system. *Journal of Biological Chemistry*, **263**, 4704–4711.
32. H.Z. Chae, K. Robison, L.B. Poole *et al.* (1994) Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proceedings of the National Academy of Sciences of the USA*, **91**, 7017–7021.
33. L. Ernster and G. Dallner (1995) Biochemical, physiological and medical aspects of ubiquinone function. *Biochimica et Biophysica Acta*, **1271**, 195–204.
34. R.A. Ehrenkranz (1989) Vitamin E and retinopathy of prematurity: still controversial. *Journal of Pediatrics*, **114**, 801–803.
35. Editorial (1991) Retinopathy of prematurity. *Lancet*, **337**, 83–84.
36. B. Halliwell and M. Dizdaroglu (1992) The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. *Free Radical Research Communications*, **16**, 75–87.
37. W.G. Stillwell, H.X. Xu, J.A. Adkins *et al.* (1989) Analysis of methylated and oxidized purines in urine by capillary gas chromatography-mass spectrometry. *Chemical Research in Toxicology*, **2**, 94–99.
38. B.N. Ames (1989) Endogenous oxidative DNA damage, aging, and cancer. *Free Radical Research Communications*, **7**, 121–128.
39. E.R. Stadtman and C.N. Oliver (1991) Metal-catalyzed oxidation of proteins. Physiological consequences. *Journal of Biological Chemistry*, **266**, 2005–2008.
40. R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, S. Shaltiel and E.R. Stadtman (1990) Determination of carbonyl content

in oxidatively modified protein. *Methods in Enzymology*, **186,** 464–487.
41. A.Z. Reznick and L. Packer (1994) Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods in Enzymology*, **233,** 357–363.
42. M.C. Wells-Knecht, T.G. Huggins, D.G. Dyer *et al.* (1993) Oxidized amino acids in lens proteins with age. Measurement of *o*-tyrosine and dityrosine in the aging human lens. *Journal of Biological Chemistry*, **268,** 12348–12352.
43. H. Ohshima, M. Friesen, I. Brouet and H. Bartsch (1990) Nitro-tyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food and Chemical Toxicology*, **28,** 647–652.
44. B. Halliwell and S. Chirico (1993) Lipid peroxidation: its mechanism, measurement and significance. *American Journal of Clinical Nutrition*, **57,** 715S–725S.
45. J.D. Morrow and L.J. Roberts II (1994) Mass spectrometry of prostanoids: F_2-isoprostanes produced by non-cyclooxygenase free radical-catalyzed mechanism. *Methods in Enzymology*, **233,** 163–174.
46. H. Kaur and B. Halliwell (1990) Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chemico-Biological Interactions*, **73,** 235–247.
47. H. Ogihara, T. Ogihara, M. Miki, H. Yasuda and M. Mino (1995) Plasma copper and antioxidant status in Wilson's disease. *Pediatric Research*, **37,** 219–226.
48. B. Demple and L. Harrison (1994) Repair of oxidative damage to DNA: enzymology and biology. *Annual Reviews of Biochemistry*, **63,** 915–948.
49. B.N. Ames, M.K. Shigenaga and T.M. Hagen (1993) Oxidants, antioxidants and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the USA*, **90,** 7915–22.
50. J.R. Totter (1980) Spontaneous cancer and its possible relationship to oxygen metabolism. *Proceedings of the National Academy of Sciences of the USA*, **77,** 1763–1767.
51. P. Cerutti (1994) Oxy-radicals and cancer. *Lancet*, **344,** 862–863.
52. W.C. Orr and R.S. Sohal (1994) Extension of life-span by over-expression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science*, **263,** 1128–1130.
53. G. Block, B. Patterson and A. Subar (1992) Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer*, **18,** 1–29.
54. I.T. Johnson, G. Williamson and S.R.R. Musk (1994) Anticarcinogenic factors in plant foods. A new class of nutrients? *Nutrition Research*, **7,** 175–204.
55. W.C. Willett (1994) Diet and health: what should we eat? *Science*, **264,** 532–537.
56. Y. Zhang, T.W. Kensler, C.G. Cho *et al.* (1994) Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proceedings of the National Academy of Sciences of the USA*, **91,** 3147–3150.
57. K.F. Gey (1995) Ten-year retrospective on the antioxidant hypothesis of arteriosclerosis. *Journal of Nutritional Biochemistry*, **6,** 206–236.
58. A.T. Diplock (1994) Antioxidants and disease prevention. *Molecular Aspects of Medicine*, **15,** 293–376.
59. T. Prestera and P. Talalay (1995) Electrophile and antioxidant regulation of enzymes that detoxify carcinogens. *Proceedings of the National Academy of Sciences of the USA*, **92,** 8965–8969.
60. M.G. Traber, R.J. Sokol, S.P. Ringel *et al.* (1987) Lack of tocopherol in peripheral nerves of vitamin E-deficient patients with peripheral neuropathy. *New England Journal of Medicine*, **317,** 262–265.
61. E.B. Rimm, M.J. Stampfer, A. Ascherio *et al.* (1993) Vitamin E consumption and the risk of coronary heart disease in men. *New England Journal of Medicine*, **328,** 1450–1456.
62. R.A. Riemersma (1994) Epidemiology and the role of antioxidants in preventing coronary heart disease: a brief overview. *Proceedings of the Nutrition Society*, **53,** 59–65.
63. G.W. Burton and M.G. Traber (1990) Vitamin E: antioxidant activity biokinetics and bioavailability. *Annual Reviews of Nutrition*, **120,** 357–382.
64. A. Bendich, L.J. Machlin, O. Scandurra *et al.* (1986) The anti-oxidant role of vitamin C. *Free Radical Biology and Medicine*, **2,** 419–44.
65. B. Halliwell (1990) How to characterize a biological anti-oxidant. *Free Radical Research Communications*, **9,** 1–32.
66. M.M. Mathews-Roth (1987) Photoprotection by carotenoids. *Federation Proceedings*, **46,** 1890–1893.
67. J.M. Gaziano, A. Hatta, M. Flynn *et al.* (1995) Supplementation with β-carotene in vivo and in vitro does not inhibit low density lipoprotein oxidation. *Atherosclerosis*, **112,** 187–195.
68. B. Halliwell (1996) Antioxidants in human health and disease. *Annual Reviews of Nutrition*, **16,** 33–50.
69. G. von Poppel, H. Poulsen, S. Loft and H. Verhagen (1995) No influence of Beta Carotene on oxidative DNA damage in male smokers. *Journal of the National Cancer Institute*, **87,** 310–311.
70. J.S. Bertram (1993) Inhibition of chemically induced neoplastic transformation by carotenoids. *Annals of the New York Academy of Sciences*, **686,** 161–176.
71. T. Nikawa, W.A. Schulz, C.E. van der Brink *et al.* (1995) Efficacy of all-*trans*-β-carotene, canthaxanthin and all-*trans*-9-*cis*- and 4-oxoretinoic acids in inducing differentiation of an F9 embryonal carcinoma RAR β-*lac* Z reporter cell line. *Archives of Biochemistry and Biophysics*, **316,** 665–672.
72. X.D. Wang, G.W. Tang, J.G. Fox *et al.* (1991) Enzymatic conversion of β-carotene into β-apocarotenals and retinoids by human, monkey, ferret and rat tissues. *Archives of Biochemistry and Biophysics*, **285,** 8–16.
73. N.I. Krinsky (1993) Action of carotenoids in biological systems. *Annual Reviews of Nutrition*, **13,** 561–587.
74. J.P. Eiserich, A. van der Vliet, G.J. Handelman, B. Halliwell and C.E. Cross (1995) Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. *American Journal of Clinical Nutrition*, **62** (suppl), 14905–15005.
75. A.K. Ratty and N.P. Das (1988) Effects of flavonoids on non-enzymatic lipid peroxidation: structure-activity relationship. *Biochemical Medicine and Metabolic Biology*, **39,** 69–79.
76. N. Salah, N.J. Miller, G. Paganga, L. Tijburg, G.P. Bolwell and C. Rice-Evans (1995) Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives of Biochemistry and Biophysics*, **322,** 339–346.
77. M.J. Laughton, P.J. Evans, M.A. Moroney *et al.* (1991) Inhibition of mammalian 5-lipoxygenase and cyclooxygenase by flavonoids and phenolic dietary additives. *Biochemical Pharmacology*, **42,** 1673–1681.
78. B. Stavric (1994) Quercetin in our diet: from potent

mutagen to probably anticarcinogen. *Clinical Biochemistry*, **27**, 245–248.
79. M.G.L. Hertog, E.J.M. Feskens, P.C.H. Hollman *et al.* (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet*, **342**, 1007–1011.
80. N.P. Das (1971) Studies on flavonoid metabolism. Absorption and metabolism of (+)-catechin in man. *Biochemical Pharmacology*, **20**, 3435–3445.
81. J. Kanner, E. Frankel, R. Granit *et al.* (1994) Natural antioxidants in grapes and wines. *Journal of Agricultural and Food Chemistry*, **42**, 64–69.
82. D.M. Goldberg (1995) Does wine work? *Clinical Chemistry*, **41**, 14–16.
83. N.J. Miller and C. Rice-Evans (1995) Antioxidant activity of resveratrol in red wine. *Clinical Chemistry*, **41**, 1–2.
84. W.F. Hodnick, F.S. Kung, W.J. Roettger, C.W. Bohmont and R.S. Pardini (1986) Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. *Biochemical Pharmacology*, **35**, 2345–2357.
85. A. Rahman, S. Shahabuddin, S.M. Hadi, J.H. Parish, K. Ainley (1989) Strand scission in DNA induced by quercetin and Cu(II): role of Cu(I) and oxygen free radicals. *Carcinogenesis*, **10**, 1833–1839.
86. H. Chen and A. Tappel (1995) Vitamin E, selenium, Trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q, β-carotene, canthaxanthin, and (+)-catechin protect against oxidative damage to kidney, heart, lung and spleen. *Free Radical Research*, **22**, 177–186.
87. J.H. Weisburger (1995) Tea antioxidants and health. In E. Cadenas, L. Packer (eds), *Handbook of antioxidants*. Marcel Dekker Inc, New York.
88. A. Laires, J. Gaspar, H. Borba, M. Proença *et al.* (1993) Genotoxicity of nitrosated red wine and of the nitrosatable phenolic compounds present in wine: tyramine, quercetin and malvidine-3-glucoside. *Food Chemistry and Toxicology*, **31**, 989–994.
89. J.D. Cook, B.S. Skikne, S.R. Lynch and M.E. Reusser (1986) Estimates of iron sufficiency in the US population. *Blood*, **68**, 726–731.
90. M.J. Burt, J.W. Halliday and L.W. Powell (1993) Iron and coronary heart disease. *British Medical Journal*, **307**, 575–576.
91. C.Q. Edwards, L.M. Griffen, D. Goldgar *et al.* (1988) Prevalence of hemochromatosis among 11 065 presumably healthy blood donors. *New England Journal of Medicine*, **318**, 1355–1362.
92. J.M.C. Gutteridge, D.A. Rowley, E. Griffiths and B. Halliwell (1985) Low-molecular-weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. *Clinical Science*, **68**, 463–467.
93. C.J. McLaran, J.H.N. Bett, J.A. Nye and J.W. Halliday (1982) Congestive cardiomyopathy and haemochromatosis – rapid progression possibly accelerated by excessive ingestion of ascorbic acid. *Australian and New Zealand Journal of Medicine*, **12**, 187–188.
94. B. Rowbotham and H.P. Roeser (1984) Iron overload associated with congenital pyruvate kinase deficiency and high dose ascorbic-acid ingestion. *Australian and New Zealand Journal of Medicine*, **14**, 667–679.
95. H.M. Berger, S. Mumby and J.M.C. Gutteridge (1995) Ferrous ions detected in iron-overloaded cord blood plasma from preterm and term babies: implications for oxidative stress. *Free Radical Research*, **22**, 555–559.
96. H.J. Powers, A. Loban, K. Silvers and A.T. Gibson (1995) Vitamin C at concentrations observed in premature babies inhibits the ferroxidase activity of caeruloplasmin. *Free Radical Research*, **22**, 57–65.
97. B. Halliwell, J.M.C. Gutteridge and C.E. Cross (1992) Free radicals, antioxidants, and human disease: where are we now? *Journal of Laboratory and Clinical Medicine*, **119**, 598–620.
98. B. Halliwell (1995) Antioxidant characterization, Methodology and mechanism. *Biochemical Pharmacology*, **49**, 1341–1348.
99. B. Halliwell, R. Aeschbach, J. Löliger and O.I. Aruoma (1995) The characterization of antioxidants. *Food and Chemical Toxicology*, **33**, 601–617.
100. R. Schreck, K. Albermann and P.A. Bauerle (1992) Nuclear factor κB: an oxidative stress-responsive transcription factor of eukaryotic cells. *Free Radical Research Communications*, **17**, 221–237.
101. S.B. Kong, M.B. Yim, E.R. Stadtman and P.B. Chock (1995) Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism by nitrating the tyrosine residue: tyrosine kinase fails to phosphorylate nitrated tyrosine. *FASEB Journal*, **9**, A1303 (abstract).
102. R.J. Ruch and J.E. Klaunig (1988) Inhibition of mouse hepatocyte intercellular communication by paraquat-generated oxygen free radicals. *Toxicology and Applied Pharmacology*, **94**, 427–436.
103. J. Hu, L. Engman and I.A. Cotgreave (1995) Redox-active chalcogen-containing glutathione peroxidase mimetics and antioxidants inhibit tumour-promoter induced downregulation of gap junctional intercellular communication between WB-F344 liver epithelial cells. *Carcinogenesis*, **16**, 1815–1824.
104. R.H. Burdon, D. Alliangana and V. Gill (1994) Endogenously generated active oxygen species and cellular glutathione levels in relation to BHK-21 cell proliferation. *Free Radical Research*, **21**, 121–134.
105. D.I. Feig, T.M. Reid and L.A. Loeb (1994) Reactive oxygen species in tumorigenesis. *Cancer Research*, **54**, (suppl): 1890s–1894s.
106. T.A. Sarafian and D.E. Bredesen (1994) Is apoptosis mediated by reactive oxygen species? *Free Radical Research*, **21**, 1–8.
107. S. Steenken (1989) Purine bases, nucleosides and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical cations and e^- and $OH^\bullet$ adducts. *Chemical Reviews*, **89**, 503–520.
108. M. Dizdaroglu (1993) Chemistry of free radical damage to DNA and nucleoproteins. In B. Halliwell and O.I. Aruoma (eds), *DNA and free radicals*. Ellis Horwood, Chichester, UK, 19–39.
109. M.N. Routledge, D.A. Wink, L.K. Keefer and A. Dipple (1994) DNA sequence changes induced by two nitric oxide donor drugs in the *supF* assay. *Chemical Research in Toxicology*, **7**, 628–632.
110. B. Epe (1993) DNA damage induced by photosensitization. In B. Halliwell and O.I. Aruoma (eds), *DNA and free radicals*. Ellis Horwood, Chichester UK, pp 41–65.
111. J. Cadet, J.L. Ravant, G.W. Buchko *et al.* (1994) Singlet oxygen DNA damage: chromatographic and mass spectrometric analysis of damage products. *Methods in Enzymology*, **234**, 79–88.
112. T de Rojas-Walker, S. Tamir, H. Ji, J.S. Wishnock and S.R. Tannenbaum (1995) Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. *Chemical Research in Toxicology*, **8**, 473–477.

113. M.K. Shigenaga, S. Christen, H.C. Yeo and B.N. Ames (1995) Nitration of guanine and its nucleoside derivatives at the C-8 position by nitronium ion, nitrogen dioxide and peroxynitrite. *Abstracts of the Meeting of the Bay Area Oxygen Club*, p 122.
114. V. Yermilov, J. Rubio, M. Becchi, M.D. Friesen, B. Pignatelli and H. Ohshima (1995) Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite *in vitro*. *Carcinogenesis*, **16**, 2045–2050.
115. V. Yermilov, J. Rubio and H. Oshima (1995) Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal by depurination. *FEBS Letters*, **376**, 207–210.
116. R.A. Floyd, J.J. Watson, P.K. Wong *et al.* (1986) Hydroxy-free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. *Free Radical Research Communications*, **1**, 163–172.
117. Z. Nackerdien, R. Olinski and M. Dizdaroglu (1992) DNA base damage in chromatin of γ-irradiated cultured human cells. *Free Radical Research Communications*, **16**, 259–273.
118. J.S. Beckman, J. Chen, H. Ischiropoulos, J.P. Crow (1994) Oxidative chemistry of peroxynitrite. *Methods in Enzymology*, **233**, 229–240.
119. A van der Vliet, C.A. O'Neill, B. Halliwell, C.E. Cross and H. Kaur (1994) Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite: evidence for hydroxyl radical production from peroxynitrite. *FEBS Letters*, **339**, 89–92.
120. L.P. Candeias, K.B. Patel, M.R.L. Stratford and P. Wardman (1993) Free hydroxyl radicals are formed upon reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. *FEBS Letters*, **333**, 151–153.
121. K. Thorstensen and I. Romslo (1984) Uptake of iron from transferrin by isolated hepatocytes. *Biochimica et Biophysica Acta*, **804**, 200–208.
121a. J.T. Wolfe, D. Ross and G.M. Cohen (1994) A role for metals and free radicals in the induction of apoptosis in thymocytes. *FEBS Letters*, **352**, 58–62.
122. J.P.E. Spencer, A. Jenner, K. Chimel *et al.* (1995) DNA damage in human respiratory tract epithelial cells. Damage by gas phase cigarette smoke apparently involves attack by reactive nitrogen species in addition to oxygen radicals. *FEBS Letters*, **375**, 179–182.
123. D.C. Malins and R. Haimanot (1991) Major alterations in the nucleotide structure of DNA in cancer of the female breast. *Cancer Research*, **51**, 5430–5432.
124. R. Olinski, T. Zastawny, J. Budzbon *et al.* (1992) DNA base modifications in chromatin of human cancerous tissues. *FEBS Letters*, **309**, 193–198.
125. S. Loft, K. Vistisen, M. Ewertz *et al.* (1992) Oxidative DNA-damage estimated by 8-hydroxydeoxyguanosine excretion in man: influence of smoking, gender and body mass index. *Carcinogenesis*, **13**, 2241–2247.
126. S. Loft, A. Fischer-Nielsen, I.B. Jeding *et al.* (1993) 8-Hydroxydeoxyguanosine as a urinary marker of oxidative DNA damage. *Journal of Toxicology and Environmental Health*, **40**, 391–404.
127. M.K. Shigenaga, E.N. Aboujaoude, Q. Chen and B.N. Ames (1994) Assays of oxidative DNA damage biomarkers. *Methods in Enzymology*, **234**, 16–33.
128. A.J.R. Teixeira, J.H. Gommers-Ampt, G. van de Werken *et al.* (1993) Method for the analysis of oxidized nucleosides by gas chromatography/mass spectrometry. *Analytical Biochemistry*, **214**, 474–483.
129. K. Sakumi, M. Furuichi, T. Tsuzuki *et al.* (1993) Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *Journal of Biological Chemistry*, **268**, 23524–23530.
130. J.Y. Mo, H. Maki and M. Sekiguchi (1992) Hydrolytic elimination of a mutagenic nucleotide, 8-oxodGTP, by human 18-kilodalton protein; sanitization of nucleotide pool. *Proceedings of the National Academy of Sciences of the USA*, **89**, 11021–11025.
131. S. Loft, E.J.M. Velthuis-te Wierik, H. van den Berg and H.E. Poulsen (1995) Energy restriction and oxidative DNA damage in humans. *Cancer Epidemiology Biomarkers and Prevention*, **4**, 515–519.
132. V. Verhagen, H.E. Poulsen, S. Loft *et al.* (1995) Reduction of oxidative DNA-damage in humans by Brussels sprouts. *Carcinogenesis*, **16**, 969–970.
133. M. Dizdaroglu (1993) Quantitative determination of oxidative base damage to DNA by stable isotope-dilution mass spectrometry. *FEBS Letters*, **315**, 1–6.
134. J. Lunec, K. Herbert, S. Blount *et al.* (1994) 8-Hydroxydeoxyguanosine, marker of oxidative DNA damage in systemic lupus erythematosus. *FEBS Letters*, **348**, 131–138.
135. P.W. Turk and S.A. Weitzman (1995) Free radical DNA adduct 8-OH-deoxyguanosine affects activity of HPAII and MSPI restriction endonucleases. *Free Radical Research*, **23**, 255–258.
136. H. Wiseman, H. Kaur and B. Halliwell (1995) DNA damage and cancer: measurement and mechanism. *Cancer Letters*, **93**, 113–120.
137. J.L. Ravanat, R.J. Turesky, E. Gremaud, L.J. Trudel and R.H. Stadler (1995) Determination of 8-oxoguanine in DNA by gas chromatography-mass spectrometry and HPLC-electrochemical detection: overestimation of the background level of the oxidized base by the gas chromatography-mass spectrometry assay. *Chemical Research in Toxicology*, **8**, 1039–1045.
138. M. Hamberg and L.Y. Zhang (1995) Quantitative determination of 8-hydroxyguanine and guanine by isotope dilution mass spectrometry. *Analytical Biochemistry*, **229**, 336–344.
139. H.G. Claycamp (1992) Phenol sensitization of DNA to subsequent oxidative damage in 8-hydroxyguanosine assays. *Carcinogenesis*, **13**, 1289–1292.
140. A.R. Collins, S.J. Duthie and V.L. Dobson (1993) Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis*, **14**, 1733–1735.
141. G. Harris, S. Bashir and P.G. Winyard (1994) 7,8-Dihydro-8-oxo-2'-deoxyguanosine present in DNA is not simply an artefact of isolation. *Carcinogenesis*, **15**, 411–413.
142. M. Dizdaroglu, G. Rao, B. Halliwell and E. Gajewski (1991) Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. *Archives of Biochemistry and Biophysics*, **285**, 317–324.
143. K. Umegaki, S. Ikegami and T. Ichikawa (1993) Influence of dietary vitamin E on the 8-hydroxydeoxyguanosine levels in rat liver DNA. *Journal of Nutritional Science and Vitaminology*, **39**, 303–310.
144. H. Esterbauer, H. Puhl, M. Dieber-Rothender, G. Waeg and H. Rabl (1991) Effect of antioxidants on oxidative modification of LDL. *Annals of Medicine*, **23**, 573–581.

145. H. Esterbauer, J. Gebicki, H. Puhl and G. Jurgens (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biology and Medicine*, **13**, 341–390.
146. T. Douki and B.N. Ames (1994) An HPLC-EC assay for 1,N^2-propano adducts of 2′-deoxyguanosine with 4-hydroxynonenal and other α,β-unsaturated aldehydes. *Chemical Research in Toxicology*, **7**, 511–518.
147. F. El Ghissassi, A. Barbin, J. Nair and H. Bartsch (1995) Formation of 1,N^6-ethenoadenine and 3,N^4-ethenocytosine by lipid peroxidation products and nucleic acid bases. *Chemical Research in Toxicology*, **8**, 278–283.
148. A.K. Chaudhary, M. Nokubo, G.R. Reddy *et al.* (1994) Detection of endogenous malondialdehyde-deoxyguanosine adducts in human liver. *Science*, **265**, 1580–1582.
149. C.E. Vaca, J.L. Fang, M. Mutanen and L. Valsta (1995) ^{32}P-postlabelling determination of DNA adducts of malonaldehyde in humans: total white blood cells and breast tissue. *Carcinogenesis*, **16**, 1847–1851.
150. S. Agarwal, J.J. Wee, M. Hadley and H.H. Draper (1994) Identification of a deoxyguanosine-malondialdehyde adduct in rat and human urine. *Lipids*, **29**, 429–432.
151. E.N. Frankel, E.J. Parks, R. Xu, B.O. Schneeman, P.A. Davis and J.B. German (1994) Effect of n-3 fatty acid-rich fish oil supplementation on the oxidation of low density lipoproteins. *Lipids*, **29**, 233–236.
152. B. Halliwell (1992) Reactive oxygen species and the central nervous system. *Journal of Neurochemistry*, **59**, 1609–1623.
153. K. Akasaka, A. Ohata, H. Ohrui and H. Meguro (1995) Automatic determination of hydroperoxides of phosphatidylcholine and phosphatidylethanolamine in human plasma. *Journal of Chromatography*, **B665**, 37–43.
154. B. Frei, Y. Yamamoto, D. Niclas and B.N. Ames (1988) Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. *Analytical Biochemistry*, **175**, 120–130.
155. A.E. Holley and T.F. Slater (1991) Measurement of lipid hydroperoxides in normal human blood plasma using HPLC-chemiluminescence linked to a diode array detector for measuring conjugated dienes. *Free Radical Research Communications*, **15**, 51–63.
156. S. Chirico, C. Smith, C. Marchant *et al.* (1993) Lipid peroxidation in hyperlipidaemic patients. A study of plasma using an HPLC-based thiobarbituric acid test. *Free Radical Research Communications*, **19**,51–57.
157. J. Noourooz-Zadeh, N.K. Gopaul, S. Barrow *et al.* (1995) Analysis of F_2-isoprostanes as indicators of non-enzymatic lipid peroxidation in vivo by gas chromatography-mass spectrometry: development of a solid-phase extraction procedure. *Journal of Chromatography*, **B667**, 199–208.
158. J.D. Morrow, B. Frei, A.W. Longmire *et al.* (1995) Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. *New England Journal of Medicine*, **332**, 1198–1203.
159. D. Pratico, J.A. Lawson and G.A. FitzGerald (1995) Cyclooxygenase-dependent formation of the isoprostane, 8-Epiprostaglandin $F_{2\alpha}$. *Journal of Biological Chemistry*, **270**, 9800–9808.
160. N.K. Gopaul, J. Nourooz-Zadeh, A.I. Mallet and E.E. Anggard (1994) Formation of F_2-isoprostanes during aortic endothelial cell-mediated oxidation of low density lipoprotein, *FEBS Letters*, **348**, 297–300.
161. R.F. Burk and T.M. Ludden (1989) Exhaled alkanes as indices of *in vivo* lipid peroxidation. *Biochemical Pharmacology*, **38**, 1029–1032.
162. L.G. McGirr, M. Hadley and H.H. Draper (1985) Identification of N^{α}-acetyl-ε-(2-propenal) lysine as a urinary metabolite of malondialdehyde. *Journal of Biological Chemistry*, **260**, 15427–15431.
163. S.N. Dhanakoti and H.H. Draper (1987) Response of urinary malondialdehyde to factors that stimulate lipid peroxidation in vivo. *Lipids*, **22**, 643–646.
164. E.D. Brown, V.C. Morris, D.G. Rhodes *et al.* (1995) Urinary excretion of malondialdehyde in subjects fed meat cooked at high or low temperatures. *Lipids*, **30**, 1053–1056.
165. J.M.C. Gutteridge and T.R. Tickner (1978) The characterisation of thiobarbituric acid reactivity in human plasma and urine. *Analytical Biochemistry*, **91**, 250–257.
166. H. Kosugi, T. Kojima and K. Kikugawa (1993) Characteristics of the thiobarbituric acid reactivity of human urine as a possible consequence of lipid peroxidation. *Lipids*, **28**, 337–343.
167. H. Kosugi, Y. Asano, T. Nagayama, M. Beppu and K. Kikugawa (1995) Urinary excretion of thiobarbituric acid reactive substances of healthy subjects supplemented with a high dose of d-α-tocopherol. *Biological and Pharmaceutical Bulletin*, **18**, 1275–1278.
168. C.M.F. Kneepkens, G. Lepage and C.C. Roy (1994) The potential of the hydrocarbon breath test as a measure of lipid peroxidation. *Free Radical Biology and Medicine*, **17**, 127–160.
169. J.R. Springfield and M.D. Levitt (1994) Pitfalls in the use of breath pentane measurements to assess lipid peroxidation. *Journal of Lipid Research*, **35**, 1497–1504.
170. C.R. Wade and A.M. van Rij (1985) *In vivo* lipid peroxidation in Man as measured by the respiratory excretion of ethane, pentane and other low-molecular-weight hydrocarbons. *Analytical Biochemistry*, **150**, 1–7.
171. D. Kohlmüller and W. Kochen (1993) Is *n*-pentane really an index of lipid peroxidation in humans and animals? A methodological reevaluation. *Analytical Biochemistry*, **210**, 268–276.
172. M. Phillips, J. Greenberg and M. Sabas (1994) Alveolar gradient of pentane in normal human breath. *Free Radical Research*, **20**, 333–337.
173. H. Wiseman and B. Halliwell (1996) Damage to DNA by reactive oxygen and nitrogen species: Role in inflammatory disease and progression to cancer. *Biochemical Journal*, **313**, 17–29.
174. A. van der Vliet, J.P. Eiserich, H. Kaur *et al.* (1996) Nitrotyrosine as a biomarker for reactive nitrogen species. *Methods in Enzymology*, (in press).
175. J.P. Eiserich, B. Halliwell, C.E. Cross and A. van der Vliet (1995) Formation of a nitrating species by reaction of nitrite with hypochlorous acid: a mechanism of tyrosine nitration independent of peroxynitrite. *Free Radical Biology and Medicine*, Abstracts of the Annual Meeting of the Oxygen Society, November 1995, p116.
176. K.S. Ambe and A.L. Tappel (1961) Oxidative damage to amino acids, peptides and proteins by radiation. *Journal of Food Science*, **26**, 448–451.
177. S. Fu, S. Gebicki, W. Jessup, J.M. Gebicki and R.T. Dean (1995) Biological fate of amino acid, peptide and protein hydroperoxides. *Biochemical Journal*, **311**, 821–827.
178. Z.Y. Jiang, A.C.S. Woolland and S.P. Wolff (1992) Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the

presence of xylenol orange: Comparison with the TBA assay and an iodometric method. *Lipids*, **26,** 853–856.
179. M. Hermes-Lima, W.G. Willmore and K.B. Storey (1995) Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. *Free Radical Biology and Medicine*, **19,** 271–280.
180. S.M. Thomas, W. Jessup, J.M. Gebicki and R.T. Dean (1989) A continuous-flow automated assay for iodometric estimation of hydroperoxides. *Analytical Biochemistry*, **176,** 353–359.
181. S.P. Gieseg, J.A. Simpson, T.S. Charlton, M.W. Duncan and R.T. Dean (1993) Protein-bound, 3,4-dihydroxyphenylalanine is a major reductant formed during hydroxyl radical damage to proteins. *Biochemistry*, **32,** 4780–4786.
182. Z. Maskos, J.D. Rush and W.H. Koppenol (1992) The hydroxylation of tryptophan. *Archives of Biochemistry and Biophysics*, **296,** 514–520.
183. H.R. Griffiths, J. Lunec and D.R. Blake (1992) Oxygen radical induced fluorescence in proteins; identification of the fluorescent tryptophan metabolite, N-formylkynurenine, as a biological index of radical damage. *Amino Acids*, **3,** 183–194.
184. K. Uchida and S. Kawakishi (1993) 2-Oxo-histidine as a novel biological marker for oxidatively modified proteins. *FEBS Letters*, **332,** 208–210.
185. K. Uchida and S. Kawakshi (1994) Identification of oxidized histidine generated at the active site of Cu,Zn-superoxide dismutase exposed to H_2O_2. *Journal of Biological Chemistry*, **269,** 2405–2410.
186. C. Giulivi and K.J.A. Davies (1993) Dityrosine and tyrosine oxidation products are endogenous markers for the selective proteolysis of oxidatively modified red blood cell hemoglobin by (the 19S) proteasome. *Journal of Biological Chemistry*, **268,** 8752–8759.
187. T.G. Huggins, M.C. Wells-Knecht, N.A. Detorie *et al.* (1993) Formation of *o*-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. *Journal of Biological Chemistry*, **268,** 12341–12347.
188. K. Kikugawa, T. Kato and A. Hayasaka (1991) Formation of dityrosine and other fluorescent amino acids by reaction of amino acids with lipid hydroperoxides. *Lipids*, **26,** 922–929.
189. L.S. Karam, D.S. Bertgold and M.G. Simic (1991) Biomarkers of OH radical damage *in vivo*. *Free Radical Research Communications*, **12/13,** 11–16.
190. H. Kaur and B. Halliwell (1994) Aromatic hydroxylation of phenylalanine as an assay for hydroxyl radicals. Measurement of hydroxyl radical formation from ozone and in blood from premature babies using improved HPLC methodology. *Analytical Biochemistry*, **220,** 11–15.
191. G. Lubec, M. Weninger and S.R. Anderson (1994) Racemization and oxidation studies of hair protein in the *Homo tirolensis*, *FASEB Journal*, **8,** 1166–1169.
192. A. Amici, R.L. Levine, L. Tsai and E.R. Stadtman (1989) Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *Journal of Biological Chemistry*, **264,** 3341–3346.
193. R.L. Levine, J.A. Williams, E.R. Stadtman and E. Shacter (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology*, **233,** 346–357.
194. P.J. Shaw, P.G. Ince, G. Falkous and D. Mantle (1995) Oxidative damage to protein in sporadic motor neurone disease spinal cord. *Annals of Neurology*, **38,** 691–695.
195. C.N. Oliver, B.A. Ahn, E.J. Moerman *et al.* (1987) Age-related changes in oxidized proteins. *Journal of Biological Chemistry*, **262,** 5488–5491.
196. A.C. Bowling, J.B. Schulz, R.H. Brown Jr and M.F. Beal (1993) Superoxide dismutase activity, oxidative damage and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *Journal of Neurochemistry*, **61,** 2322–2325.
197. G. Cao and R.G. Cutler (1995) Protein oxidation and aging. Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine. *Archives of Biochemistry and Biophysics*, **320,** 106–114.
198. L. Lyras, P.J. Shaw, P.J. Evans and B. Halliwell (1996) Oxidative damage and motor neurone disease. Difficulties in the measurement of protein carbonyls in human brain tissue. *Free Radical Research*, in press.
199. J. Keller, N.C. Halmes, J.A. Hinson and N.R. Pumford (1993) Immuno-chemical detection of oxidized proteins. *Chemical Research in Toxicology*, **6,** 430–433.
200. A. Wennmalm, G. Benthin, A. Edlund *et al* (1993) Metabolism and excretion of nitric oxide in humans. *Circulation Research*, **73,** 1121–1127.
201. B. Jilma, J. Kastner, C. Mensik *et al* (1996) Sex differences in concentrations of exhaled nitric oxide and plasma nitrate. *Life Science*, **58,** 469–476.
202. H. Moshage, B. Kok, J.R. Huizenga and P.L.M. Jansen (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. *Clinical Chemistry*, **41,** 892–896.
203. A. Wennmalm, G. Benthin, L. Jungersten, A. Edlund and A.S. Petersson (1994) Nitric oxide formation in man as reflected by plasma levels of nitrate, with special focus on kinetics, confounding factors and response to immunological challenge. In *The Biology of Nitric Oxide*, vol. 4, eds. S. Moncada, M. Feelisch, R. Busse and E.A. Higgs, Portland Press, pp. 474–476.
204. W. Brever, S. Epsztejn and Z.I. Cabantchik (1996) Dynamics of the cytosolic chelatable iron pool of K562 cells. *FEBS Letters*, **382,** 304–308.
205. K.E. Herbert, M.D. Evans, M.T.V. Finnegan *et al* (1996) A novel HPLC procedure for the analysis of 8-oxoguanine in DNA. *Free Radical Biology and Medicine*, **3,** 467–473.
206. R. Hasegawa, T. Chujo, K. Sai-Kato *et al* (1995) Preventive effects of green tea against liver oxidative DNA damage and hepatotoxicity in rats treated with 2-nitropropane. *Food Chemistry and Toxicology*, **33,** 961–970.
207. M.T.V. Finnegan, K.E. Herbert, M.D. Evans, H.R. Griffiths and J. Lunec (1996) Evidence for sensitization of DNA to oxidative damage during isolation. *Free Radical Biology and Medicine*, **20,** 93–98.
208. H. Mahmoodi, M. Hadley, Y.X. Chang and H.H. Draper (1995) Increased formation and degradation of malondialdehyde-modified proteins under conditions of peroxidative stress. *Lipids*, **30,** 963–966.
209. Z. Wang, G. Ciabattoni, C.Creminon *et al* (1995) Immunological characterization of urinary 8-epi-prostaglandin $F_{2\alpha}$ excretion in Man. *Journal of Pharmacology and Experimental Therapeutics*, **275,** 94–100.
210. C.S.I. Nobel, M. Kimland, B. Lind, S. Orrenius and A.F.G. Slater (1995) Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. *Journal of Biological Chemistry*, **270,** 26202–26208.