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REVIEW

Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean?

*,[1]Barry Halliwell & [1]Matthew Whiteman

[1]Department of Biochemistry, Faculty of Medicine, National University of Singapore, MD 7 #03-08, 8 Medical Drive, Singapore 117597, Singapore

1 Free radicals and other reactive species (RS) are thought to play an important role in many human diseases. Establishing their precise role requires the ability to measure them and the oxidative damage that they cause.

2 This article first reviews what is meant by the terms free radical, RS, antioxidant, oxidative damage and oxidative stress.

3 It then critically examines methods used to trap RS, including spin trapping and aromatic hydroxylation, with a particular emphasis on those methods applicable to human studies.

4 Methods used to measure oxidative damage to DNA, lipids and proteins and methods used to detect RS in cell culture, especially the various fluorescent 'probes' of RS, are also critically reviewed.

5 The emphasis throughout is on the caution that is needed in applying these methods in view of possible errors and artifacts in interpreting the results.
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Keywords: Cell culture; free radical; reactive species; antioxidant; oxidative stress; oxidative damage; fluorescent probe; lipid peroxidation; superoxide

Abbreviations: 8OHdG, 8-hydroxy-2′-deoxyguanosine; APF, 2-[6-(4′amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid; BMPO, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; CuZnSOD, copper and zinc-containing superoxide dismutase; DCFDA, dichlorofluorescin diacetate; DCF, dichlorofluorescein; DCFH, dichlorofluorescin; DECPO, 5,5-diethylcarbonyl-1-pyrroline *N*-oxide; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide; EPPN, *N*-2-(2-ethoxycarbonyl-propyl)-α-phenylnitrone; ESR, electron spin resonance; GC, gas chromatography; GSH, reduced glutathione; HPF, 2-[6-4′hydroxy]phenoxy-3H-xanthen-3-on-9-yl]benzoic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; L-DOPA, L-dihydroxyphenylalanine; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-∝]pyrazin-3-one; MDA, malondialdehyde; MnSOD, manganese-containing superoxide dismutase; MRI, magnetic resonance imaging; MS, mass spectrometry; PBN, *N*-*tert*-butyl-∝-phenylnitrone; TBA, thiobarbituric acid; TMINO, 1,1,3-trimethyl-isoindole *N*-oxide

Introduction

Free radicals and other 'reactive oxygen (ROS)/nitrogen/chlorine species' (for an explanation of these terms see Table 1) are widely believed to contribute to the development of several age-related diseases, and perhaps, even to the aging process itself (Halliwell & Gutteridge, 1999; Sohal *et al*., 2002) by causing 'oxidative stress' and 'oxidative damage' (terms explained in Table 2). For example, many studies have shown increased oxidative damage to all the major classes of biomolecules in the brains of Alzheimer's patients (Halliwell, 2001; Butterfield, 2002; Liu *et al*., 2003). Other diseases in which oxidative damage has been implicated include cancer, atherosclerosis, other neurodegenerative diseases and diabetes (Hagen *et al*., 1994; Chowienczyk *et al*., 2000; Halliwell, 2000a; 2001; 2002a, b; Parthasarathy *et al*., 2000). If oxidative damage contributes significantly to disease pathology (Table 3 lists the criteria needed to establish this), then actions that decrease it should be therapeutically beneficial (Halliwell, 2001; Lee *et al*., 2002a; Liu *et al*., 2003). If the oxidative damage is involved in the origin of a disease, then successful antioxidant treatment should delay or prevent the onset of that disease (Halliwell, 1991; 2002a, b; Galli *et al*., 2002; Steinberg & Witztum, 2002). To establish the role of oxidative damage (Table 3), it is therefore essential to be able to measure it accurately. For example, the failure of interventions with antioxidants such as vitamin E, β-carotene or ascorbate to decrease disease incidence in several human intervention trials may have simply been due to the failure of these compounds to decrease oxidative damage in the subjects tested (Halliwell, 1999a; 2000c; Levine *et al*., 2001; Meagher *et al*., 2001). In this review, we will examine the methods available to measure reactive species (RS) and oxidative damage, with a particular emphasis on those applicable to human studies.

Measuring RS *in vivo*: basic principles

Some fascinating techniques such as L-band electron spin resonance (ESR) with nitroxyl probes and magnetic resonance

*Author for correspondence; E-mail: bchbh@nus.edu.sg

Table 1 Nomenclature of reactive species

Free radicals[a]	*Nonradicals*
Reactive oxygen species (ROS)	
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $OH^{\bullet}$	Hypobromous acid, HOBr[b]
Hydroperoxyl, $HO_2^{\bullet}$	Hypochlorous acid, HOCl[c]
	Ozone O_3
Peroxyl, $RO_2^{\bullet}$	Singlet oxygen ($O_2^1\Delta g$)
Alkoxyl, $RO^{\bullet}$	Organic peroxides, ROOH
Carbonate, $CO_3^{\bullet -}$	Peroxynitrite, $ONOO^-$ [d]
Carbon dioxide, $CO_2^{\bullet -}$	Peroxynitrous acid, ONOOH[d]
Reactive chlorine species (RCS)	
Atomic chlorine, $Cl^{\bullet}$	Hypochlorous acid, HOCl[c]
	Nitryl (nitronium) chloride, NO_2Cl[e]
	Chloramines
	Chlorine gas (Cl_2)
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\bullet}$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$[d]
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO_2^+
	Alkyl peroxynitrites, ROONO
	Nitryl (nitronium) chloride, NO_2Cl[e]

ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O_3, $ONOO^-$, $^1O_2$, H_2O_2). In other words, *all oxygen radicals are ROS, but not all ROS are oxygen radicals*. Peroxynitrite and H_2O_2 are frequently erroneously described in the literature as free radicals, for example. RNS is also a collective term including nitric oxide and nitrogen dioxide radicals, as well as nonradicals such as HNO_2 and N_2O_4. 'Reactive' is not always an appropriate term: H_2O_2, $NO^{\bullet}$ and $O_2^{\bullet -}$ react quickly with only a few molecules, whereas $OH^{\bullet}$ reacts quickly with almost everything. $RO_2^{\bullet}$, $RO^{\bullet}$, HOCl, HOBr, $NO_2^{\bullet}$, $ONOO^-$, NO_2^+ and O_3 have intermediate reactivites.

[a]A free radical is any species that contains one or more unpaired electrons, that is, electrons singly occupying an atomic or molecular orbital.

[b]HOBr could also be regarded as a 'reactive bromine species'.

[c]HOCl is often included as an ROS.

[d]$ONOO^-$ and ONOOH are often included as ROS.

[e]NO_2Cl is a chlorinating and nitrating species that can be formed by reaction of HOCl with NO_2^-.

imaging spin trapping are under development to measure RS directly in whole animals (e.g. Berliner *et al*., 2001; Han *et al*., 2001; Utsumi & Yamada, 2003), but no probes are currently suitable for human use. Most RS persist for only a short time *in vivo* and cannot be measured directly. There are a few exceptions: examples include H_2O_2 (discussed below), and perhaps, $NO^{\bullet}$, in the sense that serum levels of NO_2^- have been claimed to measure vascular endothelial $NO^{\bullet}$ synthesis (Kelm *et al*., 1999), despite the fact that NO_2^- is quickly oxidized to NO_3^- *in vivo* (Kelm *et al*., 1999; Oldreive & Rice-Evans, 2001). Essentially, there are two approaches to detecting transient RS:

- attempting to *trap* these species and measure the levels of the trapped molecules and
- measuring the levels of the damage done by RS, that is, the amount of oxidative damage.

Sometimes other approaches are used. They include measurements of erythrocyte antioxidant defences and of total antioxidant activity of body fluids; falls in these parameters are often taken as evidence of oxidative stress. Erythrocytes cannot synthesize proteins, however, and their antioxidant enzyme levels may drop as they 'age' in the circulation (Denton *et al*., 1975). Thus changes in their levels are more likely to reflect changes in the rates of red blood cell turnover: if this slows down, the circulating erythrocytes will be older on average and so levels of antioxidant enzymes in them will appear to fall. *Vice versa*, if an intervention accelerates red cell removal or increases erythropoiesis, levels of antioxidants in red cells will seem to rise. Hence, such data should be interpreted with caution.

Depending on the method that is used to measure it, the plasma or serum 'total antioxidant capacity' (TAC) usually involves major contributions from urate, ascorbate and sometimes albumin −SH groups (Benzie & Strain, 1996; Halliwell & Gutteridge, 1999; Prior & Cao, 1999; Rice-Evans, 2000; Bartosz, 2003), although different methods measure different things (Schlesier *et al*., 2002; Bartosz, 2003). Thus, for example, if plasma albumin levels fall, TAC will fall. If urate levels rise, TAC will rise. The multiple changes in blood chemistry that occur in sick people mean that TAC changes should be interpreted with caution. TAC is also influenced by diet, often because consumption of certain foods may produce changes in plasma ascorbate and/or urate levels (Halliwell, 2003b).

Trapping of RS

The only technique that can 'see' free radicals directly and specifically is electron spin resonance (ESR), because it detects the presence of unpaired electrons. However, ESR detects only fairly unreactive radicals, since reactive ones do not accumulate to high-enough levels to be measured. One solution to this problem is to add 'traps' or 'probes', agents that intercept reactive radicals, reacting with them to form a stable radical that can be detected by ESR. Whole-body ESR techniques are being used on animals (Berliner *et al*., 2001; Han *et al*., 2001; Utsumi & Yamada, 2003), but are currently inapplicable to humans due to the lack of any human safety data on the probes. A wide range of traps is available for use in animals and cell culture systems, not only the tried and tested *N*-*tert*-butyl-$\propto$-phenylnitrone (PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Khan *et al*., 2003) but also such relative newcomers as 1,1,3-trimethyl-isoindole *N*-oxide (TMINO) (Bottle *et al*., 2003), 5,5-diethylcarbonyl-1-pyrroline *N*-oxide (DECPO) (Karoui *et al*., 2004), *N*-2-(2-ethoxycarbonyl-propyl)-α-phenylnitrone (EPPN) (Stolze *et al*., 2003), 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) (Liu *et al*., 1999; Khan *et al*., 2003) and 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO) (Zhao *et al*., 2001a). Potential problems in their use are summarized by Rosen *et al*. (1999), Halliwell & Gutteridge (1999) and Khan *et al*. (2003). One generally underappreciated problem is that the reaction products giving the ESR signal can be rapidly removed *in vivo* and in cultured cells, both by enzymic metabolism and by direct reduction by such agents as ascorbate. For example, when DMPO is used to trap $OH^{\bullet}$, any ascorbate present can directly reduce the DMPO−$OH^{\bullet}$ adduct to an ESR-silent species (Halliwell & Gutteridge, 1999). Many papers have

Table 2 Some key definitions

Oxidative stress
The term oxidative stress is vaguely defined. In essence, it refers to a serious imbalance between production of reactive species and antioxidant defence. Sies (1991) defined it as *a disturbance in the pro-oxidant–antioxidant balance in favor of the former, leading to potential damage*.
Oxidative stress can result from:
1. *Diminished levels of antioxidants*, for example, mutations affecting the activities of antioxidant defence enzymes such as CuZnSOD, or glutathione peroxidase, or toxins that deplete antioxidant defences. For example, many xenobiotics are metabolized by conjugation with GSH; high doses can deplete GSH and cause oxidative stress even if the xenobiotic is not itself a generator of reactive species. Deficiencies in dietary minerals (e.g. Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Se) and/or antioxidants can also cause oxidative stress.
   And/Or
2. *Increased production of reactive species*, for example, by exposure of cells or organisms to elevated levels of O_2 or to other toxins that are themselves reactive species (e.g. $NO^{\bullet}_2$) or are metabolized to generate reactive species (e.g. paraquat), or excessive activation of 'natural' systems producing such species (e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases).

Oxidative damage
The biomolecular damage that can be caused by direct attack of reactive species during oxidative stress.
Consequences of oxidative stress can include:
1. *Adaptation* of the cell or organism by upregulation of defence systems, which may (a) completely protect against damage; (b) protect against damage to some extent but not completely; or (c) 'overprotect' (e.g. the cells is then resistant to higher levels of oxidative stress imposed subsequently).
2. *Cell injury*: This involves damage (*oxidative damage*) to any or all molecular targets: lipids, DNA, protein, carbohydrate, etc. Oxidative damage can also occur during adaptation (point 1 above). *Not all damage caused by oxidative stress is oxidative damage*: damage to biomolecules can result from oxidative stress-related changes in ion levels (e.g. Ca^{2+}) or activation of proteases, for example.
3. *Cell death:* The cell may (a) recover from the oxidative damage by repairing it or replacing the damaged molecules, or (b) it may survive with persistent oxidative damage or (c) oxidative damage, especially to DNA, may trigger cell death, by apoptosis or necrosis.

Antioxidant
'Antioxidant' is a much-misused word. One can make almost any chemical exert antioxidant effects *in vitro* by choosing appropriate assay conditions. To reflect the fact that calling something an antioxidant means little without specifying the assay methodology used, Halliwell & Gutteridge (1999) defined an antioxidant as *any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate*. The term 'oxidizable substrate' includes every type of molecule found *in vivo*. This definition emphasizes the importance of the damage target studied and the source of reactive species used when antioxidant actions are examined.

Table 3 Criteria for implicating RS as a significant mechanism of tissue injury in human disease

1. The RS (or the oxidative damage it causes) should always be demonstrable at the site of injury.
2 The time course of formation of the RS (or of the oxidative damage it causes) should be consistent with the time course of tissue injury, preceding or accompanying it.
3 Direct application of the RS over a relevant time course (point 2 above) to the tissue at concentrations within the range found *in vivo* should reproduce most or all of the tissue injury and oxidative damage observed.
4 Removing the RS or inhibiting its formation should diminish the tissue injury to an extent related to the degree of inhibition of the oxidative damage caused by the RS.

described alleged antioxidant actions of various compounds evaluated *in vitro* by measuring decreases in the ESR signal that results when the compound is added to DMPO (or other spin trap) in the presence of radical-generating systems (frequently a Fenton system, $H_2O_2 + Fe^{2+}$). These decreases are attributed to scavenging of $OH^{\bullet}$ by the added compound, in competition with DMPO for $OH^{\bullet}$. Rarely do these papers report the essential controls (Halliwell, 1995): does the added compound interfere with the radical-generating system (e.g. decomposing H_2O_2 or chelating iron in the Fe^{2+}/H_2O_2 system) or does it interact directly with the trap spin adduct, reducing it to an ESR-silent species?

Rizzi *et al* (2003) have introduced triarylmethyl free radical, TAM OX063, as a probe for the detection of $O^{\bullet}_2{}^{-}$ in aqueous solution. In this case, the $O^{\bullet}_2{}^{-}$ reacts with the probe to cause the loss of the ESR signal. One advantage of TAM OX063 is that it is not subject to reduction by such agents as ascorbate or reduced glutathione (GSH).

Ex vivo *trapping* Presently available spin traps cannot be administered to humans because of unknown toxicity at the high levels that would be required for radical trapping *in vivo*, in order for the trap to complete with endogenous molecules for a reactive free radical. However, traps can be used on body fluids and tissue samples. For example, DMPO was used to measure free radicals in human skin biopsies (Haywood *et al*., 1999). A hydroxylamine probe has been used to measure free radicals in liver biopsies (Valgimigli *et al*., 2002). PBN has been employed to demonstrate free radical production by adding it to coronary sinus blood drawn during coronary bypass surgery (Tarkka *et al*., 2000; Clermont *et al*., 2002). Of course, very highly reactive radicals such as $OH^{\bullet}$, and highly reactive nonradical species (such as peroxynitrous acid) that may be formed *in vivo* (Sun *et al*., 1993; Halliwell & Gutteridge, 1999; Ferdinandy & Schulz, 2003), will simply not survive to be detected in *ex vivo* material. For example, $OH^{\bullet}$ reacts with whatever is in its immediate vicinity within microseconds (Halliwell & Gutteridge, 1999) and ONOOH breaks down in seconds (Radi *et al*., 2001). ESR of *ex vivo* samples to which traps have been added probably detects secondary radicals resulting from reaction of RS with biomolecules. These secondary radicals will include lipid-derived radicals (alkoxyl, peroxyl, etc.) and possibly also protein radicals (Pantke *et al*., 1999; Ostdal *et al*., 2002).

Ascorbic acid (vitamin C) reacts with a wide range of free radicals and other RS (Buettner, 1993; Halliwell, 1999c) and one of its oxidation products, semidehydroascorbate radical, can easily be detected by ESR (Buettner & Jurkiewicz, 1993). Measurement of semidehydroascorbate has been used as an indication of free radical production in organs, blood plasma and skin (Buettner & Jurkiewicz, 1993; Sharma & Buettner, 1993; Sharma *et al*., 1994; Jurkiewicz & Buettner, 1996; Haywood *et al*., 1999). It is only semiquantitative, since ascorbate radicals quickly react with each other to generate ESR-silent species (ascorbate and dehydroascorbate) (Bielski *et al*., 1975; Kobayashi *et al*., 1991). The ascorbate radical is also reduced by enzymes *in vivo* (May *et al*., 2001).

Traps, both the ESR 'spin traps' described above and other types of trap (see below), are likely to perturb the biological system under investigation. If traps scavenge RS, and these RS are important mediators of cell or tissue injury, it follows logically that the traps should protect against damage, provided, of course, that they are scavenging enough of the RS (Table 3). Indeed, some compounds derived from spin traps have entered clinical trials, for example, for stroke (Lapchak & Araujo, 2003). However, one must never assume that any beneficial effects that traps exert are due to free radical scavenging without direct evidence for this mechanism, since these compounds can exert other pharmacological effects (Pietri *et al*., 1998; Floyd *et al*., 1999; Kotake, 1999).

Aromatic traps Potentially more useful than spin traps *in vivo* in humans are aromatic 'free radical' traps known to be acceptable for human consumption, including salicylate and phenylalanine (Kaur *et al*., 1996; Grootveld & Halliwell, 1986; Kaur & Halliwell, 1994; Halliwell & Kaur, 1997; Coudray & Favier, 2000; Themann *et al*., 2001). Salicylate is hydroxylated by $OH^{\bullet}$ to yield, among other products, 2-3 dihydroxybenzoate, which is not apparently produced enzymically *in vivo* (Ingelman-Sundberg *et al*., 1991; Dupont *et al*., 1999). Both L- and D-phenylalanine are hydroxylated by $OH^{\bullet}$ to yield *ortho*- and *meta*-tyrosines, which again seem not to be enzymically produced in humans, although the evidence supporting this statement is weak and more studies are required (Kaur & Halliwell, 1994; Halliwell and Kaur, 1997; Li *et al*., 2003). Both salicylate and phenylalanine have been used to measure *ex vivo* radical formation in blood from patients with rheumatoid arthritis (Kaur *et al*., 1996) or in humans exposed to ozone (Frischer *et al*., 1997; Liu *et al*., 1997a). Phenylalanine was used to detect $OH^{\bullet}$ formation in saliva (Nair *et al*., 1995). Several human studies have also used salicylate to detect $OH^{\bullet}$ *in vivo*, with some success. Examples include studies on diabetes (Ghiselli *et al*., 1992), alcoholism (Thome *et al*., 1997) and myocardial infarction (Tubaro *et al*., 1992). Endogenous levels of free (Lubec *et al*., 1997; Ogihara *et al*., 2003) or protein-bound (Fu *et al*., 1998; Lamb *et al*., 1999; Pennathur *et al*., 2001) *ortho*- or *meta*-tyrosines have also been used to implicate $OH^{\bullet}$ formation *in vivo*.

The success of aromatic compounds (or spin traps) in detecting $OH^{\bullet}$ will depend on their concentration at sites of free radical generation in relation to that of other $OH^{\bullet}$ scavengers (virtually all other biomolecules react fast with $OH^{\bullet}$ and will compete), and so it is unlikely that any trap can intercept more than a small percentage of any $OH^{\bullet}$ generated. 2,3-Dihydroxybenzoate can be metabolized in the liver, and possibly other organs, causing its loss (unpublished data). It follows that aromatic hydroxylation techniques are not *quantitative* measures of $OH^{\bullet}$ generation, especially if the end products are measured in the plasma, where their origin is unclear. Specificity of the traps for $OH^{\bullet}$ is uncertain: $ONOO^{-}$ can hydroxylate both salicylate and phenylalanine, perhaps, in part, by direct reaction with ONOOH (Kaur *et al*., 1997). Simultaneous determination of nitration products can distinguish the two (Kaur *et al*., 1997; Ferger *et al*., 2001), since $OH^{\bullet}$ cannot nitrate aromatic compounds whereas $ONOO^{-}$ usually does. Myeloperoxidase can also hydroxylate aromatic compounds, although the favored product from salicylate is 2,5-dihydroxybenzoate (Kettle & Winterbourn, 1994; Kawakami *et al*., 1999). Hypochlorous acid produced by myeloperoxidase can, however, react with $O_2^{\bullet-}$ to produce $OH^{\bullet}$ (Candeias *et al*., 1993).

Another problem is that high-performance liquid chromatography (HPLC) analysis of *meta*-tyrosine in human samples can be confused by the frequent presence of a coeluting peak of unknown identity (Reddy *et al*., 1999). Be suspicious if you see an apparent *meta*-tyrosine peak on HPLC much greater than that of *ortho*-tyrosine: this is suggestive of an artifact since both products should be formed in comparable amounts when $OH^{\bullet}$ attacks phenylalanine. Mass spectrometry (MS) can be used to avoid this problem (Pennathur *et al*., 2001). As an additional probe, the compound antipyrene is hydroxylated by $OH^{\bullet}$ into *para*- and *ortho*-hydroxylation products, the latter again not apparently formed enzymically in humans. Antipyrene has been used to measure $OH^{\bullet}$ production during exercise (Meijer *et al*., 2001).

Urate as a trap An endogenous molecule might also function as a trap (indeed, measurement of *meta*- and *ortho*-tyrosines in body proteins as 'endogenous traps' would fit into this category), although it can be argued that measuring specific end products of the trapping of RS by endogenous molecules is the same as measuring 'biomarkers' (see below). Ascorbate reaction with free radicals is one example, as discussed above. Another example is urate, which is readily oxidized by a range of RS (Kaur & Halliwell, 1990), including peroxynitrite (Whiteman *et al*., 2002b). Several groups have used urate as a 'selective' scavenger of $ONOO^{-}$ in animal studies, neglecting the fact that it reacts with many species (Kaur & Halliwell, 1990). One of urate's oxidation products, allantoin, can be measured in human body fluids and its plasma levels are elevated in conditions associated with oxidative stress, such as chronic inflammation, diabetes, premature birth, iron overload, chronic heart failure and exercise (Grootveld & Halliwell, 1987; Moison *et al*., 1997; Ogihara *et al*., 1998; Benzie *et al*., 1999; Mikami *et al*., 2000; Doehner *et al*., 2002; Pavitt *et al*., 2002; Yardim-Akaydin *et al*., 2004). Allantoin can also be measured in urine (Mikami *et al*., 2000) and cerebral microdialysis fluid (Marklund *et al*., 2000). Levels of allantoin rise in the human muscle during exhaustive exercise, presumably due to oxidation of urate by RS generated during exercise (Hellsten *et al*., 2001). Allantoin measurement may be one of the more promising methods for human use, since human urate levels *in vivo* are high and urate reacts with a wide range of RS.

Measuring stable species in body fluids: hydrogen peroxide

Several laboratories have demonstrated the presence of H_2O_2 in freshly voided human urine (Varma & Devamanoharan, 1990; Kuge *et al*., 1999; Long *et al*., 1999; Laborie *et al*., 2000; Long & Halliwell, 2000; Hiramoto *et al*., 2002; Kirschbaum, 2002; Yuen & Benzie, 2003). It has also been measured in rabbit urine (Pi *et al*., 2003). Since assay of urinary H_2O_2 can be performed rapidly and simply (Long *et al*., 1999), the question has been raised if its levels in urine (after creatinine standardization) can be related to rates of generation of RS *in vivo*. Caution must be exercised, since drinking coffee artifactually raises urinary H_2O_2 levels (Long & Halliwell, 2000; Hiramoto *et al*., 2002), apparently because hydroxyhydroquinone in coffee is absorbed into the body, excreted into the urine and autooxidizes therein to produce H_2O_2 (Hiramoto *et al*., 1998; Halliwell *et al*., 2004a). Patients with cancer showed elevated urinary H_2O_2 levels (Banerjee *et al*., 2003). The currently available data are insufficient to support the use of urinary H_2O_2 as a biomarker of oxidative stress and more work is required (Yuen & Benzie, 2003; Halliwell *et al*., 2004a).

Hydrogen peroxide can also be detected in exhaled air and breath condensate, and the levels appear to increase during inflammation. The origin of the H_2O_2 is unclear: release by respiratory tract cells and oral bacteria are possibilities (Jobsis *et al*., 2001; Geiszt *et al*., 2003; Rahman & Kelly, 2003).

Measuring changes in blood pressure and vascular tone

The free radical $NO^{\bullet}$ plays a key role in vasodilatation. Its action can be antagonized by $O_2^{\bullet-}$, which removes $NO^{\bullet}$ by reacting fast with it to give $ONOO^-$ (reviewed by Beckman & Koppenol, 1996). In the healthy arterial wall, there is a balance between $NO^{\bullet}$ and $O_2^{\bullet-}$, but increased oxidative stress can diminish the bioactivity of $NO^{\bullet}$ (Darley-Usmar *et al*., 1995; Kojda & Harrison, 1999). Sources of $O_2^{\bullet-}$ in and around the vessel wall include the vascular endothelium (especially in atherosclerotic lesions), fibroblasts, lymphocytes, phagocytes and the enzyme xanthine oxidase (Darley-Usmar & Halliwell, 1996; Reth, 2002; Rey & Pagano, 2002). Upregulation of $O_2^{\bullet-}$-generating NAD(P)H oxidases may play a key role in causing impaired vascular tone and hypertension (Harrison *et al*., 2003; Lassegue & Clempus, 2003; Murphey *et al*., 2003).

Many papers have claimed that high doses of infused or orally administrated antioxidants (ascorbate being most commonly used) can scavenge ROS, restore $NO^{\bullet}$ bioavailability and ameliorate human endothelial dysfunction (for examples see Plotnick *et al*., 1997; Duffy *et al*., 1999; Gokce *et al*., 1999; Wilkinson *et al*., 1999; May, 2000; Beckman *et al*., 2001; Duffy *et al*., 2001; Pleiner *et al*., 2002; Heiss *et al*., 2003), although negative reports are now accumulating (e.g. Darko *et al*., 2002; Singh *et al*., 2002; Svetkey & Loria, 2002; Widlansky *et al*., 2004; our unpublished date). The timing of antioxidant administration and the state of the vascular bed (healthy, mildly diseased or severely diseased) may be critical variables (Carroll & Schade, 2003). Hence it is possible that examination of short-term vascular effects, which is easily achievable in human studies by measurements of forearm blood flow, can gather evidence about localized scavenging of RS in blood vessel walls by antioxidants.

However, it must not be assumed that any beneficial effects of antioxidants on vascular function are *necessarily* due to free radical scavenging. For example, ascorbate may have a direct stimulating effect on eNOS activity by increasing endothelial tetrahydrobiopterin levels (Huang *et al*., 2000; d'Uscio *et al*., 2003).

Fingerprinting of RS

If a RS combines with a biological molecule to leave a unique chemical 'fingerprint', then the presence of that fingerprint (which is of course an example of oxidative damage) can be used to infer that the RS has been formed (Halliwell & Gutteridge, 1999). Such 'biomarkers' can then be used to investigate effects of dietary supplements or synthetic antioxidants on oxidative damage, hopefully leading to experimentally verifiable predictions of the likely effects of these compounds on oxidative stress-related diseases arising subsequently. Indeed, since methods currently available for the direct measurement of RS are of limited applicability to humans (see above), most clinical studies focus on the measurement of oxidative damage. This is to some extent logical, since it is the damage caused by RS that is important rather than the total amount of such species generated. For example, if highly reactive $OH^{\bullet}$ radicals are generated within a cell, many of them may react with unimportant targets. The small fraction that reacts with DNA (to cause strand breakage and formation of mutagenic base or sugar oxidation products), with key proteins, or with lipids (initiating lipid peroxidation) may be the important fraction.

Table 4 summarizes the requirements of the ideal biomarker. No currently used biomarker has yet fulfilled the key criterion (A); the experiments have not been carried out. It is sad that biomarkers were never built into all the human intervention trials with antioxidants that have been carried out; they could have been of great value in interpreting the confusing and conflicting results (Halliwell, 1999a; 2000b, c). No biomarker meets all parts of criterion (B), but some are better than others. We have already considered small molecules such as ascorbate and urate, so now will comment briefly on currently available biomarkers of oxidative damage to the three main classes of macromolecules: nucleic acids, proteins and lipids.

Lipids

Lipids can be oxidized, chlorinated and nitrated by a range of RS (not including H_2O_2, $NO^{\bullet}$ or $O_2^{\bullet-}$, which are essentially unreactive with lipids; Halliwell & Gutteridge, 1999). Techniques for the measurement of chlorinated and nitrated lipids are being developed, and species such as nitrated linoleate have been detected in human blood plasma (Hazen *et al*., 1996; Lim *et al*., 2002; Lima *et al*., 2002; 2003; Thukkani *et al*., 2003). We await further studies on these compounds with interest.

Lipid peroxidation is a complex process, and a wide range of products is formed in variable amounts (Halliwell & Gutteridge, 1999). Lipid oxidation can be measured in many ways, but commonly used methods such as diene conjugation and thiobarbituric acid (TBA)-reactive material are of questionable

validity (Halliwell & Gutteridge, 1999). In particular, the simple TBA test should be dismissed as unacceptable in modern research, simply because most TBA-reactive material in human body fluids is not related to lipid peroxidation. A significant improvement to the TBA test can be made by using HPLC to isolate the malondialdehyde (MDA)-TBA chromogen before analysis (Halliwell & Gutteridge, 1999). One can also assay MDA directly (e.g. Liu *et al*., 1997b), but MDA is only one of many aldehydes formed during lipid peroxidation, and MDA can also arise from free radical attack on sialic acid and deoxyribose (Halliwell & Gutteridge, 1999). Unsaturated aldehydes such as 4-hydroxynonenal and acrolein may cause considerably more cytotoxicity *in vivo* than MDA, so measuring them is perhaps more logical (Ong *et al*., 2000; Uchida, 1999; 2003). Lipid hydroperoxides and aldehydes can also be absorbed from the diet. For example, some foods contain MDA–amino-acid adducts that can be absorbed through the gut and then excreted in urine (Nelson *et al*., 1993; Richelle *et al*., 1999; Draper *et al*., 2000; Cohn, 2002; Indart *et al*., 2002; Wilson *et al*., 2002). It follows that measurements of urinary MDA can be confounded by diet and should not be used as an index of whole-body lipid peroxidation unless diet is controlled.

The isoprostanes The best available biomarker of lipid peroxidation appears to be the isoprostanes, specific end products of the peroxidation of polyunsaturated fatty acids (Roberts & Morrow, 2002; Fam & Morrow, 2003). Most work has been carried out on the F_2-isoprostanes, which arise from arachidonic acid (Roberts & Morrow, 2002), but some data are also available on isoprostanes from eicosapentaenoic (Nourooz-Zadeh *et al*., 1997) and docosahexaenoic acids. The latter are often called neuroprostanes or F_4-isoprostanes (Nourooz-Zadeh *et al*., 1999; Reich *et al*., 2001; Roberts & Morrow, 2002). Isoprostanes are best measured by MS (Lawson *et al*., 1999; Roberts & Morrow, 2002). Although some immunoassay kits for F_2-isoprostanes are commercially available, their reliability has been questioned (Proudfoot *et al*., 1999; Bessard *et al*., 2001; Roberts & Morrow, 2002). Although isoprostanes can be detected in foods, they do not appear to pass through the gut in sufficient quantities to affect plasma or urinary levels (Richelle *et al*., 1999; Gopaul *et al*., 2000). Reliable MS-based techniques have been established to detect isoprostanes and their metabolites in the plasma and urine (Lawson *et al*., 1999; Li *et al*., 1999; Fam & Morrow, 2003), although the 'work-up' techniques prior to MS are tedious.

Even the isoprostanes do not qualify as 'ideal' biomarkers. The products commonly measured (e.g. 8-*iso*-$PGF_{2\alpha}$) are often minor end products of peroxidation (Lawson *et al*., 1999), and the amounts formed can be influenced by such parameters as O_2 concentration (Morrow *et al*., 1998; Fessel *et al*., 2002; Roberts & Morrow, 2002). Hence, much work is proceeding to identify new products arising from the isoprostane pathway (Roberts & Morrow, 2002; Fessel *et al*., 2002). Isoprostanes appear to be rapidly metabolized, turning over quickly (Roberts & Morrow, 2002; Basu, 2004). Thus a rise in plasma isoprostane levels could conceivably be due not only to increased formation by oxidative damage to lipids but also by slower metabolism. Measurement of isoprostanes in plasma and urine is, of course, a 'whole-body' measurement; it is not necessarily clear from where the isoprostanes originated. They can also be measured in fluids drawn from specific sites, such as synovial fluid (Basu *et al*., 2001), pericardial fluid (Mallat *et al*., 1998), wound exudate (Sim and Stacey, 2003) and breath condensate (Montuschi *et al*., 1999).

Of especial current interest is the use of isoprostane measurements to study Alzheimer's disease (AD), a condition in which lipid peroxidation and other oxidative damage seem to play significant pathological roles (Montine *et al*., 1998; 1999; Pratico *et al*., 2000; 2001; 2002; Halliwell, 2001; Butterfield, 2002). Levels of F_2- and F_4-isoprostanes are elevated in CSF from AD patients (Montine *et al*., 1998; 2002; Pratico *et al*., 2000), and may even be elevated prior to the development of AD (Pratico *et al*., 2001; 2002), consistent with the view that peroxidation may be an important step in progressive neuronal injury leading to clinically manifested disease (Halliwell, 2001; Pratico *et al*., 2001; 2002). It has been claimed that urinary and plasma levels of isoprostanes are also elevated in AD patients to an extent correlated with the degree of cognitive impairment (Pratico *et al*., 2000; 2002), although this was not confirmed in other studies (Montine *et al*., 2002; Bohnstedt *et al*., 2003). It would be of great advantage in researching neuroprotective antioxidants to have a peripheral biomarker that could report on oxidative events in the brain, and so more work is needed to investigate urinary and plasma isoprostanes in this context and resolve the conflicting data. However, there does seem to be agreement that there are elevated CSF isoprostane levels in AD (Pratico *et al*., 2000; 2002; Montine *et al*., 2002).

Increased levels of isoprostanes have been observed in many animal (reviewed by Roberts & Morrow, 2002; Basu, 2004) and human conditions associated with oxidative stress, including cardiopulmonary bypass (Ulus *et al*., 2003), angioplasty (Guan *et al*., 1999; 2003; Iuliano *et al*., 2001) and diabetes (Gopaul *et al*., 2001; Jialal *et al*., 2002; Sampson *et al*., 2002). Hyperglycemia causes oxidative stress (Brownlee, 2001; Gopaul *et al*., 2001; Jialal *et al*., 2002), and plasma isoprostane

Table 4 Criteria for the ideal biomarker of oxidative damage

A. Core criterion
That the biomarker is predictive of the later development of disease.

B. Technical criteria
(i) The biomarker should detect a major part, or at least a fixed percentage, of total ongoing oxidative damage *in vivo*.
(ii) The coefficient of variation between different assays of the same sample should be small in comparison with the difference between subjects.
(iii) Its levels should not vary widely in the same subjects under the same conditions at different times.
(iv) It must employ chemically robust measurement technology.
(v) It must not be confounded by diet.
(vi) It should ideally be stable on storage, not being lost, or formed artifactually, in stored samples.

levels in diabetes may be determined to a considerable extent by plasma glucose levels (Sampson *et al*., 2002).

Isoprostanes have also been used to study effects of dietary antioxidant supplementation on lipid peroxidation. Plasma and urinary isoprostane levels in humans have responded to antioxidant supplementation in some studies (reviewed by Halliwell, 2000a; Roberts & Morrow, 2002), but in general responses are limited or absent in healthy well-nourished subjects, indicating that lipid peroxidation is little affected by supplements. Supplementation of healthy volunteers with vitamins C or E, for example, usually decreases isoprostane levels only slightly, if at all (Levine *et al*., 2001; Meagher *et al*., 2001), which might help to explain why these vitamins have had little effect on disease outcomes in many human intervention trials (Halliwell, 2000c). However, some positive effects have been reported, for example, supplementation with vitamins E and C did decrease urinary isoprostanes in one study upon healthy American nonsmoking subjects (Huang *et al*., 2002). Isoprostane measurements also suggest that obesity and hypercholesterolemia (known risk factors for cardiovascular disease) are associated with elevated rates of lipid peroxidation. This is consistent with the view that lipid peroxidation is an important contributor to cardiovascular disease and can help explain why weight loss and statins decrease cardiovascular events (Roberts *et al*., 2002; Keaney *et al*., 2003; Morrow, 2003; Russell *et al*., 2003; Samuelsson *et al*., 2003). There is also increasing interest in the link between hypercholesterolemia, oxidative stress and AD (Refolo *et al*., 2001; Leonarduzzi *et al*., 2002; Puglielli *et al*., 2003; Wolfrum *et al*., 2003; Ong & Halliwell, 2004).

An important question for any biomarker is the coefficient of variation between assays of the same sample and between samples taken from the same subjects at different times. There have been claims that isoprostane levels vary with time of day and from day to day (Helmersson & Basu, 2001; Kanabrocki *et al*., 2002), possibly because oxidative stress also varies (reviewed by Halliwell *et al*., 2004a). If there is such a variation, it is essential to take it into account in human studies.

Exhaled hydrocarbons Exhaled air contains not only isoprostanes (Rahman & Kelly, 2003; Montuschi *et al*., 1999) and aldehydes but also a range of hydrocarbons, including ethane and pentane (Andreoni *et al*., 1999; Phillips *et al*., 2000; Dale *et al*., 2003). The available data suggest that ethane may be a biomarker of lipid peroxidation, pentane perhaps less so. However, these hydrocarbons are minor end products of lipid peroxidation, formed to variable extents depending on the exact environment of the oxidizing lipids (Halliwell & Gutteridge, 1999). Exhaled hydrocarbons are also difficult to measure routinely in human studies, requiring cumbersome equipment, as compared to, for example, measuring isoprostanes in blood and urine samples.

DNA

Oxidative DNA damage seems to relate to an increased risk of cancer development later in life (reviewed by Halliwell, 2002b). DNA subjected to attack by hydroxyl radical generates a huge range of base and sugar modification products (Dizdaroglu *et al*., 2002). Such products can be measured by HPLC, gas chromatography (GC)–MS, liquid chromatography (LC)–MS and antibody-based techniques, and arguments rage over which is best (Halliwell, 1999b, 2000c; Dizdaroglu *et al*., 2002; Kasai, 2002; Collins *et al*., 2004). None of them is the 'gold standard' we all seek. Initial products of free radical attack upon purines, pyrimidines and deoxyribose undergo transformation into stable end products, whose relative amounts depend on reaction conditions (Dizdaroglu, 1992; Alam *et al*., 1997; Halliwell, 1999b; Dizdaroglu *et al*., 2002). Thus it is intrinsically unreliable to measure any single reaction product as an index of oxidative DNA damage, but that is what is usually done, as 8-hydroxy-2′-deoxyguanosine (8OHdG) (Collins *et al*., 2004). The advantages and artifacts of measuring 8OHdG in cellular DNA have been recently reviewed (Halliwell, 2000c; 2002b; Collins *et al*., 2004), so they need not be reiterated here. The major problems arise from artifactual oxidative damage to DNA and consequent 8OHdG formation during isolation of DNA, its preparation for analysis and the analysis itself. In general, there is no agreement even on basal levels of 8OHdG in cellular DNA, nor does an agreement seem close (Collins *et al*., 2004). In particular, the suggestion almost 10 years ago that mitochondrial DNA (mtDNA) is more oxidized than nuclear DNA does not rest on a firm experimental foundation (Beckman & Ames, 1999). Our own studies (unpublished) indicate similar or lower (for some base damage products) levels of oxidative DNA damage in rat liver mtDNA as compared with nuclear DNA. Several RS formed at sites of inflammation, such as HOCl and $ONOO^-$, can destroy 8OHdG in DNA (Whiteman *et al*., 1997; 2002a). Nevertheless, significant increases in levels of 8OHdG and other DNA base damage products have been observed in many studies on diseased human material (reviewed by Halliwell, 2000c; Evans *et al*., 2003), although (as with the isoprostanes) effects of dietary antioxidant supplementation on levels of 8OHdG or other base damage products *in vivo* seem limited (reviewed by Halliwell, 2000c; Moller & Loft, 2002). These limited effects may explain why antioxidant administration has generally not decreased cancer incidence in human intervention trials in Western countries.

One approach would be to bypass these problems of artifactual DNA oxidation during DNA isolation and analysis by measuring oxidative DNA damage in the intact cell. Antibody methods have been developed for 8OHdG (e.g. Toyokuni *et al*., 1997) and are useful for visualization of damage, but seem likely to be semiquantitative (Shimoi *et al*., 2002; Yoshida *et al*., 2002). The comet assay (Duthie *et al*., 1996) can be applied directly to cells and measures DNA strand breaks. If a digestion step with DNA repair enzymes is included in the protocol, the increased numbers of DNA strand breaks can be used to estimate the level of oxidized DNA bases in the cell. Studies with the comet assay seem more likely than other methods to show positive effects of antioxidant interventions in human volunteers, and the reasons for this need to be elucidated (Duthie *et al*., 1996; Toyokuni *et al*., 1997; Collins & Horvathova, 2001; Moller & Loft, 2002). Is it that the comet assay is more reliable because the baseline is lower, artifactual DNA oxidation having been minimized? Or does the comet assay simply just generate a different kind of artifact, in underestimating damage? It seems very unlikely that all the oxidized bases in compact DNA can be recognized by exogenously applied enzymes. Strand breaks, which are what the comet assay measures, arise not only during oxidative DNA damage but also during repair of that

damage (Spencer *et al*., 1996a). Enzymic DNA cleavage during apoptosis may also generate comets, which can lead to false conclusions about the genotoxicity of apoptosis-inducing agents. An antioxidant that suppressed apoptosis could be misinterpreted to be protecting directly against DNA damage (Choucroun *et al*., 2001).

None of the analytical methods mentioned above identifies where the oxidative DNA damage is located: is it in expressed genes, inactive genes, merely in 'junk DNA' or even in telomeres (Oikawa & Kawanishi, 1999)? Attempts are underway to address this problem (e.g. Rodriguez *et al*., 2000; Choi *et al*., 2003; Sawyer *et al*., 2003). Another problem in studying damage to DNA by RS is the limited availability of human tissues from which to obtain DNA. Most studies are performed on DNA isolated from lymphocytes or total white cells from human blood, and it is assumed (possibly erroneously: reviewed by Halliwell, 2002b) that changes here are reflected in other tissues. Sperm, buccal cells, placenta, and biopsies of muscle, skin, colon and other tissues are other potential sources of DNA.

Urinary measurements Measurement of 8OHdG in urine has been used to assess rates of 'whole-body' oxidative DNA damage. This can be achieved by HPLC and MS techniques (Bogdanov *et al*., 1999; Gackowski *et al*., 2001; Kasai, 2002; Pilger *et al*., 2002; Lin *et al*., 2004), but ELISA techniques have also been described (Shimoi *et al*., 2002; Yoshida *et al*., 2002). The presence of interfering peaks in certain urine samples is a major bugbear, and sample 'cleanup' techniques are critical (Bogdanov *et al*., 1999; Lin *et al*., 2004). The assays seem reliable (except that ELISA can give high levels sometimes) and data fairly comparable between laboratories (Bogdanov *et al*., 1999; Gackowski *et al*., 2001; Cooke *et al*., 2002; Kasai, 2002; Pilger *et al*., 2002; Shimoi *et al*., 2002; Yoshida *et al*., 2002; Lin *et al*., 2004), unlike what is seen with the measurement of 8OHdG in isolated DNA (Collins *et al*., 2004). No urinary 8OHdG appears to originate from diet (Gackowski *et al*., 2001). However, 8OHdG can arise from degradation of oxidized dGTP in the DNA precursor pool, not just from removal of oxidized guanine residues from DNA by repair processes (Cooke *et al*., 2002). Hence urinary levels may not truly reflect rates of oxidative damage to DNA. Another reason why one cannot equate 8OHdG in urine with oxidative DNA damage is that there are many other products of this process (Dizdaroglu, 1992; Dizdaroglu *et al*., 2002). Hence, urinary 8OHdG is a partial measure of damage to guanine residues in DNA and its nucleotide precursor pool. Nevertheless, 8OHdG is the best urinary measure we have at the moment. Other oxidized bases can be measured in urine (e.g. Ravanat *et al*., 1999), but a confounding by diet cannot yet be ruled out. DNA in foods can be oxidized during storage and cooking, and it is possible that oxidized bases can be absorbed and reexcreted, just as it happens with MDA. This is not a problem with 8OHdG (Gackowski *et al*., 2001).

Measurement of 8OHdG in urine gives no information about its tissue origin, although it is sometimes possible to sample from specific sites, such as cerebrospinal fluid (Rozalski *et al*., 2003). The possibility of diurnal variations in urinary 8OHdG levels has been raised, so spot measurements should be interpreted with caution (Kanabrocki *et al*., 2002). However, our laboratory has not found this to be a major problem (Lin *et al*., 2004). Products of reaction of DNA bases with end products of lipid peroxidation such as MDA can also be detected in human urine (Hanaoka *et al*., 2002; Otteneder *et al*., 2003), but a confounding effect of diet is possible since these adducts may be present in foods, for example, after cooking. DNA base–aldehyde adducts can also be measured in DNA isolated from cells and tissues (Bartsch & Nair, 2000; Marnett *et al*., 2003), but one must again be cautious because tissue homogenates can undergo rapid peroxidation *in vitro* (Halliwell & Gutteridge, 1999), leading to possible modification of DNA by aldehydes generated artifactually during the isolation process.

Reactive nitrogen and chlorine species DNA can also be damaged by reactive nitrogen species, undergoing mainly nitration and deamination of purines (Yermilov *et al*., 1995; Spencer *et al*., 1996b; Zhao *et al*., 2001b; Lee *et al*., 2002b). It was initially thought that 8-nitroguanine was a specific product of reaction of $ONOO^-$ with DNA, but this does not seem to be the case (Byun *et al*., 1999). Methods for the measurement of DNA base nitration and deamination products have been developed, but may need more refinement before they can be routinely applied to human material. However, cigarette smoking was observed to raise the level of 8-nitroguanine in peripheral lymphocytes of humans (Hsieh *et al*., 2002). Antibody-based methods have been used to demonstrate rises in 8-nitroguanine during inflammation in animals (Akaike *et al*., 2003; Pinlaor *et al*., 2003). Assays to identify chlorinated DNA bases have also been developed (Whiteman *et al*. 1997; Henderson *et al*., 2003).

Ultraviolet light Ultraviolet light produces a range of lesions in DNA, including thymine dimers (Douki *et al*., 2000), which can be measured in human urine. Levels increase after exposure to sunlight (Cooke *et al*., 2001; Le Curieux & Hemminki, 2001) and in psoriasis (Ahmad *et al*., 1999). Urinary thymine dimers might thus be a useful biomarker to assess effects of antioxidants on sunlight-induced DNA damage.

Proteins

Oxidative damage to proteins may be important *in vivo* both in its own right (affecting the function of receptors, enzymes, transport proteins, etc., and perhaps, generating new antigens that can provoke immune responses) (Halliwell, 1978; Cascio-la-Rosen *et al*., 1997) and because it can contribute to secondary damage to other biomolecules, for example, inactivation of DNA repair enzymes and loss of fidelity of damaged DNA polymerases in replicating DNA (Wiseman & Halliwell, 1996). However, analyzing protein oxidative damage products is an order of magnitude more complex than dealing with DNA: rather than four bases and one sugar there are 20 different amino acids, each of which can be attacked by RS in multiple ways (Davies *et al*., 1999; Headlam & Davies, 2003). Free radical attack on proteins can generate amino-acid radicals, which may crosslink or react with O_2 to give peroxyl radicals. These may abstract $H^{\bullet}$, triggering more free radicals and forming protein peroxides, which can decompose in complex ways, accelerated by transition metal ions, to generate yet more radicals (Headlam & Davies, 2003). Proteins can also be oxidized during food cooking, meaning that oxidized amino acids could conceivably be absorbed from the diet, which

could confound measurements of them in body fluids as putative biomarkers of oxidative damage. Individual amino-acid oxidation products that have been measured in various human tissues include kynurenines (from tryptophan), bityrosine (which appears to be metabolically stable and can be detected in urine), valine and leucine hydroxides, L-dihydroxyphenylalanine (L-DOPA), *ortho*-tyrosine, 2-oxo-histidine, glutamate semialdehyde and adipic semialdehyde (Uchida & Kawakishi, 1993; Morin *et al*., 1998; Leeuwenburgh *et al*., 1999a, b; Giulivi & Davies, 2001; Harth *et al*., 2001; Headlam & Davies, 2003). Bityrosine, easily detectable in human and other animal urines, formed by free radical attack on a wide range of proteins, and apparently not metabolized, might be a biomarker worth further development for human use (Davies *et al*., 1999; Leeuwenburgh *et al*., 1999a, b; Giulivi & Davies, 2001). Patients with sepsis were reported to have increased urinary excretion of bityrosine (Bhattacharjee *et al*., 2001), as were children with kwashiorkor (Manary *et al*., 2000), a disease which is believed to involve increased oxidative stress (Golden & Ramdath, 1987; Fechner *et al*., 2001). An immunohistochemical method to detect bityrosine in human brain has been described (Kato *et al*., 1998). Sakharov *et al*. (2003) described the use of an acetyltyramine–fluorescein probe to detect formation of tyrosine radicals in proteins in cells subjected to oxidative stress. If both the tyramine probe and tyrosine residues are oxidized, the probe can crosslink to the protein tyrosyl residues, generating a stable crosslink similar to that in bityrosine. The fluorescein-labeled proteins can then be visualized and identified by proteomic methods.

Theoretically also useful might be the measurement of methionine sulfoxide, since methionine residues appear to be a 'radical sink' in proteins (Levine *et al*., 1999), that is, oxidative attack at different sites on a protein may eventually appear as methionine sulfoxide. However, methionine sulfoxide residues are usually quickly removed by methionine sulfoxide reductase enzymes (Levine *et al*., 1999; Sharov *et al*., 1999), and this amino-acid does not appear to have been used as a biomarker in human studies.

Proteins can also be attacked by reactive chlorine, bromine and nitrogen species (defined in Table 1), giving products such as 3-chlorotyrosine, *para*-hydroxyphenylacetaldehyde, 3,5-dichlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine (Hazen & Heinecke, 1997; Wu *et al*., 1999; 2000; Winterbourn & Kettle, 2000; Greenacre & Ischiropoulos, 2001; Himmelfarb *et al*., 2001; Aldridge *et al*., 2002; Gaut *et al*., 2002a). 3-Nitrotyrosine is often thought to be a specific marker of attack of $ONOO^-$ upon proteins, but in fact it can be formed from tyrosine by a range of reactive nitrogen species and its production *in vivo* is often dependent on the production of such species as nitrogen dioxide radical ($NO_2^\bullet$) by myeloperoxidase or other peroxidases (Halliwell *et al*., 1999; Greenacre & Ischiropoulos, 2001; MacPherson *et al*., 2001; Gaut *et al*., 2002b). 3-Nitrotyrosine has been frequently measured by immunostaining, HPLC and MS in human tissues, but we have been unable to detect it in human urine (unpublished data). Nitrotyrosine is metabolized to 3-nitro-4-hydroxyphenylacetate (NHPA), which some groups have measured in urine as an estimate of nitrotyrosine production *in vivo* (Ohshima *et al*., 1990). However, some (and perhaps most) urinary NHPA arises by other mechanisms (Mani *et al*., 2003), making the assay questionable. Simple HPLC-based assays for 3-nitrotyrosine are prone to artifact (Kaur *et al*., 1998), and so MS-based techniques are preferred.

Another problem is that exposure of body fluids or tissues to acid during staining or protein hydrolysis (frequently HCl digestion is used to liberate protein-bound nitrotyrosine) can cause artifactual nitration of tyrosine by species derived from nitrous acid (HNO_2), generated when nitrite in the sample is exposed to acid (Frost *et al*., 2000). Even freezing phosphate- and nitrite-containing samples can cause artifactual nitration (Daiber *et al*., 2003). In general, the more accurate the measurement methods that are used, the lower the levels of 3-nitrotyrosine detected in human material (Frost *et al*., 2000; Gaut *et al*., 2002a; Duncan, 2003; Morton *et al*., 2003). Nitrotyrosine can be destroyed by hypochlorous acid, generated by activated neutrophils (Whiteman & Halliwell, 1999). Immunostaining for nitrotyrosine is prone to artifacts (Halliwell *et al*., 1999; Ogino *et al*., 2002; Ichimori *et al*., 2003) but, when carried out with appropriate controls, has proved useful in qualitative demonstration of increased formation of reactive nitrogen species in tissues (e.g. MacMillan-Crow *et al*., 1996; Kooy *et al*., 1997; Greenacre & Ischiropoulos, 2001). Provided that sensitive and accurate MS-based assays are used, measurements of nitro-, chloro- and *ortho*-tyrosines can be useful biomarkers of protein damage by RS and how such damage can be modulated by antioxidant or other interventions (Hazen & Heinecke, 1997; Buss *et al*., 2003). For example, statin treatment decreased levels of these products in the plasma of hypercholesterolemia patients, consistent with the view (discussed above) that hyperchloesterolemia leads to oxidative stress (Shishehbor *et al*., 2003). Proteomic methods can be used to separate proteins, followed by recognition of modified protein by blotting, and this technique was used to identify targets of nitration in muscle, for example (Kanski *et al*., 2003). Protein nitrosylation, a reversible modification involving attachment of $NO^\bullet$ to a metal site or a cysteine residue, can also be measured in human material (Mannick & Schonhoff, 2004).

The carbonyl assay The most frequently used biomarker of protein damage is the carbonyl assay, measurement of protein carbonyl groups (Chevion *et al*., 2000; Levine, 2002; Dalle-Donne *et al*., 2003). Carbonyls can arise as a result of protein glycation by sugars, by the binding of aldehyes (including many of those formed during lipid peroxidation) to proteins and by the direct oxidation of amino-acid side chains by RS to generate such products as glutamate and aminoadipic semialdehydes (Reznick *et al*., 1992; Calingasan *et al*., 1999; Chevion *et al*., 2000; Adams *et al*., 2001; Requena *et al*., 2001; Levine, 2002; Dalle-Donne *et al*., 2003). Carbonyls can be readily measured spectrophotometrically and by ELISA techniques (Buss & Winterbourn, 2002; Levine, 2002), and tissue or plasma levels have been shown to be elevated in many human diseases (reviewed by Dalle-Donne *et al*., 2003). For example, serum levels were increased during cardiopulmonary bypass (Pantke *et al*., 1999). Carbonyls can also be measured in other body fluids and in tissues (Chapman *et al*., 1989; Buss *et al*., 1997; Chevion *et al*., 2000; Frank *et al*., 2000; Schock *et al*., 2001; Levine, 2002; Tanaka *et al*., 2003). Of course, carbonyls are not specific as markers of oxidative damage because bound aldehydes and glycated protein are also measured. Indeed, immunochemical assays for protein-bound

aldehydes such as acrolein and 4-HNE are widely used (Uchida, 1999; 2003). Data from their use have shown that both aldehydes may be important neurotoxic agents in neurodegenerative diseases (Calingasan *et al*., 1999; Ong *et al*., 2000).

The carbonyl assay as applied to tissues and body fluids measures the 'average' extent of protein modification. It is informative to use proteomic techniques to identify the specific proteins damaged. Often only a small selection of proteins is oxidized. For example, in human plasma subjected to oxidative stress, carbonyls appear to reside mostly on fibrinogen (Shacter *et al*., 1994). In the brains of subjects who died with AD, several specific proteins including enolase, UCH-L1 and DRP-2 seemed to be oxidatively modified (Butterfield *et al*., 2003).

Direct measurements of glutamate and aminoadipate semialdehyde (major contributors to total protein carbonyl residues) in human plasma proteins have also been used to assess the effects of alterations in dietary antioxidant intake on plasma protein oxidation, but no decreases in their levels were observed after increased intake of flavonoid-rich foods (Nielsen *et al*., 1999; Young *et al*., 2000a, b). Whether this is due to the insensitivity of this biomarker or due to a lack of effect of flavonoids on oxidative damage in the human body (Dragsted, 2003; Halliwell *et al*., 2004b) is uncertain as yet. The possible confounding effect of uptake of oxidized amino acids from the diet also needs to be addressed.

Is there a single biomarker of oxidative stress or oxidative damage?

No, there is not (England *et al*., 2000), although (as might be expected) biomarkers of damage to several different molecules frequently rise in parallel in cells subjected to severe oxidative stress. Why is there no such correlation in healthy tissues? Levels of 8OHdG in DNA are a steady-state balance between rate of oxidative DNA damage and rate of removal of lesions by DNA repair mechanisms. Isoprostanes, once formed, are metabolized quickly. Oxidized proteins are degraded, mostly by the proteasome system, and appear to turn over more slowly. Thus even if all biomolecules are damaged, the extents and time courses of the biomarkers of such damage can be very different. Hence, no correlation was observed between levels of plasma isoprostanes and oxidative DNA damage products in healthy human subjects (England *et al*., 2000).

Measurement of RS in cells

In addition to those of us trying to study oxidative stress in humans, many groups are engaged in the apparently simpler task of trying to measure oxidative stress in cells in culture. Many methods allegedly measuring this have become widely used, but precise information on what they really measure is surprisingly sparse, as discussed below. An important first consideration is that *the cell culture process itself imposes oxidative stress*, both by facilitating generation of RS and by hindering adaptive upregulation of cellular antioxidants (reviewed by Halliwell, 2003a). For example, the 'Hayflick limit' in fibroblasts (replicative senescence after a certain number of cell divisions) may be largely an artifact of oxidative stress during cell culture (Wright & Shay, 2002). In addition, results can be confounded by free radical reactions taking place in the culture media (Grzelak *et al*., 2000; Roques *et al*., 2002; Halliwell, 2003a; Wee *et al*., 2003). For example, many reports of effects of ascorbate and polyphenolic compounds (e.g. flavonoids) on cells in culture are artifacts caused by the oxidation of these compounds in the culture media (Long *et al*., 2000; Clement *et al*., 2001; Long & Halliwell, 2001). It is essential to examine what the medium alone might do when you add compounds to it; the results can be surprising (e.g. Clement *et al*., 2001; 2002). Sometimes, indeed, the presence of cells suppresses free radical reactions occurring in the medium (Halliwell, 2003a).

Some simple principles can be used as guidelines in understanding oxidative stress/oxidative damage in cell culture. Hydrogen peroxide generally crosses cell membranes readily, probably through the aquaporins (Henzler & Steudle, 2000). Thus catalase added outside cells can exert both intracellular and extracellular effects on H_2O_2 level, the former by 'draining' H_2O_2 out of the cell by removing extracellular H_2O_2 and thus establishing a concentration gradient (Halliwell, 2003a). In contrast, $O_2^{\bullet-}$ does not generally cross cell membranes readily (Lynch & Fridovich, 1978; Marla *et al*., 1997). Thus if externally added superoxide dismutase is protective against an event in cell culture, be wary of what this means; it could be indicative of extracellular $O_2^{\bullet-}$–generating reactions. Similarly, neither the iron-chelating agent deferoxamine (which suppresses most, but not all, iron-dependent free radical reactions) nor the thiol antioxidant GSH enter cells easily, so again be wary if they have protective effects in short-term experiments: this is suggestive of extracellular effects (Meister & Anderson, 1983; Halliwell, 1989; Marla *et al*., 1997). As an example, Clement *et al*. (2002) showed that GSH protects against the cytotoxicity of dopamine simply because it reacts with dopamine oxidation products *generated in the cell culture medium*. Given long enough, however, everything can enter cells, including deferoxamine, superoxide dismutase (SOD) and GSH (Doulias *et al*., 2003; Rius *et al*., 2003).

Studies of RS production in cells can, of course, be achieved by using aromatic compounds or spin traps, described above, and oxidative damage can be measured by assaying such end products as oxidized DNA bases, protein carbonyls, isoprostanes and 3-nitrotyrosine. Spin traps have been used successfully in many cell studies, since a wider range of traps at higher concentrations can be used than could ever be employed *in vivo*. Again, one must be aware of the possibility of rapid reduction of free radical–spin trap adducts to ESR-silent species by nonenzymic antioxidants (such as ascorbate) and cellular enzymatic reducing systems. An interesting combination is 5-((2-carboxy)phenyl)-5-hydroxy-1-(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl-3-phenyl-2-pyrrolin-4-one sodium salt, a nitroxide that is nonfluorescent. When it combines with a RS, the nitroxide is removed, the ESR signal is lost and the fluorescence is restored (Pou *et al*., 1993).

Let us now comment on the use of probes, usually fluorescence based, that are alleged to detect cellular production of RS.

Dichlorofluorescin diacetate (DCFDA) DCFDA is the most popular of these probes, frequently being used to detect 'cellular peroxides'. In fact, it is unlikely to do so because it reacts only slowly with H_2O_2 or lipid peroxides (LeBel *et al*.,

1992; Ischiropoulos *et al*., 1999; Bilski *et al*., 2002; Ohashi *et al*., 2002; Wardman *et al*., 2002) (Table 5). DCFDA enters cells and accumulates mostly in the cytosol. To avoid any cytotoxicity, cells should be loaded with DCFDA at low concentrations. With a variety of cell types, we have found loading at 1–10 μM for 45 min–1 h is adequate. Serum-free media must be used since serum will contain endogenous esterase activity and de-esterified dichlorofluorescein (DCF) is less cell permeable and will generate inconsistent data. Higher levels of DCFDA or high light intensities can also result in an artifactual photochemical oxidation to fluorescent products that can be mistaken for ROS generation (Marchesi *et al*., 1999; Afzal *et al*., 2003; Bindokas *et al*., 2003) (Table 5).

DCFDA is deacetylated by esterases to dichlorofluorescin (DCFH). This nonfluorescent product is converted by RS into DCF, which can easily be visualized by strong fluorescence at around 525 nm when excited at around 488 nm. Many papers have confused dichlorofluorescin and dichlorofluorescein, so it may be best to stick to correct chemical nomenclature (Figure 1). The chemistry of the conversion is complex (Figure 1). Washing cells after loading will allow unreacted DCFDA to diffuse out again. DCFH and DCF can also diffuse out and undergo extracellular reactions (Ohashi *et al*., 2002; Subramaniam *et al*., 2002). Thus be careful if you use a plate reader: you may be measuring light emission from the medium as well as from the cells. Neither H_2O_2 nor $O_2^{\bullet-}$ can oxidize DCFH, but peroxyl, alkoxyl, $NO_2^{\bullet}$, carbonate ($CO_3^{\bullet-}$) and $OH^{\bullet}$ radicals can, as can peroxynitrite (LeBel *et al*., 1992; Ischiropoulos *et al*., 1999; Bilski *et al*., 2002; Ohashi *et al*., 2002; Wardman *et al*., 2002; Myhre *et al*., 2003) (Table 5). DCFDA can only detect cellular peroxides efficiently if they are decomposed to radicals, for example, by transition metal ions. For example, in bovine aortic endothelial cells, the generation of a signal from DCFDA upon addition of H_2O_2 required the uptake of extracellular iron from the medium (Tampo *et al*., 2003). Horseradish peroxidase, myeloperoxidase and other heme proteins can also oxidize DCFH in the presence of H_2O_2 (indeed, DCFDA was first used in biology as a detector for H_2O_2 by adding horseradish peroxidase; Keston & Brandt, 1965; LeBel *et al*., 1992). Hence cellular peroxidase level and heme protein content are other variables to consider when interpreting studies with this probe (LeBel *et al*., 1992; Ohashi *et al*., 2002). For example, Ohashi *et al* (2002) showed that manipulating the heme content of cells by adding hemin or δ-aminolevulinic acid affected rates of DCFH oxidation.

Table 5 Sensitivity of various probes to different reactive species

Reactive species	*HPF*	*APF*	*DCFH*
OH^{a}	730	1200	7400
$ONOO^{-b}$	120	560	6600
Hypochlorite[c]	6	3600	86
Singlet O_2[d]	5	9	26
Superoxide[e]	8	6	67
H_2O_2[f]	3	<1	190
$NO^{\bullet}$[g]	6	<1	150
$ROO^{\bullet}$[h]	17	2	710
Autoxidation[i] of the probe	<1	<1	2000

Fluorescence probe reagents were added to sodium phosphate buffer (0.1 M, pH 7.4) (final 10 μM; 0.1% DMF as a cosolvent). The fluorescence intensities of HPF, APF and DCFH were measured at excitation wavelength of 490, 490 and 500 nm and fluorescence emission wavelength of 515, 515 and 520 nm, respectively.
[a]100 μM of ferrous perchlorate (II) and 1 mM of H_2O_2 were added.
[b]3 μM (final) of $ONOO^-$ was added.
[c]3 μM (final) of NaOCI was added.
[d]100 μM of 3-(1,4-dihydro-1,4-epidioxy-1-naphthyl)propionic acid was added.
[e]100 μM of KO_2 was added.
[f]100 μM of H_2O_2 was added.
[g]100 μM of 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-methyl-1-triazene was added.
[h]100 μM of 2,2′-azobis(2-amidinopropane)dihydrochloride was added.
[i]Fluorescence probe reagents solutions were placed under a fluorescent lamp for 2.5 h.
Adapted from Setsukinai *et al*. (2003).
We thank Professor Tetsuo Nagano and the American Society of Biochemistry and Molecular Biology for granting permission to reproduce this table.

It follows that DCF fluorescence is an assay of generalized oxidative stress rather than of any particular RS, and is *not* a direct assay of H_2O_2, $NO^{\bullet}$, lipid peroxides, singlet O_2 or $O_2^{\bullet-}$. One-electron oxidation of DCFH by various radicals and heme proteins is likely to produce intermediate radicals (Figure 1), including phenoxyl radicals, that can interact with such cellular antioxidants as GSH and ascorbate and with NADH to create more free radicals (Rota *et al*., 1999). Lawrence *et al*. (2003) pointed out that cytochrome *c* is a powerful catalyst of DCFH oxidation, and so use of DCFDA to probe oxidative stress during apoptosis should be approached with caution, since a rise in cytosolic cytochrome *c* levels could result in a bigger 'signal' without any change in cellular peroxide levels. Chromium (V), pyocyanin, mitoxantrone and ametantrone can directly oxidize DCFH and cause an artifactual signal, and the possibility of such direct oxidations must always be checked before using DCFDA to measure oxidative stress in cells exposed to various toxins (Martin *et al*., 1998; O'Malley *et al*., 2004). Variation in cellular esterase content could also conceivably affect the use of DCFDA as a probe, but this issue has not been explored in the literature.

Hempel *et al*. (1999) have stated that dihydrofluorescein diacetate has certain advantages over DCFDA in some cellular systems, but again it detects a range of RS. 2-[6-4′hydroxy]phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4′amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid (APF) (Figure 2) have been introduced as probes of ROS. Both fluoresce after reaction with $OH^{\bullet}$, $ONOO^-$ and peroxidase-derived species, but only APF emits light after exposure to HOCl. Both appear more stable in cell systems than DCFDA (Setsukinai *et al*., 2003). Neither responds to $^1O_2$, $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$ or peroxyl radicals and both are more stable to photochemical events than DCFDA (Table 5).

Dihydrorhodamine 123 (DHR) DHR is a probe widely used to detect several RS ($OH^{\bullet}$, $ONOO^-$, $NO_2^{\bullet}$, peroxidase-derived species), but is poorly responsive to $O_2^{\bullet-}$, H_2O_2 or $NO^{\bullet}$ (Buxser *et al*., 1999). DHR is oxidized to rhodamine 123, which is highly fluorescent around 536 nm when excited at about 500 nm (Figure 3). Rhodamine 123 is lipophilic and positively charged, and tends to accumulate in mitochondria, held there by the membrane potential. As a result, once it is

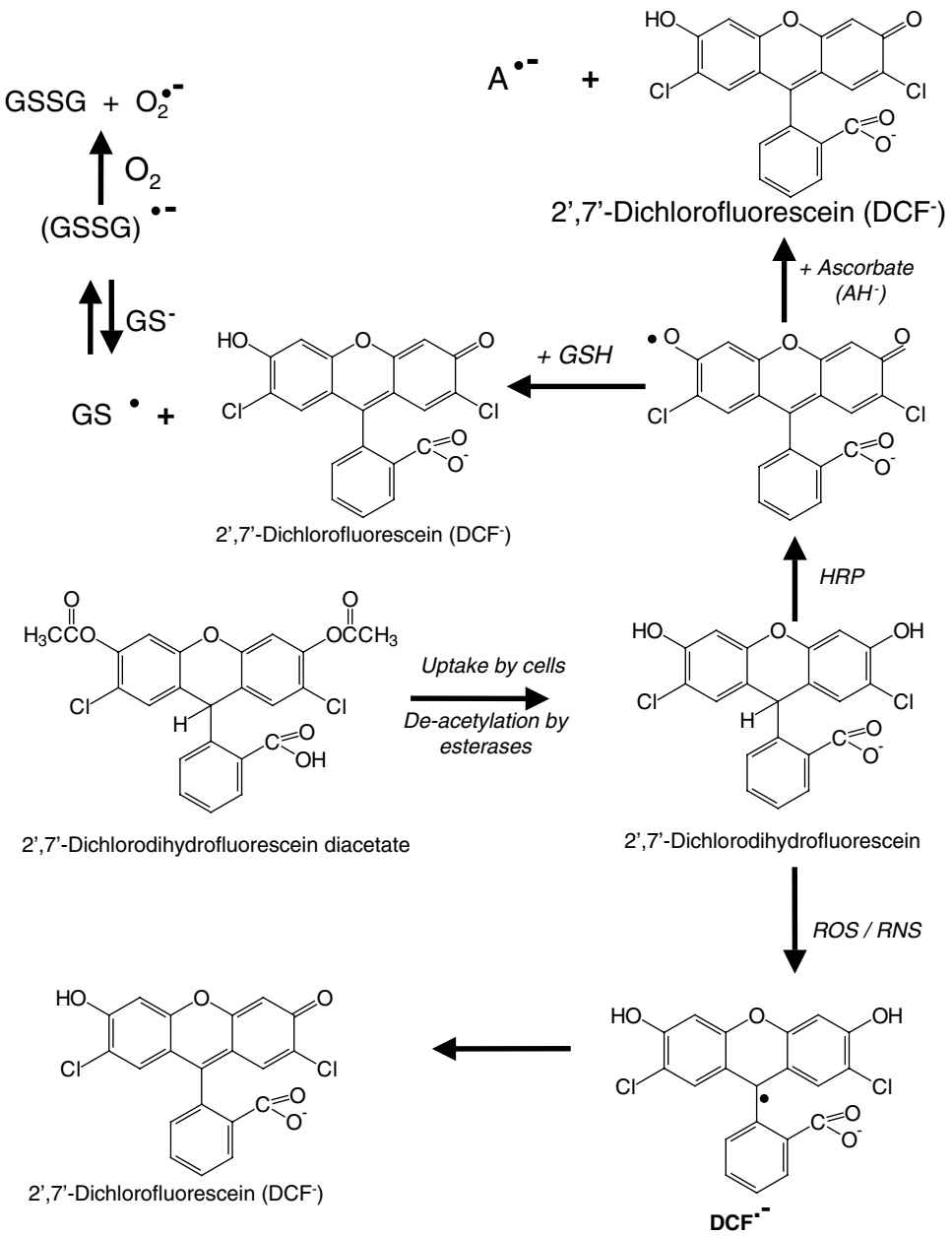

Figure 1 Conversion of DCFDA to a fluorescent product DCFDA is dichlorofluorescin (sometimes written as dichlorofluorescein) diacetate, perhaps better described by its proper chemical name as 2′,7′-dichlorodihydrofluorescein diacetate. It is hydrolyzed by cellular esterases to dichlorofluorescin (2′,7′-dichlorodihydrofluorescein), whose oxidation by several RS yields florescent DCF (dichlorofluorescein, more correctly called 2′7′-dichlorofluorescein) *via* an intermediate radial, $DCF^{\bullet -}$. Peroxidases can also convert it into a phenoxyl radical that can interact with antioxidants such as ascorbate (AH^-), reducing the phenoxyl radical and oxidizing ascorbate, or with GSH. $GS^{\bullet}$ resulting from the latter reaction can lead to $O_2^{\bullet -}$ generation. The phenoxyl radical can also be recycled by NADH (not shown), producing $NAD^{\bullet}$ radical, which reacts rapidly with O_2 to produce $O_2^{\bullet -}$:

$$NAD^{\bullet} + O_2 \rightarrow NAD^+ + O_2^{\bullet -}$$

formed, little rhodamine 123 leaks out of cells. DHR is also more sensitive at detecting HOCl than DCFDA (Buxser *et al*., 1999; Ischiropoulos *et al*., 1999). Rhodamine 123 and ethidium (see below) can be ejected from cells by drug conjugate efflux pumps, so the presence and activity of these membrane transport systems in the cells being studied is a factor that needs to be considered (Buxser *et al*., 1999).

At high levels, rhodamine 123 can sensitize singlet O_2 formation in mitochondria and cause NAD(P)H oxidation (Petrat *et al*., 2003). Palomba *et al*. (2000) found that exposure of PC12 cells to the synthetic hydroperoxide *tert*-butylhydroperoxide caused light emission from dihydrorhodamine. This appeared to be due to increased formation of $NO^{\bullet}$ leading to $ONOO^-$ formation, which in turn activated the enzyme phospholipase A_2 to raise cellular arachidonic acid levels. Arachidonic acid metabolism to RS was suggested to be the source of the DHR response.

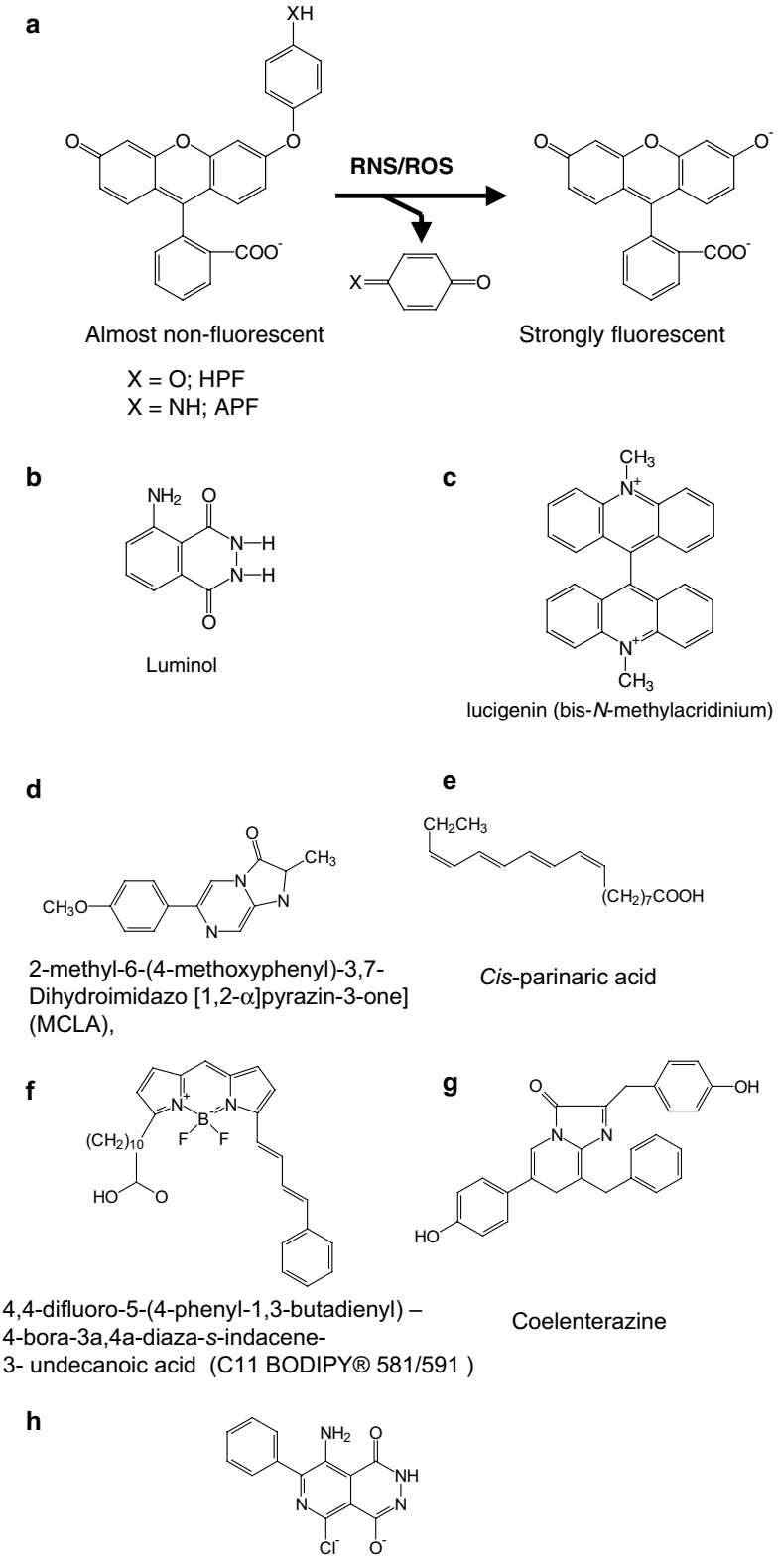

Figure 2 Structures of some common probes used for the detection of RS: (a) HPF and APF, (b) luminol, (c) LC and (d) MCLA. (e) *Cis*-parinaric acid, (f) 4,4-difluoro-5-(4-phenyl-1,3,-butadienyl)-4-bora-3a,4a-diaza-**s**-indacene-3-undecanoic acid (C11-BODIPY $^{581/591}$), (g) coelenterazine and (h) 8-amino-5-chloro-7-phenylpyridazo [3,4-*d*] pyridazine-1,4-(2H, 3H) dione (L-O12).

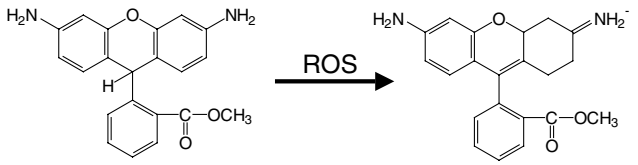

Figure 3 Conversion of dihydrorhodamine 123 to rhodamine 123.

Dihydroethidium (dihydroethidine) (DHE) This is frequently used as a probe for $O_2^{\bullet-}$, being oxidized to a fluorescent product. This is usually thought to be ethidium (Figure 4), which tends to intercalate into nuclear DNA. Ethidium fluoresces strongly at around 600 nm when excited at 500–530 nm. Recent work, however, suggests that the product is not ethidium (Zhao *et al*., 2003). Although somewhat specific for $O_2^{\bullet-}$ (Buxser *et al*., 1999; Zhao *et al*., 2003), DHE is readily spontaneously oxidized and often gives a high background (Buxser *et al*., 1999). It can also undergo a direct redox reaction with ferricytochrome *c* (Benov *et al*., 1998).

Luminol and lucigenin (LC) These two compounds (Figure 2) are often used to detect the production of RS by activated phagocytes, although they have also been used in other cell types (Faulkner & Fridovich, 1993). A luminol analogue L-012 (Figure 2) was reported to be more sensitive than luminol for the detection of $O_2^{\bullet-}$ and $ONOO^-$ or than dihydroethidine for $O_2^{\bullet-}$ detection (Daiber *et al*., 2004).The use of luminol to detect $O_2^{\bullet-}$ is a problematic area. It does *not* react directly with $O_2^{\bullet-}$ but must first be oxidized in a one-electron step (e.g. by $OH^{\bullet}$, $ONOO^-$ or peroxidase plus H_2O_2). The resulting luminol radical reacts with $O_2^{\bullet-}$ to generate a light-emitting product. Unfortunately, the luminol radical can also reduce O_2 to *generate* $O_2^{\bullet-}$, that is, the presence of luminol plus an oxidizing agent can lead to artifactual $O_2^{\bullet-}$ generation (Faulkner & Fridovich, 1993). Hence luminol is an unreliable probe; any oxidizing agent that can oxidize luminol by one electron will cause light emission inhibitable by SOD and the luminol is both the source and the detector of the $O_2^{\bullet-}$. LC is often said to be more specific for the detection of $O_2^{\bullet-}$ than luminol, but again it does not react *directly* with $O_2^{\bullet-}$ (Faulkner & Fridovich, 1993; Spasojevic *et al*., 2000). It must first be reduced to LC cation radical ($LC^{\bullet+}$), which then reacts with $O_2^{\bullet-}$ to give the fluorescent product. Conversion of LC to $LC^{\bullet+}$ cannot be achieved rapidly by $O_2^{\bullet-}$, and requires other cellular reducing systems (e.g. xanthine oxidase, the mitochondrial electron transport chain or the phagocyte NADPH oxidase), introducing an obvious complexity in interpreting results. $LC^{\bullet+}$ can also reduce O_2 to $O_2^{\bullet-}$, that is, addition of LC can artifactully *generate* more $O_2^{\bullet-}$ (Tarpey *et al*., 1999; Sohn *et al*., 2000; Spasojevic *et al*., 2000). The extent to which this artifact can interfere with accurate measurement of $O_2^{\bullet-}$ by LC continues to be debated (Munzel *et al*., 2002), but appears to be significant in some cell systems (Tarpey *et al*., 1999; Sohn *et al*., 2000; Spasojevic *et al*., 2000; Wardman *et al*., 2002). Alternative probes that might be more specific include coelenterazine (Tarpey *et al*., 1999; Munzel *et al*., 2002) and the luciferin analogue 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-$\propto$]pyrazin-3-one (MCLA) (Figure 2). However, MCLA may also react with peroxyl radicals (Ukamoto and Yonaha, 1998) and coelenterazine with $ONOO^-$ (Tarpey *et al*., 1999). Both these compounds are highly light sensitive.

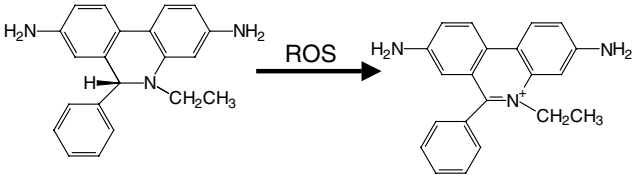

Figure 4 Conversion of DHE to ethidium.

Probes of lipid peroxidation/membrane RS Several membrane-partitioning probes have been introduced to measure lipid peroxidation or RS within membranes. One is *cis*-parinaric acid (Figure 2). When incorporated into lipids undergoing peroxidation, it is rapidly oxidized, losing its characteristic fluorescence (emission at 413 nm, excitation 324 nm) (Ritov *et al*., 1996; Drummen *et al*., 1999). Parinaric acid needs careful handling, because its polyunsaturated structure (Figure 2) makes it highly susceptible to nonspecific oxidation, and so it should be stored in the dark under a N_2 atmosphere. We have found it to work well, although the need to use the UV range is one disadvantage. For example, the required wavelengths are not always available on microplate readers or confocal/flow cytometry systems. It can be added as the free acid or incorporated into specific phospholipids (parinaroyl lipids) and used to study their relative susceptibilities to oxidative damage within membranes (Ritov *et al*., 1996; Shvedova *et al*., 2002).

Another probe is diphenyl-1-pyrenylphosphine (Takahashi *et al*., 2001), reported to react with peroxides to generate a product that fluoresces at 380 nm when excited at 351 nm (Figure 5).We have found it to be light and air sensitive and difficult to obtain reproducible results using it under our laboratory conditions. Another fatty acid probe becoming widely used is C-11-BODIPY$^{581/591}$, which upon oxidation shifts its fluorescence excitation and emission from red to green, so that the green/(red + green) ratio can be used as an estimate of membrane oxidation. Use of the ratio helps to decrease variations caused by heterogeneous probe distributions (cell thickness, uneven dye loading, compartmentalization, etc.) (Drummen *et al*., 2002). C-11-BODIPY$^{581/591}$ responds to a range of RS ($OH^{\bullet}$, peroxyl, alkoxyl, $ONOO^-$) but not to $O_2^{\bullet-}$, $NO^{\bullet}$, H_2O_2, singlet O_2 or hydroperoxides. Hence it can be used to determine RS within membranes (Drummen *et al*., 2002; Yoshida *et al*., 2003). When added to cells, C-11-BODIPY$^{581/591}$ seems to enter most membranes, with no preference for any organelle (Drummen *et al*., 2002). Again, the presence of these various probes can be expected to alter the rate, and perhaps, the mechanistic pathway, of lipid peroxidation. Serum proteins, especially albumin, avidly bind fatty acids and can deter loading of probes into cells, and may also cause slow leaching out when loaded cells are incubated in the cell culture media with added serum. Yet, serum-deprived cells suffer oxidative stress, which can elevate background production of RS (Lee *et al*., 2001).

How should we measure the output of these probes? The simplest technique is the fluorescence microplate reader, where data are presented as increases or decreases in relative fluorescence. However, the quality and sensitivity of the machines commercially available vary tremendously, and the requirement for additional excitation and emission filters often makes them expensive. Newer models are available that do not

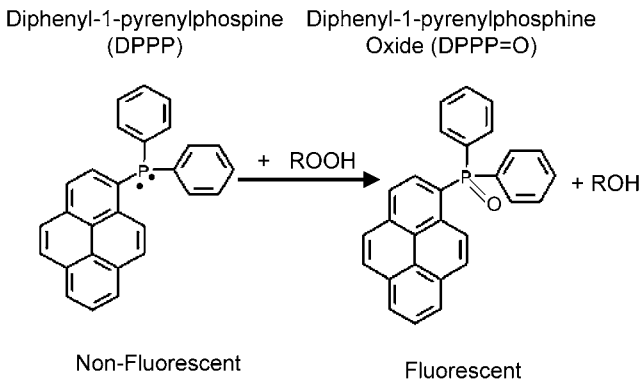

Figure 5 Conversion of diphenyl-1-pyrenylphosphine to a fluorescent product by peroxides.

require expensive filters but act on a dual monochromator principle and offer greater flexibility. Simultaneous dual wavelength excitation and emission measurements are particularly useful in determining the fluorescence ratio changes required for C-11-BODIPY$^{581/591}$ or the product of reaction of $O_2^{\bullet-}$ with DHE. It is important to check if the machine being used is a top-reading fluorescence machine, since this requires the cells to be in suspension. Bottom-reading machines have the advantage that the cells can be measured *in situ* without the need for trypsinization or cell scraping, processes that themselves generate cellular oxidative stress (Halliwell, 2003a) and result in artifactual changes in fluorescence. Plate readers measure total fluorescence, that is, they do not distinguish between intracellular and extracellular fluorescence from chemical reactions in the culture medium. We have already alluded to this problem in the case of DCFDA.

Flow cytometry offers the advantage of being able to measure the intracellular fluorescence of cells in the culture media. Quantitative data on the numbers of cells emitting fluorescence can be obtained rather than just relative fluorescence units. However, cells are required to be in suspension and require either scraping or trypsinization, which induce oxidative stress. Control experiments to optimize assay conditions must always be conducted to limit this. An additional disadvantage of flow cytometry is that often the cytometer is at room temperature rather than 37°C, which can give variable data with certain cells such as murine primary cortical neurones (Dr Nam Sang Cheung, unpublished observation).

Confocal microscopy is a powerful tool; cells can be loaded with fluorescent dyes and viewed in real time *in situ* in culture chambers at 37°C. The intracellular location of RS can be visualized, and the role of mitochondrial, endoplasmic reticulum or lysosomal events in oxidative stress may be visualized using counter stains such as MitoTracker, ER Tracker or Lysotracker dyes coloaded with dyes that detect RS. However, caution must be excercised to ensure that the signal from the organelle Tracker stain does not interfere with the measurement of the RS-sensitive dye and *vice versa*. It should also be stressed that simple overlapping of the Tracker stains with the signal from the RS-sensitive dyes only suggests involvement of a particular organelle, but does not rigorously prove it.

Effects of RS on other probes A wide range of probes is available to study many cellular events, such as pH changes and ion movements and to identify various cellular organelles (as mentioned above). For example, rises in Ca^{2+} frequently accompany oxidative stress (Halliwell & Gutteridge, 1999). Several papers have indicated that ion probes can be affected by the generation of RS (e.g. the Ca^{2+} probe Fura-2 can be degraded; Sarvazyan *et al*., 1998) or can themselves contribute to RS formation, such as calcein (Beghetto *et al*., 2000). MitoTracker Red, a fluorescent probe used to identify mitochondria, can stimulate mitochondrial ROS generation (Minamikawa *et al*., 1999), for example.

Conclusions

Whatever method you use to trap RS or measure oxidative damage, it is necessary to think carefully about *how* the method works, *what* is likely to confound it and how quantitative it can be (*how*, *what* and *how much*). With careful attention to understanding these points, erroneous interpretations can be minimized and the vibrant field of free radical research can continue to move forward.

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