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Current Status Review: Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis

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

It is difficult these days to open a medical journal without seeing some paper on the role of 'reactive oxygen species' or 'free radicals' in human disease. These species have been implicated in over 50 diseases, with atherosclerosis high on the list (Halliwell 1987). What are 'free radicals' and 'reactive oxygen species'? Do they cause disease? Are they produced in increased amounts as a result of disease and then contribute to further tissue injury? Are they merely an epiphenomenon of no relevance to clinical medicine? This review will attempt to answer such questions, with specific reference to atherosclerosis. Let us first establish some basic definitions.

What is a free radical?

Electrons in atoms occupy regions of space known as *orbitals*. Each orbital can hold a maximum of two electrons, spinning in opposite directions. A free radical can be simply defined as *any species capable of independent existence that contains one or more unpaired electrons*, an unpaired electron being one that is alone in an orbital. Most biological molecules are non-radicals, containing only paired electrons.

An electron occupying an orbital by itself has two possible directions of spin. Indeed, the technique of *electron spin resonance* detects radicals because it measures the energy changes that occur as they change their direction of spin (Cammack, 1987). Because electrons are more stable when paired together in orbitals, radicals are in general more reactive than non-radical species, although there is a considerable variation in their reactivity.

Radicals can react with other molecules in a number of ways (Slater 1984). Thus, if two radicals meet, they can combine their unpaired electrons (symbolized by $\cdot$) and join to form a covalent bond (a shared pair of electrons).

$$A\cdot + A\cdot \rightarrow A{-}A \qquad (1)$$

A radical might donate its unpaired electron to another molecule, or it might take an electron from another molecule in order to pair. However, if a radical gives one electron to, or takes one electron from, a non-radical, that non-radical becomes a radical. Thus, a feature of the reactions of free radicals is that they tend to proceed as *chain reactions*: one radical begets another and so on.

For many years, chemists have been interested in free radical reactions. Thus, many plastics, such as polythene, arise by free radical chain polymerizations (Scott 1988). Combustion is a free radical reaction. The drying and ageing of paint also involves free radical reactions: curators of museums have studied the role of free radical damage in the age-dependent deterioration of paintings and other items (Daniels 1988).

Hydroxyl radical

Chemists and biologists have examined in detail the role of free radical reactions in the

damage done to living cells by high-energy radiation. When tissues are exposed to, for example, gamma-radiation, most of the energy they take up is absorbed by the cell water, simply because there is more water there than any other molecule. The radiation causes one of the oxygen–hydrogen covalent bonds in water to split, leaving a single electron on hydrogen and one on oxygen and creating two radicals

$$\mathrm{H{-}O{-}H} \xrightarrow[\text{stages}]{\text{radiation}} \text{intermediate} \rightarrow \mathrm{H\cdot} + \cdot\mathrm{OH} \quad (2)$$

H· is a *hydrogen radical*, ·OH is a *hydroxyl radical*. The latter species is the most reactive radical known to chemistry: it can attack and damage almost every molecule found in living cells (Halliwell & Gutteridge 1985a). Since it is so reactive, ·OH does not persist for more than a few microseconds before combining with a molecule in its immediate vicinity. Because it is a radical, however, its reactions leave behind a legacy in the cell in the form of propagating chain reactions. Thus, if ·OH attacks DNA, free radical chain reactions propagate through the DNA and cause chemical alteration of the bases (that can lead to mutations) as well as strand breakage (Aruoma *et al.* 1989a). Imperfect repair of such damage can result in oncogene activation and carcinogenesis: hence high-energy radiation can lead to cancer (Breimer 1988). Hydroxyl radical generation is, paradoxically, the major mechanism by which malignant cells are killed during radiotherapy.

Perhaps the best-characterized biological damage caused by ·OH is its ability to stimulate the free radical chain reaction known as *Lipid peroxidation* (Fig. 1). This occurs when the ·OH is generated close to membranes and attacks the fatty acid side-chains of the membrane phospholipids. It preferentially attacks fatty acid side-chains with several double bonds, such as arachidonic acid. The ·OH abstracts an atom of hydrogen from one of the carbon atoms in the side-chain and combines with it to form water

$$\begin{array}{c} \mathrm{H} \\ | \\ \mathrm{-C-} \end{array} + \cdot\mathrm{OH} \rightarrow \mathrm{-\dot{C}-} + \mathrm{H\cdot} \quad (3)$$

$$\mathrm{H\cdot} + \cdot\mathrm{OH} \rightarrow \mathrm{H_2O} \quad (4)$$

Reactions (3) and (4) remove the ·OH, but they leave behind a carbon-centred radical (—Ċ—) in the membrane. Carbon-centred radicals formed from polyunsaturated fatty acid side-chains usually undergo molecular rearrangement to give conjugated diene structures, which can have various fates. Thus, if two such radicals collided in the membrane, cross-linking of fatty acid side-chains could occur as the two electrons joined to form a covalent bond. Reaction with membrane proteins is also a possibility. However, under physiological conditions, the most likely fate of carbon-centred radicals is to combine with oxygen, creating yet another radical, the *peroxyl radical* (sometimes abbreviated to the peroxy radical).

$$\mathrm{-\dot{C}-} + \mathrm{O_2} \rightarrow \begin{array}{c} \mathrm{O_2^{\cdot}} \\ | \\ \mathrm{-C-} \end{array} \quad (5)$$

Peroxyl radicals are reactive enough to attack adjacent fatty acid side-chains, abstracting hydrogen

$$\begin{array}{c} \mathrm{O_2^{\cdot}} \\ | \\ \mathrm{-C-} \end{array} + \begin{array}{c} \mathrm{H} \\ | \\ \mathrm{-C-} \end{array} \rightarrow \begin{array}{c} \mathrm{O_2H} \\ | \\ \mathrm{-C-} \\ \text{lipid hydroperoxide} \end{array} + \mathrm{-\dot{C}-} \quad (6)$$

Another carbon-centred radical is generated, and so the chain reaction (equations 5 and 6) can continue. Hence one ·OH can result in the conversion of many hundred fatty acid side-chains into *lipid hydroperoxides*. Accumulation of lipid hydroperoxides in a membrane disrupts its function and can cause it to collapse. In addition, lipid hydroperoxides can decompose to yield a range of highly cytotoxic products, the most unpleasant of which are aldehydes (Esterbauer *et al.*

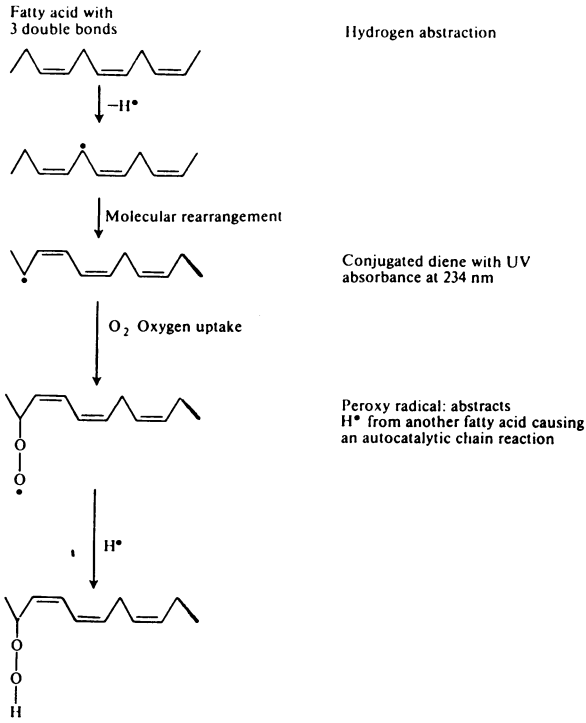

Fig. 1. The chain reaction of lipid peroxidation, initiated by hydroxyl radical. The structure shows part of a polyunsaturated fatty acid side-chain in a membrane lipid. The hydroxyl radical initiates the process of lipid peroxidation by abstracting a hydrogen atom and combining with it to form water. Since a hydrogen atom has only one electron, this leaves behind an unpaired electron on the carbon atom from which it was abstracted (symbolized by •). This carbon radical undergoes molecular rearrangement to form a conjugated diene, which then reacts with O_2 to give a peroxyl radical. The peroxyl radical abstracts a hydrogen atom from an adjacent fatty acid side-chain to continue the process, converting itself into a lipid peroxide. These are stable when pure but *in vivo* their decomposition is catalysed by copper and iron ions, and complexes, e.g. haem, methaemoglobin, cytochromes. The decomposition products include radicals that can abstract further hydrogen atoms, as well as hydrocarbon gases and cytotoxic aldehydes. Lipid peroxidation is thus a radical reaction. The antioxidant vitamin E (α-tocopherol) interferes with this chain reaction by donating hydrogen atoms to peroxyl radicals, stopping them from abstracting hydrogen (Slater 1984).

1988). Most attention has been paid in the literature to malonaldehyde (sometimes called malondialdehyde) but this is relatively harmless in comparison to such noxious products as 4-hydroxynonenal (Esterbauer *et al.* 1988; Curzio 1988). Peroxyl radicals and cytotoxic aldehydes can also cause severe damage to membrane proteins, inactivating receptors and membrane-bound enzymes (Dean *et al.* 1986).

Oxygen radicals

Biochemists (apart from those with a special interest in ionizing radiation) became interested in radicals only in the 1970s. This followed from the discovery in 1968 of an enzyme that is specific for a free radical substrate. This enzyme is *superoxide dismutase* (Fridovich 1974). It removes superoxide radical, a species that is made by adding an

extra electron onto the oxygen molecule

$$O_2 + e^- \rightarrow \underset{\text{superoxide radical}}{O_2^-} \quad (7)$$

Superoxide dismutase removes O_2^- by catalysing the dismutation reaction shown below

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

In the absence of SOD, reaction (8) occurs non-enzymically but at a rate approximately four orders of magnitude less.

The discovery of SOD led to the realization that O_2^- is formed *in vivo* in living organisms, and SOD functions to remove it. Some of the O_2^- formed *in vivo* is a chemical accident. For example, when mitochondria are functioning, some of the electrons passing through the respiratory chain leak from the electron carriers and pass directly onto oxygen, reducing it to O_2^- (Fridovich 1974, 1983). Many molecules oxidize on contact with oxygen, e.g. an adrenalin solution left on the bench 'goes off' and eventually forms a pink product. The first stage in this oxidation is transfer of an electron from the adrenalin onto O_2, giving O_2^-. Such oxidations undoubtedly proceed *in vivo* as well (Halliwell & Gutteridge 1985a). For example, several sugars, including glucose, oxidize very slowly to produce oxygen radicals. Thus it has been suggested that decades of exposure of body tissues to elevated blood glucose can result in diabetic patients suffering 'oxidative stress' that may contribute to the side-effects of hyperglycaemia (Wolff & Dean 1987). Elevated blood glucose leads to a non-enzymatic glycosylation of proteins, to which free radical generation might contribute.

Oxidants and phagocyte action

Some of the O_2^- production *in vivo* may be accidental but much is functional. Thus activated phagocytic cells generate O_2^- as has been shown for monocytes, neutrophils, eosinophils, and macrophages of all types (Curnutte & Babior 1987). Radical production is important in allowing phagocytes to kill some of the bacterial strains that they can engulf. This can be illustrated by examining patients with *chronic granulomatous disease*, an inborn condition in which the membrane-bound NADPH oxidase system in phagocytes that makes the O_2^- does not work (Curnutte & Babior 1987). Such patients have phagocytes that engulf and process bacteria perfectly normally, but several bacterial strains are not killed and are released in viable form when the phagocytes die. Thus patients suffer severe, persistent and multiple infections with such organisms as *Staphylococcus aureus*. Another killing mechanism used by neutrophils (but not by macrophages) is the enzyme *myeloperoxidase* (Weiss 1989). It uses H_2O_2 produced by dismutation of O_2^- to oxidize chloride ions into *hypochlorous acid*, HOCl, a powerful antibacterial agent (Domestos is a solution of the sodium salt of hypochlorous acid)

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^- \quad (9)$$

Superoxide and the endothelium

Another example of a useful role for O_2^- may be provided by the vascular endothelium. It has recently been shown that endothelium-derived relaxing factor (EDRF), a humoral agent that is produced by endothelium and is an important mediator of vasodilator responses induced by several pharmacological agents, including acetylcholine and bradykinin, is identical to nitric oxide (Palmer *et al.* 1988; Sneddon & Vane 1988). The endothelium also seems to produce continuously small amounts of O_2^-, which can react with NO and inactivate it. Both NO and O_2^- are free radicals (each with one unpaired electron) and so they can combine to give a non-radical product

$$NO + O_2^- \rightarrow \text{intermediates} \rightarrow \underset{\text{nitrate ion (non-radical)}}{NO_3^-} \quad (10)$$

Hence variations in the production of NO and O_2^- by endothelium may provide one mechanism for regulation of vascular tone

(Halliwell 1989). It is even possible that impaired endothelium-mediated vasodilation in diabetic patients could be related to increased radical formation *in vivo* (de Tejada *et al.* 1989).

Superoxide formed *in vivo*, whether functionally or accidentally, is disposed of by SOD (equation 8). Indeed, this enzyme seems essential to aerobic life, i.e. the removal of O_2^- is a key process. Recent studies using genetic engineering techniques to manipulate the SOD levels of organisms, or to delete the genes encoding SOD, have reinforced this point (Farr *et al.* 1986). It is interesting to note that no complete inborn deficiencies of SOD have been reported in humans, perhaps because they would be lethal mutations.

Reactive oxygen species

SOD removes O_2^- by converting it into hydrogen peroxide (H_2O_2) and oxygen (equation 8). Hydrogen peroxide itself can be quite toxic to cells. For example, incubation of cells with large amounts of H_2O_2 can cause DNA damage, membrane disruption and release of Ca^{2+} ions within the cells, causing Ca^{2+}-dependent proteolytic enzymes to become activated (reviewed in Halliwell 1987). At least some of this damage may be mediated by a reaction of H_2O_2 with O_2^- in the presence of iron ions, to form highly reactive radicals, one of which is ·OH (equation 11). We have already seen that ·OH can attack DNA, membrane lipids and proteins.

$$O_2^- + H_2O_2 \xrightarrow{\text{iron ions}} \underset{\text{hydroxyl radical}}{\cdot OH} + OH^- + O_2 \qquad (11)$$

Thus removal of H_2O_2, as well as of O_2^-, is advantageous (Halliwell & Gutteridge 1985a, b).

The SOD enzymes therefore work in conjunction with two enzymes that remove H_2O_2 in human cells: catalase and glutathione peroxidase (Chance *et al.* 1979). The study of inborn errors of metabolism shows that glutathione peroxidase is the more important of the two in removing H_2O_2, probably because it is located in the same subcellular organelles as SOD. Glutathione peroxidase has the distinction of being the only human enzyme known that requires the element selenium for its activity: a selenocysteine residue (side-chain—SeH instead of −SH, as in normal cysteine) is present at its active site. It is unlikely, however, that the sole function of selenium in humans is to act as a cofactor for glutathione peroxidase (Levander 1987). Glutathione peroxidase removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG).

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad (12)$$

Hydrogen peroxide has no unpaired electrons and thus does not qualify as a radical. Hence the term *reactive oxygen species* has been introduced to describe collectively not only O_2^- and ·OH (radicals) but also H_2O_2 (non-radical). Hypochlorous acid (HOCl) produced by neutrophil myeloperoxidase can also be included under this heading: it is a powerful oxidizing agent but not a radical, having no unpaired electrons. H_2O_2, O_2^-, ·OH and HOCl are sometimes collectively called 'oxidants'. This is a valid description for H_2O_2, ·OH and HOCl, which are oxidizing agents. However, O_2^- has both oxidizing and reducing properties. Indeed, the latter property is made use of in a popular assay for O_2^-, the SOD-inhibitable reduction of cytochrome c, often applied to measure O_2^- production by phagocytes

$$\text{cyt c } (Fe^{3+}) + O_2^- \rightarrow O_2 + \text{cyt c } (Fe^{2+}) \qquad (13)$$

Hence the term 'oxidant' is not strictly correct when applied to O_2^-.

The role of transition metals

Many transition metals have variable oxidation numbers, e.g. iron as Fe^{2+} or Fe^{3+} ions and copper as Cu^+ or Cu^{2+}. Changing

between oxidation states involves accepting and donating single electrons, e.g.

$$Fe^{3+} + e^- \rightarrow Fe^{2+} \quad (14)$$

$$Cu^{2+} + e^- \rightarrow Cu^{+} \quad (15)$$

Thus transition metal ions are remarkably good promoters of free radical reactions (Hill 1981). Polymer scientists (Scott 1988) and food chemists (Grootveld & Jain 1989) have known this for years, and biochemists have recently realized that metal ions can act in this way *in vivo* (Halliwell & Gutteridge 1985a, b; Wolff & Dean 1987).

Transition metals and lipid peroxidation

Transition metal ions can be involved in lipid peroxidation in two ways. Firstly, they can participate in *first-chain initiation* which involves attack by any species that is capable of abstracting a hydrogen atom. Highly-reactive hydroxyl radical (·OH) has been shown to do this (see above) and hydroxyl radical can be produced by reaction of $O_2^{\cdot-}$ and H_2O_2 with iron ions (equation 11). It may also be produced by reaction of H_2O_2 with Cu^{2+} ions, although there is some debate about whether this reaction generates ·OH or another highly oxidizing species, such as a copper ion–oxygen complex in which the copper has an oxidation number of three (Czapski *et al.* 1988). Several iron ion–oxygen complexes, such as perferryl, ferryl or $Fe^{2+}/Fe^{3+}/O_2$ complexes, have also been claimed to initiate peroxidation (Minotti & Aust 1987), although their ability to do this is still uncertain (Aruoma *et al.* 1989b).

However, the question of first-chain initiation is probably largely irrelevant to most, if not all, biochemical studies on the peroxidation of fatty acids, isolated membrane fractions, or purified lipoproteins. This is because the lipids studied already contain from trace to large amounts of peroxides.

Commercial fatty acids are heavily contaminated with lipid peroxides (Gutteridge 1988). Cell disruption to isolate membrane fractions not only causes increased rates of non-enzymic free radical reactions but also activates enzymes (cyclooxygenases and lipooxygenases) that produce organic peroxides. When metal ions are added to lipid systems already containing traces of peroxides, their main action is to decompose these peroxides into peroxyl and alkoxyl (lipid-O·) radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Gutteridge 1988). This may be represented, in a simplified form, by the equations below, in which lipid· symbolises a carbon-centred radical

$$\text{lipid—OOH} + \underset{(Cu^+)}{Fe^{2+}} \rightarrow \underset{\text{alkoxyl radical}}{\text{lipid—O}\cdot} + \underset{(Cu^{2+})}{Fe^{3+}} + OH^- \quad (16)$$

$$\text{lipid—OOH} + Fe^{3+} \rightarrow \underset{\text{peroxyl radical}}{\text{lipid—OO}\cdot} + Fe^{2+} + H^+ \quad (17)$$

$$\text{lipid O}\cdot + \text{lipid—H} \rightarrow \text{lipid—OH} + \text{lipid}\cdot \quad (18)$$

$$\text{lipid—OO}\cdot + \text{lipid—H} \rightarrow \text{lipid—OOH} + \text{lipid}\cdot \quad (19)$$

$$\text{lipid}\cdot + O_2 \rightarrow \text{lipid—OO}\cdot \quad (20)$$

Reducing agents such as ascorbic acid or $O_2^{\cdot-}$, can accelerate these metal ion-dependent peroxidation reactions because Cu^+ and Fe^{2+} ions seem to react with peroxides faster than do Cu^{2+} and Fe^{3+}, respectively. The end-products of these complex metal ion-catalysed breakdowns of lipid hydroperoxides include the cytotoxic aldehydes mentioned previously, as well as hydrocarbon gases such as ethane and pentane (Halliwell & Gutteridge 1985a, b).

Antioxidant defence and damage repair

Organisms use superoxide dismutases, catalase and glutathione peroxidase to protect themselves against generation of reactive oxygen species. They also take great care to keep as many iron and copper ions as possible safely bound in storage or transport proteins. Thus there is three times as much transferrin iron-binding capacity in plasma as iron needing to be transported, so that

there are essentially no free iron ions in plasma. Iron ions bound to such proteins as transferrin cannot stimulate lipid peroxidation or ·OH generation: the same is true of copper ions bound to the plasma proteins caeruloplasmin or albumin (Halliwell 1988; Halliwell & Gutteridge 1986; Aruoma & Halliwell 1987). The value of this sequestration is shown by an inspection of the pathology suffered by patients with iron overload disease, in which simple iron ion complexes circulate in the blood (Grootveld *et al.* 1989). The patients can suffer liver damage, diabetes, joint inflammation and hepatoma, among other problems (McLaren *et al.* 1983).

Alpha-tocopherol

As well as the primary defences (scavenger enzymes and metal-ion sequestration) there are secondary defences. Thus, cell membranes and plasma lipoproteins contain *α-tocopherol*, which functions as a *chain-breaking antioxidant.* It is a lipid-soluble molecule that sits in the interior of biological membranes. Attached to the hydrophobic structure of α-tocopherol is an —OH group whose hydrogen atom is very easy to remove. Thus when peroxyl and alkoxyl radicals are generated during lipid peroxidation, they combine preferentially with the antioxidant, e.g.

$$\begin{array}{l} \text{tocopherol—OH} + \text{—C(O}_2^{\cdot}\text{)—} \rightarrow \text{—C(O}_2\text{H)—} \\ \text{tocopherol—O}\cdot + \text{—C—} \end{array} \qquad (21)$$

instead of with an adjacent fatty acid. This therefore terminates the chain reaction, hence the name *chain-breaking antioxidant.* It also converts the α-tocopherol into a new radical, tocopherol-O·. This radical is poorly reactive, being unable to attack adjacent fatty acid side-chains, so the chain reaction is stopped. Evidence exists (McCay 1985) that the tocopherol radical can migrate to the membrane surface and be converted back to α-tocopherol by reaction with ascorbic acid (vitamin C). Thus both vitamin C and α-tocopherol seem to minimize the consequences of lipid peroxidation in lipoproteins and in membranes, should this process begin.

The terms α-tocopherol and vitamin E are often used synonymously. This is not quite correct: vitamin E is defined nutritionally as a factor needed in the diet of pregnant female rats to prevent resorption of the foetus (Diplock 1985) and compounds other than α-tocopherol (e.g. β-, γ- and δ-tocopherols) have some effect in this assay. However, α-tocopherol is the most effective, and it is by far the most important lipid-soluble chain-breaking antioxidant *in vivo* in humans (Ingold *et al.* 1986). Thus the content of α-tocopherol in circulating LDL helps to determine their resistance to lipid peroxidation and thus may affect the development of atherosclerosis (Esterbauer *et al.* 1989). Indeed, low plasma levels of α-tocopherol and vitamin C seem to correlate with an increased incidence of myocardial infarction and of some forms of cancer (Gey *et al.* 1987).

Although α-tocopherol is by far the most important chain-breaking antioxidant in humans, it is not the only one (Esterbauer *et al.* 1989). Figure 2 shows the depletions of compounds that occur when peroxidation is stimulated in LDL isolated from human plasma, by addition of copper ions. Not until tocopherols, β-carotene, lycopene and retinyl stearate had been oxidized did peroxidation of fatty acid side-chains begin to accelerate.

Some other compounds may also function as antioxidants *in vivo.* Recent suggestions have included uric acid and bilirubin, but it is probably too early as yet to assess their physiological importance. In addition it is clear that antioxidant defence is not perfect. Thus, cells contain systems that can repair DNA after attack by radicals (Breimer 1988), degrade proteins damaged by radicals (Marcillat *et al.* 1988) and metabolize lipid hydroperoxides (Halliwell & Gutteridge 1985a).

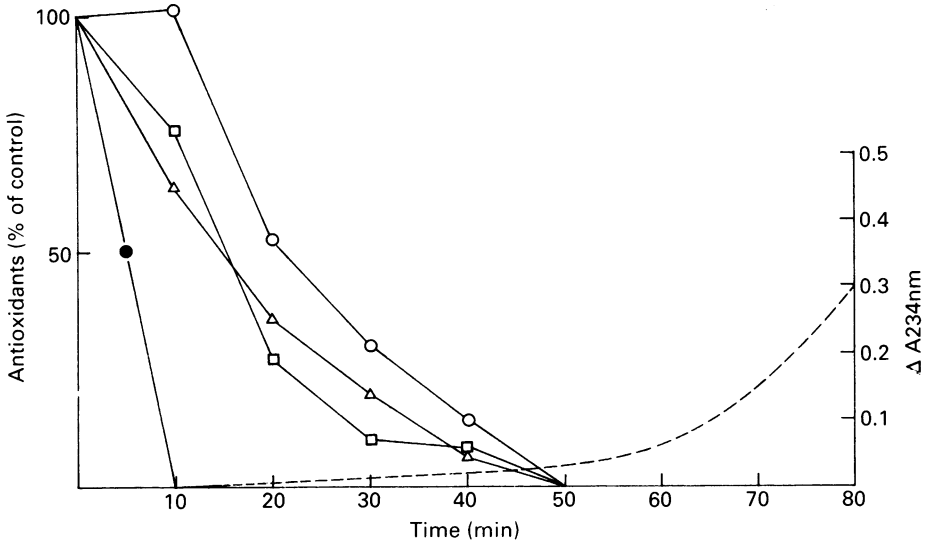

Fig. 2. Time course of the consumption of antioxidants and the onset of lipid peroxidation (as formation of conjugated dienes) during oxidation of human low-density lipoproteins (LDL) induced by copper ions. The diene increase was measured by continuously monitoring the increase of the 234 nm absorbance. ○, β-carotene, △, Retinyl stearate, □, Lycopene and ●, α, γ-tocopherols were measured by HPLC. – – –, Δ A 234 nm. Professor Dr H. Esterbauer and Harwood Academic Publishers, from *Free Radical Res. Commun.* 6, 67–75.

Free radicals and human disease: causation, consequence or triviality?

Does increased formation of free radicals and other reactive oxygen species cause any human disease? Radiation-induced carcinogenesis may be initiated by radicals, as we have seen. The signs produced by chronic dietary deficiencies of selenium (Keshan disease) or of vitamin E (neurological disorders seen in patients with inborn errors in the mechanism of intestinal fat absorption) could also be mediated by reactive oxygen species (Levander 1987; Muller *et al.* 1983). In the premature infant, exposure of the incompletely-vascularized retina to elevated concentrations of oxygen can lead to retinopathy of prematurity, which in its most severe forms can result in blindness. Several controlled clinical trials have documented the efficiency of α-tocopherol in minimizing the effect of the retinopathy (Kretzer *et al.* 1984) suggesting a role for lipid peroxidation.

For most human diseases, however, the oxidant formation is secondary to the primary disease process. For example, activated neutrophils produce $O_2^{\cdot-}$, H_2O_2 and HOCl in order to kill bacteria. However, if a large number of phagocytes become activated in a localized area, they can produce tissue damage. For example, the synovial fluid in the swollen knee joints of rheumatoid patients swarms with activated neutrophils. There is evidence that oxidants and other products derived from neutrophils are contributing to the joint injury: whether this is a major or a minor contribution to joint damage remains to be established (Halliwell *et al.* 1988a). In some forms of the adult respiratory distress system (ARDS), the lung damage seems to be mediated by an influx of neutrophils into the lung, where they become activated to produce prostaglandins, leukotrienes, proteolytic enzymes such as elastase, and reactive oxygen species (Baldwin *et al.* 1986). Among other effects, the reactive oxygen species can inactivate the

proteins (such as α_1-antiproteinase) within the lung that normally inhibit the action of enzymes such as elastase and prevent them from attacking lung elastic fibres. The precise contribution of oxidant generation to lung damage in ARDS is unknown, but deserves investigation in view of the high mortality rate.

In both ARDS and in rheumatoid arthritis, the increased generation of reactive oxygen species is secondary to the processes that caused neutrophil infiltration, but oxidants may make an important additional contribution to tissue injury.

Ischaemia/reoxygenation injury

One of the most exciting research areas in recent years has been that of ischaemia/reperfusion injury (McCord 1985). When tissues are deprived of O_2 they are injured and after a period the injury becomes irreversible and the tissue will die. How long this period is depends on the extent of O_2 deprivation (whether it is ischaemia or merely hypoxia) and the tissue in question: skeletal muscle can be rendered bloodless for hours without much injury, whereas in the brain the period is in minutes. For heart, up to about 60 min seems to be a tolerable period. Thus ischaemia injures cells and will eventually kill them, and the aim should be to restore blood flow as soon as possible: hence the use of thrombolytic agents in the treatment of myocardial infarction (Bolli 1988).

However, studies on isolated organs and in animals have shown that, provided the period of ischaemia does not itself do irreversible damage, tissue function is better preserved if antioxidants are included in the reoxygenation medium (McCord 1985; Bolli 1988; Simpson & Lucchesi 1987). Hence restoration of O_2, although obviously beneficial overall, causes increased oxidant formation in the damaged tissue and temporarily worsens the injury. Thus inclusion of antioxidants when blood flow is restored offers protective effects: antioxidants used include superoxide dismutase and desferrioxamine, a chelator that binds iron ions and usually stops them from accelerating free radical reactions (Gutteridge *et al.* 1979). Clinical trials are currently under way to assess the benefit of combining thrombolytic agents with recombinant human SOD in the treatment of acute myocardial infarction. The use of antioxidants in the treatment of shock and in the preservation of organs for transplantation is also receiving attention (Bolli 1988; Gower *et al.* 1989; Sanan *et al.* 1989).

Why should ischaemia lead to more oxidant generation when tissues are reoxygenated? The answer is not yet clear: possibilities include release of iron ions, catalytic for free radical reactions, from their normal storage sites within the cell (Halliwell 1989; Gower *et al.* 1989; Sanan *et al.* 1989), disruption of mitochondrial respiratory chains so that more electrons leak to oxygen to form $O_2^{\cdot-}$ (Marklund 1988) and increases in the activities of certain enzymes such as xanthine oxidase, that generate $O_2^{\cdot-}$ (McCord 1985). However, there is considerable doubt about the role of xanthine oxidase in human myocardial injury (Kehrer 1989; Halliwell 1989).

Traumatic injury

There are some other examples in which tissue injury, by a non-radical mechanism, can lead to increased free radical reactions. Thus mechanical (e.g. crushing) or chemical injury to tissues can cause cells to rupture and release their contents into the surrounding area. These contents will include transition metal ions. Thus administration of cytotoxic drugs to patients with acute myeloid leukaemia has been shown to create a temporary 'iron overload' state, probably due to extensive drug-induced lysis of the leukaemic cells. This increased iron availability could contribute to the side-effects of cytotoxic chemotherapy (Halliwell *et al.* 1988b).

Perhaps the greatest interest in this area lies in the sequelae of traumatic or ischaemic injury to the brain. Some areas of the human

brain are rich in iron and cerebrospinal fluid has no significant iron-binding capacity, since its content of transferrin is low. Thus it has been proposed (Halliwell & Gutteridge 1985c) that injury to the brain by mechanical means (trauma) or by oxygen deprivation (stroke) can result in release of iron ions into the surrounding area. These ions can facilitate further damage to the surrounding areas by accelerating free radical reactions. This proposal has been given some support from animal studies, using chelating agents that bind iron ions and prevent them from catalysing radical reactions. Promising results have been obtained with aminosteroid-based iron chelators. Thus one such chelator, U74006F, has been observed to decrease the effects of reperfusion injury upon the brain of cats (Hall & Yonkers 1988), to decrease post-traumatic spinal cord degeneration in cats (Hall 1988) and to minimize neurological damage after head injury in mice (Hall *et al.* 1988).

Free radicals in human disease: a triviality

Tissue destruction and degeneration can result in increased oxidant damage, by such processes as metal ion release, phagocyte activation and disruption of mitochondrial electron transport chains (so that more electrons 'escape' to oxygen to form $O_2^{\cdot-}$). It follows that almost any disease is likely to be accompanied by increased formation of reactive oxygen species. It is not therefore surprising that the list of diseases in which their formation has been implicated is long and is growing longer (Halliwell 1987). For atherosclerosis (see below), rheumatoid arthritis, some forms of ARDS, reoxygenation injury and traumatic or ischaemic damage to the central nervous system, there is reasonable evidence to suggest that free radical reactions make a significant contribution to the disease pathology. As has been stressed previously, however (Halliwell & Gutteridge 1984), it is equally likely that in some (perhaps most) diseases, the increased oxidant formation is an epiphenomenon that makes no significant contribution to the progression of the disease. Each proposal must be subjected to stringent examination.

Reactive oxygen species and atherosclerosis

Interest in the role of lipid peroxidation in atherogenesis was raised when it was found that probucol has a marked anti-atherogenic effect in hyperlipidaemic rabbits (Carew *et al.* 1987). Probucol, like α-tocopherol, has easily-donatable hydrogen atoms (Fig. 3) and is a powerful chain-breaking inhibitor of lipid peroxidation. For example, Fig. 4 shows that probucol, at micromolar concentrations, can inhibit peroxidation of rat liver microsomes, a popular 'substrate' for studies of lipid peroxidation *in vitro*.

What roles could be played by reactive oxygen species in atherogenesis? The origin of atherosclerosis is uncertain, but a popular current theory is that it begins with damage, by some mechanism (possibly haemodynamic), to the vascular endothelium (Ross 1986; Babiak & Rudel 1987). Endothelial cells are known to be sensitive to damage by reactive oxidizing species and lipid hydroperoxides (Silverman & Santucci 1988; Whorton *et al.* 1985; Hennig *et al.* 1986). Thus any lipid hydroperoxides present in the plasma lipoproteins (e.g. resulting from a high intake of dietary fat and/or a low intake of antioxidant vitamins) could conceivably contribute to initial endothelial cell damage (Yagi 1987).

Endothelial cell damage is thought to be followed by attachment of monocytes from

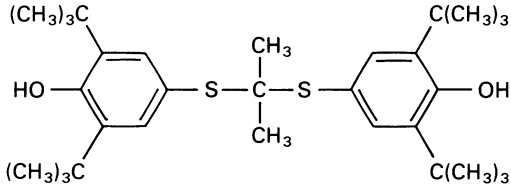

Fig. 3. Strucutre of probucol. Note the phenolic —OH groups, which can easily donate hydrogen atoms.

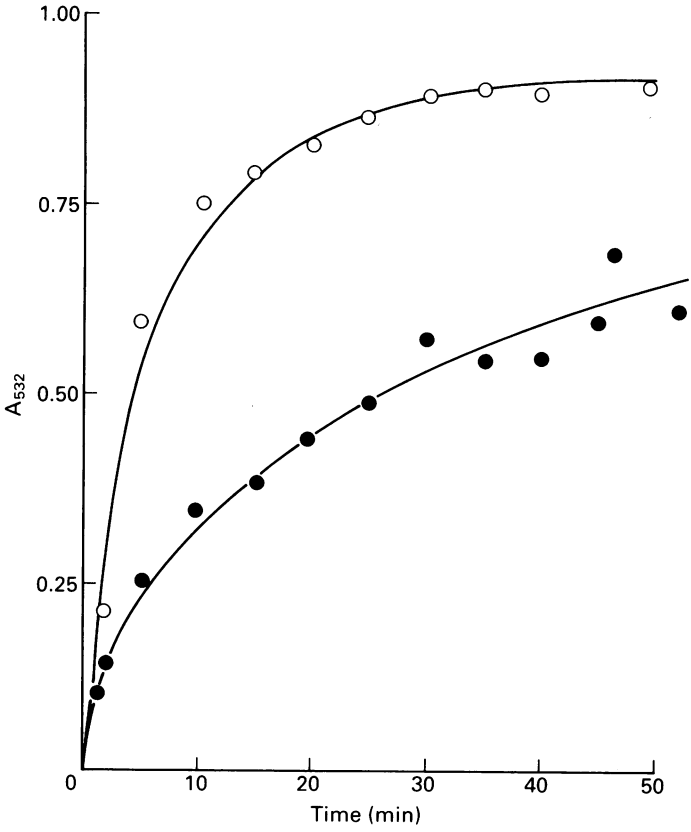

Fig. 4. Action of probucol on lipid peroxidation in rat liver microsomes. Peroxidation of rat liver microsomes in the presence of $FeCl_3$ and ascorbic acid was measured by the thiobarbituric acid (TBA) test, and results are shown as the final absorbance at 532 nm. 50 μM probucol inhibited peroxidation by >95%; the graph shows the effect of ●, 10 μM probucol. ○, No probucol control. (O.I. Aruoma, unpublished results).

the circulation (e.g. Quinn *et al.* 1988), that develop into macrophages within the vessel wall. Macrophages play an important role in the growth of the atherosclerotic lesion (Mitchinson & Ball 1987). Activated monocytes and macrophages could conceivably injure neighbouring endothelial cells by secreting O_2^-, H_2O_2 and hydrolytic enzymes, and factors released by macrophages can stimulate the proliferation of smooth muscle cells. Macrophages also release platelet-stimulating factors and adherence of platelets to injured epithelium might cause release of other agents that encourage proliferation of smooth muscle cells, although the precise role of platelets in atherosclerosis is an area of controversy (discussed by Curtiss *et al.* 1987).

Macrophages possess receptors for low-density lipoprotein (LDL), but if LDL has undergone lipid peroxidation it is recognized by a separate class of macrophage receptors known as the *scavenger receptors* (Goldstein *et al.* 1979). There may be more than one type of scavenger receptor (Sparrow *et al.* 1989). LDL bound to these receptors is taken up with enhanced efficiency, so that cholesterol rapidly accumulates within the macrophage and may convert it into a foam cell (Goldstein *et al.* 1979; Mitchinson & Ball 1987). Arter-

ial endothelial cells, smooth muscle cells and macrophages have been shown to be capable of oxidizing LDL *in vitro* so that macrophages will internalize it faster (reviewed in Steinberg *et al.* 1989). The modification process that allows recognition by the scavenger receptors involves the derivatization of lysine residues of the apoprotein B moiety of LDL by lipid peroxidation products, such as the cytotoxic aldehyde 4-hydroxynonenal (Jurgens *et al.* 1987; Quinn *et al.* 1987; Steinbrecher 1987).

Peroxidation of LDL within the vessel wall could have other deleterious effects. It has been suggested that products formed in peroxidized LDL, such as lysophosphatidyl choline (Quinn *et al.* 1988), might act as chemotactic factors for blood monocytes, encouraging their recruitment into an atherosclerotic lesion. Also low (sub-micromolar) concentrations of peroxides might accelerate cyclooxygenase and lipoxygenase-catalysed reactions in endothelium and in any platelets present, leading to enhanced formation of eicosanoids (Warso & Lands 1983; Holland *et al.* 1988). Oxidized LDL may also stimulate the production of eicosanoids by macrophages (Yokode *et al.* 1988). There have been several speculations that oxidation products of cholesterol might also be involved in atherogenesis (e.g. Bernheimer *et al.* 1987; Wilson 1976); cholesterol is oxidized into a wide variety of products in peroxidizing lipid systems (Sevanian & Peterson 1986) and oxidized cholesterol has been reported to be toxic to arterial smooth muscle cells (Bernheimer *et al.* 1987; Sevanian & Peterson 1986). However, another group has reported that cholesterol hydroperoxides *suppress* atherogenesis in rabbits (Tipton *et al.* 1987). Activation of the classical complement pathway by cholesterol or by its oxidation products has also been suggested to play a role in atherogenesis (Wilson 1976; Swartz *et al.* 1988). Clearly this is an area that deserves further study, in view of the well established relationship between LDL cholesterol and atherosclerosis.

What is the evidence that lipid peroxidation takes place in the atherosclerotic lesion? Human lesions accumulate the pigment ceroid, which is known to be an end-product of peroxidation (Wilson 1976; Ball *et al.* 1987). Antibodies directed against LDL that has undergone peroxidation or has been treated with 4-hydroxynonenal, bind to rabbit aortic atherosclerotic lesions. In addition, LDL eluted from such lesions can bind to antibody specific for malondialdehyde-treated LDL. Plasma samples from hypercholesterolaemic rabbits and from several human subjects have been found to contain antibodies that will react with peroxidized LDL, suggesting that peroxidation does indeed take place *in vivo* (Palinski *et al.* 1989; Haberland *et al.* 1988; Mowri *et al.* 1988). The action of probucol in inhibiting atherogenesis in hyperlipidaemic rabbits (Carew *et al.* 1987) is also consistent with the role of peroxidation in lesion development: trials with this and other chain-breaking antioxidants are now being performed in several centres. Previous studies on animals supplemented with α-tocopherol have given equivocal results (Wilson 1976). A relation between lipid peroxidation and atherosclerosis would provide an explanation for the observation that diabetic patients, who are often under increased oxidative stress, show enhanced atherogenesis (Wolff & Dean 1987).

Is there more plasma lipid peroxide in patients with atherosclerosis?

Studies on ceroid and experiments with monoclonal antibodies (see above) provide strong evidence that lipid peroxidation is taking place within the atherosclerotic lesion. However, is there more lipid peroxidation in the blood plasma in patients with advanced atherosclerosis? This question is relevant to suggestions that lipid hydroperoxides in plasma might cause endothelial damage (Yagi 1987).

Two problems arise. Firstly, storage or mis-handling of plasma samples can lead to peroxidation of lipoproteins and many previous studies have been led astray by this

artifact (e.g. see Schuh *et al.* 1978; Gutteridge *et al.* 1985). Thus *fresh* samples must always be used in assays. A second, and more serious, problem is that the two most popular assays used to date to measure lipid peroxidation in human body fluids, diene conjugation and the TBA test, are seriously flawed. Let us see why.

Diene conjugation

Oxidation of unsaturated fatty acid side-chains is accompanied by the formation of conjugated diene structures that absorb ultraviolet light in the wavelength range 230–235 nm (Fig. 1). Measurement of this u.v. absorbance is an extremely useful index of peroxidation in studies on pure lipids, but it often cannot be used directly upon biological materials because many of the other substances present, such as haem proteins, absorb strongly in the ultraviolet and create a high background. This can be overcome by extracting diene conjugates into organic solvents, although many such solvents have significant u.v. absorbance, thus reducing the accuracy of spectrophotometric measurements. Corongiu *et al.* (1986) have improved the sensitivity of the diene conjugation method by using second-derivative spectroscopy. In the second derivative ($d^2A/d\lambda^2$) spectrum, the 'shoulder' that appears in the ordinary (A/λ) spectrum translates into a sharp minimum 'peak' that is easily measurable and is a good index of the conjugated diene present. The increased resolution of second-derivative spectroscopy may allow discrimination between different conjugated diene structures present (Corongiu *et al.* 1986).

Although conjugated diene methods have often been successfully used to study peroxidation in animal body fluids and tissue extracts, their application to human body fluids has produced serious problems. Dormandy and Wickens (1987) used HPLC techniques to separate u.v.-absorbing 'diene conjugates' from human body fluids, and reported that the isolated material consists almost entirely of a non-oxygen-containing isomer of linoleic acid, octadeca-9 (*cis*), 11 (*trans*)-dienoic acid. They proposed that this compound is produced by reaction of the carbon-centred radicals, obtained when H is abstracted from linoleic acid, with protein. Such a reaction is certainly possible, but chemical studies show that reaction with oxygen is the preferred fate of carbon-centred radicals, except at very low O_2 concentrations. In any case, peroxidation of biological membranes produces carbon-centred radicals from several fatty acids (e.g. arachidonate), not only linoleic acid, and would not be expected to give only a 9 (*cis*), 11 (*trans*) isomer. Further, this u.v.-absorbing product is not found in the plasma of animals subjected to oxidant stress (e.g. rats given the hepatotoxin bromochloromethane, a potent inducer of lipid peroxidation). Thus Thompson and Smith (1985) and the authors (Halliwell & Gutteridge 1985b) have argued that octadeca-9,11-dienoic acid is unlikely to arise by lipid peroxidation. Instead it may be ingested in food and produced by the metabolism of gut bacteria (Thompson & Smith 1985; Fairbank *et al.* 1988).

The fact that the major u.v.-absorbing material in human body fluids does not appear to arise by lipid peroxidation means that application of any diene conjugation method to human body fluids, or to extracts of them, is probably not measuring lipid peroxidation, although diene conjugation methods still seem applicable to animal body fluids (Thompson & Smith 1985). Thus the authors cannot recommend use of the diene conjugation techniques upon human material.

The thiobarbituric acid (TBA) test

The TBA test is one of the oldest and most frequently used tests for measuring the peroxidation of fatty acids, membranes, and food products. It is the easiest method to use (the material under test is merely heated with thiobarbituric acid under acidic conditions, and the formation of a pink colour

measured at or close to 532 nm) and it can be applied to crude biological systems. Unfortunately, the simplicity of the test has led many scientists into using it as an index of peroxidation without understanding exactly what it measures, and it has been widely applied to measure 'lipid peroxides' in plasma or tissue samples from patients with several diseases (reviewed by Yagi 1987). Small amounts of free malondialdehyde (MDA) are formed during the peroxidation of most membrane systems. This MDA can react in the TBA test to generate a coloured product, an adduct of 2 moles of TBA with one of MDA. In acid solution, the product absorbs light (λ_{max} 532 nm) and fluoresces (λ_{max} 553 nm) and is readily extractable into organic solvents such as butan-1-ol (Gutteridge 1986a).

Because the TBA test is calibrated with MDA, the results are often expressed in terms of amount of MDA produced in a given time. This has sometimes given the impression that the TBA test detects only free MDA, and so measures the amount of free MDA in the peroxidizing lipid system. However, the amount of free MDA produced in most peroxidizing lipid systems is low and would be insufficient to give a significant yield in the TBA test. Indeed, it was shown many years ago that the bulk of the 'MDA' detected in the TBA test is not present in the sample being assayed, but instead forms by decomposition of lipid peroxides and further peroxidation during the acid-heating stage of the test itself (discussed in Gutteridge & Quinlan 1983; Gutteridge 1986a). Peroxide decomposition requires the presence of contaminating iron ions in the TBA reagents: this can lead to artifacts in studies of the action of metal-chelating agents on lipid peroxidation, which can also affect colour development in the TBA test itself (Gutteridge & Quinlan 1983). Some scientists add antioxidants, such as butylated hydroxytoluene, to the TBA test to minimize peroxidation during the test itself (reviewed by Buege & Aust 1978).

In addition to this problem, several compounds other than MDA give products that absorb at, or close to, 532 nm on heating with TBA. For example, bile pigments in plasma react with TBA to produce coloured products (Gutteridge & Tickner 1978a), urine contains several TBA-reactive substances (Gutteridge & Tickner 1978b) and various unsaturated aldehydes also react in the TBA test (Kosugi *et al.* 1987). Thus simple measurement at 532 nm after a TBA test could include contributions from these substances. Some distinction can be achieved by separating the $(TBA)_2$–MDA adduct from the reaction mixture before measurement. This is best done by modern HPLC or gas-chromatographic methods (e.g. Bird *et al.* 1983). Even so, it must be noted that exposure of several carbohydrates and amino acids to hydroxyl radicals, produced by ionizing radiation or metal-ion H_2O_2 systems, yields products that give a *genuine* $(TBA)_2$–MDA adduct on heating with TBA (e.g. Gutteridge 1981). The exact amount of colour developed with these alternative compounds depends on the type and strength of acid used in the TBA test, and on the time of heating. Application of the TBA assay to human body fluids will also measure endoperoxides produced enzymically by the prostaglandin synthesis pathway (Shimizu *et al.* 1981).

The lack of specificity of the TBA assay when applied to human body fluids, such as plasma, is clearly illustrated by the work of several groups. Marshall *et al.* (1985), using a specific enzyme method based on the ability of lipid peroxides to activate the enzyme cyclooxygenase, measured a mean peroxide content in human plasma of around 0.5 μM, whereas expression of the results from a TBA test in terms of 'peroxide equivalents' gave a mean value of 38 μM. Largilliere and Melancon (1988) found considerable TBA reactivity, but no free MDA, in human plasma. Thus the author does not recommend the TBA test for use to measure 'lipid peroxides' or 'aldehydes' in human biological material.

It should be noted that measurements of the peroxide content of human body fluids by assays that have greater specificity have not given consistent results to date. Thus the

cyclooxygenase-based assay found 0.5 μM 'total lipid peroxide' in human plasma (Marshall *et al.* 1985) whereas an HPLC-based separation followed by a microperoxidase-dependent determination found no peroxides at all in human plasma, even in patients with adult respiratory distress syndrome (upper limit 0.03 μM; Frei *et al.* 1988).

The results of the application of assays of greater specificity to samples from patients with atherosclerosis are awaited with interest. At the moment it may be stated that there is no good evidence for substantial amounts of circulating lipid hydroperoxides in human plasma in atherosclerosis or in any other disease state. This could be because lipid hydroperoxide is not formed, or because it is efficiently cleared from the circulation, e.g. by the liver (Steinberg *et al.* 1989). In any case, it follows that peroxidation *within the atherosclerotic lesion* may be playing a much more important role in atherosclerosis than is peroxidation in the 'bulk' plasma. Hence the idea that peroxidation of LDL occurs within the vessel wall seems very reasonable (Steinberg *et al.* 1989). LDL also contains chain-breaking antioxidants (Fig. 2); the dietary intake of these will presumably affect the resistance of LDL to peroxidation (Gey *et al.* 1987). Thus, for example, smokers may be more susceptible to atheroma development because the 'oxidant stress' created by smoking (Pryor 1987) leads to consumption of α-tocopherol and ascorbic acid, decreasing their concentrations in body fluids (Pacht *et al.* 1986).

The role of metal ions

Incubation of LDL with macrophages, monocytes, smooth muscle cells or endothelial cells has been reported to stimulate peroxidation (Henriksen *et al.* 1981; Quinn *et al.* 1987; Jurgens *et al.* 1987; Heinecke *et al.* 1986; Cathcart *et al.* 1989). In such studies traces of transition metal ions were added to the reaction mixture, or were probably present as contaminants in the reagents used. Indeed, merely incubating isolated LDL with copper ions can lead to peroxidation (Fig. 2). The cells probably act by providing reducing agents (e.g. $O_2^{\cdot-}$ in the case of monocytes and macrophages) to keep the metal ions in the reduced forms.

Studies upon the peroxidation of LDL *in vitro*, using systems with added or contaminating iron or copper ions, can be considered to be biologically relevant only if sources for such metal ions can be identified *in vivo*. Plasma iron in humans is largely bound to the iron-transport protein transferrin, with some in ferritin. Iron ions attached to ferritin or transferrin are not effective in catalysing lipid peroxidation or $\cdot OH$ formation (Aruoma & Halliwell 1987; Halliwell & Gutteridge 1986). The excess of transferrin over iron to be transported in normal human plasma means that the concentration of non-transferrin-bound low molecular-mass iron complexes is essentially zero (Gutteridge & Halliwell 1987). However, $O_2^{\cdot-}$ can mobilize iron ions from ferritin (Biemond *et al.* 1984) and both H_2O_2 and lipid hydroperoxides can cause release of iron ions from haemoglobin (Gutteridge 1986b). H_2O_2 also reacts with haemoglobin to form a 'reactive oxygen species', possibly ferrylhaemoglobin, that is not identical to $\cdot OH$ but may be able to initiate lipid peroxidation (Puppo & Halliwell 1988). Thus, iron ions to stimulate free radical reactions are *not* available in the bulk plasma. However, iron proteins trapped within an atherosclerotic lesion and subjected to attack by $O_2^{\cdot-}$ and H_2O_2 from monocytes and macrophages might release iron in forms that can stimulate lipid peroxidation and $\cdot OH$ formation.

Patients suffering from the inborn disease idiopathic haemochromatosis often present for treatment with extensive body iron overload, including the presence of high (up to 30 μM) concentrations of non-transferrin-bound iron in plasma (Gutteridge & Halliwell 1987; Aruoma *et al.* 1988). However, simple clinical observation suggests that these patients do not suffer any increased inci-

dence of atherosclerosis-related diseases such as strokes or myocardial infarction. This perhaps suggests that iron is not the metal that favours atherogenesis *in vivo*.

Copper ions are especially effective in promoting peroxidation of lipoproteins *in vitro* (Fig. 2; Jurgens *et al.* 1987), probably because of their ability to efficiently decompose traces of lipid hydroperoxides in lipoprotein preparations (equations 16–19). Thus, are copper ions available *in vivo*? According to the literature, 90–95% of plasma copper belongs to the protein caeruloplasmin, a physiological inhibitor of lipid peroxidation (Gutteridge & Stocks 1981). The rest is believed to consist of both albumin-bound copper ions and a low-molecular-mass copper pool in which copper ions are bound to such ligands as histidine or short peptides. The size of this pool is uncertain: application of a specific assay to detect non-caeruloplasmin bound copper ions (the 'phenanthroline assay') in freshly-prepared human plasma shows that the concentration of non-caeruloplasmin copper is much lower than has previously been supposed, and may be zero (Gutteridge 1984; Evans *et al.* 1989). However, storage of plasma, or subjecting it to column chromatography, appears to cause release of copper ions from caeruloplasmin (Gutteridge 1984) and the released copper ions can stimulate the peroxidation of plasma lipoproteins (Gutteridge *et al.* 1985).

Thus, copper ions to stimulate free radical reactions do not seem to be generally available in plasma. However, release of copper ions from caeruloplasmin, a labile protein, within the atherosclerotic lesion is one possibility. If transition metal ions largely act by decomposing traces of preformed hydroperoxide in LDL (equations 16, 17), then a source for these hydroperoxides must be identified. Initiation of peroxidation by $\cdot OH$, formed within the lesion by reaction of phagocyte-derived O_2^- and H_2O_2, could occur. Another possibility is that lipoxygenase enzymes introduce peroxides into LDL (Parthasarathy *et al.* 1989).

Conclusion

The suggestion that the free radical reaction of lipid peroxidation is important in the development of atherosclerosis (Steinberg *et al.* 1989) is a fascinating one and is rapidly leading to direct experimental tests of the protective action of such antioxidants as probucol (e.g. Carew *et al.* 1987). The results of these tests will tell us how important peroxidation really is. It seems clear, however, that events within the lesion itself are more important than events in the plasma, and it seems unlikely at the moment that plasma lipid peroxides act as initiators of atherosclerosis.

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