Tell me about free radicals, doctor: a review

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It is difficult these days to open a medical journal without seeing some paper on the role of 'oxidants', 'reactive oxygen species' or 'free radicals' in human disease. A recent review lists the diseases in which some involvement of these species has been claimed[1]. What are these 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? Before attempting to answer these questions, it is wise to establish some basic definitions.

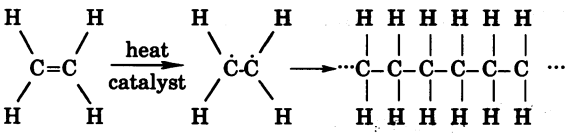
What is a free radical?

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. An electron occupying an orbital by itself can choose its direction 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. Because electrons are more stable when paired together in orbitals, radicals are on the whole more reactive than non-radical species, although they have a wide range of reactivity, as we shall see. Radicals can react with other molecules in a number of ways. 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)

$$A\cdot + A\cdot \rightarrow A\text{-}A$$

A radical might donate its unpaired electron to another molecule, or it might steal an electron from another molecule in order to pair. However, if a radical gives one electron to, or takes one electron from, another molecule, that other molecule itself 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 one, 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[2]:



ethene, precursor of polythene — a covalent bond breaks, leaving one unpaired electron on each carbon — The radicals join up to form a long chain of polythene

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[3].

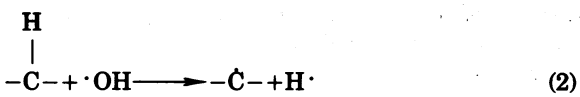
Hydroxyl radical

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, say, 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, so creating two radicals

$$H\text{-}O\text{-}H \xrightarrow{\text{radiation}} \text{intermediate stages} \longrightarrow H\cdot + \cdot OH \quad (1)$$

H$\cdot$ is a *hydrogen radical*, $\cdot$OH is a *hydroxyl radical*. The latter species is the most reactive radical species known to chemistry: it can attack and damage almost every molecule found in living cells. Since it is so reactive, $\cdot$OH does not stay around 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 $\cdot$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. Imperfect repair of such damage can lead to oncogene activation and carcinogenesis: hence high-energy radiation can lead to cancer[4]. Hydroxyl radical generation is, paradoxically, the major mechanism by which malignant cells are killed during radiotherapy.

Perhaps the best-characterized biological damage caused by $\cdot$OH is its ability to stimulate the free radical chain reaction known as *lipid peroxidation*. This occurs when the $\cdot$OH is generated close to membranes, and attacks the fatty acid side chains of the membrane phospholipids. It prefers to attack fatty acid side-chains with several double bonds, such as those of arachidonic acid. The $\cdot$OH pulls off an atom of hydrogen from one of the carbon atoms in the side chain, and combines with it to form water



$$-\overset{H}{\underset{|}{C}}- + \cdot OH \longrightarrow -\dot{C}- + H\cdot \quad (2)$$

$$H\cdot + \cdot OH \longrightarrow H_2O \quad (3)$$

Reactions 2 and 3 remove the $\cdot$OH, but leave behind a *carbon-centred radical* in the membrane. Under

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physiological conditions, this is most likely to combine with oxygen, creating yet another radical, the *peroxyl radical*

$$-\dot{C}- + O_2 \longrightarrow -\overset{\cdot O_2}{\underset{|}{C}}- \quad \text{(4)}$$

peroxyl radical

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

$$-\overset{\cdot O_2}{\underset{|}{C}}- + -\overset{H}{\underset{|}{C}}- \longrightarrow -\overset{O_2H}{\underset{|}{C}}- + -\dot{C}- \quad \text{(5)}$$

lipid hydroperoxide

Another carbon-centred radical is generated, and so the chain reaction (equations 4 and 5) can continue. Hence one ·OH can result in conversion of many hundred fatty acid side-chains into *lipid hydroperoxides*. Accumulation of lipid hydroperoxides in a membrane disrupts its functioning 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[5].

Toxicologists have also been interested in free radical reactions. For example, repeated exposure to halothane can damage the liver. Halothane is metabolized in the liver to produce free radicals that can attack membranes, leading to lipid peroxidation[6]. They can also attack proteins[6]. Free-radical-modified proteins have altered antigenicity and can provoke immune responses.

Oxygen radicals

Biochemists (apart from those with a special interest in ionizing radiation) only became interested in radicals 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*[7]. It removes superoxide radical, a species that is made by adding an extra electron onto the oxygen molecule.

$$O_2 + e^- \longrightarrow O_2^{\cdot -} \quad \text{(6)}$$

superoxide radical

Superoxide dismutase, known as SOD to the people who work with it, removes $O_2^{\cdot -}$ by catalyzing the reaction

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(7)}$$

Its discovery led to the realization that $O_2^{\cdot -}$ is formed in vivo in living organisms, and SOD functions to remove it. Some of the $O_2^{\cdot -}$ formation 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 $O_2^{\cdot -}$. Many molecules oxidize on contact with oxygen, eg 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 molecule onto O_2, giving $O_2^{\cdot -}$. Such oxidations undoubtedly proceed in vivo as well[8].

Oxidants and phagocyte action

Some of the $O_2^{\cdot -}$ production in vivo is, however, deliberate. Thus activated phagocytic cells generate $O_2^{\cdot -}$ and use it to kill some of the bacterial strains that they can engulf[9]. The importance of this killing mechanism can be illustrated in patients with *chronic granulomatous disease*, an inborn condition in which the enzyme system in phagocytes that makes the $O_2^{\cdot -}$ does not work. 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 *Staph. aureus*[9]. Another killing mechanism used by neutrophils is an enzyme known as *myeloperoxidase*. It oxidizes chloride ions into *hypochlorous acid*, HOCl, a powerful antibacterial agent (Domestos® is a solution of the sodium salt of hypochlorous acid). Effective functioning of the myeloperoxidase system[10] also involves $O_2^{\cdot -}$.

Superoxide and the endothelium

Another example of a useful role for $O_2^{\cdot -}$ may be 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 the compound nitric oxide, NO[11]. The endothelium also produces small amounts of $O_2^{\cdot -}$, which can react with NO and inactivate it. Both NO and $O_2^{\cdot -}$ are free radicals (each with one unpaired electron) and so they combine to give a non-radical product

$$NO + O_2^{\cdot -} \longrightarrow \text{intermediates} \longrightarrow NO_3^- \quad \text{(8)}$$

nitrate ion (non-radical)

Hence controlled production of NO and $O_2^{\cdot -}$ by endothelium may provide one mechanism for regulation of vascular tone[11,12].

Superoxide formed in vivo, either deliberately or accidentally, is disposed of by SOD (eqn 7). Indeed, this enzyme seems essential to aerobic life, ie the removal of $O_2^{\cdot -}$ 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[13]. It is interesting to note that no human complete inborn deficiencies of SOD have been reported, perhaps because they would be lethal mutations.

Reactive oxygen species

SOD removes $O_2^{\cdot -}$ by converting it into hydrogen peroxide (H_2O_2) and oxygen (eqn 7). 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. At least some of this damage seems to be mediated by a reaction of H_2O_2 with $O_2^{\cdot -}$ in the presence of iron or copper ions[1,8], to form several highly-reactive radicals, one of which is ·OH (eqn 10). We have already seen that ·OH can attack DNA, membrane lipids and proteins.

$$O_2^{\cdot -} + H_2O_2 \xrightarrow[\text{ions}]{\text{iron or copper}} \cdot OH + OH^- + O_2 \quad \text{(9)}$$

hydroxyl radical

Thus removal of H_2O_2, as well as of $O_2^{\cdot -}$, is advantageous.

The SOD therefore works in conjunction with two enzymes that remove H_2O_2 in human cells: catalase and glutathione peroxidase. The study of inborn errors of metabolism shows that the latter 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[14]. It is unlikely, however, that the sole function of selenium in humans is to act as a cofactor for glutathione peroxidase[14]. Glutathione peroxidase removes H_2O_2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \quad (10)$$

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 $O_2^{\cdot -}$ and $\cdot OH$ (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, $O_2^{\cdot -}$, $\cdot OH$ and HOCl are sometimes also collectively called 'oxidants'.

The role of transition metals

Many transition metals have variable valencies, eg iron as Fe^{2+} or Fe^{3+} ions and copper as Cu^+ or Cu^{2+}. Changing between valency states involves accepting or donating single electrons

$$Fe^{3+} + e^- \rightleftharpoons Fe^{2+} \quad (11)$$
$$Cu^{2+} + e^- \rightleftharpoons Cu^+ \quad (12)$$

Thus transition metal ions are remarkably good promoters of free radical reactions. Polymer scientists[2,3] and food chemists have known this for years, and biochemists have recently realized that metal ions can act in this way in vivo[1,8,15]. Thus iron and copper ions can convert $O_2^{\cdot -}$ and H_2O_2 into highly-damaging $\cdot OH$ (eqn 10). Metal ions can also accelerate lipid peroxidation by decomposing lipid hydroperoxides into new radical species

$$2\ -\overset{O_2H}{\underset{|}{C}}- \xrightarrow{\text{iron, copper ions}} -\overset{O\cdot}{\underset{|}{C}}- \text{ (alkoxyl radical)} + -\overset{O_2\cdot}{\underset{|}{C}}- \text{ (peroxyl radical)} + 2H^+ \quad (13)$$

Both peroxyl (eqn 5) and alkoxyl radicals can then further propagate the chain reaction of lipid peroxidation by abstracting hydrogen atoms from adjacent fatty acid side chains.

Atherosclerosis

As an example, both iron and copper ions are remarkably effective in accelerating the peroxidation of phospholipids in plasma low density lipoproteins (LDL). Peroxidized LDL molecules can be taken up by macrophages, which become converted into foam cells, many of which are found in atherosclerotic plaques. Thus there is considerable interest in the role of lipid peroxidation and metal ions in the development of atherosclerosis, a major clinical problem[16].

Antioxidant defence and damage repair

Organisms use SOD, catalase and glutathione peroxidase to protect themselves against generation of reactive oxygen species. They also take great care to keep as much iron and copper ion 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 is essentially no free iron ion in plasma. Iron bound to such proteins as transferrin cannot stimulate lipid peroxidation or $\cdot OH$ generation: the same is true of copper bound to the plasma proteins caeruloplasmin or albumin[15,19]. The value of this sequestration is shown by an inspection of the pathology suffered by patients with iron overload disease, in which free iron ions do circulate in the blood[17,18]. The patients can suffer liver damage, diabetes, joint inflammation and hepatoma, among other problems[17].

α-Tocopherol

As well as these primary defences, there are secondary defences. Thus cell membranes, and LDL circulating in the plasma, contain α-tocopherol, which functions as a chain-breaking antioxidant. It is a lipid-soluble molecule that sits in the interior of the biological membrane or LDL particle. 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 (eqns 4 and 13), they combine preferentially with the antioxidant, eg

$$\text{Tocopherol}-OH + -\overset{O_2\cdot}{\underset{|}{C}}- \longrightarrow \text{Tocopherol-}O\cdot + -\overset{O_2H}{\underset{|}{C}}- \quad (14)$$

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-$O\cdot$. This radical is poorly reactive, being unable to attack adjacent fatty acid side-chains, so the chain reaction is stopped. Evidence exists that the tocopherol radical can migrate to the membrane surface and be converted back to α-tocopherol by reaction with ascorbic acid (vitamin C)[23]. Thus both vitamin C and α-tocopherol seem to contribute to minimizing the consequences of lipid peroxidation in LDL and in membranes, should this process begin. The terms α-tocopherol and vitamin E are often used synonomously. This is not quite correct: vitamin E is defined nutritionally as a factor needed in the diet of pregnant female rats to prevent reabsorption of the fetus[23], and compounds other than α-tocopherol (eg β-, γ- and δ-tocopherols) have some effect in this assay. However, α-tocopherol is the most effective and is by far the most important lipid-soluble chain-breaking antioxidant in vivo in humans. 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[20]. 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[21]. Interest in the role of

lipid peroxidation in atherogenesis was raised when it was discovered that a synthetic chain-breaking antioxidant, probucol, had a marked antiatherogenic effect in hyperlipidaemic rabbits[22].

Other antioxidants

It is not only biochemists and polymer scientists[2] who are interested in chain-breaking antioxidants. Food technologists have developed many such compounds (eg BHA, BHT, propyl gallate) to inhibit peroxidation of food lipids on storage: peroxidized lipids and their aldehyde decomposition products smell and taste unpleasant ('rancidity' or 'off flavour'). These and other inhibitors of free radical reactions will become increasingly important in the future with the growing vogue for sterilizing food by the use of ionizing radiation[25].

Some other compounds may also function as antioxidants in vivo. Recent suggestions have included uric acid, β-carotene 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 systems exist within cells that can repair DNA that has been attacked by radicals[4], degrade proteins damaged by radicals[24] and metabolize lipid hydroperoxides[8].

Free radicals and human disease: causation or consequence?

Does increased formation of free radicals and other reactive oxidizing 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[14] (Keshan disease) or of vitamin E (neurological disorders seen in patients with inborn errors in the mechanism of intestinal fat absorption[23,26]) could also be mediated by oxidants. In the premature infant, exposure of the incompletely-vascularized retina to elevated concentrations of oxygen can lead to retinopathy of prematurity, which in its worst forms can result in blindness. Several controlled clinical trials have documented the efficiency of α-tocopherol in minimizing the severity of the retinopathy[27], suggesting a possible role for lipid peroxidation. The precise relation of LDL peroxidation to the initiation and progression of atherosclerosis is uncertain, but it does seem to play an important role[16,20-22].

For most human diseases however, the oxidant formation is secondary to the primary disease process. For example, activated neutrophils produce O_2^-, H_2O_2 and HOCl in order to kill bacteria. However, if a large number of phagocytes is 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 minor contribution to joint damage remains to be established[28]. 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 release prostaglandins, leukotrienes, proteolytic enzymes such as elastase and reactive oxygen species[29]. 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, preventing it 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/reperfusion injury

One of the most exciting research areas in recent years has been that of ischaemia/reperfusion injury. 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 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.

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. Hence restoration of O_2, although obviously beneficial overall, causes increased oxidant formation in the damaged tissue and temporarily worsens the injury[30]. Thus inclusion of antioxidants when blood flow is restored offers protective effects: antioxidants used include SOD and desferrioxamine, a chelator that binds iron ions and stops them accelerating free radical reactions[19]. Clinical trials are currently underway to assess the benefit of combining thrombolytic agents with recombinant human SOD in the treatment of acute myocardial infarction. The use of antioxidants in preservation of organs for transplantation is also receiving attention[30,31].

Why should ischaemia lead to more oxidant generation when tissues are reoxygenated? The answer is not yet clear: possibilities include release of iron ions from their normal storage sites within the cell[12,31], disruption of mitochondrial respiratory chains so that more electrons leak to oxygen to form $O_2^{\cdot -}$, and increases in the activities of certain enzymes, such as xanthine oxidase, that generate $O_2^{\cdot -}$ during their normal operation. Neutrophils infiltrating into a previously-ischaemic tissue might also become activated to release oxidants.

Traumatic injury

There are some other examples where tissue injury, by a non-radical mechanism, can lead to increased free radical reactions. Thus mechanical (eg crushing) or chemical injury to cells can cause them 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', apparently due to massive lysis of leukaemic cells. This increased iron availability could contribute to the side effects of cytotoxic chemotherapy[32]. 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[33] that injury to the brain by mechanical means (trauma) or O_2 deprivation (stroke) can result in release of iron ions into the surrounding area, which can facilitate further damage, to these surrounding areas, by accelerating free radical reactions. This proposal has been given considerable support in animal studies, using chelating agents that bind iron ions and prevent them from catalyzing radical reactions. Promising results have been obtained with aminosteroid-based iron chelators. Thus one such chelator, U74006F, has been observed to minimize the effects of reperfusion injury upon the brain of cats[34], to decrease post-traumatic spinal cord degeneration in cats[35] and to minimize neurological damage after head injury in mice[36].

Free radicals and human disease: a triviality?

It seems likely that tissue destruction and degeneration results in increased oxidant damage, by such processes as metal ion release and disruption of mitochondrial electron transport chains, so that more electrons 'escape' to oxygen to form $O_2^{\cdot -}$. If so, it follows that almost any disease is likely to be accompanied by increased free radical formation. It is not therefore surprising that the list of diseases in which their formation has been implicated is long[1], and is growing longer. For atherosclerosis, 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[37], 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.

Conclusions

Oxidant generation is a part of normal human metabolism. When produced in excess, oxidants can cause tissue injury. However, tissue injury can itself cause more oxidant generation which may (or may not, depending on the situation) contribute to a worsening of the injury. Expanding free radical theories to an ever-increasing list of diseases is not the way forward. The careful use of a range of antioxidants, combined with new methods for measuring oxidant generation in humans[16,38], is needed to evaluate the exact contribution of oxidant generation to disease pathology.

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