Review

Free radicals and antioxidant strategies in sports

Okezie I. Aruoma

The Pharmacology Group, University of London King's College, London, UK

Reactive oxygen species are continuously produced in the human body. Some of these have beneficial effects but when produced in excess, beyond the antioxidant capacity of the body, they can cause tissue damage. Exercise causes an increased oxygen consumption in vivo; however, a small part of the oxygen consumed leads to formation of reactive oxygen species. This review discusses the possibility that use of a range of antioxidants combined with new methods for measuring oxidant generation would help to delineate the contribution of nutrients to the modulation of the consequences of free radicals in the human body during exercise. (J. Nutr. Biochem. 5:370–381, 1994.)

Keywords: free radicals; antioxidants; exercise; biomarkers; antioxidant supplementation

Introduction

A free radical may be defined as any species capable of independent existence and possessing one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. The conventional radical dot (·) designates the presence of one or more of the unpaired electrons. Examples of free radicals are hydroxyl ($OH^{\cdot}$), superoxide ($O_2^{\cdot -}$, an oxygen-centered radical), peroxyl ($RO_2^{\cdot}$, radical intermediates arising as a result of lipid oxidation), trichloromethyl ($^{\cdot}CCl_3$, a carbon-centered radical), and nitric oxide ($NO^{\cdot}$). Examples of nonradical oxygen-derived species are hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and ozone (O_3). Radicals can react with other molecules in many ways.[1] The net effect is that the radical donates its unpaired electron to another molecule and this molecule then becomes a radical. Free radicals and other reactive oxygen species are constantly formed in the human body.[2–7] It is worthwhile commenting briefly on some of the species.

Nitric oxide

NO is a free radical. It is widely thought that the endothelium derived-relaxing factor (EDRF) produced by vascular endothelium, which is an important mediator of vascular responses induced by several pharmacological agents (including bradykinin and acetylcholine) is identical to NO.[8,9] Vascular endothelial cells that line the blood vessels also seem to produce small amounts of $O_2^{\cdot -}$, some of which could react with NO, producing nonradical products. Thus, variation in the production of NO and $O_2^{\cdot -}$ by endothelium might provide one mechanism for the regulation of vascular tone and hence blood pressure.[10] Nitric oxide is synthesized by the oxidation of a guanidino nitrogen atom of L-arginine by the enzyme NO synthase.[9,11]

$NO^{\cdot}$ reacts at an almost diffusion-controlled rate (6.7 ± 0.9) $\times$ $10^9 M^{-1}s^{-1}$) with $O_2^{\cdot -}$ to give peroxynitrite.[12] The reaction of NO with $O_2^{\cdot -}$ has been argued to have a cytoprotective function.[13] However, the peroxynitrite may decompose at physiological pH to give $OH^{\cdot}$,[14] and/or nitronium ion (NO_2^+), a powerful nitrating agent that attacks aromatic ring structures. The view is growing that peroxynitrite and toxic agents derived from it may mediate some of the deleterious effects of $NO^{\cdot}$. For example, modification of low-density lipoprotein,[15] oxidation of sulfhydryl groups[16] and depletion of antioxidant status of plasma[17] are mediated.

It has recently been shown that the decrease in oxygen concentration as a result of superoxide generation was responsible for the inhibition of bovine cerebellum NO synthase activity.[18] Van der Vliet et al.[19] have shown that the decomposition product of peroxynitrite could form hydroxylated and nitro-aromatic compounds, suggesting $OH^{\cdot}$ formation. Sessa et al.[20] recently demonstrated that chronic exercise (in dogs) increases EDRF/NO production and the endothelial cell NO synthase gene expression and suggests that this may contribute to the beneficial effects of exercise on the cardiovascular system.

Superoxide radical

The discovery by McCord and Fridovich of the enzyme superoxide dismutase (SOD), which accelerates the dismuta-

Address reprint requests to Dr. Aruoma at The Pharmacology Group, University of London King's College, Manresa Road, London, SW3 6LX, UK.
Received November 30, 1993; accepted March 18, 1994.

© 1994 Butterworth–Heinemann

tion of $O_2^{\cdot -}$ and converts it to hydrogen peroxide (H_2O_2) and oxygen (O_2), has been a pivotal catalyst for the rapid growth of interest in free radical research relating to human diseases. When an O_2 molecule accepts a single electron, it forms the superoxide radical $O_2^{\cdot -}$ (this species has one unpaired electron). $O_2^{\cdot -}$ is formed in vivo in a variety of ways. A major source is the activity of electron transport chains in mitochondria and endoplasmic reticulum. Some of the electrons passing through these chains "leak" directly from intermediate electron carriers onto O_2. Because O_2 accepts electrons one at a time, $O_2^{\cdot -}$ is formed. The rate of leakage at physiological O_2 concentrations is probably <5% of total electron flow through the chains, but it rises as O_2 concentration is increased.[21,22] Hence, the toxicity of excess O_2 may be due to increased formation of $O_2^{\cdot -}$ (e.g., by faster electron leakage and accelerated autoxidations of such molecules as catecholamines) beyond the ability of antioxidant defenses to cope. Activated phagocytic cells produce $O_2^{\cdot -}$, which plays an important part in the mechanism by which engulfed bacteria are killed.[23] Phagocytes able to produce $O_2^{\cdot -}$ include monocytes, neutrophils, eosinophils, and macrophages of various types. Excessive activation of phagocytic cells (as in chronic inflammation) can lead to free radical damage.[2,24,25] Several cell types such as fibroblasts, lymphocytes, and the vascular endothelial cells have been reported to produce and release small amounts of $O_2^{\cdot -}$.[26,27] Although the number of targets within mammalian cells that are known to be sensitive to $O_2^{\cdot -}$ is small, the evidence supporting the superoxide theory of oxygen toxicity abounds. The $O_2^{\cdot -}$ itself has limited reactivity.[2,21,22,28,29]

Peroxyl radicals

These are found during lipid oxidation chain reactions, such as the oxidation of polyunsaturated fats resulting in deterioration of lipid-containing foods.[30,31] Lipid peroxidation may be initiated by any species that has sufficient reactivity to abstract a hydrogen atom from a polyunsaturated fatty acid side chain (such as those of arachidonic acid and linolenic acid, for example) in membrane lipids. Arachidonic acid is a precursor of prostaglandins and leukotrienes. It contains a number of methylene-interrupted double bonds that are particularly prone to hydrogen atom abstraction.[31] Although much has been written about the mechanism and biological significance of lipid peroxidation, there seems no unanimity in the methods for its measurement. Delineation of lipid peroxidation as a major pathway in most degenerative diseases depends on adequate standardization and control of measurement conditions.[31–34] The link between DNA damage, faulty repair of DNA, protooncogene activation, and the ability of some of the end products of lipid peroxidation to act as promoters of carcinogenesis is of considerable interest.[32,35,36]

Hydrogen peroxide

H_2O_2 can act as an oxidizing agent, although it is poorly reactive. Unlike $O_2^{\cdot -}$, however, H_2O_2 crosses cell membranes easily.[29] H_2O_2 does not qualify as a radical because it contains no unpaired electrons, and it can be removed within human cells by the action of two types of enzyme, catalases and selenium-dependent glutathione peroxidases *(Figure 1)*. Several enzymes, including glucose oxidase, D-amino acid oxidase, glycollate oxidase, and monoamine oxidase, can produce H_2O_2. H_2O_2 generates hydroxyl radicals in the presence of transition metal ions.

Oxidative deamination of dopamine by monoamine oxidase is the main catabolic pathway for dopamine within dopamine nerve terminals. Cohen[37] suggested that an accelerated turnover of dopamine in patients with Parkinson's disease, leading to increased H_2O_2 formation, may provoke an oxidative stress (i.e., an increase in the generation of oxygen-derived species beyond the ability of antioxidant defenses to cope with them) within surviving dopamine terminals, thus accelerating their destruction.

Hydroxyl radical

This is a very reactive radical with an estimated half life in cells of only 10^{-9} seconds. Most studies in free radical chemistry prior to the discovery of SOD were done by radiation chemists.[38] One feature of the hydroxyl radical is that it begets another radical, i.e., when it reacts with a molecule, the result is the formation of another radical species. The resulting species usually has lower reactivity than the $OH^{\cdot}$. Hydroxyl radical attacks all proteins, DNA, polyunsaturated fatty acids in membranes, and almost any biological molecule it touches.

The interrelationship between reactive oxygen species (ROS) and antioxidants is shown in *Figure 1*. The protective mechanisms (see also *Table 5*) do not act independently of one another but rather, they tend to function co-operatively in the form of a cascade. The scavenging enzymes and antioxidants can inhibit free radical production by: (a) chelating the transition metal catalysts, e.g. transferrin, (b) breaking chain reactions, e.g. alpha tocopherol, (c) reducing concentrations of ROS, e.g. glutathione and (d) scavenging initiating radicals, e.g. superoxide dismutase. Cytoprotective enzymes are located within both the hydrophillic and hydrophobic compartments of the cell, while antioxidants are both intra- and extra-cellular. The tissue damage alluded to arises as a consequence of oxidative stress. Oxidative stress may be mediated by (1) increased activity of the radical generating enzymes (e.g., xanthine oxidase) and/or their substrates (e.g., hypo-xanthine); (2) activation of phagocytes; (3) activation of phospholipases, cyclooxygenases and lipoxygenases; (4) dilution and destruction of antioxidants; (5) release of "free" metal ions from sequestered sites and/or muscle; (6) release of heme proteins (hemoglobin, myoglobin); and (7) disruption of electron transport chains and increased electron leakage for $O_2^{\cdot -}$.

These activities could be amplified by such factors as heat, trauma, ultrasound, environmental pollution, radiation, hyperoxia, toxins, and exercise to excess.

Sports and free radicals

ROS are implicated in the molecular and tissue damage arising from increased oxidative metabolism associated with strenuous exercise.[39–58]

Exercise causes an increased oxygen (O_2) consumption in vivo. O_2 is a toxic gas. Humans and other aerobes can

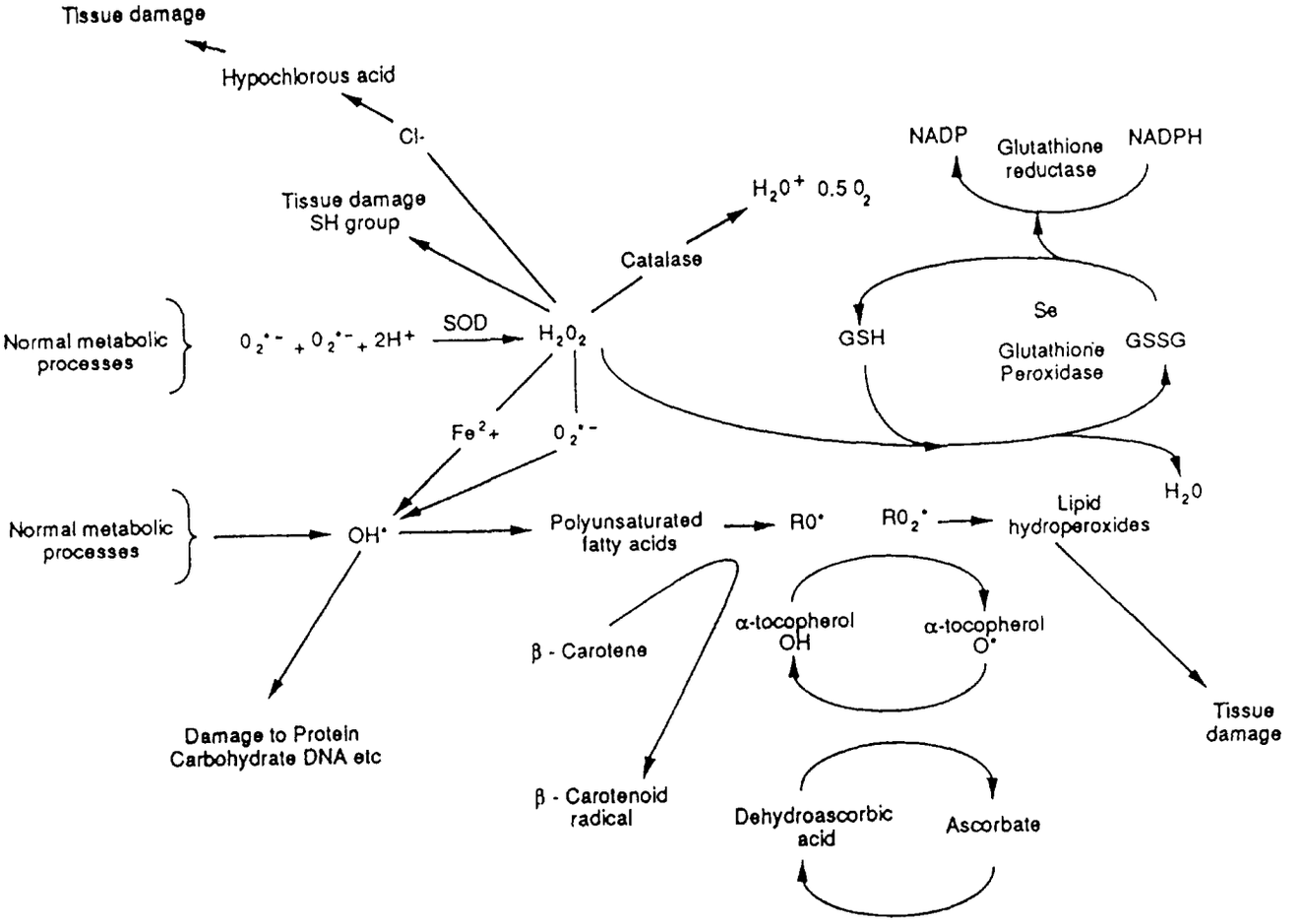

Figure 1 The interrelationship between antioxidants (courtesy of Dr. R.J. Ward).

tolerate it because antioxidant defenses to protect against the toxic effects of O_2 evolved in line with the evolution of the electron transport chains. A small part of the oxygen consumed by the human body leads to the formation of reactive oxygen species.

Iron and copper in free radical reactions

It is generally accepted that much of the toxicity of $O_2^{\cdot -}$ and H_2O_2 in living organisms is due to their conversion into $OH^{\cdot}$ and into reactive radical metal ion complexes. These processes are often referred to as either the iron catalyzed Haber-Weiss reaction (Equation 1) or the superoxide-driven Fenton reaction (Equation 2).[2,17,59–61]

Haber-Weiss:

$$H_2O_2 + O_2^{\cdot -} \xrightarrow[\text{catalyst}]{\text{Fe/Cu}} O_2 + OH^{\cdot} + OH^- \quad (1)$$

Fenton:

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate} \rightarrow Fe^{3+} + OH^{\cdot} + OH^- \quad (2)$$

Pure $O_2^{\cdot -}$ and H_2O_2 are poorly reactive in aqueous solutions. They do not oxidize membrane lipids or degrade DNA.[62] On production in vivo, $OH^{\cdot}$ reacts at its site of formation. Thus, in the case of $OH^{\cdot}$ generation by Fenton-type chemistry, the extent of $OH^{\cdot}$ formation is largely determined by the availability and location of the metal ion catalyst. Iron salts have been widely studied as catalysts of free radical reactions in vivo but copper ions may also be important. When the roles of copper ions and iron ions in causing damage to DNA in systems containing H_2O_2[63] are compared, added copper ions were significantly more reactive in causing DNA damage. This catalytic ability suggests that the availability of "free" iron and copper ions in the human body should be carefully controlled.

One of the potentially devastating effects of $OH^{\cdot}$ is its action on membrane lipids.[31,32] *Figure 2* shows the mechanism of a typical peroxidation reaction occurring with arachidonic acid in a lipid membrane. Various species that are generated on mixing $O_2^{\cdot -}$ and H_2O_2 and iron or copper ions are capable of initiating the process of lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid side chain (those with two or more carbon double bonds) in a membrane lipid *(Figure 2)*. Abstraction of a hydrogen atom leaves behind a carbon-centered radical ($L^{\cdot}$) in the membrane (Equation 3).

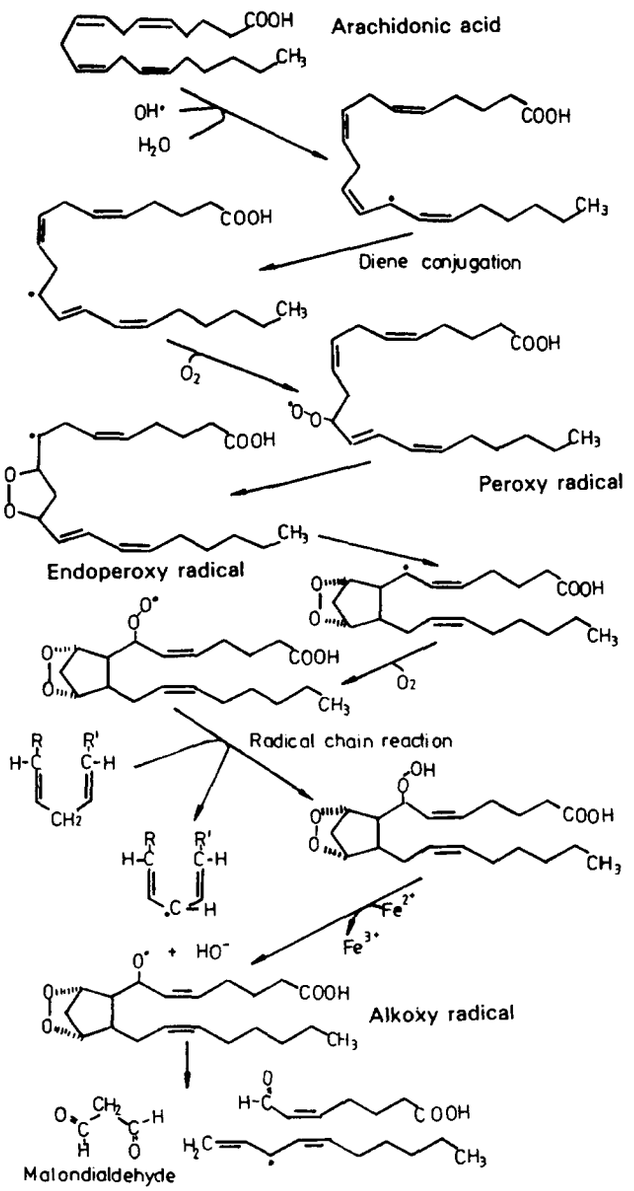

Figure 2 Initiation and propagation of lipid peroxidation of arachidonic acid resulting in the formation of malondialdehyde. The reaction sequence shown is highly hypothetical.[31]

$$\text{L-H} + \text{radical} \rightarrow \text{L}^{\cdot} + \text{radical-H} \quad (3)$$

$$\text{L}^{\cdot} + \text{O}_2 \rightarrow \text{LO}_2^{\cdot} \quad (4)$$

$$\text{LO}_2^{\cdot} + \text{LH} \rightarrow \text{LO}_2\text{H} + \text{L}^{\cdot} \quad (5)$$

The most likely fate of carbon-centered radicals in vivo is reaction with O_2 to form peroxyl radicals (often shortened to "peroxy" radicals).

Peroxyl radicals can attack membrane proteins (damaging receptors and enzymes) and can also abstract hydrogen atoms from adjacent fatty acid side chains.

Thus, abstraction of a single hydrogen can set off a free radical chain reaction (Equations 4 and 5) that leads to conversion of many membrane lipids into lipid hydroperoxides (lipid-O_2H). The existence of lipid peroxides within a membrane severely disrupts its functioning, altering (usually decreasing) fluidity and allowing ions such as Ca^{2+} to leak across the membrane. This is in addition to the damage produced by the attack of peroxyl radicals on membrane proteins.

Iron and copper ions can contribute to lipid peroxidation in two ways. First, they catalyze formation of initiating (H-abstracting) species. Second, they stimulate peroxidation by reacting with lipid hydroperoxides and decomposing them to peroxyl radicals and alkoxyl radicals (lipid-O·), which can abstract H and lead to further peroxidation.[2,31] Products of these complex decomposition reactions include hydrocarbon gases and a wide range of toxic carbonyl compounds, including aldehydes. Of these aldehydes, much attention in the literature is usually devoted to malonaldehyde (sometimes called malondialdehyde or MDA), but this is much less noxious than such highly cytotoxic unsaturated aldehydes as 4-hydroxy-2,3-*trans*-nonenal.[64] Proteins essential for membrane function can thus be damaged not only by attack of peroxyl radicals, but also by covalent modification by aldehyde end products of peroxidation.

The nature of the damage done to cells by excess formation of H_2O_2 and $O_2^{\cdot -}$ will be affected by the location of metal ion catalysts of reaction within the cells. It also follows that if no catalytic metal ions are available $O_2^{\cdot -}$ and H_2O_2 will have limited, if any, damaging effects. These fundamental principles underlie the importance of examining the availability and distribution of "catalytic" metal ions in explaining oxidative damage to cells.

Metal-ion metabolism during human exercise

The condition known as "sports anemia," which is characterized by sub-normal hemoglobin and hematocrit values, has been reported to occur during hard physical training.[65–70] The prevalence of anemia in athletes has been argued.[68,71–76] It would seem that physiological processes that could cause an imbalance in the distribution of iron and/or copper might be of some significance in oxidative damage.

The average human body contains much less copper (0.08 g) than iron (4.5 g). Iron ions are absorbed from the gut and transported to iron-requiring cells by the protein transferrin. In contrast, most or all of the plasma copper in humans is attached to the protein ceruloplasmin. Iron specifically bound to transferrin will not participate in free radical reactions.[77] The protein ceruloplasmin similarly does not stimulate free radical reactions.[78] The consequences of uncontrolled iron availability can be seen in "iron-overload" diseases, such as idiopathic hemochromatosis. In this disease, there is a progressive increase in total body iron stores of iron in the parenchymal cells of the liver.[79] Iron metabolism influences the pathology of a number of tropical diseases such as thalassaemia, sickle cell disease, malaria, and Leishmaniasis and trypanosomiasis.[80] Hard training athletes and endurance performers have greatly accelerated sweat loss and sometimes, especially for women, develop iron-deficiency anemia. By comparing the plasma levels of zinc, iron, and copper before and after a period of controlled exercise with losses of the metals in sweat, it may be possible to gain an insight into the significance of metal metabolism

in exercise.[72,76,81-87] The amounts of iron lost during exercise varies between different parts of the human anatomy *(Table 1)*. Appreciable amounts of copper are also lost via the sweat route. Given the relatively low pool of copper in vivo, these losses are biologically significant. It can be seen that there is a tendency for losses of copper in sweat from the abdomen, arm, chest, and back to be greater than losses of iron, although there is considerable variation between subjects. These variabilities between subjects are also reflected in *Table 2*. The variability, even between male subjects of similar age and body mass in such parameters as sweat rate, plasma volume changes, and changes in the metal contents of plasma suggests that "real sports" anemia may be a feature only of certain individuals.[81] Gender differences in amounts of iron lost via sweat have been reported.[84] If the loss of iron in sweat were a significant contributor to sports anemia, then depletion of copper would also be expected because the body pool of copper is less than that of iron.

Sports anemia has recently been described as a misnomer[75] on the grounds that the low hemoglobin concentration in an endurance athlete is a false anemia. The increase in the baseline plasma volume was suggested to be an adaptation to acute loss in plasma volume that accompanies a period of exhaustive exercise. "Pseudoanemia" was advocated by Weight et al.[73] after they investigated the red cell indices and iron status of male distance runners and ballet dancers compared with gender matched non-exercising controls. Hallberg and Magnusson[88] advocated that sports anemia "was a physiological response of the regulatory system controlling hemoglobin concentration in the blood, reacting to unphysiologically heavy and prolonged exercise load."

In normal cells, antioxidant defenses are afforded by the enzymes superoxide dismutase, catalase, and glutathione peroxidase and its substrate glutathione (GSH) (*Figure 1* for the interrelationship). When these cells are damaged (as may be the case in foot-strike hemolysis),[74] the antioxidant capacity of the cells is lost. Haem may also be degraded releasing iron which is a prooxidant. Biological systems must sequester iron to prevent it from circulating around in free form. In vivo, iron is normally bound to transport and storage proteins, transferrin, lactoferrin, ferritin, and hemosiderin. At pH 7.4, apotransferrin and apolactoferrin are able to bind iron ions and protect against $OH^{\cdot}$ radical generation promoted by iron added as $FeCl_3$ or by iron released from ferritin in the presence of ascorbate or a superoxide radical generating system, supporting the proposal that under normal conditions, iron binding proteins function as antioxidants in vivo.[2,77]

Table 1 Sweat metal content as a function of body site

Site studied	μmol/L (mean ± SD) of Fe	μmol/L (mean ± SD) of Cu
Abdomen	8.8 ± 7.0 (14)*	14.0 ± 9.9 (8)
Arm	5.0 ± 2.5 (10)	8.2 ± 7.5 (4)
Chest	8.9 ± 10.8 (15)	11.5 ± 11.6 (8)
Back	3.6 ± 3.2 (19)	8.8 ± 6.8 (14)

*Numbers of samples analysed are given in parentheses. Exercise lasting 30 or 40 minutes was performed on a cycle ergometer by healthy male athletes. The levels of Fe and Cu were measured as described in Aruoma *et al.*[81]

Table 2 Plasma metal concentration before and after exercise

Subject	Copper (μmol/L) Before	Copper (μmol/L) After	Iron (μmol/L) Before	Iron (μmol/L) After
A	18.0	16.0	21.0	17.0
B	19.0	16.0	14.8	9.0
C	18.0	23.0	14.8	16.0
D	27.0	22.0	21.5	14.0
E	20.5	23.0	21.5	19.8
F	24.0	17.0	17.5	12.5
G	22.0	20.5	21.4	14.5
H	20.0	17.0	16.6	22.0
I	16.0	16.0	10.5	11.8
J	23.0	19.0	19.0	13.7
K	27.0	24.0	8.9	11.3
L	20.0	20.0	8.9	13.6
Mean ± SD of results	21.2 ± 3.5	19.5 ± 3.0	16.4 ± 4.8	14.6 ± 3.7

Normal plasma values (μmol/L; mean ± SD); Fe 17.9 ± 6.7, Cu 16.6 ± 2.9. (data abstracted from Aruoma *et al).*[81]

Sports and reactive oxygen species: methodology considerations

Hydroxyl radicals $OH^{\cdot}$ produced by the Fenton-type reaction in biological systems can cause damage to DNA, lipids, proteins, and carbohydrates. Generation of the highly reactive oxygen species requires catalytic metal complexes, especially those of iron. However, proving that oxidative damage in biological systems is due to the $OH^{\cdot}$ radical is extremely difficult. This highly reactive radical, once generated, will combine very quickly with adjacent molecules and is almost impossible to scavenge. It is not surprising that the detection and measurement of lipid peroxidation is often cited as evidence in support of the involvement of free radical reactions in toxicology and in human disease. It would be necessary to understand how lipid peroxidation of cellular importance relates to mechanisms of cell damage, including oxidation of proteins, inactivation of enzymes, loss of ionic homeostasis, and damage to genetic material.[62,89] Lipid peroxidation damages cells directly by attacking membrane structures and indirectly by releasing reactive products.[32]

While it remains to be fully established whether oxidative stress makes a significant contribution to the pathology of a given disease or whether it is merely an epiphenomenon, specific assays applicable to human subjects would greatly contribute to progress in our understanding of the role played by free radicals in normal physiology and in human diseases including sports, with the main premise being that although many of the ROS can serve useful physiological functions, they can be toxic when generated in excess, and this toxicity is often aggravated by the presence of ions of such transition metals as iron and copper.

Table 3 identifies a number of products of hydroxyl radical attack on aromatic compounds.[90] The development of new and widely available methodology for measuring oxidant generation in humans would contribute to a greater

Table 3 Products of hydroxyl radical attack on aromatic compounds

Compound	Major product(s)	Hydroxyl radical generation system	Comments
Salicylate (2-hydroxybenzoate)	2, 3- and 2,5-Dihydroxy-benzoates	Fenton reaction at pH 7.4	2,3 isomer is a product of free radical attack
Phenylamine (aniline)	*p*- and *o*-Aminophenol	Hypoxanthine + $FeCl_3$ + xanthine oxidase, pH 7.4	*p*-Aminophenol formation from aniline is catalyzed by a wide range of hemoproteins
4-Nitrophenol	4-Nitrocatechol	Several	Other products may form. Ethanol-inducible cytochrome P_{450} also converts 4-nitrophenol into 4-nitrocatechol
Phenylalanine	*o*-, *m*-, and *p*-Tyrosines	Hypoxanthine + xanthine oxidase, pH 5.5, or oxidation of 6, 7-dimethyl-5,6,7,8-tetra-hydropteridine	All three tyrosines are formed *in vivo* in the rat by enzymic-hydroxylating systems
*Deoxyguanosine (free or in DNA)	8-Hydroxydeoxyguanosine	X-irradiation. UV-H_2O_2 or Fe^{2+}-H_2O_2 systems at pH 7.4; ascorbate plus Fe^{2+}/EDTA at pH 6.8	Reaction may be involved in damage to DNA by ·OH
Dopamine	2-, 5-, and 6-Hydroxy-dopamines in the approximate ratio 3:2:1	H_2O_2 + Fe^{2+}-chelate or Fe-EDTA + ascorbate at pH 7.2	Of potential use to measure ·OH radical formation in dopaminergic neurones
Tryptophan	Six products produced	Radiolysis and photolysis	Method could be used to distinguish ·OH from singlet oxygen

*These methods may be particularly useful in sports research to delineate involvement of OH· reactions and assessments of antioxidant status. Gas chromatography-mass spectroscopy measures modified DNA bases and would be a useful tool for assessing DNA damage resulting from oxidative stress in exercise (see text for details).

understanding of the role of reactive oxygen species in disease pathology. Further discussions in methodology may be found in Rice-Evans *et al.*,[91] Aruoma[34] and in Greenwald.[92]

Measurement of DNA damage by free radicals using the technique of gas chromatography with selected ion monitoring has been discussed in the literature (Halliwell and Aruoma,[62,89] Dizdaroglu,[93] Aruoma *et al.*[63,94]). Methods involving measurement of single base products such as 8-hydroxydeoxyguanosine[95–98] and assays involving repair endonucleases[99,100] have also been described.

One of the methods that could be applied to sports research for the measurement of free radical reaction in vivo, aromatic hydroxylation, involves the use of a nontoxic aromatic compound (aspirin, for example).

The aromatic hydroxylation assay is based on the fact that hydroxyl radicals generated under physiological conditions react with aromatic compounds at diffusion-controlled rates, giving rise to predominantly hydroxylate end products. Salicylates and the aromatic amino acid phenylalanine are used as sources of aromatic moieties in this assay.[101,102] In the case of salicylates, 2-hydroxybenzoate products measured include 2,3dihydrobenzoate and 2,5dihydroxybenzoate. The latter product is also a product of a reaction involving actions of cytochrome P450. The 2,3 isomer is a product of free radical attack. In the case of phenylalanine, only the L isomer is recognized in vivo by phenylalanine hydroxylase to form L-p-tyrosines. Hydroxyl radicals, unable to distinguish between the L and D isomer, attack them to produce a mixture of *o*-, *m*-, and *p*-tyrosines. Formation of these tyrosines have been used to measure hydroxyl radical production by activated neutrophils[102] in dogs after myocardial ischemia[103] *(Figure 3)*.

Phenylalanine concentrations present in the human body fluids and cells are probably too low to intercept ·OH, and administration of high doses of phenylalanine to humans may be difficult to justify. Phenylalanine, unlike salicylate, is less toxic and does not interfere with arachidonic acid metabolism.

Uric acid is an end product of purine metabolism because the enzyme urate oxidase is not present in humans. Uric acid has been suggested to act as an antioxidant in vivo.[104] Measurement of products of attack of oxygen-derived species on uric acid might be a potential marker of oxidative damage in humans.[105,106] Products of uric acid oxidation include allantoin, oxonic acid, oxaluric acid, cyanuric acid, and parabanic acid. The measurement of the levels of uric acid[58,107–109] and its oxidation product allantoin *(Table 4)* has been applied to exercise studies.

Oxidative damage and antioxidant strategies

The premise that the antioxidant status of the exercising subjects and/or training athletes is important, and that biological systems must tend toward avoidance of environments harboring excessive ROS formation is well established. There is a need to delineate the contribution of nutrients to modulation of the pathological consequences of free radicals in the human body. It seems that use of a range of antioxidants combined with new methods for measuring oxidant generation is one way.[110]

Table 5 identifies some biologically important antioxidants. For the training athlete or exercising individuals, antioxidant status is important. Do antioxidants develop during exercise? Perhaps dietary supplementation may be significant as a preventative measure should the answer be "no." It is encouraging that work has begun to address these questions.[45–49,57,58,75,111–115]

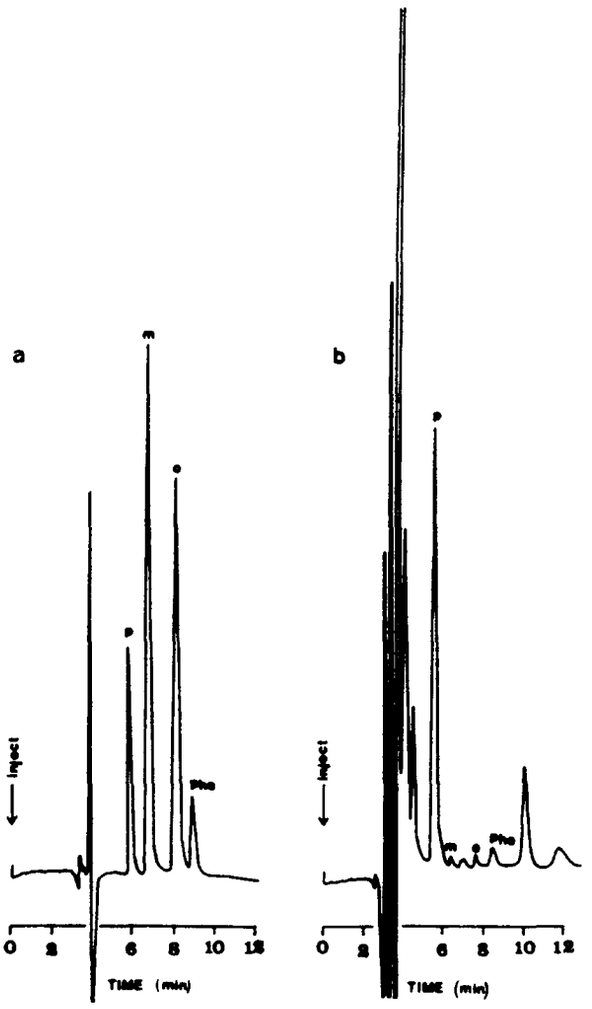

Figure 3 High-performance liquid chromatographic separation of the hydroxylated products of phenylalanine (Phe) on a Nucleocil 5-μm C-18 column (25 cm × 4.6 mm) (HPLC Technology, Macclesfield, UK) with a guard (Hibar from Merck, Poole, UK, with a C-18 cartridge). The eluent was 90% 5 mM KH_2PO_4/H_3PO_4 (pH 3.0) with 10% methanol (vol/vol) at a flow rate of 1 mL/min through an Applied Chromatography Systems pump; the ultraviolet detector was set at 274 nm (sensitivity, 0.02). *o* indicates *ortho*-tyrosine; *m*, *meta*-tyrosine; and *p*, *para*-tyrosine. Using this technique the detection limits are 0.05 μM for tyrosines and 0.1 mM for Phe. *a*, separation of a mixture of standards of *o*-, *m*-, and *p*-tyrosines (40 μM each) and Phe (2.0 mM) is shown. This mixture is stable at room temperature for up to 2 weeks. *b*, detection of tyrosines and Phe in a sample of dog plasma is shown. Based on a calibration plot, this sample was calculated to contain 63 μM *p*-tyrosine, 0.75 μM *m*-tyrosine, 1.00 μM *o*-tyrosine, and 0.40 mM Phe. The detected peaks were confirmed by spiking. (Reprinted from Sun *et al.* 1993. Reproduced with permission from Sun et al., *Circulation Research*, volume 73, pages 534–539, American Heart Association).

Maxwell et al.[58] recently observed significant rises in plasma antioxidant capacity in response to 1 hour of eccentric exercise *(Table 6)*, and that this change can be influenced by vitamin supplementation. Prior to exercise, eight subjects (group A) received no medication, eight (group C) received 400 mg of vitamin C daily for 3 weeks before and 1 week after exercise. Group E received 400 mg of vitamin E for

Table 4 Uric acid and allantoin analysis

Subject	Uric Acid μM	Uric Acid Allantoin	Uric Acid μM	Uric Acid Allantoin
	Before exercise		*After exercise*	
A	300	10.8	359.5	16.8
B	309.5	24.4	342.9	31.5
C	243	13.6	345.2	20.9
D	359	10.1	304.2	24.0
E	442.9	18.2	504.8	26.3
Mean ± SD	330.9 ± 75	15.4 ± 5.9	371.3 ± 77.4	23.9 ± 5.5

Healthy male athletes performed 30 to 40 min hard exercise on a cycle ergometer. All subjects had given informed consent. A work load of 210 watts was used, but this was determined by the ability of the subject to sustain the exercise intensity. Plasma samples were subjected to analysis as described in Grootveld and Halliwell.[105]

the same period. The antioxidant levels rose significantly with exercise and remained elevated during the first 60 minutes of recovery *(Figure 4)*. Antioxidant therapy to minimize the consequences of ROS to human clinical conditions has been suggested.[17,110,116–120]

An antioxidant may be defined as a substance that when present at low concentrations compared with that of an oxidizable substrate significantly delays or prevents the oxidation of that substrate. Food manufacturers aim to produce foods that maintain their shelf life and nutritional quality over a defined period. Thus the use of antioxidants to minimize the oxidation of lipids in food materials is extensively practiced. Antioxidants act at different levels in the oxidative sequence involving lipids (see *Figure 2* for a mechanism of lipid peroxidation). They may act, for example, by: (1) decreasing localized oxygen concentration; (2) preventing first-chain initiation by scavenging initial radicals such as hydroxyl radicals; (3) binding metal ions in forms that will not generate the lipid peroxidation initiating species; (4) decomposing peroxides by converting them to nonradical products such as alcohols; and (5) chain-breaking whereby intermediate radicals such as peroxyl and alkoxyl radicals are scavenged to prevent continued hydrogen abstraction.

The extent to which oxidation of fatty acids and their esters occurs in foods depends on the chemical structure of the fatty acid, the nature of food processing, and the temperature at which the food has been stored and/or cooked. For reviews on antioxidants see St. Angelo,[121] Aruoma and Halliwell,[122] Hudson,[123] Aruoma,[117,124] Shahidi *et al.*,[125] Rice-Evans and Diplock,[116] and Ong and Packer.[126] Antioxidants that protect lipids against free radical damage may actually accelerate damage to other molecules such as DNA, carbohydrates, and proteins under certain conditions. It is therefore important to examine suggested antioxidant activity or the activity of components within a proposed "antioxidant cocktail."[34,110,117,127,128] It is important to consider the following issues.

What biomolecule is the compound supposed to protect? An inhibitor of lipid peroxidation is unlikely to be useful if it can mediate oxidative attack on proteins, DNA, and/or carbohydrates. In other words, can the antioxidant cause

Table 5 Some biologically important antioxidants

Site	Mode of action
Extracellular	
Transferrin	Binds ferric ions.
Lactoferrin	Secreted by phagocyclic cells, binds ferric ions and retains them at low pH.
Haptoglobins	Bind hemoglobin and deter hemoglobin from decomposing lipid peroxides.
Hemopexin	Binds heme and prevents it from decomposing lipid peroxides.
Albumin	Binds copper ions and heme tightly and iron ions weakly. Probably a site-specific sacrificial antioxidant. Scavenges hypochlorous acid and protects α-antiproteinase against it.
Ceruloplasmin	Catalyzes oxidation of ferrous ions and ferrous complexes to the ferric state for binding to transferrin (ferroxidase I activity). Ferroxidase activity involves the four-electron reduction of O_2 to H_2O with no reactive oxygen intermediates released. Can react with O_2^- stoichiometrically and utilize H_2O_2 for reoxidation of certain reduced copper sites. Binds copper ions nonspecifically and inhibits copper-dependent radical reactions.
Extracellular superoxide dismutases (EC-SOD)	Large molecular mass glycoprotein catalytically removes O_2^-, probably from endothelial cell surface. (Only trace amounts present in bulk fluid.)
Extracellular glutathione peroxidase (EC-GSHpase)	Large molecular mass selenium containing glycoprotein that can remove H_2O_2 and lipid peroxides (low concentration, function not yet clear).
Urate (uric acid)	Scavenges organic and inorganic oxygen radicals and can bind iron and copper ions and stop or slow their catalysis of free radical reactions.
Glucose	Rate constant for reaction with ·OH radicals $\sim 1.0 \times 10^9\ M^{-1}s^{-1}$. Present at 4–6 mM or higher after a carbohydrate-rich meal.
Bilirubin	Scavenges peroxyl radicals; may protect albumin-bound fatty acid molecules from oxidation.
Mucins	Scavenge ·OH radicals with high rate constant ($\sim 5 \times 10^9\ M^{-1}s^{-1}$) and bind metal ions.
Membrane	
Vitamin E	Lipid soluble, chain-breaking antioxidant. May also protect lipoprotein lipids in the plasma.
β-Carotene	Singlet oxygen and ·OH radical scavenger; inhibitor of lipid peroxidation under certain conditions.
Coenzyme Q	In the reduced state it may act as an antioxidant in addition to its major roles in energy metabolism.
Intracellular	
Superoxide dismutases (Cu and Mn enzymes in animals)	Catalytic removal from cells of O_2^-.
Catalase (contains four NADPH molecules)	Catalytic removal of H_2O_2 is at high concentrations (catalytic activity). Has a peroxidatic activity when methanol, ethanol, formate, and nitrite are electron donors.
Glutathione peroxidase (selenium enzyme)	Catalytic removal of H_2O_2 and lipid hydroperoxides. Can effectively remove low steady-state levels of H_2O_2.

(Courtesy of Professor Halliwell.)

damage in biological systems different from those in which it exerts protection?

Can the antioxidants become available to the desired target at physiologically meaningful concentrations?

Is antioxidant protection the primary biological role of the molecule or a secondary one? This applies to naturally occurring antioxidants (e.g., flavonoids and other nutrient components).

If the antioxidants act by scavenging an ROS, can the antioxidant derived radicals themselves do biological damage?

It is also important to consider the physiological state of the athlete during antioxidant supplementation. Bioavailability of drugs, for example, is determined by their pharmacokinetics; absorption, metabolism, distribution, and elimination. Although antioxidant supplements are not drugs within the broad definition of drugs, they must become available to the consumer following ingestion. Thus, how much of the consumed antioxidant supplement becomes available to the athlete depends on the chemical nature of the antioxidant, the effect of other nutrient components in the antioxidant cocktail, diet, and the physiological state of the subject. It is generally accepted that based on toxicity studies and relative biologic antioxidant activities, α-tocopherol, ascorbic acid, and β-carotene may become the best candidates for antioxidant therapy.[129,132] The reader is also referred to the report of Haumann[133] on the health implications of antioxidants and the recent report of the Finnish study[134,135] which in general argues for a moratorium on health claims about antioxidant vitamins. However, foods (fruits, grains, and vegetables) are the primary dietary sources of α-tocopherol, ascorbic acid, and a number of other nutrient components.[110,130,131,135] The mechanisms of tissue injury in humans are very complex and it may not be possible to clearly identify the role played by free radicals in the process. This should not inhibit further research. From the standpoint of antioxidant supplementation to minimize tissue injury, it is necessary to first validate the index of such injury and then develop universal methods for measuring free radical generation in humans. This is the direction of current research.

Table 6 The acute effect of 60 minutes of eccentric exercise on antioxidant status and plasma biochemistry

	Group A	Group C	Group E
Vitamin C (μmol/L)			
basal pre-exercise	53.3 ± 4.4	112.5 ± 7.4	99.9 ± 16.4
post-exercise	59.6 ± 7.5	140.4 ± 7.3***	110.4 ± 20.0*
Vitamin E (μmol/L)			
basal pre-exercise	22.7 ± 1.2	23.7 ± 1.3	32.7 ± 3.0
post-exercise	22.7 ± 1.3	25.4 ± 1.2	35.7 ± 4.2**
Uric Acid (μmol/L)			
basal pre-exercise	305.1 ± 24.9	308.6 ± 28.0	287.3 ± 9.7
post-exercise	325.3 ± 26.8**	329.1 ± 27.2	324.9 ± 12.1**
Total AOC (μmol/L)			
basal pre-exercise	397.5 ± 27.8	400.3 ± 33.5	369.0 ± 17.5
post-exercise	435.4 ± 23.7*	464 ± 33.0***	418.5 ± 21.1***
Ammonia (μmol/L)			
basal pre-exercise	—	34.7 ± 5.8	32.6 ± 3.6
post-exercise	—	65.5 ± 12.0**	81.2 ± 11.8**
Lactate (mmol/L)			
basal pre-exercise	1.10 ± 0.05	0.86 ± 0.12	0.81 ± 0.08
post-exercise	2.48 ± 0.50*	2.48 ± 0.43**	1.71 ± 0.26**
Malondialdehyde (μmol/L)			
basal pre-exercise	0.89 ± 0.12	0.98 ± 0.15	1.26 ± 0.13
post-exercise	1.23 ± 0.20	1.47 ± 0.21	2.33 ± 1.37

(Mean ± SE; significance differences from basal pre-exercise levels within groups $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). All biochemical analyses have been corrected for changes in plasma volume. (AOC = antioxidant capacity). Full experimental details may be found in Maxwell *et al.*[58] The authors observe that the intensity of exercise and extent of prior training are important variables that might explain data emanating from similar studies (reproduced with permission from Maxwell et al., *Free Radical Research Communications*, volume 19(3), pages 191–202, Harwood Academic Publishers).

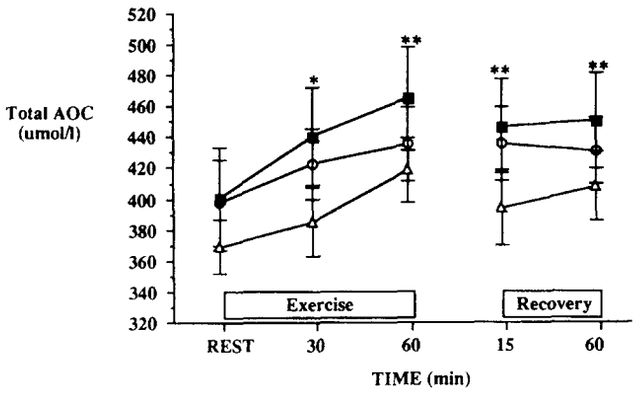

Figure 4 Changes in total antioxidant capacity during exercise and after 1 hour of recovery. All time points are illustrated as ± SE. Significant changes from resting values within each group are denoted by **. (From Maxwell *et al.*[58] Reproduced with permission from Maxwell et al., *Free Radical Research Communications*, volume 19(3), pages 191–202, Harwood Academic Publishers). ○, group A; ■, group C; Δ, group E; and ⊕ mean ± SE. $^{**}P < 0.05$ from rest for all groups; $^*P < 0.05$ from rest for group C.

Acknowledgments

I am grateful to the UK Sports Council (particularly for a research scholarship 1985–1987), UK Ministry of Agriculture, Fisheries and Food, and Nestec SA for research support.

References

1 Buettner, G.R. (1993). The pecking order of free radicals and antoxidants: lipid peroxidation, α-tocopherol and ascorbate. *Arch. Biochem. Biophys.* **300,** 535–543
2 Halliwell, B. and Gutteridge, J.M.C. (1989). *Free Radicals in Biology and Medicine* 2nd ed., Clarendon Press, Oxford, UK
3 Slater, T.F. (1989). Free radicals in medicine. *Free Rad. Res. Commun.* **7,** 119–390
4 Sies, H. (1985). *Oxidative Stress,* Academic Press, London, UK
5 Aruoma, O.I. (1993). *Free Radicals in Tropical Diseases,* Harwood Publishers, London UK
6 Slater, T.F. and Cheeseman, K.H. (1993). *Free Radicals in Medicine,* Special issue British Medical Bulletin Vol. 49., Churchill Livingstone, London, UK
7 Cerutti, P.A., Fridovich, I., and McCord, J.M. (eds) (1988). *Oxyradicals in Molecular Biology and Pathology,* Alan R Liss, New York, NY USA
8 Sneddon, J.W. and Vane, J.R. (1988). Endothelium-derived relaxing factor reduces platelet adhesion to bovine endothelium cells. *Proc. Natl. Acad. Sci. U.S.A.* **85,**1341–1344
9 Palmer, R.M.J., Ashton, D.S., and Moncada, S. (1988). Vascular endothelium cell synthesize nitric oxide from L-arginine. *Nature* **333,** 664–666
10 Halliwell, B. (1989). Superoxide, iron, vascular endothelium and reperfusion injury. *Free Rad. Res. Commun.* **5,** 315–318
11 Schmidt, H.H.H., Nauh, H., Wiltfoht, W., Gerlach, J., Prescher, K.E., Klein, M.M., Niroomand, F., and Bohme, E. (1988). Arginine is a physiological precurser of endothelium-derived nitric oxide. *Eur. J. Pharmacol.* **154,** 213–216
12 Huie, R. and Padmaja, S. (1993). The reaction of NO with superoxide. *Free Rad. Res. Commun.* **18,** 195–200
13 Rabanyi, G.M., Ho, E.H., Cantor, E.H., Lumma, W.C., and Parker-Botelho, L.H. (1991). Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* **181,** 1392–1397
14 Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., and Freeman, B.A. (1990). Apparent hydroxyl radical production by peroxy-

nitrite: implications for endothelium injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci U.S.A.* **87,** 1620–1624
15 Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V., and Moncada, S. (1993). Peroxynitrite modification of low density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **330,** 181–185
16 Radi, R., Beckman, J.S., Bush, K.M., and Freeman, B.A. (1991). Peroxy-nitrite oxidation of sulfhydryls. *J. Biol. Chem.* **266,** 4244–4250
17 Halliwell, B., Gutteridge, J.M.C., and Cross, C.E. (1992). Free radicals, antioxidants and human disease: where are we now? *J. Lab. Clin. Med.* **119,** 598–620
18 Rengasamy, A. and Johns, R.A. (1993). Inhibition of nitric oxide synthase by a superoxide generating system. *J. Pharmacol. Exp. Ther.* **267,** 1024–1027
19 Van der Vliet, A., O' Neill, C.A., Halliwell, B., Cross, C.E., and Kaur, H. (1994). Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite: evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett.* **339,** 89–92
20 Sessa, W.C., Pritchard, K., Seyedi, N., Wang, J., Hintze, T.H. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* **74,** 349–353
21 Fridovich, I. (1989). Superoxide dismutases. An adaptation to a paramagnetic gas. *J. Biol. Chem.* **264,** 7761–7764
22 McCord, J.M. (1993). Human disease, free radicals and the oxidant/antioxidant balance. *Clin. Biochem.* **26,** 351–367
23 Curnutte, J.T. and Babior, B.M. (1987). Chronic granulomatous disease. *Adv. Hum. Genet.* **16,** 229–297
24 McCord, J.M. (1987). Oxygen-derived radicals. A link between reperfusion injury and inflammation. *Fed. Proc.* **46,** 2402–2406
25 Babior, B.M. (1991). The respiratory burst oxidase and the molecular basis of chronic granulomatous disease. *Am. J. Hematol.* **37,** 263–266
26 Maly, F.E. (1990). The B-lymphocytes: a newly-recognized source of reactive oxygen species with immunoregulatory potential. *Free Rad. Res. Commun.* **8,** 143–148
27 Murrell, G.A.C., Francis, M.J.O., and Bromley, L. (1990). Modulation of fibroblast proliferation by oxygen free radicals. *Biochem. J.* **265,** 659–665
28 Fridovich, I. (1978). The biology of oxygen radicals. *Science* **209,** 875–877
29 Fridovich, I. (1983). Superoxide radical: an endogenous toxicant. *Ann. Rev. Pharmacol. Toxicol.* **23,** 239–257
30 Chan, H.-W. (ed) (1987). *Autoxidation of Unsaturated Lipids,* Academic Press, London, UK
31 Kappus, H. (1991). Lipid peroxidation: mechanism and biological relevance. In *Free Radicals and Food Additives,* p 59–75, (O.I. Aruoma and B. Halliwell, eds). Taylor & Francis, London, UK
32 Cheeseman, K.H. (1993). Lipid peroxidation and cancer. In *DNA and Free Radicals,* (B. Halliwell and O.I. Aruoma, eds.), p 109–144, Ellis Horwood, London, UK
33 Halliwell, B. and Chirico, S. (1993). Lipid peroxidation: its mechanism, measurement and significance. *Am. J. Clin. Nutr.* **57,** 715S–725S
34 Aruoma, O.I. (1993). Experimental tools in free radical biochemistry. In *Free Radicals in Tropical Diseases,* (O.I. Aruoma, ed.), p 233–267, Harwood Academic Publishers, London, UK
35 Cerutti, P.A. (1985). Pro-oxidant states and tumour activation. *Science* **227,** 375–381
36 Morrero, R. and Marnett, L.J. (1993). The role of organic peroxyl radicals in carcinogenesis. In *DNA and Free Radicals* (B. Halliwell and O.I. Aruoma, eds.), p 145–161, Ellis Horwood, London, UK
37 Spina, M.B. and Cohen, G. (1989). Dopamine turnover and glutathione oxidation: implications for Parkinson diseases. *Proc. Natl. Acad. Sci. USA.* **86,** 1398–1400
38 Von Sonntag, C. (1987). *The Chemical Basis of Radiation Biology,* Taylor and Francis, London
39 Packer, L. (1984). Vitamin E, physical exercise and tissue damage in animals. *Med. Biol.* **62,** 105–109
40 Packer, L. (1986). Oxygen radicals and antioxidants in endurance exercise. In *Biochemical Aspects of Physical Exercise,* (G. Benzi, L. Packer, and N. Siliprandi, eds.), p 73–92, Elsevier, Amsterdam, The Netherlands
41 Davies, K.J.A., Quintanilha, A.T., Brooks, G.A., and Packer, L. (1982). Free radicals and tissue damage produced by exercise. *Biochem. Biophys. Res. Commun.* **107,** 1198–1205
42 Kagan, V.E., Bakalova, R.A., Rangelova, D.S., Stofanovsky, D.A., Koynova, G.M., and Wolinsky, I. (1989). Oxidative stress leads to inhibition of calcium transport by sarcoplasmic reticulum in skeletal muscle. *Proc. Soc. Exp. Biol. Med.* **190,** 365–368
43 Laughlin, M.H., Simpson, T., Sexton, W.L., Brown, O.R., Smith, J.K., and Korthuis, R.J. (1990). Skeletal muscle oxidative capacity, antioxidant enzymes and exercise training. *J. Appl. Physiol.* **66,** 2337–2343
44 Barga de Quiroga, G. (1992). Brown fat thermogenesis and exercise: two examples of physiological oxidative stress. *Free Rad. Biol. Med.* **13,** 325–340
45 Duthie, G.G., Robertsen, J.D., Maughan, R.J., and Morrice, P.C. (1990). Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Arch. Biochem. Biophys.* **282,** 78–83
46 Bendich, A. (1991). Exercise and free radicals: effects of antioxidant vitamins. *Med. Sport Sci.* **32,** 59–78
47 Goldfarb, A.H. (1993). Antioxidants: role of supplementation to prevent exercise-induced oxidative stress. *Med. Sci. Sports Exerc.* **25,** 232–236
48 Viguie, C.A., Frei, B., Shigenaga, M.K., Ames, B.N., Packer, L., and Brooks, G.A. (1993). Antioxidant status and indexes of oxidative stress during consecutive days of exercise. *J. Appl. Physiol.* **75,** 566–572
49 Kauter, M.M., Nolte, L.A., and Holloszy, J.D. (1993). Effects of an antioxidant vitamin mixture on lipid peroxidation at rest and post-exercise. *J. Appl. Physiol.* **74,** 965–969
50 Jenkins, R.R. (1988). Free radical chemistry: relationship to exercise. *Sports Med.* **5,** 156–170
51 Starnes, J.G., Cantu, R., Farrar, R., and Kehrer, J. (1989). Skeletal muscle lipid peroxidation in exercised and food restricted rats during aging. *J. Appl. Physiol.* **67,** 69–75
52 Dernbach, A.R., Sherman, W.M., Simonsen, J.C., Flowers, K.M., and Lamb, D.R. (1993). No evidence of oxidant stress during high-intensity rowing training. *J. Appl. Physiol.* **74,** 2140–2145
53 Alessio, H. and Goldfarb, A. (1988). Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. *J. Appl. Physiol.* **64,** 1333–1336
54 Jenkins, R.R., Krause, K., and Schofield, L.S. (1993). Influence of exercise on clearance of oxidant stress products and loosely bound iron. *Med. Sci. Sports Exerc.* **25,** 213–217
55 Alessio, H.M. (1993). Exercise-induced oxidative stress. *Med. Sci. Sports Exerc.* **25,** 218–224
56 Ji, L.L. (1993). Antioxidant enzyme response to exercise and aging. *Med. Sci. Sports Exerc.* **25,** 225–231
57 Meydani, M., Evans, W.J., Handelman, G., Biddle, L., Fielding, R.A., Meydani, S.N., Burrill, J., Faitarone, M.A., Blumberg, J.B., and Cannon, J.G. (1993). Protective effect of vitamin E on exercise induced oxidative damage in young and older adults. *Am. J. Physiol.* **264,** R992–R998
58 Maxwell, S.R.J., Jakeman, P., Thomsen, H., Leguen, C., and Thorpe, G.H.G. (1993). Changes in plasma antioxidant status during eccentric exercise and the effect of vitamin supplementation. *Free Rad. Res. Commun.* **19,** 191–202
59 Fenton, H.J.H. (1894). Oxidation of tartaric acid in the presence of iron. *J. Chem. Soc.* **65,** 899–910
60 Haber, F. and Weiss, J.J. (1943). The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. Roy. Soc. London Ser. A* **147,** 332–352
61 Walling, C. (1975). Fenton's reagent revisited. *Acc. Chem. Res.* **8,** 125–131
62 Halliwell, B. and Aruoma, O.I. (1991). DNA damage by oxygen derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett.* **281,** 9–19
63 Aruoma, O.I., Halliwell, B., Gajewski, E., and Dizdaroglu, M. (1991). Copper ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide. *Biochem. J.* **273,** 601–604
64 Esterbauer, H. (1985). Lipid peroxidation products: formation, chemical properties and biological activities. In *Free Radicals in Liver Injury,* (G. Poli, K.H. Cheeseman, M.V. Dianzani, and TF Slater, eds.) IRL Press: Virginia p 29–47

65 Yoshimura, H. (1970). Anemia during physical training (sports anemia). *Nutr. Rev.* **28,** 251–253

66 Yoshimura, H., Inove, T., Yamada, T., and Shiraki, K. (1980). Anemia during hard and physical training (sports anemia) and its causal mechanism with special reference to protein nutrition. *World Rev. Nutr. Diet* **35,** 1–86

67 Pate, R. (1983). Sports anemia: a review of the current research literature. *Physician Sports Med.* **11,** 115–126

68 Pelliccia, A. and Di Nucci, G.B. (1987). Anemia in swimmers: facts or fiction? Study of hematologic and iron status in male and female top-level swimmers. *Int. J. Sports Med.* **8,** 227–230

69 Magnussen, B., Hallberg, L., Rossander, I., and Swolin, B. (1984). Iron metabolism and "sports anemia" I. a study of several iron parameters in elite runners with differences in iron status. *Acta Med. Scand.* **216,** 149–155

70 Magnussen, B., Hallberg, L., Rossander, L., and Swolin, B. (1984). Iron metabolism and "sports anemia" II. A hematological comparison of elite runners and control subjects. *Acta Med. Scand.* **216,** 157–164

71 Miller, B.J., Pate, R.R., and Burgess, W. (1988). Foot impact force and intravascular hemolysis during distance running. *Int. J. Sports Med.* **9,** 56–60

72 Monton, G., Sluse, E.E., Bertrand, A., Welter, A., Cobay, J.L., and Camus, G. (1990). Iron status in runners of various running specialities. *Arch. Intern. Physiol. Biochem.* **98,** 103–109

73 Weight, L.M., Klein, M., Noakes, T.D., and Jacobs, P. (1992). Sports anemia: a real or apparent phenomenon in endurance-trained athletes. *Int. J. Sports Med.* **13,** 344–347

74 Eichner, E.R. (1985). Runner's macrocytosis: a clue to footstrike hemolysis. Runner's anemia as a benefit versus runner's hemolysis as a detriment. *Am. J. Med.* **78,** 321–325

75 Eichner, E.R. (1994). Sports anemia, iron supplements, and blood doping. *Med. Sci. Sports Exerc.* **24,** S315–S318 (suppl 9)

76 Balaban, E.P., Cox, J.V., Snell, P., Vaughan, R.H., and Frenkel, E.P. (1989). The frequency of anemia and iron deficiency in the runner. *Med. Sci. Sports Exerc.* **21,** 643–648

77 Aruoma, O.I. and Halliwell, B. (1987). Superoxide dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl radical generation? *Biochem. J.* **241,** 273–283

78 Halliwell, B. and Gutteridge, J.M.C. (1990). Role of free radicals and catalytic metal ions in human diseases: an overview. *Methods Enzymol.* **186,** 1–85

79 Lauffer, R.B. (ed) (1992). *Iron and Human Disease,* CRC Press, Boca Raton, FL USA

80 Bomford, A., Ward, R.J., and Crichton, R.R. (1993). Influence of tropical disease on iron metabolism. In *Free Radicals in Tropical Diseases,* (OI Aruoma, ed.), p 13–33, Harwood Academic Publishers, London, UK

81 Aruoma, O.I., Reilly, T., MacLaren, D., and Halliwell, B. (1988). Iron, copper and zinc concentrations in human sweat and plasma: the effect of exercise. *Clin. Chim. Acta* **177,** 81–88

82 Resina, A., Fedi, S., Gatteschi, L., Rubenni, M.G., Giamberardino, M.A., Trabassi, E., and Imreh, F. (1990). Comparison of some serum copper parameters in trained runners and control subjects. *Int. J. Sports Med.* **11,** 58–60

83 Lukaski, H.C., Hoverson, B.S., Gallagher, S.K., and Bolonchuk, W.W. (1990). Physical training and copper, iron and zinc status in swimmers. *Am. J. Clin. Nutr.* **51,** 1093–1099

84 Lamanca, J.J., Hymes, E.M. Daly, J.A., Moffatt, R.J., and Waller, M.F. (1988). Sweat iron loss of male and female runners during exercise. *Int. J. Sports Med.* **9,** 52–55

85 O'Toole, M.L., Hiller, A.B., Roalstad, M., and Douglas, P.S. (1988). Hemolysis during triathlon races: its relation to race distance. *Med. Sci. Sports Exerc.* **20,** 272–275

86 Gutteridge, J.M.C., Rowley, D.A., Halliwell, B., Cooper, D.F., and Heeley, D.M. (1985). Copper and iron complexes catalytic for oxygen radical reactions in sweat from human athletes. *Clin. Chim. Acta* **145,** 267–273

87 Diehl, D.M., Lohman, T.G., Smith, S.C., and Kertzer, R. (1986). Effects of physical training and competition on the iron status of female field hockey players. *Int. J. Sports Med.* **7,** 264–270

88 Hallberg, L. and Magnusson, B. (1984). The etiology of "sports anemia". *Acta Med. Scand.* **216,** 145–148

89 Halliwell, B. and Aruoma, O.I. (eds) (1993). *DNA and Free Radicals,* Ellis Horwood, London, UK

90 Halliwell, B., Grootveld, M., and Gutteridge, J.M.C. (1989). Methods for the measurements of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. *Meth. Biochem. Anal.* **33,** 59–90

91 Rice-Evans, C., Diplock, A.T., and Symons, M.C.R. (eds). (1991). *Techniques in Free Radical Research.* Elsevier Applied Science, London, UK

92 Greenwald, R.A. (1985). *Handbook of Methods for Oxygen Radical Research,* CRC Press, Boca Raton, FL USA

93 Dizdaroglu, M. (1991). Chemical determination of free radical induced damage to DNA. *Free Rad. Biol. Med.* **10,** 225–242

94 Aruoma, O.I., Halliwell, B., and Dizdaroglu, M. (1989). Iron ion-dependent modification of bases in DNA by the superoxide radical generating system hypoxanthine/xanthine oxidase. *J. Biol. Chem.* **264,** 13024–13028

95 Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H., and Rickard, R.C. (1986). Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanism of formation. *Free Rad. Res. Commun.* **1,** 163–172

96 Kasai, H., Nishimura, S., Kurokawa, Y., and Hayashi, Y. (1987). Oral administration of the renal carcinogen, potassium bromate, specifically produces 8-hydroxydeoxyguanosine in rat target organ DNA. *Carcinogenesis* **8,** 1959–1961

97 Loft, S., Vistisen, K., Ewertz, M., Tjonneland, A., Overvad, K., and Paulson, H.E. (1992). Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* **13,** 2441–2447

98 Shigenago, M.K., Gimeno, C.J., and Ames, B.N. (1989). Urinary 8-hydroxy 2′deoxyguanosine as a biological marker of in vivo oxidative DNA damage. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9697–9701

99 Hegler, J., Bittner, D., Boiteux, S., and Epe, B. (1993). Quantitation of oxidative DNA modification in mitochondria. *Carcinogenesis* **14,** 2309–2312

100 Collins, A.R., Duthie, S.J., and Dobson, V.L. (1993). Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* **14,** 1733–1735

101 Kaur, H. and Halliwell, B. (1994). Detection of hydroxyl radicals by aromatic hydroxylation. *Methods Enzymol.* **233,** 67–82

102 Halliwell, B. Grootveld, M., Kaur, H., and Fagerheim, I. (1988). Aromatic hydroxylation and uric acid degradation as methods for detecting and measuring oxygen radicals *in vitro* and *in vivo.* In *Free Radicals, Methodology and Concepts,* (C. Rice-Evans and B. Halliwell, eds), p 33–59, Richelieu Press, London, UK

103 Sun, J.-Z., Kaur, H., Halliwell, B., Li, X.-Y., and Bolli R (1993). Use of aromatic hydroxylation of phenylalanine to measure production of hydroxyl radicals after myocardial ischemia in vivo. Direct evidence for a pathogenetic role of the hydroxyl radical in myocardial stunning. *Circ. Res.* **73,** 534–549

104 Ames, B.N. (1989). DNA damage as related to cancer and aging. *Mutat. Res.* **214,** 41–46

105 Grootveld, M. and Halliwell, B. (1987). Measurement of allantoin and uric acid in human body fluids. A potential index of free radical reactions in vivo. *Biochem. J.* **243,** 803–808

106 Kaur, H. and Halliwell, B. (1990). Action of biologically-relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chem. Biol. Interac.* **73,** 235–247

107 Hellsten, Y., Ekblom, B., and Sjodin, B. (1989). The metabolic relation between hypoxanthine and uric acid in man following maximal short distance running. *Acta Physiol. Scand.* **137,** 341–345

108 Green, H.J. and Fraser, I.G. (1988). Differential effects of exercise intensity on serum uric acid concentration. *Med. Sci. Sports Exerc.* **16,** 275–277

109 Sahlin, K., Ekberg, K., and Cizinsky, S. (1991). Changes in plasma hypoxanthine and free radical markers during exercise in man. *Acta Physiol. Scand.* **142,** 257–281

110 Aruoma, O.I. (1994). Nutrition and health aspects of free radicals and antioxidants. *Fd. Chem. Toxicol.* **32,** 671–683

111 Murray, R. (1993). Nutrition for the marathon and other endurance sports: environmental stress and dehydration. *Med. Sci. Sports Exerc.* **24,** S319–S323 (suppl 9)

112 Miyashita, M. and Nishibata, I. (1993). Nutritional supplements and athletic performance: with special reference to vitamin E. *In Vitamin*

E. Its Usefulness in Health and in Curing Diseases, (M Mino, H Nakamura, AT Diplock and HJ Kayden eds) Karger: Basel p 153–161

113 Simon-Schnass, I. (1993). Vitamin requirements for increased physical activity: vitamin E. *World Rev. Nutr. Diet.* **71,** 144–153

114 Van der Beek, E.J. (1991). Vitamin supplementation and physical exercise performance. *J. Sports Sci.* **9,** 77–90

115 Burke, L.M. and Read, R.S.D. (1993). Dietary supplements in sport. *Sports Med.* **15,** 43–65

116 Rice-Evans, C.A. and Diplock, A.T. (1993). Current status of antioxidant therapy. *Free Rad. Biol. Med.* **15,** 77–96

117 Aruoma, O.I. (1993). Free radicals: their role in nutrition. In *Free Radicals in Tropical Diseases*, (O.I. Aruoma, ed.), p. 187–217, Harwood Academic Publishers, London, UK

118 Halliwell, B., Evans, P.J., Kaur, H., and Aruoma, O.I. (1994). Free radicals, tissue injury and human disease: A potential for therapeutic use of antioxidants. In *Organ Metabolism and Nutrition Ideas for Future Critical Care* (J.M. Kinney and H.N. Tucker, eds), pp. 425–445, Raven Press, New York

119 Riemersma, R.A., Wood, D.A., Macintyre, C.C., Elton, R.A., Gey, K.F., and Oliver, M.F. (1991). Risk of angina pectoris and plasma concentrations of vitamins A, C and E and carotene. *Lancet* **337,** 1–5

120 Waldron, K.W., Johnson, I.T., and Fenwick, G.R. (eds) (1993). Food and Cancer Prevention: Chemical and Biological Aspects, Special Publication 123, Royal Society of Chemistry Publication, London, UK

121 St. Angelo, A.J. (eds.) (1992). *Lipid Oxidation in Food*, American Chemical Society: Series 500, Washington, DC USA

122 Aruoma, O.I. and Halliwell, B. (1991). *Free Radicals and Food Additives*, Taylor and Francis, London

123 Hudson, B.J.F. (eds.) (1990). *Food Antioxidants*, Elsevier, Amsterdam, The Netherlands

124 Aruoma, O.I. (1993). Free radicals and food. *Chem. Brit.* **29,** 210–214

125 Shahidi, F., Janitha, P.K., and Wanasundera, P.D. (1992). Phenolic antioxidants. *Crit. Rev. Fd. Sci. Nutr.* **32,** 67–103

126 Ong, A.S.H. and Packer, L. (1992). *Lipid Soluble Antioxidants: Biochemistry and Clinical Applications*, Birkhauser Verlag, Basel, Switzerland

127 Halliwell, B. (1990). How to characterize a biological antioxidant. *Free Rad. Res. Commun.* **9,** 1

128 Bonorden, W.R. and Pariza, M.W. (1994). Antioxidant nutrients and protection from free radicals. In *Nutritional Toxicology* (F.N. Katsonis, M. Mackey, and J.J. Hjelle, eds), pp. 19–48, Raven Press, New York

129 Packer, L.S. (1993). Health effects of nutritional antioxidants. *Free Rad. Biol. Med.* **15,** 685–686

130 Block, G., Patterson, B., and Subar, A. (1992). Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **18,** 1–29

131 Bal, D.G. and Forester, S.B. (1991). Changing the American diet. Impact on cancer prevention policy recommendations and program implications for the American Cancer Society. *Cancer* **67,** 2671–2680

132 Papas, A.M. (1993). Vitamin E and exercise: aspects of biokinetics and bioavailability. *World. Rev. Nutr. Diet* **72,** 165–176

133 Haumann, B.F. (1994). Antioxidants: health implications. *INFORM* **5,** 242–252

134 Heinonen, O.P. and Albanes, D. (1994). The effect of vitamin E and β-carotene on the incidence of lung cancer and other cancers in male smokers. *N. Engl. J. Med.* **330,** 1029–1035

135 Nowak, R. (1994). Beta-carotene: helpful or harmful? *Science* **264,** 500–501

136 Begley, S. (1994). Beyond vitamins. *Newsweek* April 25, 45–49.