Immunopharmacology of Free Radical Species

EDITED BY

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Paul G Winyard

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Immunopharmacology of Free Radical Species

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Series Preface

The consequences of diseases involving the immune system such as AIDS, and chronic inflammatory diseases such as bronchial asthma, rheumatoid arthritis and atherosclerosis, now account for a considerable economic burden to governments worldwide. In response to this, there has been a massive research effort investigating the basic mechanisms underlying such diseases, and a tremendous drive to identify novel therapeutic applications for the prevention and treatment of such diseases. Despite this effort, however, much of it within the pharmaceutical industries, this area of medical research has not gained the prominence of cardiovascular pharmacology or neuropharmacology. Over the last decade there has been a plethora of research papers and publications on immunology, but comparatively little written about the implications of such research for drug development. There is also no focal information source for pharmacologists with an interest in diseases affecting the immune system or the inflammatory response to consult, whether as a teaching aid or as a research reference. The main impetus behind the creation of this series was to provide such a source by commissioning a comprehensive collection of volumes on all aspects of immunopharmacology. It has been a deliberate policy to seek editors for each volume who are not only active in their respective areas of expertise, but who also have a distinctly *pharmacological* bias to their research. My hope is that *The Handbook of Immunopharmacology* will become indispensable to researchers and teachers for many years to come, with volumes being regularly updated.

The series follows three main themes, each theme represented by volumes on individual component topics. The first covers each of the major cell types and classes of inflammatory mediators. The second covers each of the major organ systems and the diseases involving the immune and inflammatory responses that can affect them. The series will thus include clinical aspects along with basic science. The third covers different classes of drugs that are currently being used to treat inflammatory disease or diseases involving the immune system, as well as novel classes of drugs under development for the treatment of such diseases.

To enhance the usefulness of the series as a reference and teaching aid, a standardized artwork policy has been adopted. A particular cell type, for instance, is represented identically throughout the series. An appendix of these standard drawings is published in each volume. Likewise, a standardized system of abbreviations of terms has been implemented and will be developed by the editors involved in individual volumes as the series grows. A glossary of abbreviated terms is also published in each volume. This should facilitate cross-referencing between volumes. In time, it is hoped that the glossary will be regarded as a source of standard terms.

While the series has been developed to be an integrated whole, each volume is complete in itself and may be used as an authoritative review of its designated topic.

I am extremely grateful to the officers of Academic Press, and in particular to Dr Carey Chapman, for their vision in agreeing to collaborate on such a venture, and greatly hope that the series does indeed prove to be invaluable to the medical and scientific community.

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Preface

In this volume of *The Handbook of Immunopharmacology* devoted to the role of free radicals in disease, we have solicited contributions, not only from scientists working in many different fields of medicine, but scientists of very diverse backgrounds. All, however, have one thing in common, that is their research is very much orientated towards developing novel therapeutic strategies, having a distinctly pharmacological bias to their research.

As can be seen from even a cursory assessment of the various chapters that make up this volume, free radicals have been implicated in a whole host of different human disease states implying that they perhaps do not have a pivotal aetiological role in any, but are primarily involved in the perpetuation of disease. Over the past few years it has become very clear that, although free radicals most certainly do have this role in perpetuating inflammatory reactions, they have perhaps a far more important role in acting as second messenger systems maintaining normal cell function and it is the perturbations of these reactions that pose the most intriguing therapeutic challenges.

In this volume centred around different disease processes, various basic mechanisms of free radical processes and injury are dissected. In each case there is an emphasis given to potential therapeutic strategies that develop from this new knowledge. We would like to thank all our contributors for their efforts and beg their forgiveness for our mild eccentricities.

D. Blake
P.G. Winyard

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1. *Methods for the Detection and Measurement of Reactive Radical Species* in vivo *and* in vitro

Martin Grootveld[1] *and* **Christopher J. Rhodes**[2]

1. *Introduction*

The considerable interest aroused in the involvement and roles of reactive oxygen-derived free radicals in the pathogenesis of a very wide range of clinical conditions has stimulated many researchers to develop and subsequently apply a variety of useful (and not so useful) analytical methods for the detection and quantification of such species in biological systems, providing evidence for their activity therein. Since the concentrations of such radicals [e.g. $^{\cdot}OH$ radical, superoxide anion ($O_2^{\cdot -}$)] and their non-radical precursors (e.g. H_2O_2) *in vivo* are very low, the level of chemical modifications arising from their reactivity with biomolecules present in living organisms is usually very small. Hence, the first problem for those involved in the development of tests for radical activity *in vivo* is the requirement of a highly sensitive analytical technique that permits the determination of very low levels of selected individual components. Fortunately, recent developments in chemical methods of analysis have led to the ability to measure concentrations of parts per billion (p.p.b.) and below.

With the above considerations in mind, the task for the analytical chemist is one of deciding which methods are likely to yield acceptable indices of free radical activity. This problem is further complicated by cost restraints and by the fact that the method to be employed is, in general, critically dependent on the nature of the radical investigated and the sample to be tested.

A number of the techniques that have been employed have the ability to directly monitor free-radical species either *in vitro* or *in vivo* [predominantly those involving electron spin resonance (e.s.r.) spectroscopy]. However, since many physiologically relevant free radicals have extremely short half-lives (e.g. 10^{-9} s for $^{\cdot}OH$), the majority of the methods utilized detect products arising from their reactions with chemical components present (i.e. indirect methods). These indirect methods for

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determining oxygen radical activity *in vivo* can be broadly classified as either quantitative or qualitative. A quantitative test system is one in which an increase or a decrease in the concentration of one or more chemical components is monitored, and is only applicable if the differences observed between samples in which radical activity has been implicated and appropriate controls are large enough to be declared highly statistically significant. However, in view of the existing wide variation in the chemical content of any class of biofluid or tissue sample, it is clear that this type of indirect test does not have the advantages associated with a qualitative one.

A qualitative test is one that involves the detection (and, of course, subsequent quantification) of new "unnatural" components (or characteristic patterns of several of such products) that is/are generated solely by selected radical species, and hence is a more desirable type of test not only for its "diagnostic" capacity but also for any deleterious toxicological properties that may be associated with the adduct(s) monitored. Briefly, if a "target" compound reacts with a reactive oxygen species [e.g. $^{\cdot}OH$ radical, similarly reactive oxo–iron complexes such as the ferryl ion (FeO^{2+}), $O_2^{\cdot-}$, H_2O_2, etc.] to generate a specific pattern of products that can be accurately measured in body fluids and/or tissue sample extracts in addition to chemical model systems, and one or more of these products are clearly distinguishable from those arising from enzyme-catalysed reactions or alternative biotransformations, then formation of these so-called "unnatural" products could conceivably be utilized to evaluate the rate and level of reactive oxygen-radical production *in vivo*. This assumes that the "detector" molecules selected are present at sites of radical generation at concentrations sufficient to compete with any other components that might scavenge the radical investigated, and that the "unnatural" product(s) are not consumed immediately by metabolic processes. Molecules employed as "detectors" might be foreign (i.e. exogenous) substances administered to the whole organism orally or via injection, or they may be present endogenously.

In this communication we review and discuss both existing and newly developed "state of the art" methods for detecting reactive oxygen-derived radical species and their related non-radical oxidants and precursors (e.g. singlet oxygen and H_2O_2) both in *in vitro* systems and *in vivo*. For many of the methods described here, attention is drawn to any limitations or potential sources of error that may be associated with their operation, particularly the artefactual generation of radicals in biological samples during periods of collection, storage and manipulation prior to analysis, a process that often gives rise to erroneous and hence misleading experimental data. In view of the authors' research interests, some emphasis is placed on the methodological applications of modern spectroscopic techniques, predominantly high-resolution nuclear magnetic resonance (NMR) and e.s.r. analysis.

2. *Direct Observation of Free-radical Species in Biological Samples by Electron Spin Resonance Spectroscopy*

Electron spin resonance spectroscopy is a technique whereby free radicals may be detected directly and structural assignments are provided for them. The principle underlying this technique is that the initial degeneracy of the two possible spin states of an unpaired electron may be lifted by the application of an external magnetic field such that an energy separation is created between the $+\frac{1}{2}$ and $-\frac{1}{2}$ levels. Having provided an energy level separation in this manner, the basic spectroscopic requirement is fulfilled, i.e. $E = h\nu$, so that the sample will absorb radiation with an energy equivalent to E, which falls in the microwave region of the electromagnetic spectrum and is supplied by either a klystron or a Gunn diode. It is fortunate that the energy-level splitting is, to some extent, modified by the local environment of the unpaired electron and hence the resonance field depends on the molecular nature of the radical examined. This phenomenon is generally expressed in terms of the 'g factor', a dimensionless constant in the equation $E = h\nu - g\mu_\beta\beta$, where μ_β is the Bohr magneton and β the strength of the externally applied magnetic field. The shifts in g value from that arising from a free electron (i.e. $g = 2.0023$) are relatively small for most organic radicals since they depend on spin-orbit coupling, and the spin-orbit coupling constants are also rather small for typical carbon-centred radicals. Thus, the g factor provides only a limited amount of information concerning the molecular nature of organic free radicals. However, such information is obtainable from "hyperfine coupling", which is analogous to "spin–spin coupling" in NMR spectroscopy and, indeed, the same principles regarding coupling patterns and coupling constants apply to both techniques. The familiar "$n+1$" rule predicts the number of lines that would be expected from a set of n equivalent protons and for the methyl radical ($^{\cdot}CH_3$) we find a total of 4 lines in its e.s.r. spectrum. Another more complex example is the ethyl radical ($CH_3CH_2^{\cdot}$), which gives rise to a pattern of 12 lines from coupling of the unpaired electron with the $n=2$, $n'=3$ set of protons yielding $(n+1)(n'+1)=12$ lines.

It is unfortunate that typical concentrations of free-radical species present in biological systems are only at the limit of e.s.r. detection sensitivity and, of course, there are major technical difficulties in studying whole animals in this manner. Therefore, the most successful e.s.r. experiments have adopted the approach of "spin trapping" in which very reactive and thus transient radical species are converted to long-lived adducts via reaction with a "trap" such as a nitrone, e.g. Equation 1.1:

$$^{\cdot}OH + R_2C{=}N(O)R \rightarrow R_2C(OH)N(O^{\cdot})R \quad (1.1)$$

It is, of course, inappropriate to administer such nitrones to living systems, since some of these compounds exert cardiac toxicity (Li *et al.*, 1993) and the major work conducted thus far has been that involving perfused isolated organs. The exception to this would appear to be the e.s.r. detection of the ubiquitous nitric oxide (NO$^{\cdot}$, which forms a relatively stable complex with haemoglobin termed "nitrosylhaemoglobin" and this adduct is detectable in many types of damaged tissue, implicating it in the mechanisms associated with a range of clinical conditions. Interestingly, we have recently detected nitrosylhaemoglobin in knee-joint synovial fluids collected from patients with rheumatoid arthritis: the spectrum shown in Fig. 1.1 contains e.s.r. signals characteristic of this species ($g_1 = 2.083$, $g_2 = 2.040$ and $g_3 = 2.003$).

Further applications of e.s.r. spectroscopy to the analysis of biological samples or related chemical model systems concern the use of "spin probes" or "spin labels". These are relatively stable nitroxide radicals that are reacted with biomolecules (e.g. proteins), the functions of which it is desired to investigate (Berliner, 1976). The most commonly used spin probe is di-*tert*-butyl nitroxide (TBN), the ^{14}N-coupling constant for which varies with its environment and hence it is possible to distinguish between its uptake in aqueous or hydrophobic environments. There is a very wide range of spin labels available that are now commonly employed to study the molecular mobility of protein segments during their interactions with selected reagents, e.g. that arising in an enzyme as it reacts with a substrate.

Spin probes are quite readily metabolized reductively by cells and have been utilized to monitor metabolic activity (Iannore *et al.*, 1990) The line widths of the e.s.r. signals are also sensitive to the presence of O_2 in both intracellular and extracellular environments and hence O_2 concentrations may be determined with TBN or alternative spin probes (Glockner and Swartz, 1991).

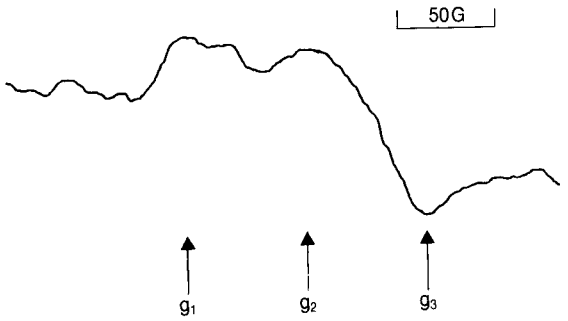


Figure 1.1 A typical e.s.r. spectrum of knee-joint synovial fluid obtained from a patient with rheumatoid arthritis. The signals detectable have *g* values that correspond to the nitrosylhaemoglobin adduct ($g_1 = 2.083$, $g_2 = 2.040$ and $g_3 = 2.003$).

3. *Detection of Protein Damage Arising from the Actions of Oxygen-derived Radical Species*

Early chemical investigations of the reactions of $^{\cdot}$OH radical with proteins in dilute aqueous solution (Barron *et al.*, 1955; Okada and Gehrmann, 1957; Drake *et al.*, 1957; Romani and Tappel, 1959; Lange and Pihl, 1960) provided conclusive evidence that aromatic and heterocyclic residues, together with the methionine and cysteine/cystine moieties, are the amino acids most susceptible to oxidative damage in this manner. However, the importance of the peptide main-chain C–H bonds as major sites of $^{\cdot}$OH radical attack was established subsequently (Garrison *et al.*, 1962). Reactions of $^{\cdot}$OH radical with proteins at the thiol group of cysteine (Lichtin *et al.*, 1972; Adams and Redpath, 1974) [predominantly yielding its corresponding disulphide (cystine) for the free amino acid (Equations 1.2–1.4)], the imidazole ring of histidine (Baverstock *et al.*, 1974; Redpath *et al.*, 1975), the aromatic rings of aromatic amino acids such as phenylalanine (Aldrich *et al.*, 1969; Adams *et al.*, 1972) (Section 6) and the C–H bonds of the peptide main chain (Lichtin *et al.*, 1972) have all been investigated in some detail by the pulse radiolysis–spectrophotometric technique.

$$RSH \rightleftharpoons RS^- + H^+ \qquad (1.2)$$

$$RS^- + {}^{\cdot}OH \rightarrow RS^{\cdot} + OH^- \qquad (1.3)$$

$$2RS^{\cdot} \rightarrow RSSR \qquad (1.4)$$

Although these investigations have provided much valuable information concerning the relative reactivities of proteins with $^{\cdot}$OH radical in O_2-free solutions, data concerning the molecular nature and yields of the products arising from these reactions remains limited. Chemical investigations of model peptide systems, however, have demonstrated that secondary radicals generated from the attack of $^{\cdot}$OH radical at the peptide main-chain and side-chain loci give rise to further radicals, which, in the majority of cases, preferentially dimerize to produce α,α'-diamino acid adducts (e.g. Equations 1.5 and 1.6) (Garrison, 1981).

$$H_3\overset{+}{N}CH(R)CONHCH(R)CO_2^- + {}^{\cdot}OH \rightarrow H_3\overset{+}{N}CH(R)CONHC^{\cdot}(R)CO_2^- + H_2O \qquad (1.5)$$

$$2H_3\overset{+}{N}CH(R)CONHC^{\cdot}(R)CO_2^- \rightarrow \begin{matrix} H_3\overset{+}{N}CH(R)CONHC(R)CO_2^- \\ | \\ H_3\overset{+}{N}CH(R)CONHC(R)CO_2^- \end{matrix} \qquad (1.6)$$

With the exception of cystine, α,α'-diamino acids represent a class of compounds not found naturally in biological samples. However, it should be noted that such compounds have been isolated previously from certain

bacteria and products derived therefrom (Greenstein and Winitz, 1961).

A wide range of plasma proteins are damaged by the oxidizing actions of reactive oxygen species *in vitro*, and these include albumin (Wolff *et al.*, 1986), glyceraldehyde-3-phosphate dehydrogenase (Hyslop *et al.*, 1988; Baker *et al.*, 1989), caeruloplasmin (Winyard *et al.*, 1989) and low-density lipoprotein (LDL) (Steinberg *et al.*, 1989), together with collagen (Greenwald *et al.*, 1976) and proteoglycans (Greenwald *et al.*, 1976; Wolff *et al.*, 1986). Oxidative modification of immunoglobulin G (IgG) by oxygen-derived radical species induces a curious fluorescence in this protein, which arises from kynurenine and *N*-formyl-kynurenine (Griffiths *et al.*, 1988), products derived from damage to tryptophan residues. Intriguingly, such fluorescence has been detected in IgG isolated from fresh samples of rheumatoid synovial fluids and sera (Blake *et al.*, 1989).

Alpha-1-antiprotease (α_1-AP) limits tissue damage arising from the actions of the leucocyte protease, elastase (Carrell and Travis, 1985), and there is much evidence available for the oxidative inactivation of this protein by oxygen-derived free-radical species and hypochlorous acid/hypochlorite anion ($HOCl/OCl^-$). The mechanism of this inactivation appears to involve the oxidation of a critical methionine residue (Met-358) to its corresponding sulphoxide and methionine sulphoxide has been detected in α_1-AP samples isolated from the lungs of cigarette smokers (Carp *et al.*, 1982) and rheumatoid synovial fluids (Wong and Travis, 1980).

Further oxidative modifications of proteins include the transformation of histidine to asparaginyl or aspartyl residues, and the conversion of proline and arginine to pyroglutamate or glutamic semialdehyde. Such oxidations have recently been shown to give rise to the conversion of amino acid side chains to carbonyl derivatives. Hence, the carbonyl content of proteins serves to provide useful information regarding the level of oxidative damage to macromolecules in biological systems and a suitable assay system has been developed. Briefly, biofluid proteins are precipitated with 10% (w/v) trichloroacetic acid and any carbonyl groups present reacted with 2,4-dinitrophenylhydrazine (2,4-DNPH). Subsequent to further protein precipitation and removal of excess 2,4-DNPH, the protein precipitate is solubilized in 6 mol/dm^3 guanidine hydrochloride, the mixture centrifuged, and the supernatant removed for spectrophotometric detection and measurement of 2,4-dinitrophenylhydrazones (λ_{max} 365 nm). The concentrations of protein carbonyl adducts have been shown to increase with age (Oliver *et al.*, 1987) and in animals exposed to 100% O_2 (Starke *et al.*, 1987). Moreover, Chapman *et al.* (1989) have reported an elevated carbonyl content of proteins in knee-joint synovial fluids obtained from patients with rheumatoid arthritis.

4. *DNA Damage*

DNA is an important target for attack by $^{\cdot}OH$ and possibly other (less reactive) radicals, and the identification and quantification of "unnatural" species produced in this manner serves as a useful index of "oxidative stress" in biological systems. Implication of oxygen-derived free-radical species as agents damaging DNA has been achieved by the determination of unique patterns of products that are known to arise from the actions of specified oxidants, i.e. the so-called "fingerprint" approach (Halliwell and Aruoma, 1992). Such studies have focused on radical-mediated oxidative damage to the purine and pyrimidine bases of this macromolecule, since products arising from oxidation of the 2-deoxyribose moiety are much less chemically distinctive (Von Sonntag, 1987).

Although physiologically relevant concentrations of $O_2^{\cdot-}$ and H_2O_2 do not themselves induce any base damage in DNA, the $^{\cdot}OH$ radical is highly reactive towards all four DNA bases (adenine, guanine, cytosine and thymine), giving rise to an extensive range of products (Dizdaroglu, 1991). For example, the addition of $^{\cdot}OH$ radical to guanine residues at the C4, C5 and C8 positions generates hydroxyguanine radicals, and that derived from attack at the C8 position can be oxidized to 8-hydroxyguanine (8-OH-Gua), reduced to 8-hydroxy-7,8-dihydroguanine, or can undergo ring opening followed by single-electron reduction and protonation to yield 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). In a similar manner, the $^{\cdot}OH$ radical can add on to the C4, C5 or C8 positions of adenine, and the fate of the resulting C8 hydroxyadenine radical includes conversion to 8-hydroxyadenine (8-OH-Ade) via oxidation, or ring opening followed by single-electron reduction to generate 5-formamido-4,6-diaminopyrimidine (FapyAde). The attack of $^{\cdot}OH$ radical on pyrimidines yields a multitude of products: e.g. 5-hydroxy-5-methylhydantoin, 5,6-dihydrothymine, 5-hydroxymethyluracil and *cis*- and *trans*-thymine glycols from thymine; and cytosine glycol and 5,6-dihydroxycytosine from cytosine. Interestingly, generation of an $^{\cdot}OH$ radical within whole cells or isolated chromatin can result in the formation of cross-links between DNA bases and the amino acid residues of nuclear proteins. Indeed, thymine–tyrosine, thymine–aliphatic amino acid, and cytosine–tyrosine cross-links have been detected in isolated calf thymus chromatin after exposure to a source of γ-radiation (Dizdaroglu, 1991).

Fortunately, the unique pattern of base-damage products generated appear to be restricted to the actions of $^{\cdot}OH$ radical. Although the reaction of singlet oxygen ($^1O_2$) with DNA gives rise to limited strand breakage (unlike $^{\cdot}OH$ radical, which causes a high level of both single- and double-strand breakages), and the production of small amounts of 8-OH-Gua and

FapyGua from isolated DNA, no further chemical modifications to the purine and pyrimidine bases have been observed (Halliwell and Aruoma, 1992).

Gas chromatography coupled with mass spectrometric detection is a technique that provides a complete characterization of oxygen radical-mediated oxidative damage to DNA and can be applied to DNA–protein complexes (e.g. chromatin) as well as DNA itself. Primarily, samples are hydrolysed and the products separated by gas chromatography and then identified by mass spectrometry. Operation of the mass spectrometric detector in the selected ion monitoring (SIM) mode yields a high level of detection sensitivity. This technique has recently been employed to detect patterns of products characteristic of $^{\cdot}OH$ radical attack in murine hybridoma cell DNA pretreated with H_2O_2 (Dizdaroglu, 1991), and in DNA from primate tracheal epithelial cells exposed to ozone or cigarette smoke (Halliwell and Aruoma, 1992). It has also been utilized to solve a number of intriguing biochemical problems such as the nature of "site-directed" damage to DNA by mixtures of copper(II) with H_2O_2 and $O_2^{\cdot-}$ or ascorbate (Aruoma *et al.*, 1991), and the mechanism of DNA cleavage by the antitumour antibiotic bleomycin in the presence of O_2, iron(III) and ascorbate, or iron(III) and H_2O_2 (Gajewski *et al.*, 1991).

High-performance liquid chromatography (HPLC) coupled with highly sensitive amperometric detection has also been employed to measure products arising from free radical attack on DNA bases, and the determination of 8-hydroxydeoxyguanosine in this manner has been extensively utilized to provide information regarding DNA damage in intact cells and whole organisms (Ames, 1989; Floyd, 1990; Fraga *et al.*, 1990). Indeed, the 8-OH-Gua content of DNA present in numerous cell types has been reported to increase following their exposure to episodes of "oxidative stress" (Kasai *et al.*, 1986; Floyd, 1990). However, much caution must be exercised when interpreting such data since there are mechanisms of forming 8-OH-Gua DNA that may not involve oxygen-derived free-radical species (Kohda *et al.*, 1987). Further, as outlined above, determination of only a single DNA base-damage product is only of a limited value when attempting to correlate such measurements with the level of $^{\cdot}OH$ radical activity *in vivo* (Halliwell and Aruoma, 1991)

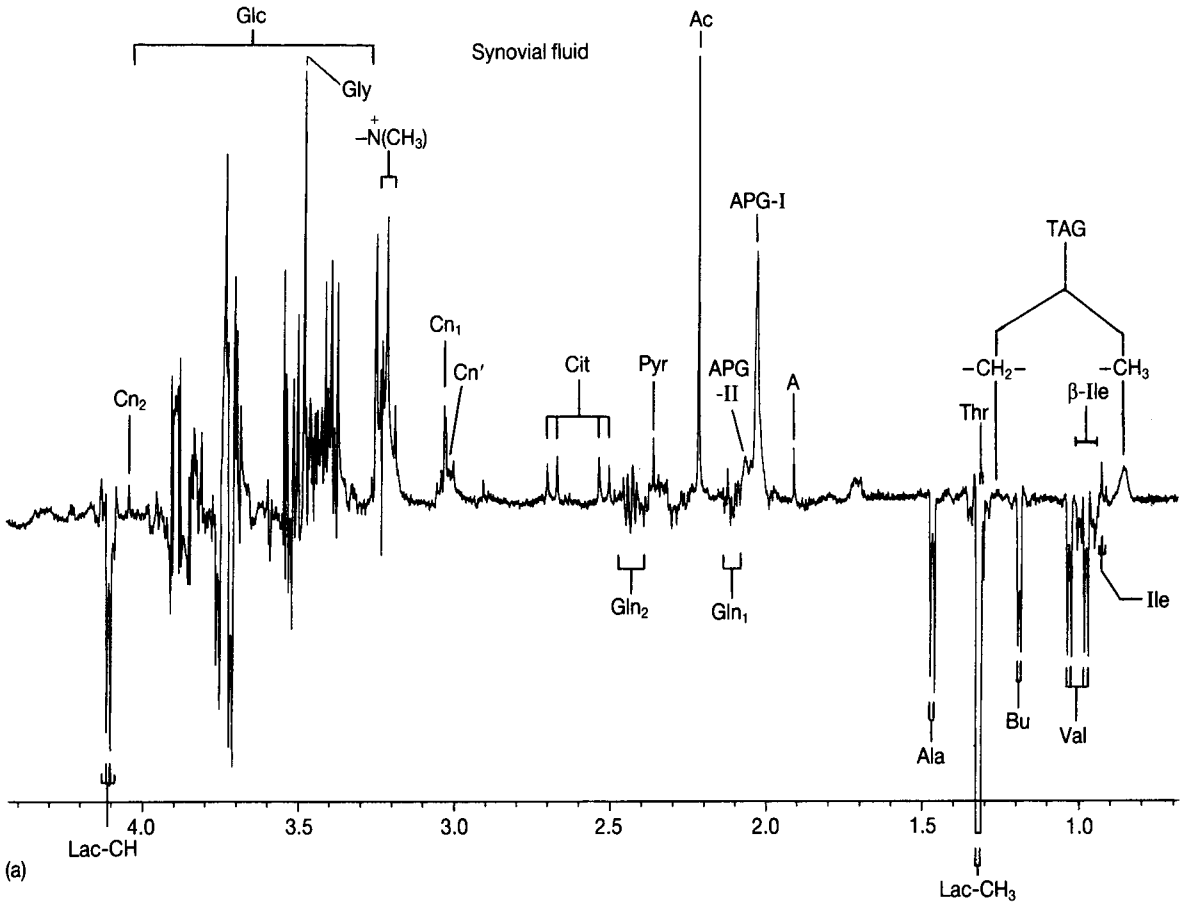
5. *Oxidative Damage to Carbohydrates*

Attack of the $^{\cdot}OH$ radical on carbohydrates of low molecular mass gives rise to a variety of products. Indeed, the reaction of radiolytically-generated $^{\cdot}OH$ radical with lower hexose sugars produces lower saccharides (for di- and higher saccharide species), uronic and aldonic acids, and 3-, 2- and 1-carbon aldehydic fragments, e.g. malondialdehyde (MDA) from deoxysugars such as 2-deoxyribose (Phillips, 1972). Moreover, formate and CO_2 are generated in the final stages of the $^{\cdot}OH$ radical-mediated oxidative degradation of simple mono- and disaccharide species (Phillips and Moody, 1960a, 1960b). Grootveld *et al.* (1990, 1991) have previously employed high-resolution proton (^1H) NMR spectroscopy to ascertain the nature and levels of such products arising from the exposure of various carbohydrate species of low molecular mass to $^{\cdot}OH$ radical fluxes in chemical model systems.

The glycosaminoglycan hyaluronate is a linear repeating disaccharide, β-D-glucuronyl-β-D-*N*-acetylglucosamine, which has a high molecular mass ($>4\times10^6$ Da) and is a major component of the proteoglycan aggregates required for the functional integrity of extracellular matrices such as articular cartilage (Laurent, 1982).

Hyaluronate is continually secreted in its unaggregated form by the type II synoviocytes and is largely responsible for the very high viscosity of knee-joint synovial fluid (Balazs, 1982). It has been demonstrated that in patients with rheumatoid arthritis or alternative inflammatory joint diseases, synovial fluid hyaluronate is depolymerized with an associated modification in the viscoelastic properties of this biofluid (Kofoed and Barcelo, 1978). This fragmentation has been attributed to the oxidizing actions of reactive oxygen radical species, since (1) hyaluronidase activity is absent from both normal and inflammatory synovial fluid samples (Greenwald and Moak, 1986), and (2) there is currently a wealth of experimental evidence that implicates the involvement of oxygen-derived free-radical species in the pathogenesis of inflammatory joint diseases (Rowley *et al.*, 1984; Blake *et al.*, 1989).

Methods using high-resolution ^1H-NMR spectroscopy have been developed to characterize products arising from oxygen radical-mediated oxidative damage to hyaluronate (Grootveld *et al.*, 1991). This powerful multicomponent analytical technique has the advantage of allowing a rapid, detailed and simultaneous analysis of a wide range of biomolecules present in complex biofluids with minimal manipulation of the sample. Attack of radiolytically-generated $^{\cdot}OH$ radical on pure hyaluronate *in vitro* gave rise to the production of formate and oligosaccharide species of low molecular mass (*c.* 10^3 Da). Proton ^1H-NMR analysis has also shown that such hyaluronate-derived oligosaccharides are present in certain synovial fluid samples obtained from patients with rheumatoid arthritis. Both hyaluronate saccharide fragments and formate are either generated or increased in concentration after γ-radiolysis (5.00 kGy) of inflammatory synovial fluid samples (Fig. 1.2). However, the only carbohydrate-derived product detectable in γ-irradiated normal serum samples was formate, which arises from $^{\cdot}OH$ radical-mediated oxidative damage to the low molecular mass sugars present (predominantly glucose).


Glc
Synovial fluid
Ac
Gly
$-\overset{+}{N}(CH_3)$
APG-I
TAG
Cn_1
Cn′
Cit
Pyr
APG
-II
A
$-CH_2-$
$-CH_3$
Thr
β-Ile
Cn_2
Gln_2
Gln_1
Ile
Ala
Bu
Val
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3.5
3.0
2.5
2.0
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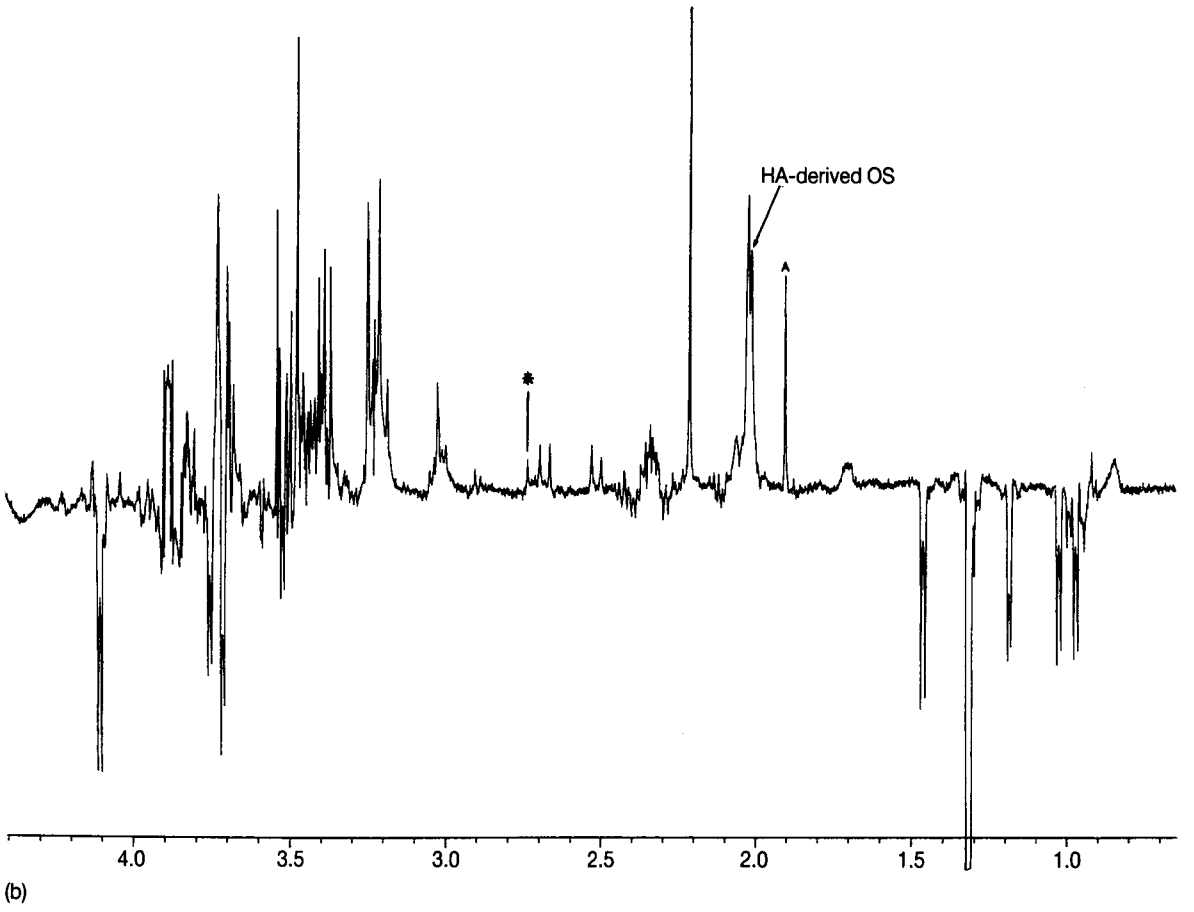
Lac-CH
Lac-CH_3
(a)


HA-derived OS
*
4.0
3.5
3.0
2.5
2.0
1.5
1.0
(b)

6. *Analysis of Products Arising from the Interactions of Reactive Oxygen Species with Further Biomolecules of Low Molecular Mass*

6.1 AROMATIC HYDROXYLATION

Endogenous or exogenous aromatic compounds such as phenols and phenolic acids react extremely rapidly with ·OH radicals to form a mixture of hydroxylated products (Halliwell *et al.*, 1988). Indeed, aromatic hydroxylation serves as an effective method for evaluating ·OH radical activity both *in vitro* (Moorhouse *et al.*, 1985; Grootveld and Halliwell, 1986a) and *in vivo* (Grootveld and Halliwell, 1986b).

If an aromatic compound reacts with an ·OH radical to form a specific set of hydroxylated products that can be accurately identified and quantified in biological samples, and one or more of these products are not identical to naturally occurring hydroxylated species, i.e. not produced by normal metabolic processes, then the identification of these "unnatural" products can be used to monitor ·OH radical activity therein. This is likely to be the case if the aromatic "detector" molecule is present at the sites of ·OH radical generation at concentrations sufficient to compete with any other molecules that might scavenge ·OH radical.

There is a very wide range of aromatic compounds present in living systems, e.g. the amino acids phenylalanine and tyrosine, and catecholamines such as norepinephrine. Although these species are very useful for *in vitro* investigations of ·OH radical generation, their applicability as suitable aromatic detector molecules for ·OH radical *in vivo* largely depends on their concentration (i.e. their ability to compete with alternative scavengers present) and the nature and extent of their chemical modification (hydroxylation and/or decarboxylation) following reaction with ·OH radical. For example, the attack of an ·OH radical upon salicylate (administered as *O*-acetylsalicylate, aspirin) produces three products. The major products are 2,3-dihydroxybenzoate (49%) and 2,5-dihydroxybenzoate (40%), but a small quantity of catechol (11%) is also formed via a decarboxylation process (Grootveld and Halliwell, 1986b) (Fig. 1.3). Although identification and quantification of the 2,3-dihydroxybenzoate isomer may serve as a useful indicator of ·OH radical activity in the blood plasma and/or knee-joint synovial fluid of subjects treated with sufficient doses of aspirin, measurement of catechol is of no practical use in view of its known occurrence in a large number of foodstuffs of plant origin.

The resulting "unnatural" hydroxylated aromatic species are readily separated from other phenolic constituents present in appropriate extracts of biofluids by reversed-phase HPLC, and are detected and quantified by electrochemical oxidation at a glassy carbon or gold working electrode. The technique has the necessary selectivity and sensitivity required to establish a suitable system for the detection of ·OH radical activity. The methodology involves the prior isolation of acidic, neutral and basic phenolic species into one or more groups using extraction into organic solvents such as diethyl ether or ethyl acetate at a controlled pH. After removal of the organic solvent by evaporation to dryness, the extracts are reconstituted in a suitable aqueous medium and then subjected to analysis by reversed-phase HPLC. Initial identity assignments of the peaks in the resulting chromatograms are obtained by direct comparison of retention time values of sample components with those of authentic compounds. However, it must be emphasized that in view of the large number of diethyl ether- or ethyl acetate-soluble species likely to be present in biological

Figure 1.2 High-field (aliphatic) region of 500 MHz ^{1}H Hahn spin-echo NMR spectra of (a) a typical inflammatory synovial fluid sample; (b) as (a) but following γ-radiolysis (5.00 kGy). Typical spectra are shown. A, acetate-CH_3; Ac, acetone-CH_3; Ala, alanine-CH_3; APG-I and II; *N*-acetyl sugars present in the 5,5′ and 2,7 positions, respectively, of the molecularly mobile carbohydrate side chains of "acute-phase" glycoproteins (predominantly α_1-acid glycoprotein); Bu, 3-D-hydroxybutyrate-CH_3; Cit, citrate-CH_2; Cn_1 and Cn_2, creatinine-N-CH_3 and -CH_2 groups, respectively; Cn′, creatine-N-CH_3; Glc, glucose carbohydrate ring proton resonances; Gln_1, and Gln_2, β- and γ-CH_2 groups of glutamine, respectively; Gly, glycine-CH_2; HA-derived OS, *N*-acetyl-CH_3 groups of *N*-acetylglucosamine residues present in oligosaccharide fragments arising from the radiolytic depolymerization of hyaluronate; Ile and β-Ile, isoleucine terminal-CH_3 and β-CH_3 groups, respectively; Lac-CH_3 and Lac-CH, lactate-CH_3 and -CH groups; -$\overset{+}{N}(CH_3)_3$, -$\overset{+}{N}(CH_3)_3$ groups of betaine, carnitine and choline; Pyr: pyruvate-CH_3; TAG-CH_3 and TAG-CH_2-, acyl chain terminal-CH_3 and bulk $(-CH_2-)_n$ groups, respectively, of fatty acids (predominantly triacylglycerols) associated with chylomicron- and very low-density lipoprotein (VLDL); Thr, threonine-CH_3; Val, valine-CH_3. The asterisk in spectrum (b) denotes a radiolytically-generated 2.74 p.p.m. singlet resonance.

The intense water signal and the broad protein resonances were suppressed by a combination of continuous secondary irradiation at the water frequency and the Hahn spin-echo sequence (D[90°x-t-180°y-t-collect]). Chemical shift values were referenced to external sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propionate (δ = 0.00 p.p.m.).

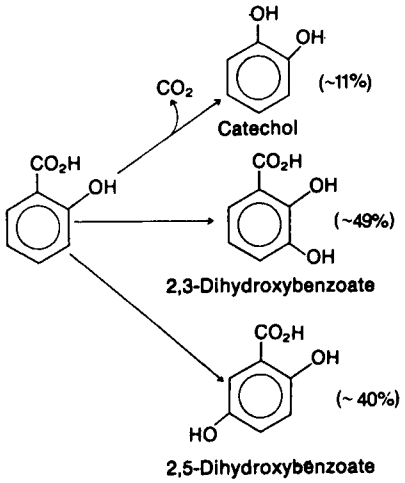


Figure 1.3 Products arising from the attack of 'OH radical on salicylate (2-hydroxybenzoate). Generation of 'OH was conducted in phosphate buffer, pH 7.4 (2.00×10^{-2} mol/dm^3), using H_2O_2 (3.30×10^{-3} mol/dm^3) and Fe(II) (aqueous) (as $FeSO_4$, 1.00×10^{-3} mol/dm^3), made up fresh immediately prior to use, in the presence of salicylate (1.00×10^{-3} mol/dm^3). The concentrations quoted are the final concentrations of each component present in the reaction mixture.

matrices, a retention time is insufficient evidence to attribute an observed peak to a suspected component. The identity of putative peaks can be confirmed in three ways. Firstly, an aliquot of the reconstituted extract is "spiked" with the suspected component at a concentration that is exactly equal to that estimated from its peak height or area in the chromatogram. If the identity of the putative peak is the same as that of the suspected component, then it will remain perfectly symmetrical and increase in height or area by a factor of two. Secondly, the electrochemical detector can be employed to obtain a voltammetric characterization of eluting species. Comparisons of the current–potential responses (plots of peak height or area versus electrochemical detector potential, known as hydrodynamic voltammograms) of sample components and authentic standards provides further confirmation of the initial identity assignment. Thirdly, the HPLC eluant composition can be varied (for example, by including different proportions of methanol or other solvent) to produce substantial modifications in retention times. If a putative peak present in a chromatogram of the extract has the same identity as a suspected component, the variable retention times observed for it will always be identical to those given by an authentic standard.

One compound that has been quite widely used as an aromatic detector molecule *in vitro* is the naturally occurring amino acid, phenylalanine (Ishimitsu *et al.*, 1984). However, human blood and tissue levels of this amino acid are normally too low for it to scavenge enough of the 'OH radical thought to be formed *in vivo*, and hence its application as a detector in living systems has been limited. Attack of 'OH radical on phenylalanine produces three products, *o*-, *m*-, and *p*-tyrosine (Kaur *et al.*, 1988). Based on the assumption that of these three products, only the *p*-isomer is a naturally occurring species present in foodstuffs (i.e. those containing high levels of protein and/or free amino acids), a test for determining their irradiation status which involves measurement of the "unnatural" amino acids *o*- and *m*-tyrosine using gas chromatography coupled with mass spectrometric detection has been developed (Karam and Simic, 1986). Unfortunately, some *o*- and *m*-tyrosine has been detected in samples collected from healthy humans and animals, but this is not totally unexpected in view of the known production of these so-called "unnatural" species in animal tissues by the action of the enzyme tyrosine hydroxylase (Ishimitsu *et al.*, 1986).

Further aromatic detector molecules that have been used for the detection of 'OH radicals in *in vitro* model systems include benzoate (release of $^{14}CO_2$ from the carboxyl-labelled molecule) (Sagone *et al.*, 1980), *p*-nitrosodimethylaniline (bleaching of its yellow colour) (Bors *et al.*, 1979), tryptophan (producing a characteristic pattern of products which differs from that arising from reaction with singlet O_2) (Singh *et al.*, 1981), phenylamine (generating *o*- and *p*- aminophenols) (Ingelman-Sundberg and Ekstrom, 1982), 4-nitrophenol (spectrophotometric detection of chromophoric 4-nitrocatechol) (Florence, 1984) and dopamine (producing 2-, 5- and 6-hydroxydopamines in the approximate ratio of 3 : 2 : 1; analysis by HPLC coupled with electrochemical detection) (Slivka and Cohen, 1985).

Intriguingly, Naughton *et al.* (1993) have utilized the principles of aromatic hydroxylation to develop a novel fibre-optic 'OH radical sensor. An 'OH radical-sensitive reagent phase containing 4-nitrophenol was immobilized on to XAD-7 methacrylate beads, which were then attached to the distal end of a polymethyl methacrylate fibre optic. 4-Nitrocatechol arising from the attack of 'OH radicals on the nitrophenol "target" molecule was detected by reflectance spectroscopy at a wavelength of 510 nm. The sensor exhibited excellent stability and linearity of response to an 'OH radical flux generated by a Fenton reaction system (Fe(II), EDTA and H_2O_2). The lower detection limit of 'OH radical concentration was that generated from a Fenton system containing only 10^{-7} mol/dm^3 H_2O_2. However, the *in vivo* applications of this sensor (i.e. for clinical research and diagnostic purposes) remain to be ascertained.

6.2 HIGH-RESOLUTION ^{1}H-NMR SPECTROSCOPY

The recent development of high-field NMR spectrometers with increased resolution, dynamic range and

sensitivity has permitted the rapid and simultaneous determination of a wide range of components present in biological samples or alternative complex multicomponent systems. The technique is generally noninvasive, since it involves only minimal sample preparation prior to analysis, and previous high-resolution proton (^{1}H) NMR investigations of human and animal biofluids (e.g. blood plasma, urine and knee-joint synovial fluid), cell culture media and perchloric acid ($HClO_4$) extracts of cultured cells have provided much useful biochemical and clinical information. Moreover, the multicomponent analytical ability of high-field NMR analysis offers many advantages over alternative methods, since chemical shifts, coupling patterns and coupling constants of resonances detectable in spectra of such samples offer much valuable information regarding the structures of biomolecules present. The broad overlapping resonances that arise from any macromolecules present in untreated samples are routinely suppressed by the application of spin-echo pulse sequences, resulting in spectra that contain many well-resolved, sharp signals attributable to a variety of low molecular mass metabolites together with the mobile portions of macromolecules (typically >40 metabolites per spectrum of human plasma at an operating frequency of 600 MHz).

Although there is currently a wide diversity of literature available dealing with the nature, mechanisms and extent of reactive oxygen species-mediated oxidative damage to various biomolecules (e.g. DNA and polynucleotides, proteins, carbohydrates and lipids), such studies are only of limited value since they involve assessments of the reactions of selected reactive oxygen species with isolated, single-component chemical model systems. However, high-resolution NMR spectroscopy has the capacity to evaluate simultaneously oxidative damage to a wide range of endogenous (or, where appropriate, exogenous) components present in intact biofluids or tissue sample extracts, providing much useful molecular information regarding the relative radical- or H_2O_2-scavenging activities of antioxidants therein.

Grootveld *et al.* (1994) employed this technique to investigate radiolytic, damage to biomolecules present in human body fluids. γ-Radiolysis of healthy or rheumatoid human serum (5.00 kGy) in the presence of atmospheric O_2 gave rise to reproducible elevations in the concentration of NMR-detectable acetate, which are predominantly ascribable to the prior oxidation of lactate to pyruvate by $^{\cdot}OH$ radical followed by oxidative decarboxylation of pyruvate by radiolytically generated H_2O_2 and/or further $^{\cdot}OH$ radicals (Equations 1.7 and 1.8).

$$\underset{\text{Lactate}}{CH_3CHOHCO_2^-} \xrightarrow{(^{\cdot}OH)} \underset{\text{Pyruvate}}{CH_3COCO_2^-} \quad (1.7)$$

$$\underset{\text{Pyruvate}}{CH_3COCO_2^-} \xrightarrow{(^{\cdot}OH/H_2O_2)} \underset{\text{Acetate}}{CH_3CO_2^-} + CO_2 \quad (1.8)$$

Typical spectra obtained are shown in Fig. 1.2. Moreover, substantial radiolytically-mediated elevations in the concentration of serum formate, arising from the oxidation of carbohydrates present by $^{\cdot}OH$ radical, were also detectable. In addition to the above modifications, γ-radiolysis of inflammatory knee-joint synovial fluid generated an oligosaccharide species of low molecular mass derived from the radiolytic fragmentation of hyaluronate as outlined in the previous section dealing with oxidative damage to carbohydrates. The radiolytically-mediated production of acetate in synovial fluid samples was markedly greater than that observed in serum samples, a consequence of the much higher levels of $^{\cdot}OH$ radical-scavenging lactate present. Indeed, increases in synovial fluid acetate concentration were detectable at doses as low as 48 Gy. In most of the biofluids examined, a singlet resonance located at 5.40 p.p.m., attributable to the single NMR-detectable proton of allantoin, was produced following γ-radiolysis, consistent with the ability of endogenous urate to scavenge $^{\cdot}OH$ radical as previously suggested by Grootveld and Halliwell (1987).

Hence, high-field ^{1}H-NMR analysis provides much useful information regarding the relative radioprotective abilities of endogenous components and the nature, status and levels of radiolytic products generated in intact biofluids. Interestingly, NMR-detectable radiolytic products with associated toxicological properties (e.g. formate) may play an important role in contributing to the deleterious effects observed following exposure of living organisms to sources of ionizing radiation.

In a further study, Herz *et al.* (1994) utilized high-field proton (^{1}H) NMR spectroscopy to assess simultaneously the hydrogen peroxide-scavenging antioxidant capacities of a range of polar, water-soluble endogenous metabolites of low molecular mass present in healthy human blood serum and inflammatory knee-joint synovial fluid. ^{1}H-NMR analysis demonstrated that prolonged equilibration (24 h) of normal serum ultrafiltrates with 2.00×10^{-4} mol/dm^3 H_2O_2 gave rise to the oxidative decarboxylation of pyruvate to acetate (Fig. 1.4), the rise in acetate concentration being quantitatively accounted for by the reduction in that of pyruvate for all samples investigated. Approximately one half of the synovial fluid ultrafiltrate samples examined also showed an increase in acetate concentration that was equivalent to the decrease in that of pyruvate following treatment with 2.00×10^{-4} mol/dm^3 H_2O_2 in the above manner. However, for *c.* 35% of the synovial fluid ultrafiltrates the increase in acetate concentration was greater than the reduction in that of pyruvate observed, results indicating that certain of these samples contain a "catalytic" source with the ability to promote the production of hydroxyl radical ($^{\cdot}OH$) from added H_2O_2, consistent with previous reports by Rowley *et al.* (1984) and Gutteridge (1987) that approximately 40% of inflammatory synovial fluids contain trace levels of bleomycin-detectable iron complexes with the

capacity to generate 'OH radicals from phagocytically-generated H_2O_2. Indeed, the elevation in acetate concentration, which cannot be accounted for by the direct reaction of the added H_2O_2 with pyruvate observed, appears to arise from the consecutive two-step reaction sequence detailed in Equations 1.7 and 1.8.

As expected, treatment of lactate with H_2O_2 in aqueous solution did not give rise to any NMR-detectable products. However, exposure of lactate to sources of 'OH radical generated pyruvate, and subsequently acetate and CO_2, providing evidence for its powerful 'OH radical scavenging ability. In view of the high levels of lactate present in inflammatory synovial fluid and the extremely high second-order rate constant

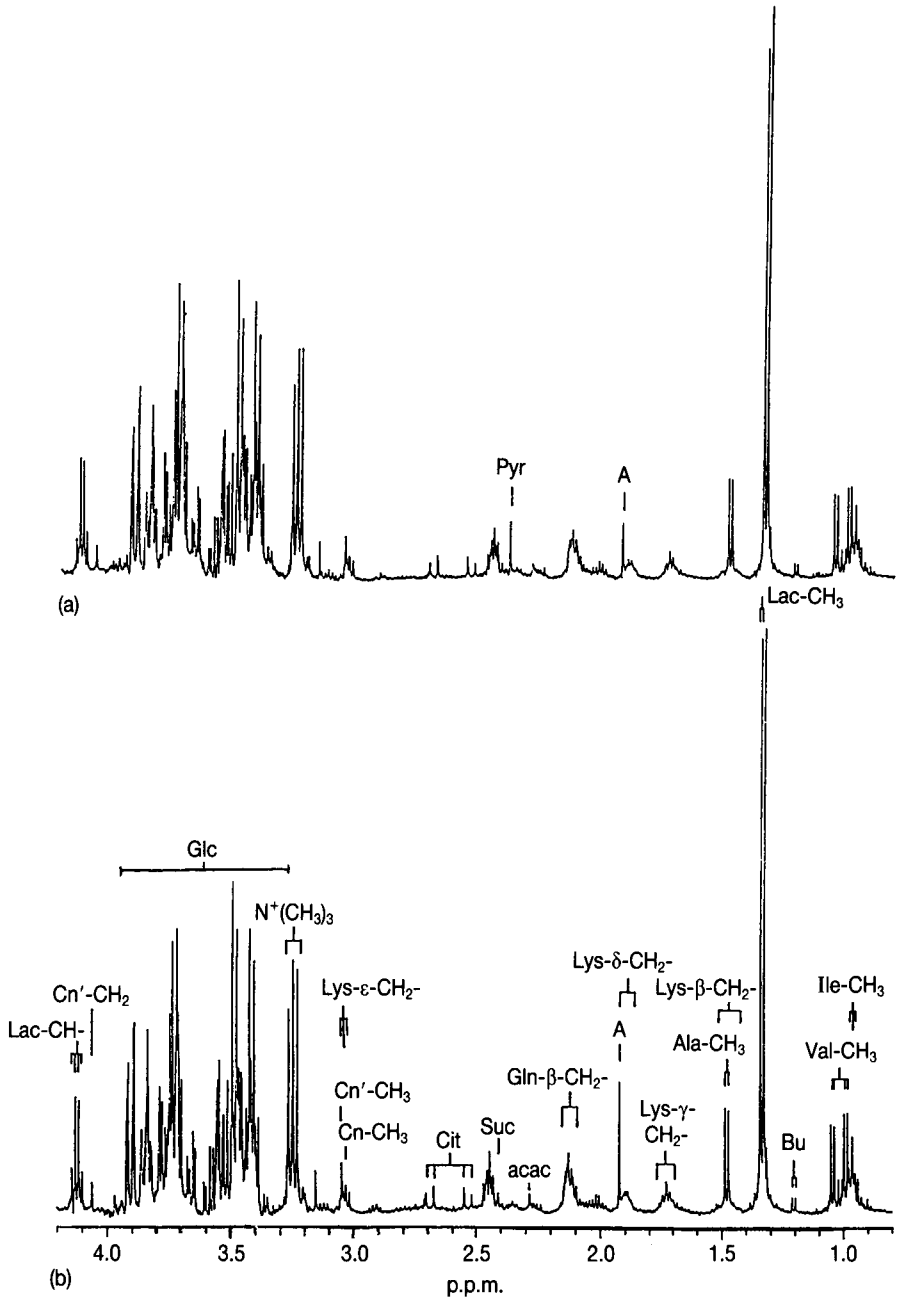


Figure 1.4 High-field (aliphatic) regions of 500 MHz single-pulse ^{1}H-NMR spectra of an inflammatory synovial fluid ultrafiltrate sample obtained before (a) and after equilibration (b) with 2.00×10^{-4} mol/dm^3 H_2O_2 at ambient temperature for a period of 24 h. Typical spectra are shown. For abbreviations, see Fig. 1.2 with acac, acetoacetate–CH_3; Lys, lysine.

for its reaction with $^{\cdot}OH$ radical [4.8×10^9 mol/dm^3/s), Anbar and Neta (1967)], this scavenger may play an important role in neutralizing the toxic effects of $^{\cdot}OH$ radicals arising from any residual H_2O_2 (i.e. that which escapes consumption by pyruvate) in this matrix.

Exposure of the amino acid methionine to H_2O_2 or an $^{\cdot}OH$ radical flux generates methionine sulphoxide as a major product, and Stevens *et al.* (1992) have recently demonstrated the applications of high-field ^{1}H-NMR analysis to the detection of methionine sulphoxide in neutrophil- or endothelial cell-conditioned culture media subsequent to stimulation with phorbol esters (Fig. 1.5). These data have supplied valuable information regarding (1) the nature and levels of reactive oxygen species

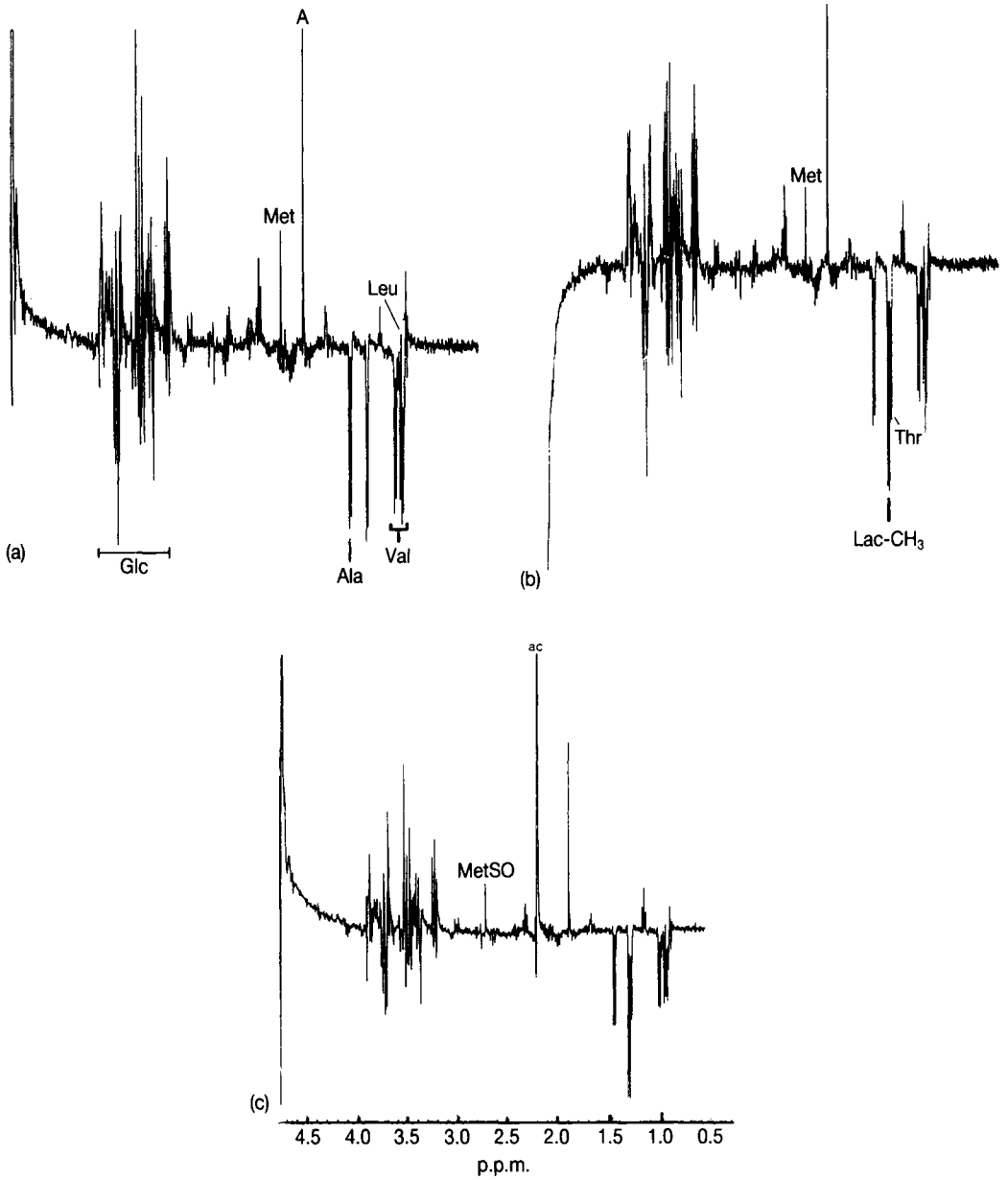
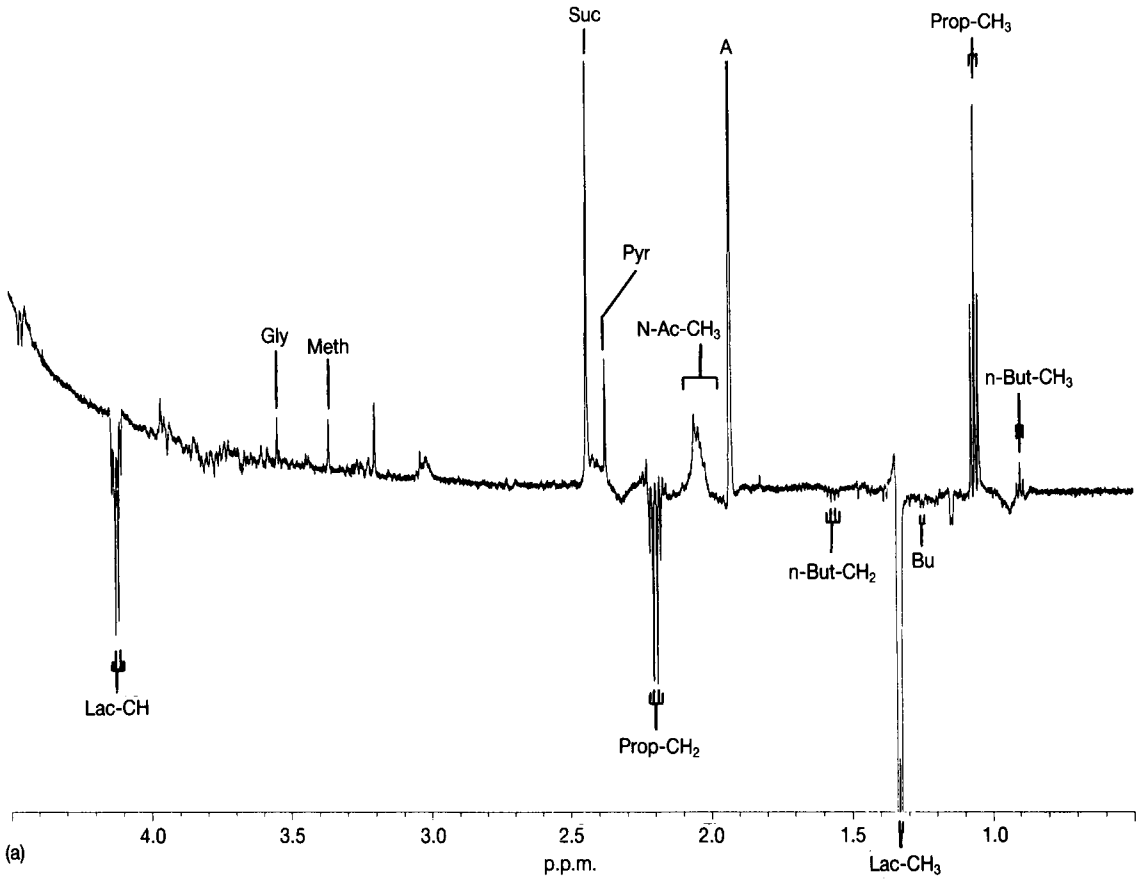


Figure 1.5 High-field (aliphatic) regions of 400 MHz ^{1}H Hahn spin-echo NMR spectra of: (a) E-199 culture medium; (b) as (a) but following a 2 h incubation with neutrophils at 37°C; (c) as (b) but incubated in the presence of 1.00×10^{-3} mol/dm^3 phorbol 12-myristate 13-acetate (PMA). For abbreviations, see Fig. 1.2 with Met, methionine-S-C$\underline{H}_3$ group resonance; MetSO, methionine sulphoxide-SO-C$\underline{H}_3$ group resonance. The 2.245 p.p.m. singlet detectable in spectrum (c) arises from the -CH_3 groups of acetone, the solvent in which PMA was solubilized. Leu: leucine-CH_3.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Prop-CH3
Pyr
N-Ac-CH3
Gly
Meth
n-But-CH3
n-But-CH2
Bu
Lac-CH
Prop-CH2
4.0
3.5
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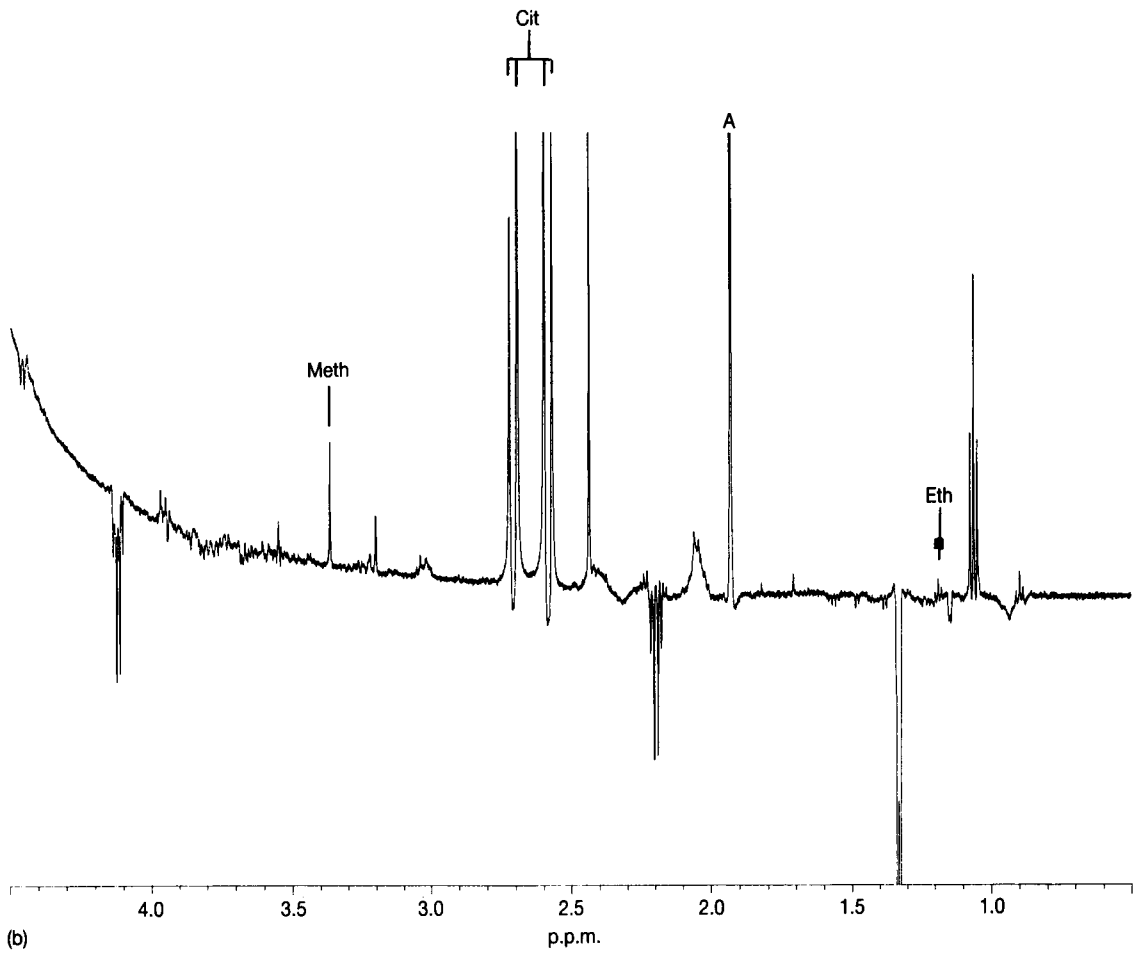
2.0
1.5
1.0
(a)
p.p.m.
Lac-CH3


Cit
A
Meth
Eth
4.0
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(b)
p.p.m.

generation by these cells, and (2) the relative susceptibility of culture medium components to oxidative damage.

Intriguingly, the therapeutic applications of the oxidizing free radical chlorine dioxide ($ClO_2^{\cdot}$, stabilized via an effective delocalization of its unpaired electron) to periodontal diseases such as marginal gingivitis and halitosis has recently attracted much interest from free-radical chemists and biochemists engaged in clinical research. Indeed, a commercially available oral rinse preparation containing an admixture of this stable free-radical species with chlorite anion (predominantly the latter) has been formulated. Chlorite anion (ClO_2^-) has the capacity to generate further $ClO_2^{\cdot}$ at pH values that reflect those of the acidotic oral environment, a process occurring via a reaction sequence that involves the disproportionation of chlorous acid (Equations 1.9 and 1.10).

$$ClO_2^- + H^+ \rightleftharpoons HClO_2 \quad (1.9)$$

$$4HClO_2 \rightarrow 2ClO_2^{\cdot} + ClO_3^- + Cl^- + 2H^+ + H_2O \quad (1.10)$$

We have recently applied high-resolution ^{1}H-NMR spectroscopy to demonstrate the oxidative consumption of salivary pyruvate by this product (Fig. 1.6), a reaction consistent with Equation 1.11 (Lynch *et al.*, unpublished data).

$$4CH_3COCO_2^- + 2ClO_2^{\cdot} \rightarrow 4CH_3CO_2^- + 4CO_2 + Cl_2 \quad (1.11)$$

However, at pH values closer to neutrality (i.e. at the mean salivary pH of 5.97), chlorite anion itself can effect the oxidative decarboxylation of pyruvate to acetate and CO_2 (Equation 1.12).

$$CH_3COCO_2^- + ClO_2^- \rightarrow CH_3CO_2^- + CO_2 + OCl^- \quad (1.12)$$

Although NMR spectrometers of operating frequencies > 400 MHz are costly and require specialist technical support staff, the technique provides a broad "picture" of the chemical modifications arising from the reactions of free radicals or related oxidants in complex, multicomponent systems such as intact biofluids, tissue sample extracts or foodstuffs, and hence enables the analyst to decide which products (i.e. "marker" molecules) to determine by cheaper, alternative techniques where required. The sensitivity of the spectroscopic technique can be improved by a factor of 100-fold or greater by the application of preconcentration procedures (e.g. solid-phase or organic solvent extraction, where appropriate) prior to analysis.

The applications of high-resolution ^{1}H-NMR spectroscopy to the analysis of products arising from the lipid peroxidation process are described in Section 7.

7. *Detection of Products Arising from the Radical-mediated Peroxidation of Polyunsaturated Fatty Acids*

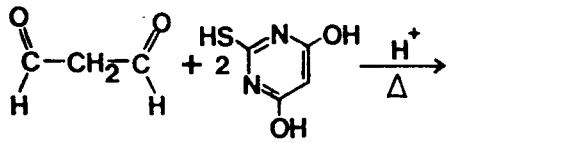
The peroxidation of polyunsaturated fatty acids (PUFAs) is an autocatalytic, self-propagating reaction that can give rise to cell dysfunction associated with a loss of membrane function and integrity. This process has been implicated in a wide variety of pathological conditions including cardiac and cerebral ischaemic/reperfusion injury, and inflammatory joint diseases amongst others [reviewed in Gutteridge (1988)]. Peroxidation of the PUFA component of LDLs and the subsequent production of foam cells from macrophages appear to play a critical role in the pathogenesis of atherosclerosis (Steinberg *et al.*, 1989; Witzum and Steinberg, 1991). Initiation of the lipid peroxidation process occurs by the abstraction of a *bis*-allylic methylene group hydrogen atom of a PUFA by radical species of sufficient reactivity (e.g. $^{\cdot}OH$) followed by reaction of the resulting resonance-stabilized carbon-centred pentadienyl lipid radical with O_2 to form a peroxyl radical, which in turn abstracts a hydrogen atom ($H^{\cdot}$) from an adjacent PUFA to produce a conjugated diene lipid hydroperoxide and a further pentadienyl lipid radical species. The peroxidation process is hence self-perpetuating unless terminated by a

Figure 1.6 0.50–4.50 p.p.m. Regions of 600 MHz ^{1}H Hahn spin-echo NMR spectra of a human saliva sample before (a) and after treatment (b) with an equivalent volume of a commercially available oral rinse preparation (RetarDEX®) containing an admixture of chlorine dioxide ($ClO_2^{\cdot}$) and chlorite anion (ClO_2^-). Typical spectra are shown. For abbreviations, see Fig. 1.2, with n-But-CH_3 and $-CH_2$, *n*-butyrate-CH_3 and β-CH_2; Prop-CH_3 and $-CH_2$ propionate-CH_3 and $-CH_2$; N-Ac-CH_3, *N*-acetyl-methyl groups of both high and low molecular mass components (e.g. mobile portions of *N*-acetylated glycoproteins and saccharide fragments derived from the depolymerization of hyaluronate, respectively); Pyr, pyruvate-CH_3; Suc, succinate-CH_2; Meth, methanol-CH_3; Eth, ethanol-CH_3. The oral rinse preparation employed also contained citrate (as a buffering agent), and acetate, methanol and ethanol as trace impurities. Hence the elevations in their ^{1}H-NMR resonance intensities observed in spectrum (b) are ascribable to the addition of this therapeutic agent (the increase in the acetate-CH_3 group signal intensity is also partially attributable to the $ClO_2^{\cdot}/ClO_2^-$-mediated oxidative decarboxylation of pyruvate described in the text). The methanol detectable in spectrum (a) arises from the inhalation of cigarette smoke (direct or passive) by the subject from whom the sample was collected and is generated via the combustion of tobacco lignin, which contains many methoxy functional groups in its complex macromolecular structure.

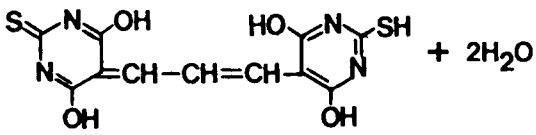
chain-breaking antioxidant. Such antioxidants limit the peroxidation reaction and α-tocopherol (α-TOH), vitamin E, exerts its biological effects via this mechanism. Conjugated hydroperoxydienes are degraded to a wide variety of secondary peroxidation products, which include saturated and unsaturated aldehydes, di- and epoxyaldehydes, lactones, furans, ketones, oxo- and hydroxy acids, and saturated and unsaturated hydrocarbons. Moreover, during the later stages of the peroxidation process, many polymerization reactions occur.

The detection and quantification of one or more of the above lipid peroxidation products (primary and/or secondary) in appropriate biofluids and tissue samples serves to provide indices of lipid peroxidation both *in vitro* and *in vivo*. However, it must be stressed that it is absolutely essential to ensure that the products monitored do not arise artifactually, a very difficult task since parameters such as the availability of "catalytic" trace metal ions and O_2, temperature and exposure to light are all capable of promoting the oxidative deterioration of PUFAs. Indeed, one sensible precaution involves the treatment of samples for analysis with sufficient levels of a chain-breaking antioxidant [for example, butylated hydroxytoluene (BHT)] immediately after collection to retard or prevent peroxidation occurring during periods of storage or preparation.

The simple spectrophotometric thiobarbituric acid (TBA) test has been frequently used for many years as an indicator of the peroxidation of PUFAs present in biological matrices. This test involves the reaction of aldehydes in the sample with TBA at *c.* 100°C under acidic conditions (Equation 1.13) to produce a pink-coloured chromogen, which absorbs light strongly at a wavelength of 532 nm (Nair and Turner, 1984).



MDA TBA



Chromogen (1.13)

Although MDA, an end-product of the lipid peroxidation process is thought to be the major contributor to the chromogen, it has now become clear that a number of other species such as sucrose, urea, proteins and other aldehydes also react with TBA to produce chromogens that absorb at a wavelength near to 532 nm (Marshall *et al.*, 1985). Moreover, it should also be noted that since only a small proportion (1–2%) of the lipid peroxidation end-products is actually MDA, a large amount of the chromogen produced in the TBA test is ascribable to the further degradation of lipid peroxides when heated with acid in the presence of trace amounts of "catalytic" transition metal ions (Sinnhuber *et al.*, 1958; Dahle *et al.*, 1962). A further major problem with this assay system is the thermally induced autoxidation of PUFAs, as outlined below. Hence, in view of its inherent lack of specificity and the high level of problems arising from artefactual peroxidation, the TBA test is of little or no practical value as an index of lipid peroxidation.

A relatively new procedure for monitoring the course of free radical-mediated damage to PUFAs is the detection and quantification of conjugated diene species (i.e. hydroperoxydienes and hydroxydienes) in appropriate extracts of human body fluids or tissues. These species and the conjugated ketodienes arising from their degradation have ultraviolet (UV) maxima at various wavelengths in the 230–270 nm range (Robards *et al.*, 1988; Merry *et al.*, 1991). However, although lipids and their corresponding peroxidation products are readily isolated from biological samples by a simple chloroform extraction process, direct measurement of absorbance at a specified wavelength in this region of the spectrum is of limited utility since the absorption maxima of conjugated diene species appear as a poorly defined shoulder superimposed on the high absorbance of other endogenous components present in the lipid–chloroform extracts. Conjugated diene absorption maxima can be resolved and clearly identified by monitoring minima present in the corresponding second-derivative (2D) spectra of the extracts (Merry *et al.*, 1991) (absorption maxima appear as absorption minima in 2D spectra).

Derivative spectrophotometry is an analytical technique of great utility for obtaining both qualitative and quantitative information from spectral curves composed of unresolved absorption bands. In general, the derivatization procedure discriminates against broad absorption bands while emphasizing sharper features of the spectrum to an extent that increases with increasing derivative order. A new generation of UV-visible spectrophotometers equipped with suitable differentiation units permits the precise recording of derivative spectra of variable order. Higher sensitivity (an increase in the signal to noise ratio) can be achieved by an electronic amplification of the signals with digital smoothing or by a numerical treatment of the recorded spectra.

Second-derivative spectrophotometry has been used to monitor the time-dependent production of *cis, trans*-(λ_{max} 242 nm) and *trans, trans*- (λ_{max} 232 nm) diene conjugates of microsomal PUFAs following the exposure of rats to carbon tetrachloride (CCl_4) (Corongui *et al.*, 1986). These signals have been postulated to be derived from mixtures of peroxidized substrates. Previous studies using chemical model systems have established that autoxidation of linolenic or arachidonic acid results in the production of *cis, trans*- and *trans, trans*-conjugated diene

species that have λmax values of 236 and 232 nm, respectively (Chan and Levett, 1977; Porter *et al.*, 1979).

The concentrations of PUFA-derived conjugated hydroperoxydienes and oxodienes in biological samples can also be determined by a modification of a spectrophotometric method originally developed by Fishwick and Swoboda (1977). This assay system involves: (1) reduction of conjugated hydroperoxydienes and oxodienes to their corresponding hydroxydienes with sodium borohydride ($NaBH_4$); and (2) dehydration of the resulting hydroxydienes to strongly chromophoric triene- and tetraene-containing adducts by a 10% (w/v) solution of sulphuric acid in ethanol. Dienoic PUFA oxidation products (e.g. 9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoic acid) yield a conjugated triene chromophore with intense absorption bands located at 258, 268 and 278 nm, whilst those of trienoic PUFAs (e.g. 9-hydroperoxy-*trans*-10, *cis*-12, *cis*-15-octadecadienoic acid) form a tetraene chromophore (λ_{max} 277, 288, 301.5 and 316 nm). When coupled with 2D spectrophotometric techniques, this method has the ability to distinguish between different classes of peroxidized PUFAs in test samples (i.e. those arising from linoleic and linolenic acid substrates), and pilot studies previously conducted in the laboratory of one of the authors have demonstrated its application to the analysis of human body fluids and tissues (e.g. Fig. 1.7). Since absorption maxima (and corresponding 2D minima) arising from conjugated hydroperoxydienes are shifted from 234 and 242 nm (*trans*-, *trans*- and *cis*, *trans*-isomers, respectively) to the lower energy regions of spectra, any interferences arising from conjugated diene species that are not derived from PUFA peroxidation processes (specifically octada-*cis*-9, *trans*-11-dienoic acid) are avoided by application of this method. Moreover, the concentration of conjugated oxodiene species present in samples can be estimated separately by monitoring the decrease in absorbance at 275 nm observed during the $NaBH_4$ reduction step.

Gas chromatography coupled with mass spectrometric detection has also been applied to the detection of

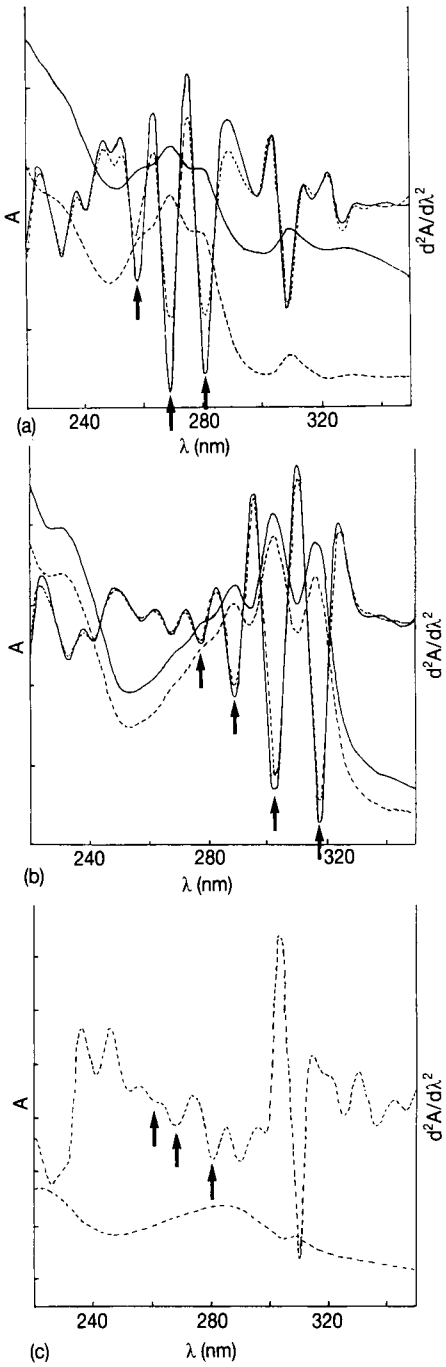
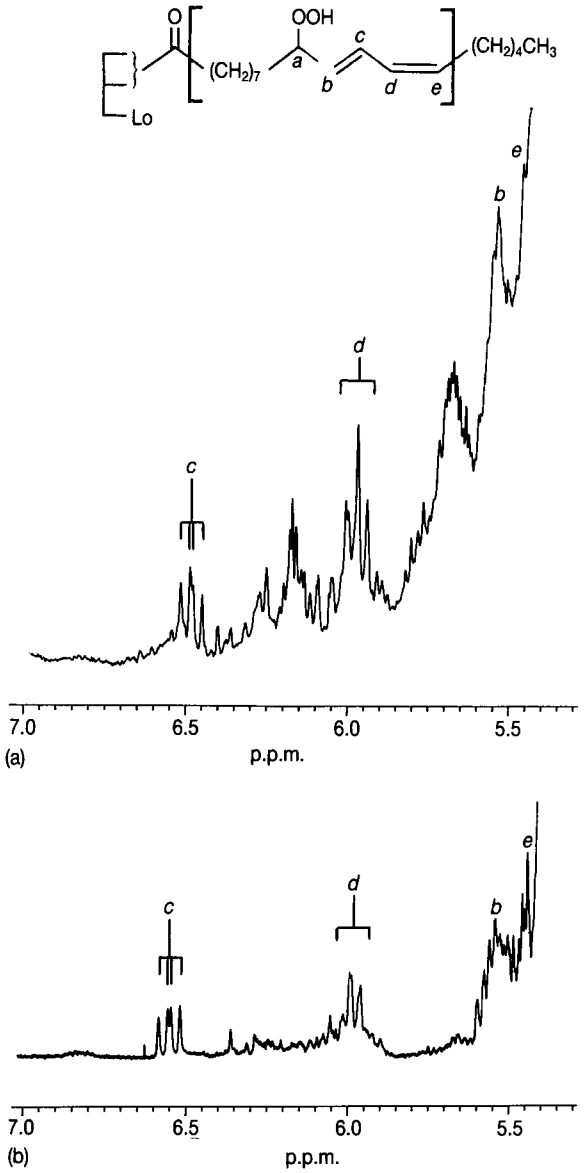


Figure 1.7 Typical zero-order and corresponding second-derivative electronic absorption spectra of ethanol-reconstituted lipid/chloroform extracts of autoxidized model polyunsaturated fatty-acid compounds and inflammatory synovial fluid obtained after (1) reduction with $NaBH_4$ and (2) dehydration with alcoholic H_2SO_4. (a) Methyl linoleate subsequent to autoxidation in air at ambient temperature for a period of 72 h (---), or exposure to a Fenton reaction system containing EDTA (5.75×10^{-3} mol/dm^3), H_2O_2 (1.14×10^{-2} mol/dm^3) and Fe(II) (5.75×10^{-3} mol/dm^3) as an aqueous suspension (—); (b) as (a) but with methyl linolenate; (c) untreated rheumatoid knee-joint synovial fluid.

With the above procedure, dienoic fatty acid peroxidation products yield a conjugated triene chromophore with second-derivative absorption minima located at 258, 269.5 and 281 nm, whilst trienoic fatty-acid peroxidation products give rise to a conjugated tetraene chromophore with minima at 278, 289, 303 and 318 nm. The arrows in (c) denote second-derivative absorption minima corresponding to the conjugated triene adduct arising from linoleate-derived peroxidation products (conjugated hydroperoxydienes, hydroxydienes and oxodienes).

PUFA-derived oxidation products in biological samples. In addition to detecting and quantifying individual conjugated hydroxydiene species, the method also serves to provide useful information concerning the nature and levels of cholesterol/cholesterol ester oxidation products. Indeed, Carpenter *et al.* (1993) have utilized this assay system to determine conjugated hydroperoxy/hydroxydienes, and oxysterols such as (cholest-5-en-3β, 26-diol and cholest-5-en-3β, 7β-diol in samples of human atheroma (necrotic gruel isolated from the interior of advanced atherosclerotic plaques in the aorta). Recent high-resolution ^{1}NMR investigations conducted by one of the authors (MG) have confirmed the presence of isomeric conjugated hydroperoxydiene species in such samples (Fig. 1.8).



Studies conducted by Barenghi *et al.* (1990) and Lodge *et al.* (1993) independently have demonstrated the facile, multicomponent analysis of a wide range of PUFA-derived peroxidation products (e.g. conjugated dienes, epoxides and oxysterols) in samples of oxidized LDL by high-field ^{1}H-NMR spectroscopy. Figure 1.9 shows the applications of this technique to the detection of cholesterol oxidation products (7-ketocholesterol and the 5α,6α and 5β,6β-epoxides) in isolated samples of plasma LDL pretreated with added copper(II) or an admixture of this metal ion with H_2O_2, an experiment conducted in the authors' laboratories.

The identification and quantification of potentially cytotoxic carbonyl compounds (e.g. aldehydes such as pentanal, hexanal, *trans*-2-octenal and 4-hydroxy-*trans*-2-nonenal, and ketones such as propan- and hexan-2-ones) also serves as a useful "marker" of the oxidative deterioration of PUFAs in isolated biological samples and chemical model systems. One method developed utilizes HPLC coupled with spectrophotometric detection and involves precolumn derivatization of peroxidized PUFA-derived aldehydes and alternative carbonyl compounds with 2,4-DNPH followed by separation of the resulting chromophoric 2,4-dinitrophenylhydrazones on a reversed-phase column and spectrophotometric detection at a wavelength of 378 nm. This method has a relatively high level of sensitivity, and has been successfully applied to the analysis of such products in rat hepatocytes and rat liver microsomal suspensions stimulated with carbon tetrachloride or ADP–iron complexes (Poli *et al.*, 1985).

High-resolution ^{1}H-NMR analysis has recently been employed to ascertain the nature and levels of aldehydes arising from the peroxidation of PUFAs. Indeed, chemical shifts, coupling patterns and coupling constants of the aldehydic (-CHO) and, for α,β-unsaturated

Figure 1.8 5.30–7.00 p.p.m. Regions of a 400 MHz ^{1}H-NMR spectra of a deuterated chloroform (C^2HCl_3) extract of a sample of human atheroma (a). The atheroma (91.7 mg) was treated with 1.00 ml of C^2HCl_3, the mixture thoroughly rotamixed, centrifuged and the C^2HCl_3 phase removed for ^{1}H-NMR analysis. The multiplet resonances b, c, d and e correspond to those of the olefinic protons of the conjugated diene system of *cis, trans*-9- and 13-hydroperoxy-octadecadienoylglycerol species (the structure of the 9-hydroperoxide is also shown above). These conjugated diene system signals were detected in the C^2HCl_3 extracts of eight out of a total of nine atheroma samples examined. Corresponding C^2HCl_3 extracts of matched normal artery samples were not found to contain any NMR-detectable PUFA-derived conjugated diene adducts. The conjugated diene region of a spectrum of a commercially available sample of corn (maize) oil heated at 180°C for a period of 90 min (in C^2HCl_3 solution) is shown in (b) for purposes of comparison.

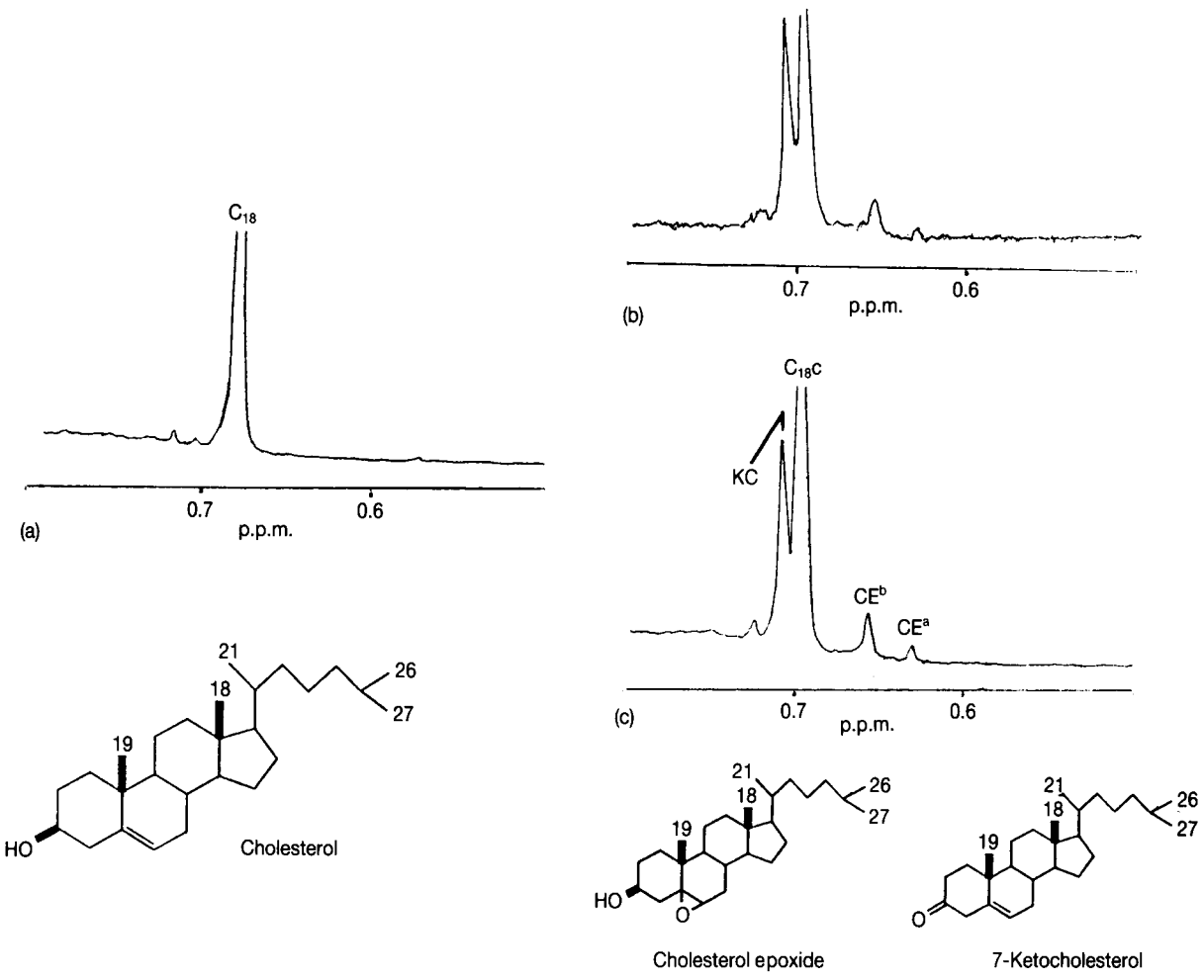


Figure 1.9 0.50–0.80 p.p.m. Regions of 600 MHz ^{1}H-NMR spectra acquired on Folch extracts of control (untreated) and oxidized LDL preparations. Samples were either untreated (a) or oxidized with 3.00×10^{-5} mol/dm^3 Cu(II) (b), or a combination of 1.00×10^{-3} mol/dm^3 Cu(II) and 2.00×10^{-3} mol/dm^3 H_2O_2 (c), and then extracted with $C^2HCl_3/C^2H_3O^2H$ (3 : 1 v/v). The singlet resonances detectable arise from the C-18 methyl group protons of unreacted cholesterol ($C_{18}c$), and in (b) and (c) three of its oxidation products [7-ketocholesterol (KC)], and the 5α, 6α- and 5β, 6β-epoxides (CE^a and CE^b, respectively).

aldehydes, the olefinic (>CH=CH<) proton resonances provide much valuable information regarding the molecular structures of these species. One novel application of this technique is the detection of products derived from the thermally induced autoxidation of PUFAs present in intact culinary frying oils (Haywood *et al.*, 1994). Figure 1.10 shows the aldehydic regions of 400 MHz ^{1}H-NMR spectra of control (unheated) and repeatedly used samples of frying oil obtained from a typical fast-food/take-away establishment, revealing high levels of potentially toxic aldehydes in the used material. Spectra acquired on heated culinary oils also contain multiplet olefinic resonances arising from unsaturated aldehydes (e.g. *trans*-2-octenal), together with their conjugated hydroperoxydiene precursors, and also provide evidence for the thermally induced consumption of PUFAs therein.

Selley *et al.* (1992) have recently employed gas chromatography combined with mass spectrometric detection to determine levels of the cytotoxic monounsaturated aldehyde 4-hydroxy-*trans*-2-nonenal in the blood plasma of healthy human subjects, and patients with rheumatoid and osteoarthritis. Intriguingly, this lipid peroxidation end-product is present at a concentration of *c.* 1×10^{-7} mol/dm^3 in healthy and osteoarthritic human plasma samples (but significantly elevated in those collected from rheumatoid arthritis patients). Although at least some of this could originate from the oxidative degradation of PUFAs *in vivo*, there may be a relationship existing between these levels and the frequency of thermally/

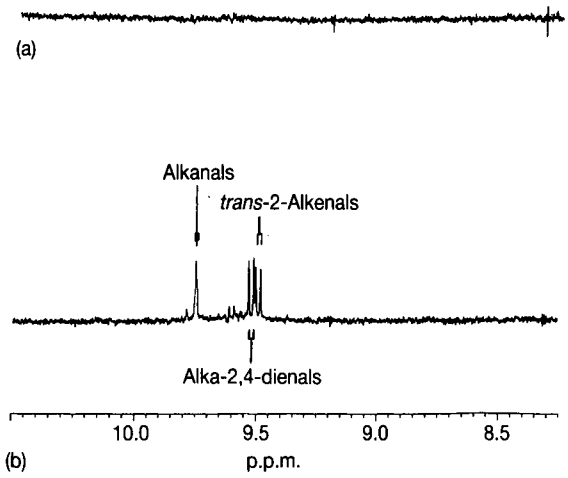


Figure 1.10 Aldehydic regions of 400 MHz ^{1}H-NMR spectra of unheated (a) and repeatedly used samples (b) of culinary frying oil obtained from a fast-food/take-away establishment. A total of 0.30 ml of each sample was diluted to a final volume of 0.90 ml with C^2HCl_3. High levels of *n*-alkanals, *trans*-2-alkenals and alka-2,4-dienals are detectable in spectrum (b).

oxidatively stressed culinary oil or fat consumption in the diet.

A further "marker" of the oxidative deterioration of PUFAs is octanoate, a residue that arises via hydrogen atom abstraction by the alkyl radical remaining after cleavage of *cis*, *trans*- and/or *trans*, *trans*-deca-2, 4-dienal from the alkoxyl radical derived from the 9-hydroperoxide of linoleate. This residue remains bound to the glycerol moiety of the parent triglyceride subsequent to the decomposition of the above conjugated hydroperoxydiene. Peers and Swoboda (1979) have utilized a gas chromatographic method to determine octanoate in samples of thermally stressed culinary oils.

Volatile hydrocarbon gases such as pentane and ethane are well-known end-products of the lipid peroxidation process, and their detection and measurement in the exhaled breath of animals or the "head-space" of selected biological samples may serve as an indicator of oxygen radical-mediated PUFA oxidation. However, it should be noted that they are only minor end-products (e.g. about 2.0×10^{-3} mol of pentane generated per mol of lipid hydroperoxide) and their production is highly dependent upon the availability of catalytic, redox-active transition metal ions, which promote the decomposition of conjugated hydroperoxydienes (Tappel and Dillard, 1981). Pentane arises from selected hydroperoxides of either linoleate or arichidonate via a mechanism involving β-scission of preformed alkoxyl radicals followed by hydrogen atom abstraction of the resulting *n*-pentyl radical [$CH_3(CH_2)_3CH_2^{\cdot}$]. Similarly, ethane is generated from linolenate. These hydrocarbon gases are absorbed and concentrated by passage through silica gel at low temperatures. On desorption, they are easily measured by gas chromatography, a technique that combines the ability to separate complex mixtures with a high level of detection sensitivity. However, the applications of this method are complicated by bacterial hydrocarbon gas production and hence there are stringent requirements for the analysis of adequate control samples.

8. Conclusions

Analytical test systems for measuring free-radical species and their activities *in vivo* are subject to a series of important considerations and requirements, including: (1) definition of a critical threshold level at which the analytes monitored are detectable; (2) the effects, if any, of potential interferences (e.g. anticoagulants previously added to biofluid sample collection tubes); (3) the ability to perform the test in the absence of a suitable control sample, where appropriate; and, perhaps most importantly of all, (4) the artefactual generation of radicals and products derived therefrom in samples during their collection, storage, handling and analysis. With reference to item (4) above, a typical and common example is the instability of endogenous thiols in neutral aqueous solutions, biofluids or tissue samples containing trace or higher levels of O_2, a problem often neglected in many experiments. If precautions are not taken promptly, significant levels of both oxygen- and sulphur-centred radical species, and H_2O_2, will be detectable in samples obtained for analysis, together with any further oxidation products [e.g. disulphides arising from the combination of two thiyl radicals (Equation 1.4)]

A major effort is required to evaluate further the potential of the newer methods outlined here.

9. Acknowledgements

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2. Lipid Peroxidation and Cardiovascular Disease

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1. Introduction

The biological membranes that surround cells and form the boundaries of intracellular organelles contain polyunsaturated fatty acids, which are susceptible to oxidation. This reaction is used under controlled conditions by enzymes, such as the lipoxygenases or cyclooxygenases, within cells to produce oxygenated lipids, which can act as mediators of inflammation (Smith and Marnett, 1991; Yamamoto, 1992). Such compounds are characterized by their high potency and specificity in their interaction with cells (Salmon, 1986). While these enzymatic reactions may involve the formation of potentially toxic free radicals, these are stabilized within the enzyme active site and the non-radical product released is not usually pro-oxidant (Schewe *et al.*, 1986). In contrast, non-enzymatic lipid peroxidation is non-specific with respect to the lipids oxidized and products formed, and is autocatalytic. In many biological systems, lipids are protected from the consequences of this reaction by the presence of antioxidants, which scavenge peroxyl radicals and detoxify peroxides, together with other enzymes designed to prevent the formation of the oxidants capable of initiating lipid peroxidation (Farber *et al.*,

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1990; Sies, 1993). One might reasonably conclude, therefore, that it is unlikely that non-enzymatic lipid peroxidation could have any role to play in human disease (Farber *et al.*, 1990).

However, peroxidation can also occur in extracellular lipid transport proteins, such as low-density lipoprotein (LDL), that are protected from oxidation only by antioxidants present in the lipoprotein itself or the extracellular environment of the artery wall. It appears that these antioxidants are not always adequate to protect LDL from oxidation *in vivo*, and extensive lipid peroxidation can occur in the artery wall and contribute to the pathogenesis of atherosclerosis (Palinski *et al.*, 1989; Esterbauer *et al.*, 1990, 1993; Yla-Herttuala *et al.*, 1990; Salonen *et al.*, 1992). Once initiation occurs the formation of the peroxyl radical results in a chain reaction, which, in effect, greatly amplifies the severity of the initial oxidative insult. In this situation it is likely that the peroxidation reaction can proceed unchecked resulting in the formation of toxic lipid decomposition products such as aldehydes and the F_2 isoprostanes (Esterbauer *et al.*, 1991; Morrow *et al.*, 1990). In support of this hypothesis, cytotoxic aldehydes such as 4-hydroxynonenal have been detected in atherosclerotic lesions but not in normal tissue (Yla-Herttuala *et al.*, 1989). Oxidized LDL has been shown to be cytotoxic, capable of inducing smooth muscle cell proliferation and is chemotactic for monocytes. These effects are probably mediated by lipid decomposition products formed during lipid peroxidation (Quinn *et al.*, 1987; Steinbrecher *et al.*, 1989). A consistent feature of the atherosclerotic lesion is the presence of macrophage derived foam cells and this has led to two contrasting ideas: (1) that these cells may take part in the detoxification of oxidized LDL, and (2) that they actively contribute to the progression of the lesion (for reviews, see Munro and Cotran, 1988; Witztum and Steinberg, 1991; Darley-Usmar and Hassall, 1993).

The basic mechanisms of lipid peroxidation are well understood and described in the literature in many excellent reviews (e.g. Girotti, 1985; Gardner, 1989; Buettner, 1993). Here, apart from essential background information, we will restrict our discussion in this short overview to recent advances in our understanding of lipid peroxidation, emphasizing those aspects relevant to coronary heart disease. Some of the biological implications of these reactions will be discussed by others in this volume.

2. *Mechanisms of Lipid Peroxidation*

In this section, the general principles of lipid peroxidation reactions, which are well established, are discussed first and specific mechanisms, which may be relevant *in vivo*, are considered later.

2.1 General Principles of Initiation and Propagation in Lipid Peroxidation

While it is generally acknowledged that lipid peroxidation reactions can be extremely complex, involving many components and reaction products, the most important reactions are readily classified according to Scheme 2.1.

$X^{\cdot}$	+ LH	→ $L^{\cdot}$	+ XH	(2.1)	Initiation
$L^{\cdot}$	+ O_2	→ $LO_2^{\cdot}$		(2.2)	Propagation
$LO_2^{\cdot}$	+ LH	→ $L^{\cdot}$	+ LOOH	(2.3)	
$LO_2^{\cdot}$	+ $LO_2^{\cdot}$	→ Products		(2.4)	Termination

Scheme 2.1 The key reactions that occur during lipid peroxidation. In this scheme, $X^{\cdot}$ represents the initiating species, which must be a highly reactive oxidant, in order to abstract a H atom from a polyunsaturated fatty-acid chain; LH, the lipid substrate; $LO_2^{\cdot}$, the peroxyl radical; $L^{\cdot}$, the alkyl radical; LOOH, the lipid hydroperoxide.

The first step in the peroxidation reaction, initiation, is the abstraction of a hydrogen atom from an unsaturated fatty acid (Reaction 2.1). In this scheme the initiating radical is described only in general terms. In fact it is difficult to assign with any confidence a specific chemical species to "$X^{\cdot}$" in biological systems. There are, however, several potential candidates and these will be discussed in subsequent sections. The formation of a lipid alkyl radical is followed by its rapid reaction with oxygen to form a lipid peroxyl radical (Reaction 2.2). The peroxyl radical is capable of abstracting a hydrogen atom from an unsaturated fatty acid with the concomitant formation of a lipid radical and lipid hydroperoxide (Reaction 2.3). If Reactions 2.2 and 2.3 are taken together the net result is:

$$LH + O_2 \rightarrow LOOH \qquad (2.5)$$

Since the peroxyl and alkyl radicals are regenerated, the cycle of propagation could continue indefinitely or until one or other of the substrates are consumed. However, experimentally the length of the propagation chain, which can be defined as the number of lipid molecules converted to lipid peroxide for each initiation event, is finite. This is largely because the cycle is not 100% efficient with peroxyl radicals being lost through radical–radical termination reactions (Reaction 2.4 in Scheme 2.1).

2.2 Initiation Reactions

It is easier to abstract a hydrogen atom and initiate lipid peroxidation from a fatty acid that has bis-allylic centres than from a fatty-acid side chain with only one double bond. It follows, therefore, that fatty-acid composition should influence the ability of a molecule such as LDL to act as a substrate for peroxidation. The potential

biological importance of this first step of the lipid peroxidation reaction and the influence of fatty acid composition on its efficiency was recently illustrated in both humans and rabbits. It was shown that dietary enrichment of LDL with oleic acid, which has only one double bond, increases its resistance to oxidation in both humans and experimental animals (Parthasarathy *et al.*, 1990; Reaven *et al.*, 1991). In the rest of this section, some of the initiation reactions, which may be relevant to human cardiovascular disease, are reviewed.

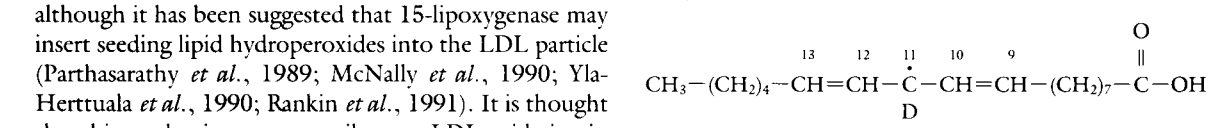
2.2.1 Enzymatic Peroxidation Catalysed by Lipoxygenases

The roles of lipoxygenases in biological systems are varied and include the formation of mediators of inflammation, such as the leukotrienes and the maturation of reticulocytes to red blood cells (Kuhn *et al.*, 1990). In most cases the lipoxygenases are probably not directly pro-oxidant, although it has been suggested that 15-lipoxygenase may insert seeding lipid hydroperoxides into the LDL particle (Parthasarathy *et al.*, 1989; McNally *et al.*, 1990; Yla-Herttuala *et al.*, 1990; Rankin *et al.*, 1991). It is thought that this mechanism may contribute to LDL oxidation in the artery wall. This hypothesis will be discussed in detail in Section 2.6, whereas the role of free radicals in the enzymatic mechanism of lipoxygenase is described here.

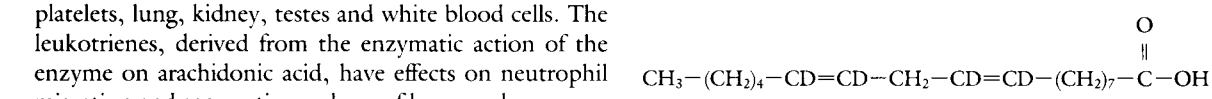
Lipoxygenases catalyse the regio-specific and stereoselective oxygenation of unsaturated fatty acids. The mammalian enzymes have been detected in human platelets, lung, kidney, testes and white blood cells. The leukotrienes, derived from the enzymatic action of the enzyme on arachidonic acid, have effects on neutrophil migration and aggregation, release of lysosomal enzymes, capillary permeability, induction of pain and smooth muscle contraction (Salmon, 1986).

The most detailed mechanistic studies of the lipoxygenase enzymes have been performed with 15-lipoxygenase isolated from soybeans. The enzyme achieves the insertion of a lipid hydroperoxide group into a fatty acid while keeping the radical intermediates of the reaction bound to the protein. This effectively prevents the peroxyl radical propagating lipid peroxidation. However, this binding of the radical intermediates is not completely efficient, allowing some propagation to occur. A striking feature of lipoxygenase reactions compared to non-specific lipid peroxidation is the stereospecific elimination of one of the two methylene hydrogen atoms from the 1,4-pentadiene structure of unsaturated fatty acids. Clearly the equivalency of the two methylene hydrogens in non-specific lipid peroxidation is removed in the enzyme–fatty acid complex. Deuterium substitution at the C-11 hydrogen of linoleic acid decreases the rate of hydroperoxide formation by soybean lipoxygenase nine-fold. This isotope effect indicates that the removal of the C-11 hydrogen is the rate-limiting step of lipoxygenase catalysis (Egmond *et al.*, 1973).

The earliest evidence that this hydrogen abstraction formed a fatty-acid free radical arose from the observation that, if oxygen was limited, lipoxygenase formed fatty-acid dimers of linoleic acid. Because the formation of dimers often occurs as the result of the self-termination of two free radicals, the formation of a linoleic acid free radical was proposed (Garssen *et al.*, 1972). In one of the first applications of electron spin resonance (e.s.r.) spin trapping to a biological problem, de Groot *et al.* (1973) showed that soybean lipoxygenase forms a linoleic acid free radical, as indicated by its trapping with 2-methyl-2-nitrosopropanol. The characteristics of the resulting e.s.r. spectrum indicated that a carbon-centred free radical had been trapped. An identical e.s.r. spectrum was obtained from *cis,cis*-9,12[11,11-D]linoleic acid, indicating that the spin adduct was not derived from the carbon-centred radical formed by the loss of one of the two deuteriums at C-11, the rate-limiting step of soybean lipoxygenase catalysis.



However, deuteration at carbons 9, 10, 12 and 13 did change the spectrum to one consisting of only a nitrogen triplet, indicating that the radical trapped had a deuterium attached to the carbon centre. This would imply that the carbon-centred radical lies at one of these four positions.



De Groot *et al.* (1973) suggested that their results were consistent with the spin trap reacting with linoleic acid radical at C-13 and/or C-9. In subsequent work, Sekiya *et al.* (1977) proposed that hydrogen abstraction at C-11 by potato lipoxygenase was followed by isomerization of the *cis* double bond between C-9 and C-10 to a *trans* double bond between C-10 and C-11, thus forming a carbon-centred free radical at C-9. It was proposed that the pentadienyl radical intermediate reacts with oxygen while bound to the enzyme to give a peroxyl radical and, ultimately, the fatty-acid hydroperoxide as the final product. The complete catalytic cycle is shown in Fig. 2.1. The active ferric lipoxygenase is reduced to the ferrous enzyme by an unsaturated fatty acid, LH (Pistorius and Axelrod, 1976). The ferric lipoxygenase is regenerated when the fatty-acid hydroperoxide forms. Chamulitrat and Mason (1989) reported, for the first time, e.s.r. evidence for the existence of the fatty-acid peroxyl radical from the lipoxygenase-catalysed peroxidation. Peroxyl free-radical formation was observed only with fatty acids containing two or more double bonds. This is again consistent with abstraction of a doubly allylic hydrogen atom from a methylene group between two double bonds forming

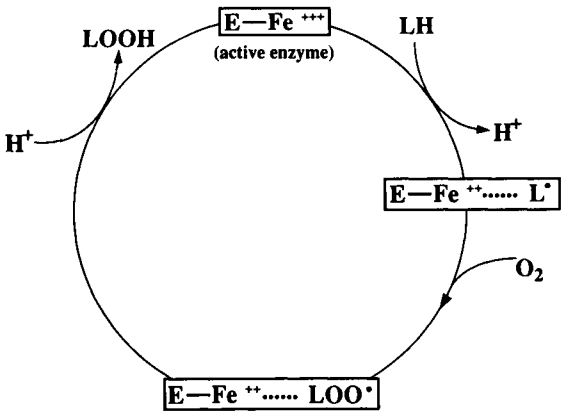


Figure 2.1 Mechanism for the oxygenation of lipids by lipoxygenase under aerobic conditions. LH, fatty acid; LOOH, fatty-acid hydroperoxide; Fe, the redox active centre of the enzyme.

a pentadienyl carbon-centred intermediate, which is the precursor of the peroxyl radical.

Evidence that either the peroxyl radical or alkyl radical can diffuse from the enzyme active site and react with chain-breaking antioxidants such as α-tocopherol or its water-soluble form, Trolox C, was also investigated using e.s.r. spectroscopy. The intensity of the arachidonic acid peroxyl radical was monitored as a function of the antioxidant concentration. Both antioxidants reacted with the peroxyl radical to form the corresponding antioxidant-derived phenoxyl radical. Such reactions may have a physiological relevance in the remodelling of mitochondrial membranes by 15-lipoxygenase during reticulocyte maturation (Kuhn *et al.*, 1990). In this case effective suppression by chain-breaking antioxidants is necessary if the peroxyl radicals are not to propagate an uncontrolled lipid peroxidation reaction.

2.2.2 Haem Proteins and Lipid Peroxidation

The cyclooxygenase enzymes catalyse the oxidation of arachadonic acid to form the prostaglandins, which have a wide range of important biological functions (Smith and Marnett, 1991). In this reaction the iron in the haem prosthetic group redox cycles between the ferric and ferryl oxidation states with minimal loss of reactive lipid-derived free radicals from the active site. Other haem-containing proteins, such as myoglobin or haemoglobin, whose normal function is to transport oxygen, may be oxidized to the ferryl oxidation state by hydrogen peroxide. The ferryl forms of haem proteins are powerful oxidants and may be able to abstract a hydrogen atom from an unsaturated fatty acid (Kanner and Harel, 1985; Galaris *et al.*, 1990; Balla *et al.*, 1991; Dee *et al.*, 1991). In addition the iron in haem proteins in the ferric oxidation state may also be able to take part in a one-electron redox cycle resulting in the decomposition of lipid hydroperoxides to alkoxyl and peroxyl radicals. This reaction is similar to that found with copper and will be described in more detail in Sections 2.4 and 2.6.

2.2.3 Superoxide, Nitric Oxide and Peroxynitrite

Not all oxidants formed biologically have the potential to promote lipid peroxidation. The free radicals superoxide and nitric oxide [or endothelium-derived relaxing factor (EDRF)] are known to be formed *in vivo* but are not able to initiate the peroxidation of lipids (Moncada *et al.*, 1991). The protonated form of the superoxide radical, the hydroperoxy radical, is capable of initiating lipid peroxidation but its low pK_a of 4.5 effectively precludes a major contribution under most physiological conditions, although this has been suggested (Aikens and Dix, 1991). Interestingly, the reaction product between nitric oxide and superoxide forms the powerful oxidant peroxynitrite (Equation 2.6) at a rate that is essentially diffusion controlled (Beckman *et al.*, 1990; Huie and Padmaja, 1993).

$$NO + O_2^- \rightarrow ONOO^- \qquad (2.6)$$

The chemistry of peroxynitrite is complex and it is clear that it may promote radical-dependent reactions and act as an oxidant in its own right (Halfpenny and Robinson, 1952; Mahoney, 1970; Beckman *et al.*, 1990; Hogg *et al.*, 1992; Koppenel *et al.*, 1992). When peroxynitrite is introduced into a biological environment containing LDL or plasma, the effects include initiation of radical-dependent chain reactions, possibly through localized decomposition of the *trans* isomer to release hydroxyl radicals and nitrogen dioxide (Equation 2.7). The extremely high and non-specific reactivity of the hydroxyl radical effectively precludes its release into free solution. The proposal, based largely upon theoretical considerations, that peroxynitrite does not decompose to release hydroxyl radicals (Koppenol *et al.*, 1992) is hard to reconcile with the published observations clearly showing radical-dependent oxidations (Halfpenny and Robinson, 1952; Mahoney, 1970; Beckman *et al.*, 1990; Hogg *et al.*, 1992).

$$ONOOH \rightarrow OH^{\cdot} + NO_2 \qquad (2.7)$$

Other reactions of peroxynitrite include the oxidation of sulphydryl groups and the formation of a nitrating agent with the chemical reactivity of the nitronium ion (Radi *et al.*, 1991; Beckman *et al.*, 1992). Most of these reactions show no requirement for transition metals, although the nitration of phenols (e.g. the amino acid, tyrosine) is favoured in solutions containing transition metal ions (Beckman *et al.*, 1992). In plasma the most susceptible target to modification by peroxynitrite appears to be the sulphydryl group, the most abundant being that on albumin (Van Der Vliet *et al.*, 1994). Since this SH group is a major contributor to the peroxyl radical scavenging capacity of plasma and its modification by peroxynitrite causes the loss of its antioxidant function, then loss of antioxidant capacity may be the most direct

consequence of the chronic exposure of plasma to peroxynitrite. Such reactions may occur *in vivo*, since humans suffering from adult respiratory distress syndrome, a disease associated with high plasma levels of nitric oxide, show a severe loss in the thiol content of plasma (Quinlan *et al.*, 1994).

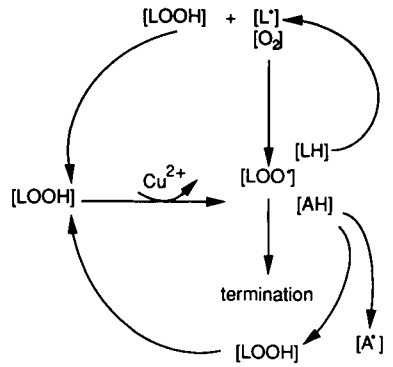
The evidence in favour of peroxynitrite formation in the vasculature while still indirect is compelling. For example, the activity of nitric oxide as a vasorelaxant (EDRF) is inhibited by high plasma cholesterol (Minor *et al.*, 1990; Ohara *et al.*, 1993) and this can be prevented by modified forms of the enzyme superoxide dismutase, which are able to penetrate the artery wall (Mugge *et al.*, 1991; White *et al.*, 1994). The superoxide appears to derive from endothelial cells, possibly xanthine oxidase, since high concentrations of oxypurinol inhibit its formation and restore EDRF-dependent relaxations of coronary vessels (Ohara *et al.*, 1993). *In vitro*, we have shown that both the simultaneous generation of nitric oxide and superoxide, and peroxynitrite, oxidizes the principal chain-breaking antioxidant in LDL, α-tocopherol, so rendering it more susceptible to oxidation by transition metals (Darley-Usmar *et al.*, 1992; Hogg *et al.*, 1993a, 1993b). Furthermore, treatment of LDL with peroxynitrite leads to the initiation of lipid peroxidation in a transition metal-independent mechanism, which may ultimately lead to the formation of a highly atherogenic form of LDL (Graham *et al.*, 1993). Since both superoxide and nitric oxide may be generated in the vasculature, the initiation of lipid peroxidation only requires that the local antioxidant defences are overwhelmed to induce a highly pro-oxidant environment.

2.3 PROPAGATION REACTIONS

In the previous section, we have described some of the mechanisms that may lead to the formation of lipid hydroperoxides or peroxyl radicals in lipids. If the peroxyl radical is formed, then this will lead to propagation if no chain-breaking antioxidants are present (Scheme 2.1). However, in many biological situations chain-breaking antioxidants are present, for example, in LDL, and these will terminate the peroxyl radical and are consumed in the process. This will concomitantly increase the size of the peroxide "pool" in the membrane or lipoprotein. Such peroxides may be metabolized by the glutathione peroxidases in a cellular environment but are probably more stable in the plasma compartment. In the next section, the promotion of lipid peroxidation if the lipid peroxides encounter a transition metal will be considered.

2.4 INTERACTIONS BETWEEN LIPID HYDROPEROXIDES AND TRANSITION METALS

The oxidation of lipids can be promoted by transition metals, such as copper or haem proteins, by a process that



Scheme 2.2 The copper-dependent decomposition of lipid peroxides and the role of antioxidants. In this scheme, LOOH represents the "pool" of peroxide; LOO˙, the peroxyl radical; LH, the lipid substrate; L˙, the lipid-derived alkyl radical; AH, a chain-breaking antioxidant; A˙, the antioxidant-derived radical. Copper is the catalyst in this reaction and would also form the alkoxy radical as shown in Reaction 2.9 (see text), which is omitted here for the sake of clarity.

has an absolute requirement for lipid hydroperoxides (Thomas and Jackson, 1991). The most likely mechanism for the promotion of lipid peroxidation by copper and haem proteins is a one-electron redox cycle between the transition metal (M) and the lipid peroxide as described in Reactions 2.8 and 2.9 below:

$$M^{(n+)} + LOOH \rightarrow LOO^{\cdot} + M^{(n-1)+} + H^{+} \quad (2.8)$$

$$M^{(n-1)+} + LOOH \rightarrow LO^{\cdot} + M^{(n+)} + OH^{-} \quad (2.9)$$

In this reaction scheme, the steady-state concentration of peroxyl radicals will be a direct function of the concentration of the transition metal and lipid peroxide content of the LDL particle, and will increase as the reaction proceeds. Scheme 2.2 is a diagrammatic representation of the redox interactions between copper, lipid hydroperoxides and lipid in the presence of a chain-breaking antioxidant. For the sake of clarity, the reaction involving the regeneration of the oxidized form of copper (Reaction 2.9) has been omitted. The first step is the independent decomposition of the lipid hydroperoxide to form the peroxyl radical. This may be terminated by reaction with an antioxidant, AH, but the lipid peroxide formed will contribute to the peroxide pool. It is evident from this scheme that the efficacy of a chain-breaking antioxidant in this scheme will be highly dependent on the initial size of the peroxide pool. In the section describing the copper-dependent oxidation of LDL (Section 2.6.1), the implications of this idea will be pursued further.

2.5 CHAIN-BREAKING ANTIOXIDANTS

As discussed earlier, the peroxyl radical has a key role to play in propagating lipid peroxidation. This molecule can be converted into a non-radical product, which cannot

propagate the chain, by the addition of an electron and proton in separate steps or a hydrogen atom, in one step. The types of compound that can act as chain-breaking antioxidants through either of these two mechanisms are, in fact, structurally quite diverse and include both natural products and synthetic molecules. In this article, four examples are considered: α-tocopherol (a form of vitamin E); probucol (a synthetic antioxidant); nitric oxide (a lipid-soluble gas); and ascorbic acid (vitamin C). A reaction of the type shown in Scheme 2.3 (reaction 2.10) is a necessary but not sufficient condition for suppression of lipid peroxidation. In fact unsaturated fatty acids can donate a hydrogen atom to the peroxyl radical as shown in Reaction 2.3 in Scheme 2.1 but, since this results in the formation of the reactive alkyl radical, no inhibition of lipid peroxidation occurs. What distinguishes this reaction from those with anti-oxidants, shown in Scheme 2.3, which do inhibit lipid peroxidation? We can immediately see from Schemes 2.1 and 2.3 at least two requirements for effective inhibition of the propagation chain:

1. The chain-breaking antioxidant must scavenge peroxyl radicals at a faster rate than they can react with another unsaturated fatty acid (reaction 2.10). The reverse reaction, whereby the antioxidant radical converts the lipid peroxide to a peroxyl radical, should also be slow (reaction 2.11 in Scheme 2.3).
2. The antioxidant radical formed must react at a lower rate with unsaturated fatty acids than the peroxyl radical (Reaction 2.12 in Scheme 2.3).

2.5.1 The Antioxidant and Pro-oxidant Reactions of α-Tocopherol

Vitamin E consists of a family of related compounds, the tocopherols. The most abundant in the American diet is γ-tocopherol and, although it is potentially superior in the detoxification of nitrogen dioxide, the biochemistry of α-tocopherol is considered more relevant to cardiovascular disease (Kayden and Traber, 1993). Vitamin E undoubtedly has several modes of action *in vivo*. The most clearly understood of these, at the chemical level, is the role of α-tocopherol as an antioxidant.

α-Tocopherol, as described above, is a chain-breaking antioxidant and will react with peroxyl radicals with a rate constant of approximately 10^6 M^{-1} s^{-1} (Niki *et al.*, 1984). This is four orders of magnitude greater than the rate constant for the reaction between an unsaturated fatty acid and peroxyl radical. α-Tocopherol is a phenolic antioxidant and, as with the synthetic analogues butylated hydroxy toluene (BHT) and probucol, donates a hydrogen atom to a lipid peroxyl radical. The net result is a lipid hydroperoxide, α-tocopheryl radical and inhibition of the propagation of lipid peroxidation.

The fate of the α-tocopheryl radical remains uncertain. Disproportionation would generate α-tocopherol quinone and α-tocopherol. The low yields of α-tocopherol quinone observed during lipid oxidation suggests that this is not a major route of radical decomposition. In studies using azo-initiators, such as ABAP and 2,2[1]-azobis (2,4-dimethylvaleronitrile) (AMVN), addition products at the 8a position of the α-tocopheryl radical have been observed. Thus with AMVN, the AMVN-peroxyl radical will add to the 8a position. Other addition products as well as dimers, trimers, epoxides and spiro compounds have been observed during oxidations by various agents using a range of conditions (reviewed in Liebler, 1993).

α-Tocopherol is the major lipid-soluble antioxidant in LDL (Smith *et al.*, 1993). There are several reports in the literature that show a protective role of α-tocopherol against lipid oxidation within the LDL particle (Dieber-Rotheneder *et al.*, 1991; Jialal and Grundy, 1992). Protection is afforded whether α-tocopherol is added directly to LDL, added to plasma before LDL isolation or incorporated by dietary means (Dieber-Rotheneder *et al.*, 1991). Such studies show a clear correlation between α-tocopherol content and resistance of LDL to oxidation initiated either by copper (II) ions or by peroxyl radicals generated by the thermolabile compound ABAP. Such a correlation is not observed, however, within a population of donors and it appears as though other variables contribute to the oxidizability of LDL that are unchanged by supplementation with α-tocopherol (reviewed in Esterbauer *et al.*, 1993).

Recently the role of α-tocopherol as an antioxidant in LDL has been questioned (Bowry *et al.*, 1992). During ABAP-dependent oxidation of LDL, peroxide accumulation was observed to be greater in the presence of α-tocopherol than in its absence. This was explained by "tocopherol-mediated peroxidation" whereby the α-tocopheryl radical reinitiates lipid peroxidation by abstracting an allylic hydrogen atom from an unsaturated fatty acid (Reaction 2.12 in Scheme 2.3). It was concluded that the small size of the LDL particle requires that the number of initiation events per particle is slow enough to give the reaction between α-tocopheryl radical and unsaturated fatty acid sufficient time to proceed. This argument, though plausible, cannot explain the observed pro-oxidant effects of α-tocopherol as the abstraction of hydrogen by an α-tocopheryl radical can, at worst, only be a chain-transfer reaction. If this reaction were infinitely fast, then oxidation would proceed at the same rate in both the presence and absence of α-tocopherol. The slower the chain transfer reaction becomes the more potent the antioxidant effect of α-tocopherol will be. A mechanism

AH	**+**	**$LOO^{\cdot}$**	**→**	**$A^{\cdot}$**	**+**	**LOOH**	**(2.10)**
$A^{\cdot}$	**+**	**LOOH**	**→**	**$LOO^{\cdot}$**	**+**	**AH**	**(2.11)**
$A^{\cdot}$	**+**	**LH**	**→**	**AH**	**+**	**$L^{\cdot}$**	**(2.12)**

Scheme 2.3 The inhibition of lipid peroxidation by antioxidants. In this scheme, AH represents an antioxidant; $A^{\cdot}$, the antioxidant-derived radical; LH, the lipid substrate; $LO_2^{\cdot}$, the peroxyl radical; $L^{\cdot}$, the alkyl radical; LOOH, the lipid hydroperoxide.

for the observed pro-oxidant effects in this system remain to be explained.

It is possible that α-tocopherol may serve to reduce copper (II) ions during copper-dependent oxidation of LDL. This reaction would accelerate oxidation as it would deplete α-tocopherol and copper (I) ions will more rapidly degrade endogenous hydroperoxides than will copper (II) ions. This effect has been observed in a model system and may well explain the long known ability of α-tocopherol, at high concentrations, to promote lipid oxidation. The effects in LDL are less clear as α-tocopherol supplementation has clearly been shown to be antioxidant (see above). It may be that in LDL there is a kinetic barrier that decreases the rate of reaction between α-tocopherol and copper (II) ions thus preventing a pro-oxidant effect. Also LDL contains other possible reducing agents for copper (II) ions, such as protein and lipid hydroperoxide (O'Leary *et al.*, 1992; Smith *et al.*, 1993; Esterbauer *et al.*, 1993).

2.5.2 The Antioxidant Reactions of Nitric Oxide

In several *in vitro* systems, nitric oxide has been shown to possess antioxidant properties. Kanner *et al.* (1991, 1992) demonstrated the ability of nitric oxide to inhibit the oxidative effects of both the Fenton reaction and ferryl myoglobin. They concluded that nitric oxide could have an antioxidant effect through ligation to ferrous iron complexes thus preventing reaction with hydrogen peroxide. Recently they have extended this observation to lipid oxidation by lipoxygenase and cyclooxygenase (Kanner *et al.*, 1992). They propose that nitric oxide could act directly with the peroxidase to reduce the ferrous enzyme to the inactive ferric form. However, Salvemini *et al.* (1993) have reported an activation of cyclooxygenase by nitric oxide in mouse macrophages that they suggest represents a direct activation of the enzyme by nitric oxide.

Nitric oxide may also be an antioxidant by virtue of the fact that it can directly inhibit NADPH oxidase and thus prevent superoxide production (Clancy *et al.*, 1992). This inhibition was reported to be independent of the reaction between nitric oxide and superoxide, which might be expected to be pro-oxidant (see Section 2.2.3).

Mouse peritoneal macrophages that have been activated to produce nitric oxide by γ-interferon and lipopolysaccharide were shown to oxidize LDL less readily than unactivated macrophages. Inhibition of nitric oxide synthesis in the same model was shown to enhance LDL oxidation (Jessup *et al.*, 1992; Yates *et al.*, 1992). It has recently been demonstrated that nitric oxide is able to inhibit lipid peroxidation directly within LDL (Hogg *et al.*, 1993c). Nitric oxide probably reacts with the propagating peroxyl radicals thus terminating the chain of lipid peroxidation. The rate constant for the reaction between nitric oxide and peroxyl radicals has recently been determined to be $1\text{–}3 \times 10^9\ M^{-1}\,s^{-1}$ (Padmaja and Huie, 1993). This antioxidant reaction may well be important in the artery wall where a low steady-state concentration of nitric oxide is present. It is also possible to postulate a causal relationship between this observation and the intriguing antiatherogenic properties of arginine in hypercholesterolaemia (Drexler *et al.*, 1991). These results suggest that in transition metal-dependent lipid peroxidation nitric oxide would be an effective antioxidant, probably by scavenging peroxyl radicals. The cell-dependent modification of LDL is a system with just such characteristics (see Section 3). In contrast, in a transition metal-independent peroxidation reaction, in an environment in which superoxide is generated, the antioxidant properties of nitric oxide will be antagonized by superoxide with the concomitant formation of peroxynitrite (see Section 2.2.3). Inflammatory sites may have these properties. *In vivo*, the effects of nitric oxide as a pro-oxidant or antioxidant cannot be predicted a priori, and both cytotoxic and protective effects have been observed.

2.5.3 Synergistic Interactions between Antioxidants

The potential for synergistic interactions between antioxidants in inhibiting lipid peroxidation is implicit in the reactions shown in Scheme 2.3. Synergy can be defined as "the co-operative action of two or more stimuli or drugs". Using this apparently simple definition it follows that, if we measure the biological or biochemical effects of two compounds in combination and the observed response is greater than we would predict from measuring and adding their independent effects, then they are interacting synergistically. Operationally, it is a simple matter to design experiments that reveal this behaviour, although providing a plausible molecular mechanism is not always straightforward.

The efficiency of the antioxidant will depend on the ratio of the rates of Reaction 2.10 to those for Reactions 2.11 and 2.12. A compound that is capable of reducing the antioxidant radical ($A^{\cdot}$) back to the parent compound (AH) will compete with Reactions 2.11 and 2.12, and so increase the efficiency of peroxyl radical scavenging (Reaction 2.10). In addition, the steady-state concentration of the antioxidant will be maintained at its initial concentration for a longer period and this should also result in more efficient suppression of the peroxidation reaction. The net result of these effects will be a synergistic enhancement of antioxidant activity.

In satisfying these criteria it is clear that a balance must be struck between a sufficiently rapid rate for Reaction 2.10 while minimizing the rates of Reactions 2.11 and 2.12. This could be achieved by decreasing the reactivity of the antioxidant by sterically hindering the functional group donating the hydrogen atom.

In the case of α-tocopherol and probucol it is possible to demonstrate synergy between these antioxidants and the water-soluble antioxidant ascorbate in both model

systems and LDL (Niki *et al.*, 1984; Sato *et al.*, 1990; Kalyanaraman *et al.*, 1992). Ascorbate is capable of scavenging peroxyl radicals directly, particularly those which are water soluble, but the rate of reaction is approximately ten times slower than the scavenging of peroxyl radicals by α-tocopherol (Niki *et al.*, 1984). The reaction of ascorbate with the α-tocopheroyl radical is, on the other hand, at least 20 times faster than the scavenging of peroxyl radicals by ascorbate. It is not surprising, therefore, that the addition of ascorbate to lipids containing α-tocopherol undergoing oxidation results in the loss of the α-tocopheroyl radical and the appearance of the ascorbate radical (Kalyanaraman *et al.*, 1992). A similar effect was observed with the interaction between probucol and ascorbate. In both cases the addition of ascorbate was also associated with a synergistic enhancement of antioxidant activity. Computer simulation of this complex sequence of reactions suggests that synergy arises from both inhibition of the pro-oxidant reactions of the α-tocopheroyl radical and an increase in its steady-state concentration. Some evidence for pro-oxidant reactions of α-tocopherol have been reported in the literature as has the predicted effects on the steady-state level of α-tocopherol in the presence of ascorbate (Bowry *et al.*, 1992; Gotoh *et al.*, 1992; Kalyanaraman *et al.*, 1992).

2.6 LIPOXYGENASE, "SEEDING PEROXIDES" AND LDL OXIDATION

Oxidation of the fatty acids in an LDL particle shares many of the characteristics associated with lipid peroxidation in other biological or chemical systems. Once initiated peroxyl radicals are formed and this results in the oxidation of α-tocopherol to give the α-tocopheroyl radical (Kalyanaraman *et al.*, 1990). This can be demonstrated by e.s.r. techniques that allow the direct observation of stable radicals such as the α-tocopheroyl radical. After the α-tocopheryl radical is consumed, lipid-derived peroxyl radicals can be detected after reaction with spin traps (Kalyanaraman *et al.*, 1990, 1991).

The reactions described so far do not require the involvement of the apo-B protein, neither would they necessarily result in a significant amount of protein modification. However, the peroxyl radical can attack the fatty acid to which it is attached to cause scission of the chain with the concomitant formation of aldehydes such as malondialdehyde and 4-hydroxynonenal (Esterbauer *et al.*, 1991). Indeed, complex mixtures of aldehydes have been detected during the oxidation of LDL and it is clear that they are capable of reacting with lysine residues on the surface of the apo-B molecule to convert the molecule to a ligand for the scavenger receptor (Haberland *et al.*, 1984; Steinbrecher *et al.*, 1989). In addition, the lipid-derived radical may react directly with the protein to cause fragmentation and modification of amino acids.

The chemical adducts formed by reaction of aldehydes with lysine residues form highly immunogenic epitopes, and antibodies have been prepared specific for malondialdehyde- and 4-hydroxynonenal-conjugated LDL (Gonen *et al.*, 1987; Yla-Herttuala *et al.*, 1989; Jurgens *et al.*, 1990). These antibodies cross-react with material in atherosclerotic lesions but not normal tissue, thus supporting the central role of lipid peroxidation in the pathogenesis of atherosclerosis (Yla-Herttuala *et al.*, 1989, 1991).

It has been suggested that the enzyme 15-lipoxygenase, present in macrophages, causes the oxidation of LDL in the artery wall (Yla-Herttuala *et al.*, 1990). In support of this idea it has been shown that: (1) purified mammalian 15-lipoxygenase can oxidize human LDL; and (2) 15-lipoxygenase and its lipid products are associated with atherosclerotic lesions but not normal tissue (Sparrow *et al.*, 1988; Parthasarathy *et al.*, 1989; McNally *et al.*, 1990; Cathcart *et al.*, 1991). Two mechanisms have been proposed by which lipoxygenase could insert lipid peroxides into LDL. In the first case it is suggested that free radicals, perhaps superoxide, hydrogen peroxide or a peroxyl radical released from the active site of the lipoxygenase enzyme diffuse into the LDL molecule and initiate peroxidation (Cathcart *et al.*, 1991). In the second mechanism, it is proposed that direct oxygenation of fatty-acid side chains in the LDL molecule results in the formation of a lipid peroxide without the formation of radical intermediates outside the active site of the enzyme. Since the lipid peroxides are stable in the absence of transition metals, then lipid peroxidation can only occur in their

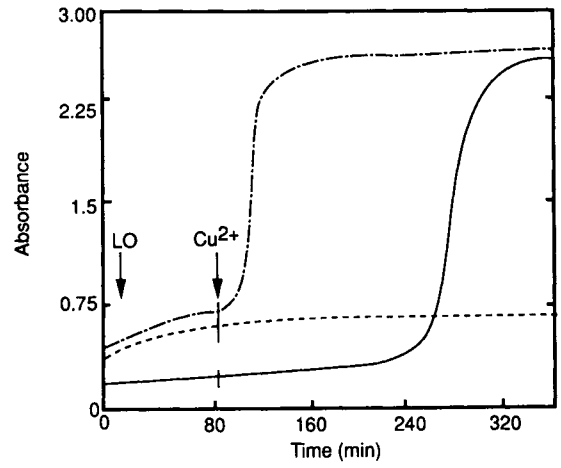


Figure 2.2 Oxidation of human LDL by lipoxygenase and exposure to copper. The oxidation of human LDL was monitored by the increase in absorbance at 234 nm after the addition of soybean lipoxygenase (LO) at $t = 0$ min (—— and ·-·) followed by the addition of Cu^{2+} (10 μM) at $t = 90$ min to one LO-treated sample (·-·) and the control (–). Other conditions were exactly as described in Jessup *et al.* (1991).

presence (Sparrow *et al.*, 1988). It is difficult to distinguish these two possibilities and data are reported in the literature that could support either. The enzyme 15-lipoxygenase is capable of introducing peroxides into LDL and indeed circulating lipoproteins appear to contain low levels of endogenous peroxides that could have been inserted by a lipoxygenase enzyme.

An example of an experiment in which LDL has been treated with 15-lipoxygenase and the oxidation monitored by the formation of conjugated diene is shown in Fig. 2.2. In the absence of transition metal, a rapid increase in absorbance occurs, with no lag phase, which ceases after a period of about 90 min under these conditions. If copper is added to promote LDL oxidation at this point, LDL treated with lipoxygenase oxidizes at a faster rate with a short lag phase when compared to the control. During this procedure there is only a minimal loss of α-tocopherol and so we may ascribe the shortened lag phase to the increase in lipid peroxides brought about by lipoxygenase treatment. A similar result was found when LDL was supplemented with preformed fatty acid hydroperoxides (O'Leary *et al.*, 1992).

2.6.1 Oxidizability and the Potency of Chain-breaking Antioxidants in LDL

As described above and in Scheme 2.2, the transition metal-dependent oxidation of LDL has an absolute requirement for the presence of seeding peroxides in the LDL particle (Thomas and Jackson, 1991). In addition, the rate of the oxidation reaction is probably dependent upon the initial concentration of lipid peroxides (O'Leary *et al.*, 1992). This method for promoting LDL oxidation has been used to determine the oxidizability of LDL isolated from individual human donors whose diets have been supplemented with α-tocopherol (Dieber-Rotheneder *et al.*, 1991; De Graaf *et al.*, 1991; Jialal and Grundy, 1992). The preliminary results, from a number of laboratories, suggest that while increased α-tocopherol does inhibit the oxidation of LDL, the efficacy of this antioxidant is highly variable between individuals (Dieber-Rotheneder *et al.*, 1991). In addition, it is clear that LDL isolated from different individuals, on a normal diet, shows considerable variation when oxidizability is assessed by the addition of copper. However, when the oxidizability of the same samples was assessed by inducing lipid peroxidation using a mechanism independent of the presence of preformed lipid hydroperoxides, little or no variation was observed (Smith *et al.*, 1993). This is consistent with the hypothesis that the endogenous lipid peroxide content of human LDL isolated from different individuals may vary. If LDL isolated from plasma truly reflected the state of LDL in the artery wall, and this is open to argument, then it is possible that the endogenous lipid peroxide content is an additional risk factor for the development of coronary heart disease. Unfortunately, at this point in time, the biological mechanism for insertion of these "seeding peroxides" is uncertain. Central to the argument that transition metals interact with lipid peroxides to promote LDL oxidation is that the metal may act in a catalytic role as shown in Equations 2.8 and 2.9 (Section 2.4). It has been argued on thermodynamic grounds that a redox cycle may occur between amino-acid residues on the protein and the reduced transition metal (Esterbauer *et al.*, 1993). This, however, appears unlikely since transition metals in such diverse forms as the iron in oxidized myoglobin (Fe III) and $CuSO_4$ are capable of oxidizing LDL by a lipid peroxide-dependent process (Dee *et al.*, 1991; Thomas and Jackson, 1991). In addition, chemical systems that contain no protein also show an organic peroxide and transition metal-dependent promotion of lipid peroxidation (Yoshida and Niki, 1992). One could reasonably question the relevance of transition metals such as copper to the oxidation of LDL in the artery wall and its use in this assay. However, it has recently been shown that the transition-metal content

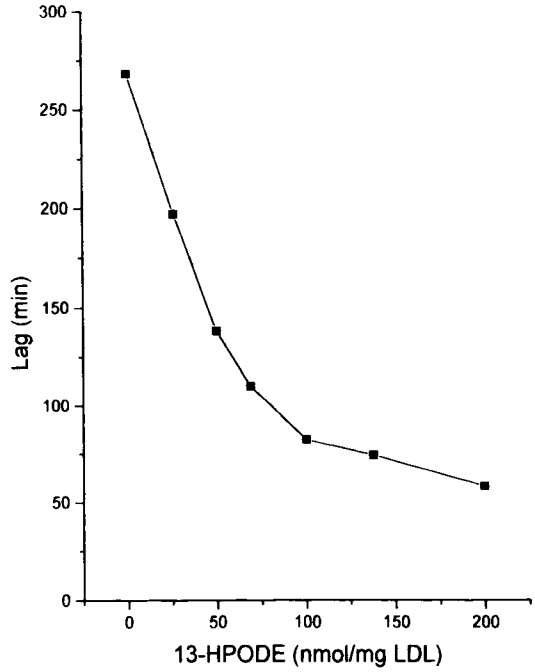


Figure 2.3 The antioxidant activity of butylated hydroxytoluene in the presence of exogenous lipid hydroperoxides. The oxidation of LDL was monitored by measuring the increase in absorbance at 234 nm as described in Fig. 2.2 and the lag phase (time before the phase of maximum rate of oxidation) estimated as described by Esterbauer *et al.* (1989). Samples of LDL were supplemented with the concentrations of 13-hydroperoxyoctadecanoic acid (13-HPODE) indicated and in the presence of 3 μM BHT. The lag phase in the absence of BHT for this preparation of LDL was 48 min. The data represented the mean of duplicate determinations.

available for pro-oxidant reactions (i.e., iron and copper) in atherosclerotic lesions is elevated (Smith *et al.*, 1992).

The potency of a chain-breaking antioxidant, which scavenges peroxyl radicals, will decrease as the concentration of lipid peroxides in the LDL particle increases (Scheme 2.2). This is illustrated in the experiment shown in Fig. 2.3 in which the antioxidant potency of a peroxyl radical scavenger (BHT) decreases as a function of added exogenous lipid hydroperoxide. If the endogenous lipid peroxide content of LDL were to vary between individuals, this could explain the observed differences in the effectiveness of α-tocopherol in suppressing lipid peroxidation promoted by copper.

In summary, in our view the principal factors that contribute to the oxidizability of LDL assessed by the addition of a transition metal such as copper are: (1) the lipid hydroperoxide content of the LDL particle; and (2) the α-tocopherol content. Other chain-breaking antioxidants such as ubiquinol and the carotenoids are present only at low concentrations in most individuals, and are unlikely to make a significant contribution.

It has to be acknowledged that the artefactual insertion of lipid peroxides during the preparation of LDL could also contribute to an apparent increased oxidizability of an individual's LDL. However, this does appear to depend on the donor, since LDL prepared under apparently identical conditions shows a transition metal-dependent variation in oxidizability (Dieber-Rotheneder *et al.*, 1991; Smith *et al.*, 1993). Clearly, an assessment of the oxidizability of LDL after addition of copper as a risk factor for coronary heart disease is needed to answer this question.

3. *Cell Culture Models of Lipoprotein Oxidation*

Lipid accumulation is a characteristic feature of atherosclerosis and is thought to require the modification of LDL to a form that is rapidly degraded and internalized by the macrophage scavenger receptor (Brown and Goldstein, 1983). This protein found in the plasma membrane of the macrophage is sometimes called "molecular flypaper", reflecting its broad specificity for large polyanionic molecules (Kreiger, 1992). The uptake of oxidized LDL by macrophages may also be mediated by other mechanisms such as phagocytosis (Sparrow *et al.*, 1989). Native LDL is a transport molecule for cholesterol, phospholipids and biological co-factors such as ubiquinol. Native LDL receptors are down-regulated as the intracellular concentration of cholesterol in the cell increases. This is in marked contrast to the scavenger receptor which does not possess such a regulatory mechanism (Brown and Goldstein, 1980, 1983; Brown *et al.*, 1980). Accordingly, for lipid accumulation to occur in a lesion, it is thought that LDL, within the arterial wall, adopts a conformation as a consequence of oxidation, which is then recognized by the macrophage scavenger receptor. To study this process, many investigators have used monocultures of smooth muscle cells, endothelial cells and macrophages to model the oxidative reactions leading to LDL oxidation *in vivo* (reviewed in Esterbauer *et al.*, 1993). These studies with cell-dependent modification of LDL have two common features: (1) the oxidation process has an absolute requirement for transition metal ions in the culture medium; and (2) oxidation is inhibited by serum. The following sections summarize the results from some of the key studies in this area with a particular emphasis on the proposed mechanisms of the initiation of lipid peroxidation.

3.1 ENDOTHELIAL CELLS

Endothelial cells are a likely source of oxidants in the vasculature and were first shown to modify LDL in 1981 (Henriksen *et al.*, 1981). Using rabbit aortic endothelial cells, it was shown that LDL preincubated with these cells was degraded by macrophages, at least in part, by the scavenger receptor and no longer recognized by the native-LDL receptor (Henriksen *et al.*, 1981). This is probably the first direct evidence that LDL could be modified by cells to a potentially atherogenic form. Later it was shown that endothelial cell modification of LDL was associated with extensive conversion of phosphatidyl choline to lysophosphatidyl choline, production of a lipid peroxidation product, malondialdehyde, and modification was inhibited by vitamin E and BHT indicating that oxidation of LDL lipids was occurring (Morel *et al.*, 1984; Steinbrecher *et al.*, 1984). It was noted that this oxidative modification of LDL was dependent upon the transition metal ion content of the cell culture medium and was, not surprisingly, similar to the oxidation promoted by copper ions. Furthermore, it was later shown that non-enzymatic fragmentation of apo-B occurs during the incubation of LDL with endothelial cells and, indeed, in human atherosclerotic lesions (Yla-Herttuala *et al.*, 1989). The role of superoxide in the endothelial cell-dependent oxidation of LDL has been controversial. For example, using rabbit endothelial cells, Steinbrecher (1988) observed inhibition of LDL oxidation by superoxide dismutase (SOD), whereas Parthasarathy *et al.* (1989) observed little inhibition of oxidation by SOD.

Cellular lipoxygenases have been implicated as possible enzymatic mediators of endothelial cell-dependent oxidation of LDL. Inhibitors of lipoxygenase, but not cyclooxygenase, have been shown to be effective inhibitors of LDL oxidation using rabbit endothelial cells (Parthasarathy *et al.*, 1989). Interestingly, a phospholipase A_2 activity intrinsic to apo-B has also been implicated in the endothelial cell-dependent modification of LDL (Parthasarathay *et al.*, 1985).

3.2 MACROPHAGES

Since macrophages in the atherosclerotic lesion take up

oxidized LDL, these cells have frequently been used to monitor the potential atherogenecity of LDL after it has undergone some form of modification *in vitro*. However, macrophages are themselves able to oxidize LDL using the conditions described above for endothelial cells (Parthasarathy *et al.*, 1986). However, as with endothelial cells, oxidation requires the presence of low levels of transition metal ions in the cell-culture medium, does not occur until the α-tocopherol content of the LDL has been depleted and peroxyl radicals are involved in the propagation reaction (Jessup *et al.*, 1990). The role of superoxide in macrophage-mediated LDL oxidation is unclear, with the most recent conclusion from one group being that there was "no secure evidence for the involvement of superoxide" (Jessup *et al.*, 1993). In the latter study it was noted that inhibition of oxidation by SOD was not necessarily diagnostic of the involvement of superoxide radicals, since the enzyme was able to chelate transition metal ions and thus had other antioxidant properties independent of its enzymatic activity. This problem of inhibitor specificity has also confounded attempts to investigate the role of lipoxygenase in macrophage-dependent modification of LDL. Rankin *et al.* (1991) demonstrated that certain lipoxygenase inhibitors were able to prevent oxidation of LDL by mouse peritoneal macrophages. However, Jessup *et al.* (1991), and Sparrow and Olszewski (1992) have shown that many of these compounds are also able to inhibit copper-dependent (cell-free) oxidation of LDL, either by scavenging peroxyl radicals or chelating copper, and thus their action cannot be attributed solely to their activities as lipoxygenase inhibitors. Moreover, specific 5-lipoxygenase inhibitors were ineffective in inhibiting oxidation and eicosatetraynoic acid, a 15-lipoxygenase inhibitor, was shown to be effective against 15-lipoxygenase at much lower concentrations than it was against LDL oxidation.

Other possible oxidants that could be generated by cells include hypochlorite and peroxynitrite. Although both are able to modify LDL oxidatively (Graham *et al.*, 1993; Hogg *et al.*, 1993b; Hazell and Stocker, 1993) neither has been demonstrated to be responsible for oxidation in cellular systems. Stimulation of nitric oxide production has been shown to inhibit macrophage-dependent LDL oxidation (Jessup *et al.*, 1992; Yates *et al.*, 1992). The mechanism for this is unknown, although it has recently been reported that nitric oxide is able to directly inhibit copper-dependent oxidation of LDL (Hogg *et al.*, 1993c; see also Section 2.5.2).

3.3 INTRACELLULAR ANTIOXIDANTS AND ATHEROSCLEROSIS

Some of the decomposition products of lipid peroxidation, such as aldehydes, are extremely cytotoxic and it has been shown that these are formed within atherosclerotic lesions (Yla-Herttuala *et al.*, 1989; Esterbauer *et al.*, 1991; Gotoh *et al.*, 1993). A number of extensive studies have shown that the most important detoxification pathway for these molecules involves the intracellular antioxidant glutathione acting in concert with a family of enzymes known as the glutathione S-transferases (Farber *et al.*, 1990; Sies, 1993). In the artery wall it is presumed that oxidized LDL is formed extracellularly by oxidants, possibly peroxynitrite or transition metals (released as a consequence of damage to haem proteins or caeruloplasmin) and "seeding lipid peroxides" present in the LDL particle (see Section 2.6). As discussed in the previous sections, oxidative modification of LDL leads to a change in the conformation of the protein to a form that is recognized by the macrophage scavenger receptor, with the consequence that macrophages may ingest large amounts of oxidized LDL leading to pools of esterified cholesterol (Brown and Goldstein, 1983). How does the macrophage respond to the concomitant exposure to high levels of toxic aldehydes such as 4-hydroxynonenal on uptake of oxidized LDL? In culture, treatment of macrophages with oxidized LDL leads to a transitory depletion of glutathione over the first few hours followed by an induction of glutathione synthesis over the subsequent 24 hours (Darley-Usmar *et al.*, 1990, 1991). Inhibition of glutathione synthesis, using buthionine sulphoximine, renders oxidized LDL highly cytotoxic to macrophages and endothelial cells (Gotoh *et al.*, 1993). Perhaps even more significant is the finding that the induction of glutathione synthesis greatly decreases the cytotoxicity of oxidized LDL (Gotoh *et al.*, 1993). Although the relevance of these experiments to events in the artery wall is currently unknown, several results suggest they may be important. For example, it has been demonstrated that hypercholesterolaemia in rabbits is associated with increased free thiol groups, probably glutathione and activity of glutathione-dependent enzymes (Del Boccio *et al.*, 1990). The essential role of glutathione-dependent detoxification in the vasculature is also supported by the results of a series of experiments examining the interactions between glutathione and ascorbic acid. In this study, it was shown that the depletion in guinea pigs of both glutathione and ascorbate was highly toxic, resulting in death preceded by both extensive and rapid atherosclerosis (Willis, 1953; Meister, 1992). The toxicity and mortality of ascorbate deficiency could be delayed by repletion with glutathione ester (Martensson *et al.*, 1993). *In vitro* experiments with macrophages in culture indicated that depletion of glutathione in the macrophage was also associated with increased cholestryl ester formation and could then be highly atherogenic (Gotoh *et al.*, 1993).

It appears then that both intracellular and extracellular antioxidants may be important in the prevention of atherosclerosis. The progression of a lesion may signify that these protective pathways are overwhelmed.

4. Summary

The mechanisms of initiation that are relevant to human cardiovascular disease remain unclear. It is possible that more than one process or combination of reactions contributes to the oxidation of LDL in the artery wall. For example, we have proposed that, in the early stages of atherosclerosis, excess production of superoxide may result in the formation of peroxynitrite. This may render LDL more susceptible to oxidation by other agents, for example, transition metals or, if produced in sufficient quantities, oxidize LDL to a pro-atherogenic form (Hogg *et al.*, 1993a). In more advanced lesions the infiltration of macrophages and the activation of enzymes like the lipoxygenases could contribute to the modification of LDL, and the formation of a fatty streak. The chemical characteristics of lipid peroxidation reactions are well understood yet the role of non-enzymatic peroxidation in human pathophysiology is only recently becoming clear. Although we have focused in this short overview on the cardiovascular aspects of lipid peroxidation, many of these processes will be relevant to other organ systems such as the central nervous system. It seems likely that it will not be sufficient to intervene only at the level of the propagation stage of the peroxidation reaction with chain-breaking antioxidants, but it will be necessary to understand in more detail the initiation mechanisms and inhibit these. Hopefully, this will result in effective therapeutic approaches to other pathophysiological conditions, which involve lipid peroxidation.

5. References

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3. Free Radicals and Antioxidants in Atherosclerosis

Catherine Rice-Evans

1. Introduction

Over the years, many theories have been proposed to account for the pathogenesis of atherosclerosis including endothelial damage, permeability changes, high concentrations of low-density lipoprotein (LDL) cholesterol, thrombogenesis, etc., but the resolution of an agreed mechanism remains to be established. Biochemical and clinical studies have suggested that oxidized LDL is atherogenic and there is increasing evidence for a role for oxidized LDL *in vivo* in the development of atherosclerotic lesions. The proposed mechanism that following change or damage by some mechanism to the endothelium, the oxidation of LDLs in the subendothelial space is one of the earliest events (Steinberg *et al.*, 1989) is rapidly being advanced. The following events are shown schematically in Fig. 3.1. Endothelial damage is proposed to be followed by adherence of blood monocytes to endothelial cells (Ross, 1981) and increased permeability by the vascular endothelium for the circulating monocytes. The release of chemotactic factors attracts monocytes from the circulation to the site of damage (Willerson and Buja, 1980). Studies have shown that oxidized LDL is a potent chemoattractant for circulating human monocytes, specifically the lyso-lecithin component of the oxidized LDL and 4-hydroxynonenal its decomposition product, encouraging their recruitment into the lesioned area (Steinbrecher *et al.*, 1990). These develop into macrophages in the vessel wall. Scavenger receptors on the macrophages recognize oxidatively modified LDL molecules and convert them to cholesterol-laden foam cells characteristic of the fatty streak. In addition, factors released by macrophages stimulate the proliferation of smooth muscle cells, which may break through the elastic lamina and form a mass of

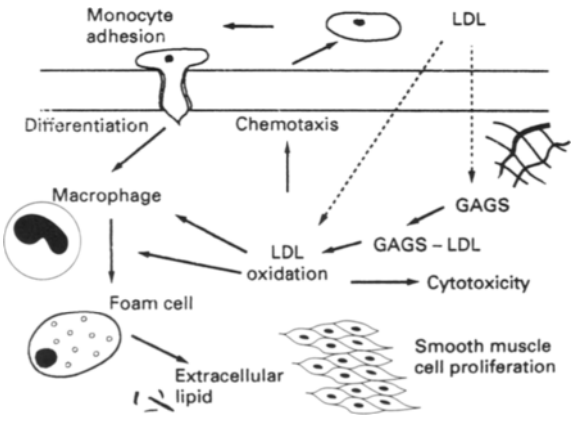

Figure 3.1 Current ideas for the scheme of events in atherosclerosis (from Rice-Evans and Bruckdorfer, 1992).

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cells that will eventually form the main mass of the atherosclerotic plaque (Ross *et al.*, 1974).

Extensive studies *in vitro* from many groups have confirmed that exposure of LDL to a variety of pro-oxidant systems, both cell-free and cell-mediated, results in the formation of lipid hydroperoxides and peroxidation products, fragmentation of apoprotein B_{100}, hydrolysis of phospholipids, oxidation of cholesterol and cholesterylesters, formation of oxysterols, preceded by consumption of α-tocopherol and accompanied by consumption of β-carotene, the minor carotenoids and γ-tocopherol.

2. *The Possible Mechanisms of the Oxidation of LDL in the Production of Lipid Hydroperoxides*

The peroxidation of a polyunsaturated fatty-acid chain in LDL (mainly 18:2 and 20:4) may be initiated by any primary free radical of sufficient reactivity to subtract an allylic hydrogen atom from a reactive methylene group of polyunsaturated fatty-acid side chains (as reviewed in Chapter 1). The formation of the initiating species is accompanied by bond rearrangement that results in stabilization by diene conjugate formation. The lipid radical then takes up oxygen to form the peroxyl radical. Propagation reactions follow leading to the formation of lipid hydroperoxides. This propagation phase can be repeated many times. Thus, an initial event triggering lipid peroxidation can be amplified, as long as oxygen supplies and unoxidized polyunsaturated fatty acid chains are available. Polyunsaturated fatty acids may also undergo enzymic oxygenation mediated by lipoxygenase enzymes, candidates being 5-, 12- and 15-lipoxygenase. It has been shown that macrophage-mediated LDL oxidation is independent of 5-lipoxygenase (Sparrow and Olszewski, 1992). The colocalization of 15-lipoxygenase in atherosclerotic lesions in the same region as oxidized LDL suggests a possible involvement (Ylä-Herttuala *et al.*, 1990) (see later), although it is not clear how the enzyme has access to LDL. The fates of lipid or fatty acyl hydroperoxides may be:

(1) Reduction by available peroxidase enzymes, e.g. glutathione peroxidase;
(2) Decomposition to aldehydic breakdown products; or
(3) Transition metal- or haem protein-mediated oxidative or reductive decomposition.

The accumulation of hydroperoxides and their subsequent decomposition to alkoxyl and peroxyl radicals can accelerate the chain reaction of polyunsaturated fatty-acid peroxidation leading to oxidative damage to cells and membranes as well as lipoproteins. It is well-recognized that transition metals or haem proteins, through their redox cycling properties, may play a role in promoting oxidative stress by catalysing the decomposition of hydroperoxides (Tappel *et al.*, 1961; O'Brien, 1969; Labeque and Marnett, 1988) generating alkoxyl radicals ($LO^{\cdot}$)

$$LOOH + HX\text{-}Fe(II) \rightarrow \quad LO^{\cdot} + HX\text{-}Fe(III) + OH^{-} \tag{3.1}$$

$$LOOH + HX\text{-}Fe(III) \rightarrow LO^{\cdot} + HX\text{-}[Fe(IV)=O] + H^{+} \tag{3.2}$$

and peroxyl radicals ($LOO^{\cdot}$)

$$LOOH + HX\text{-}Fe(III) \rightarrow LOO^{\cdot} + HX\text{-}Fe(II) + H^{+} \tag{3.3}$$

both of which can exacerbate the peroxidative process, initiating further rounds of lipid peroxidation as well as recycling the haem proteins for further oxidative events. The lipid alkoxyl and peroxyl radicals formed during the modification of the polyunsaturated fatty-acid side chains of lipids can amplify lipid peroxidation, oxidize cholesterol and can react with the protein moiety.

Cleavage of the carbon bonds during LDL peroxidation reactions may result in the formation of aldehydic decomposition products including alkanals, such as malonyldialdehyde (Tappel and Dillard, 1981), and alkenals, such as 4-hydroxynonenal (Esterbauer, 1985). It has been reported that 4-hydroxynonenal has multiple biological effects including cytotoxic properties, acting as a chemotractant, inhibition of platelet aggregation, modification of adenylate cyclase activity and acting as substrate for the glutathione transferases. Due to their lipophilic nature these aldehydic products remain associated with the LDL particle, diffusing to the apo B and may react there through Schiff's base formation with amino groups, for example, altering the charge and recognition properties (Jurgens *et al.*, 1987; Steinbrecher, 1987). Carbonyl derivatives as decomposition products of lipid peroxidation or monosaccharide oxidation can also interact with amino groups on protein amino acyl side chains, thus altering their charge and nature. This mechanism has been proposed as a contributing sequence of events in the oxidative modification of LDLs. Thus, the binding of malonyldialdehyde, 4-hydroxynonenal, etc., to lysine residues on the apoB protein portion of LDLs decreases the positive charge on the surface of the LDL and limits its uptake by the conventional LDL receptor on cells (Brown *et al.*, 1980; Haberland *et al.*, 1984; Esterbauer *et al.*, 1990). Rather, it becomes recognizable by the scavenger receptors on macrophages, forming cholesterol-laden foam cells, contributing towards the fatty streak in atherosclerosis. The apo protein B_{100} is itself also susceptible to attack from free radical intermediates of lipid peroxidation, such as alkoxyl $LO^{\cdot}$ and peroxyl $LOO^{\cdot}$ radicals. These may react with amino acids in proteins closely associated with peroxidizing lipids such as histidine. Lipid alkoxyl radicals can also cause the degradation of the apolipoprotein

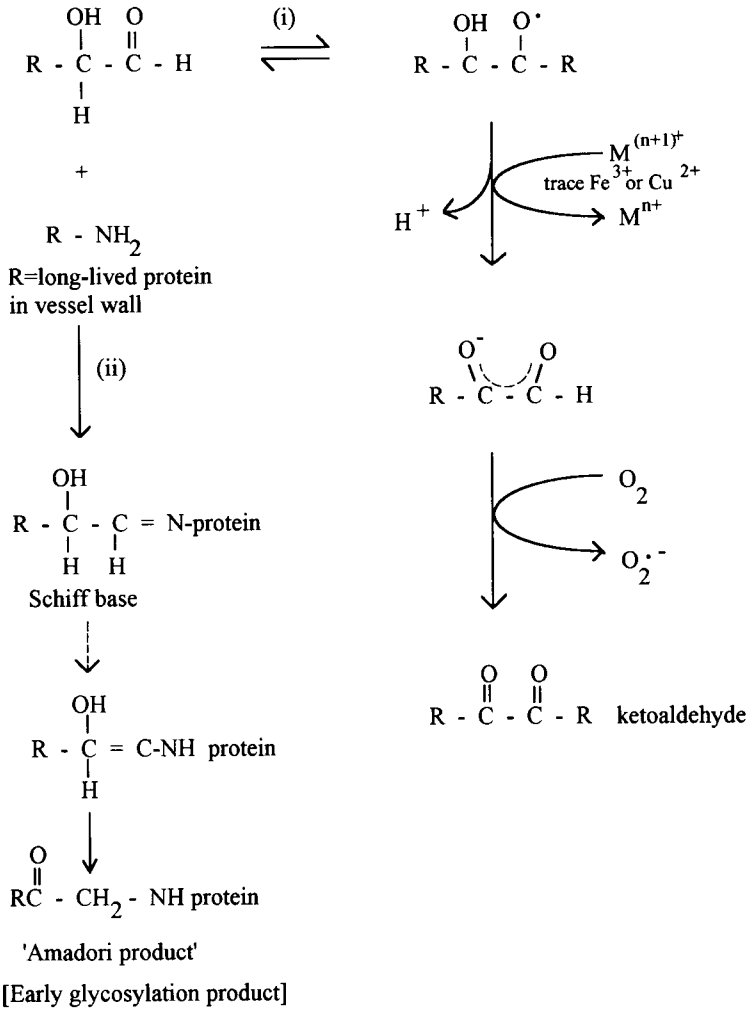

Figure 3.2 Proposed mechanisms of protein modification through carbonyl interactions involving monosaccharides [from Brownlee *et al.* (1988) and Wolff and Dean (1987)].

B_{100} to smaller peptide fragments, giving rise to new epitopes of apoB.

Protein modification via interaction with carbonyls derived from monosaccharides is also a possibility (as has been described in diabetes). There are two modes of modification by glucose (Fig. 3.2) (Wolff and Dean, 1987; Hunt *et al.*, 1988, 1993): (1) by the mechanism by which monosaccharides become oxidized when catalysed by trace amounts of transition metals, generating free radicals, hydrogen peroxide and reactive dicarbonyls directly (Hunt, 1994), and subsequent covalent binding of the carbonyl products of the process to protein components; and (2) by the Maillard reaction, which describes the non-enzymic glycation of proteins. Glucose is considered to be toxic by virtue of its ability to behave chemically as an aldehyde and is known to form chemically reversible early glycosylation products with protein at a rate proportional to the glucose concentration. These Schiff bases then rearrange to form the more stable Amadori-type early glycosylation products (Brownlee *et al.*, 1988). Protein that has been glycated *in vitro* is conformationally altered. For example, the amount of early glycosylation products *in vivo* in diabetics, whether on haemoglobin (Hb) or basement membrane, increases when blood glucose levels are normalized by treatment.

Some of the early glycosylation products on collagen and other long-lived proteins of the vessel wall do not dissociate but undergo a slow complex series of chemical rearrangements to form irreversible advanced glycosylation end-products. A number of these irreversible end-products are capable of forming covalent bonds with amino groups on other proteins, forming cross-links. It has been proposed that hyperglycaemia in diabetes may involve covalent cross-linking of extravasated plasma lipoproteins to matrix lipoproteins by advanced glycolysation end-products, and retard the rate of cholesterol efflux and accelerate the development of vascular disease.

3. *Antioxidants and their Actions*

Antioxidants are defined as any substances that, when present at low concentrations compared to those of the oxidizable substrate, significantly delay or inhibit oxidation of that substrate. The body is equipped with a range of antioxidants to protect against excessive radical generation and the consequences thereof (reviewed in Halliwell, 1990). Those located intracellularly are appropriately located for scavenging aberrant free radical species; the extracellular antioxidants have the roles of intercepting propagating radical reactions and that of removing delocalized metal ions and decompartmentalized haem proteins, by sequestering them in forms incapable of stimulating free radical reactions.

The primary antioxidant enzymes, located intracellularly, are the superoxide dismutases (Fridovich, 1978), catalase (Ogura and Yamazaki, 1983), glutathione peroxidase (Cohen and Hochstein, 1963) and other peroxidases. Other essential secondary enzymes involved in the antioxidant capacity are: those promoting the synthesis of such antioxidant compounds as glutathione, uric acid, bilirubin and ubiquinol; those enzymes preventing the formation of reactive oxygen species, e.g. glutathione transferases and DT-diaphorase; and those promoting the regeneration of antioxidant compounds, namely glutathione reductase and semiquinone reductases. The major plasma antioxidants include ascorbate, albumin, urate, bilirubin, the iron-controlling network of caeruloplasmin and transferrin, the haem-removing antioxidants haptoglobin and haemopexin, and the lipoprotein-soluble antioxidants the tocopherols, the carotenoids and ubiquinol-10.

The water-soluble antioxidant, ascorbate, is located in specific intracellular locations, particularly muscle, the adrenals and the eye, as well as in plasma (reference interval for concentration in plasma: 34–111 μM). The most important chemical property of ascorbate is its ease of oxidation either by one- or two-electron transfer. Both the 2- and 3-hydroxyl groups must be unsubstituted for antioxidant activity (Cort, 1982). During its antioxidant action, ascorbic acid undergoes a two-electron oxidation to dehydroascorbate with intermediate formation of the ascorbyl radical. Dehydroascorbic acid is relatively unstable and can be reduced back to ascorbic acid or hydrolysed to diketogulonic acid. It reacts with oxygen and hydrogen peroxide, and scavenges oxygen radicals: superoxide, hydroperoxyl, hydroxyl radical and singlet oxygen (Fessenden and Verma, 1978; Nanni *et al.*, 1980; Cabelli and Bielski, 1983). In the presence of transition metals, especially iron (III) or copper (II) ions, the autoxidation of ascorbate is catalysed forming dehydroascorbate with the production of hydrogen peroxide (Martell, 1982). The ascorbate monoanion is involved in an intermediate complex.

Albumin, the major protein of human plasma (reference interval: 535–760 μM), has the properties of copper binding (Halliwell, 1988) and protects against haem-catalysed oxidation. The sulphydryl group of albumin may also have importance in reactions with peroxyl radicals at specific localizations in intercepting hypochlorite (Wasil *et al.*, 1987).

Urate (reference interval for concentration in plasma: 180–420 μM) (Wayner *et al.*, 1985) has been proved to be a selective antioxidant (reviewed in Becker, 1993) capable of the chelation of catalytically active iron (Sevanian *et al.*, 1991), reaction with hypochlorous acid (Thomas, 1992) and can attenuate the ozone-induced oxidation of lipids, lipoproteins and unsaturated fatty acids (Meadows *et al.*, 1986; Cross *et al.*, 1992). Currently, the tendency still remains to regard uric acid as a risk factor for atherosclerosis and coronary heart disease (Persky *et al.*, 1979; Brand *et al.*, 1985; Agamah *et al.*, 1991), although recent epidemiological studies have not confirmed the predicted associations. Several studies have suggested that the urate radical might be a reactive oxidant and thus its maximal effectiveness as an antioxidant might be in the presence of a potential regenerating agent such as ascorbate (Maples and Mason, 1988), although urate has also been described as a protective antioxidant for ascorbate (Lam *et al.*, 1984). It is, however, unlikely that this would be physiologically relevant, since plasma ascorbate seems to be utilized prior to urate (Stocker and Frei, 1991).

Bilirubin (normal plasma concentration <20 μM) is able to scavenge singlet oxygen and peroxyl radicals. It has been proposed that bilirubin bound to human albumin contributes significantly to the non-enzymic antioxidant defences in human plasma (Stocker and Ames, 1987).

α-Tocopherol (reference interval for concentration in plasma: 14–44 μM) is the major lipid-soluble chain-breaking antioxidant in plasma (Burton *et al.*, 1983) and in cell membranes. It inhibits amplification of peroxidation by intercepting propagating lipid chains by reducing peroxyl radicals to hydroperoxides. α-Tocopherol reacts with peroxyl radicals with a rate constant of approximately 10^6 mol^{-1} s^{-1} (Niki *et al.*, 1984). However, in the presence of haem proteins, or iron or copper complexes, lipid peroxides can continue cycling, to peroxyl radicals, which can be intercepted by tocopherol, or alkoxyl radicals to hydroxyl derivatives, which terminate the sequence.

It has been proposed that the α-tocopheroxyl radical can be recycled back to tocopherol by ascorbate producing the ascorbyl radical (Packer *et al.*, 1979; Scarpa *et al.*, 1984). The location of α-tocopherol, with its phytyl tail in the membrane parallel to the fatty acyl chains of the phospholipids and its phenolic hydroxyl group at the membrane–water interface near the polar headgroups of the phospholipid bilayer, enables ascorbate to donate hydrogen atoms to the tocopheroxyl radical. The suitability for ascorbate and tocopherol as chain-breaking antioxidants is exemplified (Buettner,

1993) in the fact that they are effective in relatively small amounts and their radical states are relatively unreactive, in either reducing or oxidizing capacities. Recent studies in experimental chemical systems have described studies in which the α-tocopheroxyl radical can act as a pro-oxidant (Bowry *et al.*, 1992). This is perhaps not so surprising in view of the well-known characteristic of many antioxidants in their assumption of the characteristics of pro-oxidants at high concentration.

A number of polyenes and carotenoids are associated with lipid antioxidant functions (Krinsky, 1988). In addition, in humans, β-carotene, α-carotene and cryptoxanthine are converted to vitamin A, whereas lutein and lycopene are not. The antioxidant function of carotene is attributed to its molecular structure, which allows the quenching of singlet oxygen and scavenging of free radicals. β-Carotene (0.3–0.6 μM range of concentrations in human plasma) may exert its antioxidant function through its ability to interact with a radical yielding a less reactive resonance-stabilized carbon-centred radical species (Burton and Ingold, 1984), whereby the chain-carrying peroxyl radicals add covalently to the conjugated system of β-carotene. β-Carotene appears to exert its optimal effectiveness at low oxygen tensions. It has been proposed that lycopene (0.5–1.0 μM) also located in LDLs is the most efficient singlet oxygen quencher (Di Mascio *et al.*, 1989).

Ubiquinol-10 (or coenzyme Q_{10}; see Corongiu *et al.*, 1993) (0.4–1.0 μM range of concentrations in human plasma) has recently been proposed as a chain-breaking antioxidant (Beyer, 1990) in LDLs as the first line of defence (Stocker *et al.*, 1991) and in liposomal systems.

The role of ubiquinol-10 in the inhibition of the early stages of lipoprotein lipid oxidation has recently been defined. Using ultrasensitive high performance liquid chromatography (HPLC) assays for lipid hydroperoxides, Stocker *et al.* (1988) concluded that ubiquinol-10 is associated with LDL, and that under conditions of constant rate of initiation the oxidation chain length in LDL was low as long as ubiquinol-10 was present, but increased 25-fold upon its consumption even though 80–90% of α-tocopherol and carotenoids were still present. Dietary supplementation of human volunteers resulted in increased concentration of ubiquinol-10 within circulating LDL and such supplemented LDL was found to be more resistant towards the initiation of lipid oxidation, to an extent that was proportional to the initial concentration of ubiquinol-10 in the LDL.

Haptoglobin (plasma concentration range 0.5–3.6 mg/ml) binds free haemoglobin released from erythrocytes preventing iron from being made available and the haem protein from propagating lipid peroxidation. Haemopexin (0.6–1.0 mg/ml in plasma) binds free haem thus inhibiting haem-induced propagation of lipid peroxidation. The copper-containing ferroxidase, caeruloplasmin (180–400 μg/ml), catalyses iron (II) conversion to iron (III) enabling its subsequent removal by binding to transferrin. Transferrin (1.8–3.3 mg/ml) itself binds ferric iron (2 per mol of protein) with a binding constant of 10^{20}, making it unavailable for catalysing radical reactions. This iron-transporting protein of the blood delivers it to the storage sites on ferritin in cells. Normally, the iron-binding sites on transferrin are only 30% occupied explaining the lack of free or available iron in human plasma.

The hierarchy of antioxidant effectiveness in plasma has been defined from *in vitro* studies (Frei *et al.*, 1988). This, of course, must be interpreted in relation to the amount of each antioxidant as well as the rate of its reaction with the specific radical substrate and the relevance of that substrate in human plasma. The hierarchy is:

Ascorbate = Protein thiols > Bilirubin > Urate > Tocopherol

against radicals generated in the aqueous phase. Plasma depleted of ascorbate but replete in the rest is vulnerable to oxidative stress, and susceptible to lipid oxidation from radicals generated in the aqueous phase. However, plasma replete in ascorbate but deficient in the others is resistant to such oxidation until the ascorbate is consumed. The lipophilic antioxidants of plasma are located mainly in the lipoproteins. In the LDL fraction, the polyunsaturated fatty acids of the phospholipids constituting the outer monolayer are protected by α-tocopherol, with approximately 10 mol per mol of fatty acid, whereas the inner core of cholesterol esters contains the carotenoids, β-carotene, lycopene, lutein, phytofluene, zeaxanthin, and α-carotene, which have had the role of protection of the polyunsaturated fatty-acid chains against oxidation attributed to them.

Recent studies have pointed to the importance of the antioxidant nutrients vitamin C, vitamin E and β-carotene in maintaining health, in contributing to a decreased incidence of disease and in protecting against the recurrence of pathological events. The WHO cross-cultural epidemiological survey (Gey *et al.*, 1991) among 16 European countries showed an inverse correlation between vitamin E levels in the blood and mortality from ischaemic heart disease. The Harvard male physicians study supplementing with 50 mg β-carotene on alternate days showed a 44% reduction in all major coronary events (Gaziano *et al.*, 1990). The time is approaching when it is becoming possible to define the intake of antioxidant nutrients that are associated with a low subsequent incidence of coronary heart disease. The recent health professionals study (Rimm *et al.*, 1993; Stampfer *et al.*, 1993) in groups of 40,000 males and 87,000 females showed a 37% reduction in risk for males from coronary heart disease after daily supplementation with 100 IU of vitamin E for at least 2 years, and a 41% reduction in risk for females with 100 IU for the same minimum period.

A major question in considering the mechanisms relating antioxidant status in the blood with decreased risk of coronary heart disease is the relationship between plasma antioxidant levels and the total antioxidant activity, which might depend on synergistic interactions between antioxidants. Many studies *in vitro* have discussed the synergistic interactions between vitamin E and vitamin C, and of vitamin E with β-carotene and shown that the water-soluble vitamin C acts to keep vitamin E, located in a lipophilic region, in its reduced state (Packer *et al.*, 1979; Burton *et al.*, 1983; Scarpa *et al.*, 1984). In addition, based on the standard one-electron reduction potentials, Buettner (1993) has predicted a hierarchy of antioxidants from highly oxidizing to highly reducing species in terms of the ability of each reducing species to donate an electron (or hydrogen atom) to any oxidized species listed above. Thus, in general, in a cascade of free-radical reactions, each reaction in the sequence will generate less reactive radicals, with antioxidants producing the least reactive radicals of all. However, in the presence of transition metals, such as iron or copper and oxygen, this would not be the case but rather the reactivity would be amplified.

4. *The Involvement of Free Radicals in the Pathogenesis of Atherosclerosis*

The earliest recognizable lesion of atherosclerosis is the fatty streak, an aggregation of lipid-rich macrophages and T lymphocyte within the intima, the innermost layer of the artery wall. The hypothesis that LDLs that have undergone oxidative damage are considerably more atherogenic than native LDLs was promulgated by the group of Steinberg *et al.* (1989) who proposed that oxidized LDL and certain other modified forms are ligands for scavenger receptors on macrophages, and can convert them to the cholesterol-laden foam cells characteristic of the fatty streak.

Evidence is accumulating from *in vitro* studies that LDL can be oxidatively modified by a variety of systems and agents, and is subsequently recognized and rapidly taken up by scavenger receptors on target macrophages. Such systems include: cells in culture, including endothelial cells, smooth muscle cells and macrophages; ruptured erythrocytes and myocytes; co-incubation with transition metal ions, such as copper, or with haem proteins such as haemoglobin and myoglobin; interaction with haem protein-derived free radicals, with peroxynitrite with specific oxygenase enzymes or by incorporation of fatty acyl hydroperoxides (Fig. 3.3).

4.1 CELL-INDUCED MODIFICATION

Evidence from cellular studies *in vitro* initially showed how oxidative processes could play a central role in the pathological changes involved in the genesis of atherosclerosis. LDL can be oxidatively modified in culture by a range of cell types including endothelial cells (Henriksen *et al.*, 1981), arterial smooth muscle cells

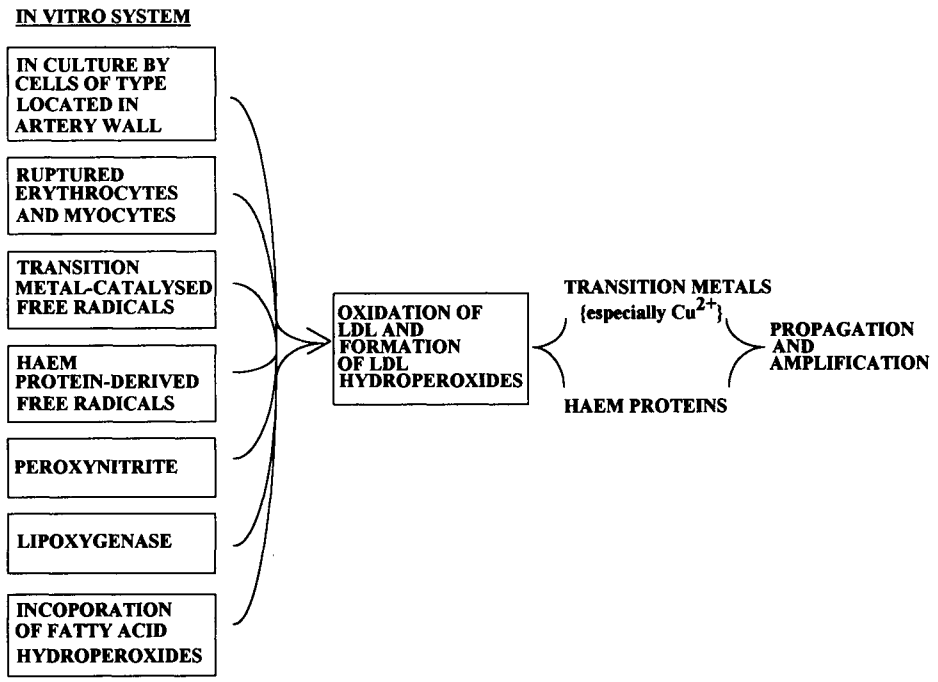

Figure 3.3 Mediators of LDL oxidation *in vitro*.

(Henriksen *et al.*, 1983; Heinecke *et al.*, 1984, 1986) as well as macrophages (Parthasarathy *et al.*, 1986; Rankin and Leake, 1987; Leake and Rankin, 1990), monocytes (Cathcart *et al.*, 1985) and is subsequently taken up by the scavenger receptors on target macrophages. Human neutrophils have also been shown to be capable of mediating LDL oxidation such that it becomes cytotoxic but these cells are not a common constituent of atheromatous lesions (Cathcart *et al.*, 1985). The relative contributions of the different cell types may achieve different levels of importance at the various stages in the development of the lesion. Normal arterial wall contains endothelial cells and smooth muscle cells, whereas atherosclerotic lesions may also contain macrophages and T lymphocytes. Thus when the atherosclerotic lesion develops, what is the mechanism by which LDL becomes oxidized *in vivo*? Stimulation of endothelial cells, smooth muscle cells or macrophages may induce the secretion of components capable of promoting mechanisms of initiation of LDL oxidation: specific free-radical species capable of initiating oxidation may be formed or propagation of peroxidation may occur subsequent to lipoxygenase-mediated hydroperoxide formation. If the former, what is the probable initiating agent, where is it located and what activates it? If the latter, are the lipoxygenases macrophage-derived or from other cell sources?

Cell-induced modification of LDL *in vitro* has been demonstrated to be mediated by free radicals. All the cell types mentioned have been shown to release superoxide radicals, albeit by different mechanisms and at different rates. However, it is known that superoxide radicals will not initiate the oxidation of polyunsaturated fatty acids (unless protonated). Thus, addition of superoxide dismutase has an inhibitory effect on the oxidative modification, although the response varies according to cell type, implicating superoxide radical in the mechanism of cell-mediated modification (Morel *et al.*, 1984; Heinecke *et al.*, 1986). The significance of superoxide radical in the initiation, but not in the propagation, of LDL oxidation in cultures of monocytes/macrophages is indicated by experiments showing inhibition of the oxidative modification by superoxide dismutase only if the antioxidant is added within a few hours of the initiation of the incubation. However, an antioxidant such as butylated hydroxytoluene, a lipid chain-breaking antioxidant as well as a hydroxyl radical scavenger, is effective in inhibiting the oxidative modification as late as 11 h after the onset of incubation. It is important to note that small amounts of iron in the medium are an absolute requirement for oxidation of LDL by cultured macrophages (Leake and Rankin, 1990) and probably for all cell systems implicated. Lipoxygenase inhibitors are effective in inhibiting the oxidative modifications induced by endothelial cells and macrophages (Parthasarathy *et al.*, 1989; Rankin *et al.*, 1991), lipoxygenase inhibitors also being substances with antioxidant properties. Studies from the laboratory of Parthasarathy *et al.* suggest that endothelial cells can initiate the oxidation of LDL through a superoxide-independent pathway that involves lipoxygenase and this pathway may predominate in endothelial cells. Chisolm's group has reported that monocyte-mediated oxidation of LDL involves monocyte lipoxygenase products, which induce release of superoxide radical from the monocytes (McNally *et al.*, 1990). Thus, of the cells present in the arterial wall, activated macrophages, endothelial cells and smooth muscle cells, all of which secrete superoxide radicals, hydrogen peroxide and hydrolytic enzymes, have been reported to oxidize LDL but, as mentioned above, the superoxide released from these cells and hydrogen peroxide generated therefrom are not very reactive *per se* towards polyunsaturated fatty acids. Their reactivity may, in principle, be amplified at lower pH values through oxidative damage through protonation of superoxide, or in the presence of: (1) available delocalized haem proteins generating ferryl haem protein-derived radicals; (2) transition metal ions generating hydroxyl radical; or (3) nitric oxide forming peroxynitrite (see Chapter 2) (Fig. 3.4).

4.2 CO-INCUBATION WITH TRANSITION METALS, OR HAEM PROTEINS AND RUPTURED ERYTHROCYTES AND MYOCYTES

Injury to cells and tissues may enhance the toxicity of the active oxygen species by releasing intracellular transition metal ions (such as iron) into the surrounding tissue from storage sites, decompartmentalized haem proteins, or metalloproteins by interaction with delocalized proteases or oxidants. Such delocalized iron and haem proteins have the capacity to decompose peroxide to peroxyl and alkoxyl radicals, exacerbating the initial lesion.

The 4 g of iron in the human body is normally compartmented into its functional locations in the haem- and non-haem-containing, and iron-binding proteins and enzymes (Fig. 3.5). The majority (65%) of the iron is in the divalent state in haemoglobin and myoglobin, which are involved in the transport and storage of oxygen in erythrocytes and myocytes, respectively. The remainder is distributed between storage sites, predominantly in the

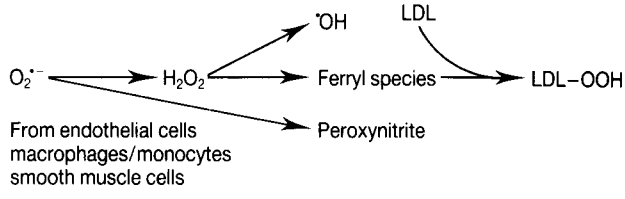

Figure 3.4 Amplification of the reactivity of superoxide and hydrogen peroxide.

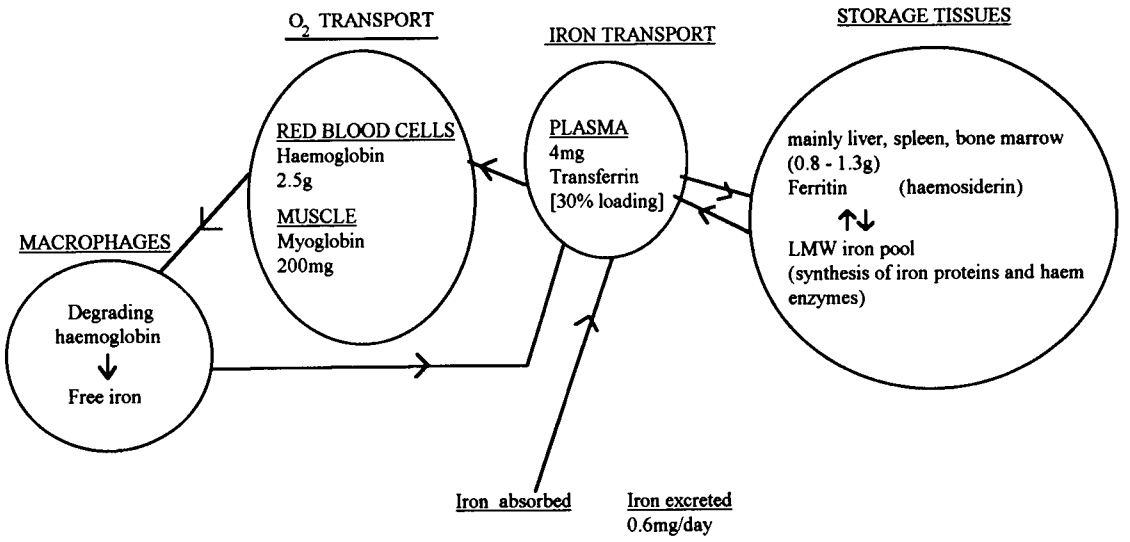

Figure 3.5 Normal functional localization of iron.

liver, spleen and bone marrow, bound to ferritin, in the low molecular weight iron pool awaiting synthesis of iron-containing proteins and enzymes, or bound to transferrin for transport and to lactoferrin. Transferrin is normally only 30% saturated, so any iron released into plasma will immediately be mopped up in the normal course of events.

Thus the question arises as to what forms of haem proteins and transition metals are available *in vivo* that are capable of mediating the formation of damaging initiating or propagating species, since the majority of the iron and haem proteins in the human body are protected *in vivo* from exerting pro-oxidant activities by their compartmentalization within their functional locations in the haem and non-haem iron-containing proteins and enzymes.

In atherogenesis, the trapping of released haem proteins from ruptured erythrocytes in the artery wall in the oxidizing locality of activated macrophages may create a scenario for the formation of propagating species. This hypothesis presupposes the presence of available haemoproteins in the subendothelial space. It should also be noted, however, that haemoglobin has been observed to occur freely in more advanced atherosclerotic plaques where haemorrhaging occurs. Delocalized haem proteins may also be significant, in the appropriate location, for converting hydroperoxides generated through a lipoxygenase-dependent or non-enzymic pathway into alkoxyl and peroxyl radical species, which are capable of initiating peroxidation in LDL in the same way as haem proteins and transition metal complexes can propagate oxidative damage in LDL. It has been shown in model systems *in vitro* that iron can be released from haemoglobin (Gutteridge, 1986) and myoglobin (Puppo and Halliwell, 1988; Rice-Evans *et al.*, 1989) by excess hydrogen peroxide formed from superoxide radical, and from myoglobin by lipid hydroperoxides in oxidizing LDLs (Rice-Evans *et al.*, 1993). This may be a potential mechanism for the origins of non-haem iron in the lesion. Thus, a source of the reported iron deposition in the artery wall in "gruel" isolated from human atherosclerotic lesions (Smith *et al.*, 1992) may be microbleeding from haemorrhaging in advanced lesions. In addition, recent studies have indicated possible relationships between the risk of myocardial infarction and serum ferritin levels (Salonen *et al.*, 1992) and others have shown associations between plasma copper levels and the progression of atherosclerotic lesions (Salonen *et al.*, 1991).

Oxidation of LDL mediated by these agents is dependent on the presence of preformed lipid hydroperoxides or minimally modified LDL. In the presence of preformed lipid hydroperoxides, induced by enzymic pathways (lipoxygenase-mediated) or non-enzymic pathways (radical-mediated), propagation of peroxidation can be effected in the vicinity of haem-containing and iron-containing species, generating alkoxyl and peroxyl radicals, which can amplify the damage by initiating further rounds of lipid peroxidation (O'Brien, 1969; Labeque and Marnett, 1988):

$$\text{LOOH} + \text{Fe(III) complex} \rightarrow \text{LOO}^{\cdot} + \text{Fe(II) complex} \quad (3.4)$$

$$\text{LOOH} + \text{Fe(III) complex} \rightarrow \text{LO}^{\cdot} + [\text{Fe(IV)}=\text{O}] \text{ complex} \quad (3.5)$$

$$\text{LOOH} + \text{Fe(II) complex} \rightarrow \text{LO}^{\cdot} + \text{Fe(III) complex} \quad (3.6)$$

$$\text{LO}^{\cdot} + \text{LH} \rightarrow \text{LOH} + \text{L}^{\cdot} \quad (3.7)$$

$$\text{L}^{\cdot} + \text{O}_2 \rightarrow \text{LOO}^{\cdot} \quad (3.8)$$

$$\text{LOO}^{\cdot} + \text{LH} \rightarrow \text{LOOH} + \text{L}^{\cdot} \quad (3.9)$$

Thus the alkoxyl radical formed is susceptible to interaction with polyunsaturated fatty acid chains, effectively reinitiating further damage, or interaction with a

chain-breaking antioxidant such as α-tocopherol or probucol, forming the hydroxy fatty acyl derivative, LOH, terminating the interaction for this species. The fate of lipid peroxyl radical passing through the same sequence of events will be the formation of lipid hydroperoxide, which can then re-enter the same propagative cycle catalysed by haem proteins or transition metal complexes, leading to further lipid peroxidation and oxidative modification of the LDL. However, this does not explain the nature and origins of the initiating species.

Esterbauer *et al.* (1992) have studied the *in vitro* effects of copper on LDL oxidation and have shown that there are three distinct stages in this process. In the first part of the reaction, the rate of oxidation is low and this period is often referred to as the lag phase; the lag phase is apparently dependent on the endogenous antioxidant content of the LDL, the lipid hydroperoxide content of the LDL particle and the fatty acid composition. In the second or propagation phase of the reaction, the rate of oxidation is much faster and independent of the initial antioxidant status of the LDL molecule. Ultimately, the termination reactions predominate and suppress the peroxidation process. The extensive studies of Esterbauer *et al.* have demonstrated the relative importance of the endogenous antioxidants within the LDL molecule in protecting it from oxidative modification.

The lag-phase measurement at 234 nm of the development of conjugated dienes on copper-stimulated LDL oxidation is used to define the oxidation resistance of different LDL samples (Esterbauer *et al.*, 1992). During the lag phase, the antioxidants in LDL (vitamin E, carotenoids, ubiquinol-10) are consumed in a distinct sequence with α-tocopherol as the first followed by γ-tocopherol, thereafter the carotenoids cryptoxanthin, lycopene and finally β-carotene. α-Tocopherol is the most prominent antioxidant of LDL (6.4 ± 1.8 mol/mol LDL), whereas the concentration of the others γ-tocopherol, β-carotene, lycopene, cryptoxanthin, zeaxanthin, lutein and phytofluene is only 1/10 to 1/300 of α-tocopherol. Since the tocopherols reside in the outer layer of the LDL molecule, protecting the monolayer of phospholipids and the carotenoids are in the inner core protecting the cholesterylesters, and the progression of oxidation is likely to occur from the aqueous interface inwards, it seems reasonable to assign to α-tocopherol the rank of the "front-line" antioxidant. *In vivo*, the LDL will also interact with the plasma water-soluble antioxidants in the circulation, not in the artery wall, as mentioned above.

In a screening study with 78 subjects, the lag phase varied from 34 to 114 min. Interestingly, only a weak correlation was found between the α-tocopherol content and the lag phase ($r = 0.2$, $P < 0.01$, $n = 78$). Increasing the α-tocopherol content of individual LDL samples *in vitro* or by oral supplementation led always to a proportional increase of oxidation resistance, according to the equation $y = kx + a$. The slope k is the efficacy of vitamin E and the intercept a represents a vitamin E-independent parameter. Strong, individual variations were observed for k and a (0.7–17 for k and 0.68–108 min for a). Thus the vitamin E content alone is not predictive for the oxidation resistance of an individual LDL but vitamin E-independent factors such as the levels of other antioxidants, the polyunsaturated fatty-acid content and the hydroperoxide level are also significant.

Other antioxidants not contained in LDL (probucol, α- and γ-tocotrienol, trolox C, ascorbate, glutathione) were also tested *in vitro*. All of them prolonged the lag phase, with probucol as the most efficient one. In contrast to ascorbate, trolox C and glutathione, probucol did not spare vitamin E in LDL. Indeed, studies of the efficacy of probucol *in vivo* in animal models of atherosclerosis have demonstrated protection from LDL oxidation and a decrease in the rate of the progression of the disease. The protective effects are independent of the antiatherogenic properties of the drug through its cholesterol-lowering properties.

In order to understand the potential for haem proteins to mediate the oxidative modification of LDLs, the interaction between ruptured erythrocytes (Paganga *et al.*, 1992) and ruptured myocytes (Bourne *et al.*, 1994) with LDL has been explored. Previous studies from this group have demonstrated that ferryl myoglobin radicals and ruptured cardiac myocytes, which generate ferryl myoglobin species on activation (Turner *et al.*, 1990, 1991), oxidatively modify LDL (Dee *et al.*, 1991; Rice-Evans and Bruckdorfer, 1992; Rice-Evans *et al.*, 1993). The interaction of ruptured erythrocytes and myocytes with LDL induces oxidative damage to the LDL, as detected by alterations in electrophoretic mobility and the peroxidation of the polyunsaturated fatty acyl chains. Difference spectroscopy reveals that the amplification of the oxidative process to the LDL by the haem proteins is apparently dependent on the transition of the oxidation state of the haemoglobin in the erythrocyte lysate from the oxy (HX-Fe(II)-O_2) to the ferryl (HX-Fe(IV) = 0) state via the deoxy form (Paganga *et al.*, 1992). The time-scale of this haem conversion is related to the antioxidant status of the LDL and that of the erythrocyte lysate. The incorporation of lipid-soluble antioxidants, such as tocopherol and butylated hydroxytoluene (BHT) at specific time points during the LDL–erythrocyte interaction, prolongs the lag phase to oxidation, eliminates the oxy to ferryl conversion of the haemoglobin and delays the oxidative modification of the LDL.

The findings here suggest that, after an initial slow phase corresponding to the antioxidant capacity of the LDL, hydroperoxides can interact with haemoglobin in a similar manner to hydrogen peroxide, forming ferryl haemoglobin, which is then rapidly reduced to mixtures consisting mainly of oxy- and met- forms, possibly by the synproportionation reaction, as proposed in the studies

of Guilivi and Davies (1990). Incorporation of the chain-breaking antioxidant arrests the rapid transition from the oxy to the ferryl form and inhibits LDL oxidation, supporting the idea that it is the interaction between lipid hydroperoxides and oxyhaemoglobin that is essential for the haemoglobin-mediated modification to LDL to take place. Enhancement of the antioxidant status of the LDL increases the resistance of LDL to oxidation and to oxidative damage induced by erythrocyte lysate; thus the antioxidant capacity of the LDL is a controlling factor in the oxidation of oxyhaemoglobin to more reactive, damaging forms.

Balla *et al.* (1991) have recently reported the destruction of haem and the release of iron on interaction of LDL with haemin/hydrogen peroxide mixtures (10- or 20-fold molar excess of peroxide). Our studies clearly show that, during interaction between LDL and erythrocyte lysate, LDL oxidation and oxidative activation of the oxyhaemoglobin occur with no requirement for exogenous oxidants, involving no haem destruction nor iron release during the time-scale studied. It has been shown that lipid-soluble antioxidants such as probucol or BHT lower the frequency of occurrence of atherosclerotic lesions in animals (Carew *et al.*, 1979; Bjorkhem *et al.*, 1991). Our examination of the incorporation of the lipid-soluble antioxidants α-tocopherol, BHT, etc., into the LDL and water-soluble antioxidants ascorbate-reduced glutathione, haptoglobin, etc., into the haem protein lysate reveals a delay in the onset of the oxidative conversion of the oxyhaemoglobin, the abolition of the transition to the ferryl and met- state and the inhibition of LDL oxidation. Thus haem proteins leaking from ruptured cells may be capable of enhancing the oxidation of LDL, which has penetrated the endothelium. This may occur by haem protein-mediated decomposition of preformed peroxides in LDL, which has already been minimally oxidized by contact with neighbouring cells or the enzymatic activity of lipoxygenases.

Iron may be released from certain haemoproteins when exposed in a local region to a relative excess of hydrogen peroxide arising continually from superoxide, perhaps released from inflammatory or other superoxide-producing cells. In addition, recent *in vitro* studies from Rice-Evans *et al.* (1993) have shown that LDL oxidized by haem protein-derived free radicals accumulates lipid hydroperoxides, which, in a time-dependent manner, destabilize the haem ring, promote haem destruction and iron release. Any released iron may, in an appropriate environment, exert catalytic effects in the generation of other highly reactive toxic-initiating species.

5. *Oxidized LDL – Is It a Reality?*

There is now strong evidence that LDL oxidation does indeed occur *in vivo* (see later) and strong clinical validation of the oxidation hypothesis has been achieved. The mechanism of oxidative modification of LDL in the artery wall *in vivo* remains uncertain but many studies from several distinguished groups have indicated that:

(1) LDL extracted from atherosclerotic lesions is in an oxidized state (Ylä-Herttuala *et al.*, 1989).
(2) LDL extracted from human or animal atherosclerotic lesions has been shown to be taken up much faster than plasma LDL by macrophages by means of their scavenger receptors.
(3) Antibodies that recognize oxidized LDL but not native LDL show positive reactivity in human or animal atherosclerotic lesions, but not the normal arterial wall (Haberland *et al.*, 1988; Palinski *et al.*, 1989, 1990; Rosenfeld *et al.*, 1990).
(4) Autoantibodies against oxidized LDL have been demonstrated in the plasma of patients and hypercholesterolaemic animals (Palinski *et al.*, 1989; Mitchinson *et al.*, 1988).
(5) Antioxidants that inhibit LDL oxidation *in vitro* prevent fatty streak formation in animal models (Carew *et al.*, 1987; Kita *et al.*, 1987; Bjorkhem *et al.*, 1991) and others are associated with protection against coronary artery disease in population studies (Gey *et al.*, 1991; Stampfer *et al.*, 1993; Rimm *et al.*, 1993).

Thus, given the evidence, there is strong experimental and epidemiological support for the "oxidized LDL" hypothesis.

More information, however, is needed concerning the *mechanisms* involved in the oxidative modification in the artery wall *in vivo*. How is LDL oxidation initiated? How is it amplified? Would supplementation with antioxidants be the answer? Knowledge of the precise mechanisms by which oxidation is mediated is required to elucidate the precise targets for antioxidant strategies. However, it must be emphasized that oxidized LDL has other properties that make it more atherogenic than native LDL, namely its chemotactic action towards monocytes, its cytotoxicity, its ability to stimulate the release of growth factors and cytokines, and its reported interference with the response of the arteries to nitric oxide. Might antioxidants protect against these effects or is their major role to protect against the oxidation *per se*?

Several studies have asked the question that if oxidized LDL is an important factor in atherogenesis, would its circulating concentration be expected to be higher in patients suffering from coronary artery disease? Recent studies from Sweden (Regnstrom *et al.*, 1992) have confirmed that the susceptibility of LDL to oxidation is associated with the severity of coronary atherosclerosis in middle-aged men. Other studies from Canada (Liu *et al.*, 1992) have demonstrated that LDL isolated from middle-aged male patients with coronary heart disease contains an increased content of oxidized cholesterol as well as displaying an increased susceptibility of the LDL to oxidation *in vitro*. Our recent studies on patients with carotid and femoral artery atherosclerosis have indicated

that LDL isolated from patients is partially oxidized and that a relationship exists between the susceptibility to oxidation of the LDL and the progression of the disease (Andrews *et al.*, 1994). LDLs were isolated from the blood of 37 patients (35 male, two female) with a demonstrable atherosclerotic plaque detected by duplex scanning. The susceptibility to oxidation of the LDL induced by an exogenous oxidative stress (5 μM metmyoglobin) was assessed by three methods: by measuring the breakdown products of lipid peroxidation; by observing spectroscopically the increased formation of conjugated dienes; and by monitoring the change in the surface charge of the apolipoprotein portion of the LDL.

The progression of the atherosclerotic plaque was assessed by measuring the maximum velocity of blood through the narrowest portion of the vessel at inclusion and after one year. A total of 29 of the 37 samples taken were found to have LDLs that were partially oxidized, whereas eight samples showed LDLs where the state of oxidation was within the normal range. Progression of the atheromatous plaque occurred in 19 of the 29 patients whose lipoproteins were partially oxidized compared with only two of the eight patients with normal lipoproteins ($P = 0.05$, Fisher's exact test). These preliminary data support an association between the progression of atherosclerotic plaques in carotid and femoral vessels, and the susceptibility to oxidation of the LDLs.

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7. *References*

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4. Oxidant Stress and the Heart: Modulation of Ion Transport Mechanisms During Ischaemia and Reperfusion

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Immunopharmacology of Free Radical Species
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1. *Reperfusion of the Heart and Oxidant Stress*

1.1 BACKGROUND

Numerous studies have sought to investigate the mechanisms underlying, and possible therapeutic approaches to, ischaemic injury in the heart. In the 1970s and 1980s, the concept of pharmacological infarct size reduction was investigated by many laboratories, and many studies suggested that ischaemic injury could be prevented and infarct size reduced *in the absence of reflow* (Maroko *et al.*, 1971; Maroko and Braunwald, 1973). It is now clear, however, that such interventions may delay rather than prevent the development of ischaemic injury and, if the myocardium within an area of severe ischaemia is to survive permanently, blood flow must be restored (Hearse and Yellon, 1984). Whilst attention was focused on the ischaemic myocardium, little attention was paid to the possible deleterious consequences of reperfusion until the recent advent of safe and effective thrombolytic and angioplasty procedures. The development of these techniques, however, has necessitated a careful reassessment of the contribution of reperfusion *per se* to cellular damage following a transient ischaemic episode (Braunwald and Kloner, 1985).

One of the earliest indications that reperfusion itself can exacerbate tissue injury was the observation of electrical disturbances and ventricular arrhythmias immediately following reflow of an ischaemic region of the dog heart (Tennant and Wiggers, 1935). In addition, in the 1970s, Hearse and colleagues (1973, 1975, 1978) demonstrated that the readmission of oxygen to the hypoxic heart can lead to membrane disruption and loss of cellular proteins. In these studies, the cellular injury induced by reoxygenation was shown to be significantly greater than if the hypoxia had been maintained. This led to the concept of the "oxygen paradox". The cellular damage on reoxygenation was shown to be not only proportional to the duration and severity of the preceding hypoxia, but also to the pO_2 of the reperfusing medium (Hearse *et al.*, 1978). Thus, it became clear that it is the readmission of oxygen *per se*, rather than the restitution of flow, that underlies reperfusion-induced injury. The central role of oxygen in mediating reperfusion-induced injury in the heart has focused attention on the possible role of oxygen-derived free radicals and oxidant stress in cellular damage during early reperfusion.

1.2 ARE FREE RADICALS PRODUCED DURING EARLY REPERFUSION IN THE HEART?

In 1984, Misra *et al.* used electron spin resonance (e.s.r.) techniques to detect carbon-centred radicals produced 15 min after reperfusion of the globally ischaemic rat heart. The production of these radicals was attenuated by superoxide dismutase and by allopurinol, and the authors suggested that the carbon-centred radicals were formed secondarily to oxyradicals produced *during ischaemia*. Since then, a number of studies have directly demonstrated that the production of oxygen-derived free radicals occurs in the early moments of reperfusion (Garlick *et al.*, 1987; Zweier *et al.*, 1987). Figure 4.1a shows the time-course of radical production during reperfusion, assessed using e.s.r. and the spin-trapping agent *N-tert*-butyl-α-phenylnitrone (PBN), following 15 min of global ischaemia in the isolated rat heart (Garlick *et al.*, 1987). It is clear from this study that there is a rapid burst of radical production during early reperfusion that peaks after 4 min and then decreases. When hearts were reperfused with anoxic buffer, Garlick *et al.* (1987) showed no radical production – radical adducts being formed only on reoxygenation (Fig. 4.1b). The prerequisite for reoxygenation rather than the restitution of flow for radical adduct formation indicates that the initiating radical species involved are likely to be oxygen centred.

1.3 FREE RADICALS AND REPERFUSION ARRHYTHMIAS

One of the most immediate and dramatic consequences of reperfusion following regional ischaemia in many animal models is the induction of severe ventricular arrhythmias. Figure 4.2 shows an epicardial electrocardiogram (ECG) recorded from an isolated rat heart at the moment of reperfusion following 10 min of regional ischaemia (Lawson, 1993). Simultaneously with the restitution of flow to the ischaemic bed, the heart shows a burst of ventricular tachycardia that rapidly degenerates into fibrillation. In the isolated rat heart model, reperfusion-induced arrhythmias are extremely reproducible and have

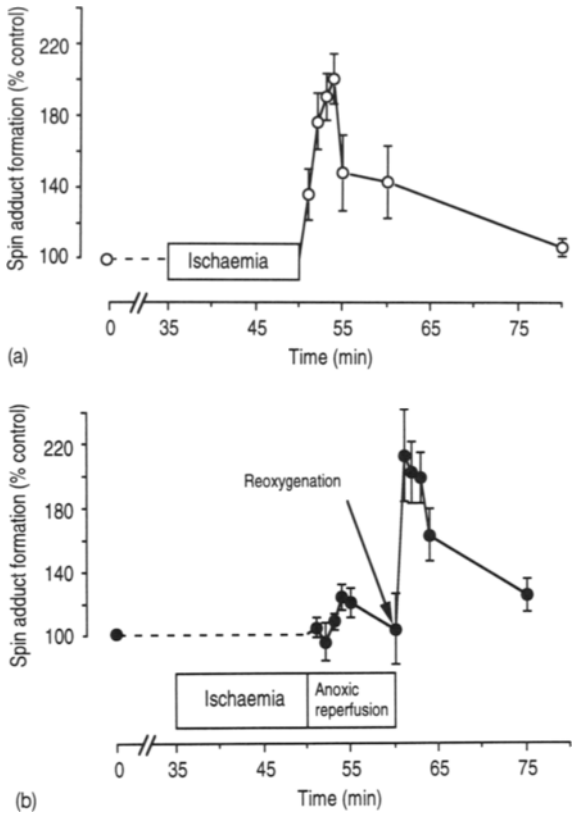

Figure 4.1 Time-course of free-radical production during aerobic (a) or anoxic (b) reperfusion of the isolated rat heart. Radical production was assessed using e.s.r. and quantified as the formation of a *N-tert*-butyl-α-phenylnitrone (PBN) spin adduct. After a 35 min stabilization period of aerobic perfusion, hearts were made globally ischaemic for 15 min. Hearts were then reperfused, either with oxygenated buffer (a) ($n = 6$), or with anoxic buffer, switching to an oxygenated buffer after 10 min (b) ($n = 5$). The bars represent the standard errors of the means. Redrawn with permission from Garlick *et al.* (1987).

been shown to occur with a peak incidence that temporally correlates with the peak in free-radical production shown in Fig. 4.1 (Manning and Hearse, 1984). More direct evidence linking these two events is provided by studies examining the anti-arrhythmic effects of free-radical scavenging agents. Woodward and Zakaria (1985) showed that allopurinol could prevent reperfusion arrhythmias in the isolated rat heart preparation. Since then, many studies, including those of Bernier *et al.* (1986) have demonstrated that a wide variety of anti-free-radical agents can reduce the susceptibility of the heart to reperfusion-induced arrhythmias (see Fig. 4.3).

Further evidence for the direct involvement of free radicals in disturbing the electrical and ionic homeostasis of the heart comes from a series of studies undertaken to investigate whether free radicals and oxidant stress, *in the absence of ischaemia and reperfusion*, can cause arrhythmias. The production of radicals under experimental conditions by many enzyme-based generating systems is slow and hence the onset of electrical changes measured in some studies has been correspondingly delayed. This has led some investigators to conclude that free radicals may not play an important role in the initiation of the very rapid electrical changes seen on reperfusion (Parratt and Wainwright, 1987; Coetzee and Opie, 1992). However, using the photoactivation of rose bengal, a very rapid and reproducible burst of oxidant stress can be induced. In aqueous solution, rose bengal can be elevated to a triplet state by illumination with light of appropriate wavelength (500–600 nm) and, in the presence of oxygen, this triplet decays producing singlet oxygen (75% of decays) and superoxide (20% of decays) (Lee and Rodgers, 1987). Using this technique, a rapid burst of oxidant stress produces changes in the epicardial ECG of rat hearts that develop within seconds and rapidly degenerate into ventricular arrhythmias (Kusama *et al.*, 1989).

In summary, therefore, the evidence implicating

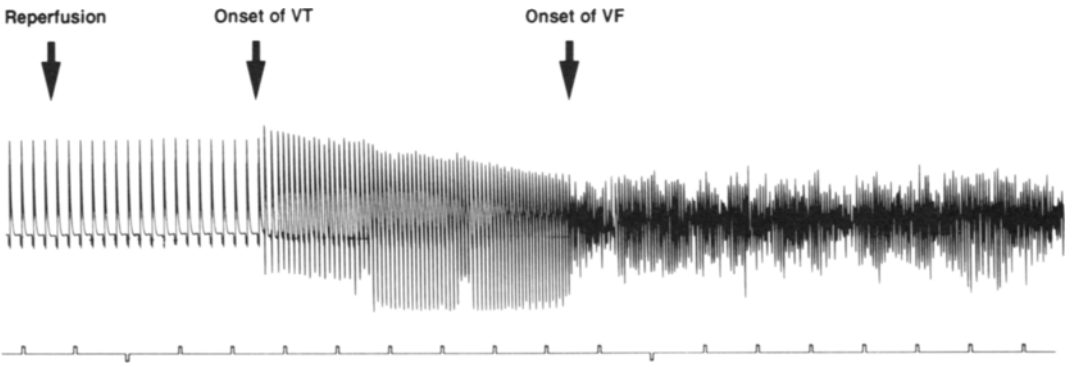

Figure 4.2 Epicardial ECG recorded from an isolated blood-perfused rat heart at the moment of reperfusion. The heart was made regionally ischaemic by occluding a snare around the left anterior descending coronary artery and, after 10 min, reperfused by releasing the snare. Note the rapid onset of ventricular tachycardia (VT) and its subsequent degeneration into ventricular fibrillation (VF). Reproduced with permission from Lawson (1993).

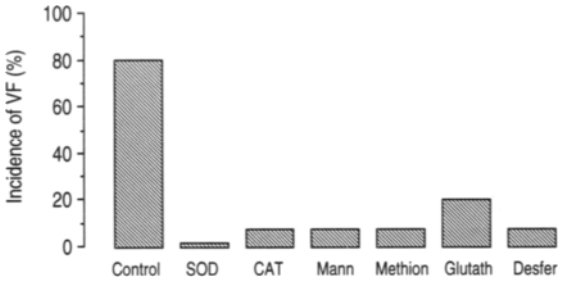

Figure 4.3 Effect of a variety of anti-free-radical interventions on reperfusion-induced ventricular fibrillation in the isolated perfused rat heart. Regional ischaemia was induced by occluding a snare around the left anterior descending coronary artery and, after 10 min, hearts were reperfused by releasing the snare. Superoxide dismutase (SOD) (1×10^5 U/l), catalase (CAT) (1×10^6 U/l), mannitol (Mann) (50 mM), L-methionine (Methion) (10 mM), glutathione (Glutath) (10 μM) or desferrioxamine (Desfer) (150 μM) were included throughout the experimental time course (n = 15/group). Redrawn with permission from Bernier *et al.* (1986).

oxidant stress in reperfusion-induced arrhythmias in the heart can be considered to be three-fold: (1) free radicals are produced during the early moments of reperfusion; (2) anti-free-radical interventions are anti-arrhythmic; and (3) free-radical generating systems, *in the absence of ischaemia and reperfusion*, are arrhythmogenic.

1.4 FREE RADICALS AND CONTRACTILE DYSFUNCTION

Hearts reperfused after short periods of reversible ischaemia show prolonged contractile dysfunction (Bolli *et al.*, 1989). This temporary decrease in contractile performance often persists for many hours or days and has been termed myocardial stunning. The contractile performance of the heart immediately following infarction is a major determinant of both short-term and long-term mortality (Pasternak *et al.*, 1988) and may also be important in determining the successful outcome of angioplasty or thrombolysis. A number of studies have shown that oxidant stress, in the early moments of reperfusion, may underlie myocardial stunning (Przyklenk and Kloner, 1986; Bolli *et al.*, 1989) – the results of such a study from Bolli *et al.* (1989) are shown in Fig. 4.4. Figure 4.4a shows that *N*-(2-mercaptopropionyl)-glycine (MPG), a free-radical scavenger and spin-trapping agent, administered 1 min *before* reperfusion can significantly alleviate myocardial contractile depression in the reperfused dog heart. Conversely, when MPG was given 1 min *after* reperfusion, myocardial stunning was unaffected (Fig. 4.4b). In this study, the early administration of MPG was also shown to produce a parallel reduction in radical generation during reperfusion. This study elegantly demonstrates the role of free radicals and

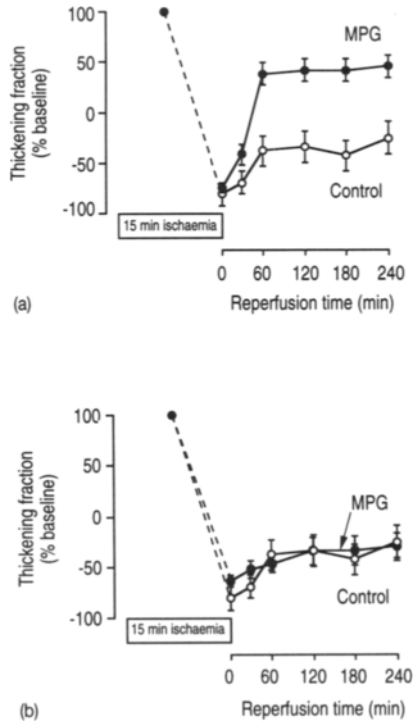

Figure 4.4 Effect of a free-radical scavenger *N*-(2-mercaptopropionyl)-glycine (MPG) on the recovery of contractile function following 15 min of regional ischaemia in the dog heart. (a) MPG infused 1 min before reperfusion. (b) MPG infused 1 min after reperfusion. Contractile function was assessed as changes in ventricular wall thickening measured using an ultrasonic pulsed-Doppler epicardial probe. Note: The free radical scavenger MPG can reduce myocardial stunning only when present during the first minute of reperfusion. Redrawn with permission from Bolli *et al.* (1989).

oxidant stress in myocardial stunning and indicates the rapidity of these effects. There appears, therefore, to be a critical "window" in the first minute of reperfusion, when these free-radical-mediated events may be manipulated. The rapidity and brevity of this "window" suggests that the mechanism by which oxidant stress contributes to the process of myocardial stunning, as for cardiac arrhythmias, must be fast-acting.

2. *How the Heart Copes with Oxidant Stress*

2.1 ANTIOXIDANTS AND FREE-RADICAL SCAVENGERS IN THE HEART

In common with many other tissues, the heart possesses a diverse array of cellular defences that act in concert to

counteract free-radical production under normal conditions and during episodes of enhanced oxidant stress such as reperfusion. This protection is afforded by enzymatic systems and low molecular weight antioxidants.

The heart has a relatively low catalase activity, which, together with the superoxide dismutase (SOD) system, acts to remove hydrogen peroxide and superoxide radicals. In addition, in man, dietary vitamin C plays an important role in the reduction of vitamin E, an intrinsic antioxidant component of biological membranes (Chen and Thacker, 1986; Niki, 1987). Both vitamins C and E can also react directly with hydroxyl and superoxide radicals (Halliwell and Gutteridge, 1989; Meister, 1992).

During ischaemia, the activity of cellular antioxidant systems may be reduced (Ferrari *et al.*, 1985; Galinanes *et al.*, 1992). In addition, a number of cellular pathways that produce free radicals are "primed" during ischaemia such as the xanthine/xanthine oxidase system (McCord, 1987), catecholamine auto-oxidation (Jackson *et al.*, 1986) and the arachadonic acid pathway (Halliwell and Gutteridge, 1989). Thus, during early reperfusion there is a burst of free radical production (see Fig. 4.1) that may overwhelm the antioxidant systems of the cells.

2.2 OXIDANT STRESS-INDUCED ALTERATIONS IN MYOCARDIAL GLUTATHIONE STATUS

Myocardial ischaemia and reperfusion are associated with a general depression in the antioxidant capacity of the heart, characterized by a reduction of mitochondrial superoxide dismutase activity and a loss of both vitamin E and vitamin C content. During oxidant stress, changes in cellular redox state result in alterations in cardiac glutathione status (Lesnefsky *et al.*, 1988; Ferrari *et al.*, 1992). While in general the heart has a relatively low antioxidant capacity compared to the liver, for example, it does possess an active glutathione cycle (Bannai and Tateishi, 1986). The role of the relatively high intracellular concentration of reduced glutathione (1–10 mM) within the cell is multifold and is thought to scavenge both superoxide and singlet oxygen directly (Halliwell and Gutteridge, 1989). Glutathione plays an important role in the removal of hydrogen peroxide and organic hydroperoxides through the activity of the glutathione peroxidase/glutathione reductase cycle (Halliwell and Gutteridge, 1989). During oxidant stress, this system operates, at the expense of NADPH from the pentose phosphate shunt, resulting in a depression in the reduced glutathione content of the myocardium and an associated rise in oxidized glutathione (GSSG) levels (Darley-Usmar *et al.*, 1989). GSSG is cytotoxic and there is evidence to suggest a specific ATP-dependent transporter exists that actively pumps GSSG out of the cell (Kondo *et al.*, 1989; Garcia Ruiz *et al.*, 1992; Heijn *et al.*, 1992). As a result, during oxidant stress there is a reduction in the ratio of reduced glutathione (GSH) to GSSG with a shift of cellular redox state towards oxidation, suggesting the occurrence of oxidant stress.

3. *Membrane Consequences of Oxidant Stress*

While many biological molecules may be targets for oxidant stress and free radicals, it is clear that the cell membrane and its associated proteins may be particularly vulnerable. The ability of the cell to control its intracellular ionic environment as well as its ability to maintain a polarized membrane potential and electrical excitability depends on the activity of ion-translocating proteins such as channels, pumps and exchangers. Either direct or indirect disturbances of the activity of these ion translocators must ultimately underlie reperfusion and oxidant stress-induced arrhythmias in the heart. A number of studies have therefore investigated the effects of free radicals and oxidant stress on cellular electrophysiology and the activity of key membrane-bound ion translocating proteins.

3.1 EFFECTS OF OXIDANT STRESS ON THE ACTION POTENTIAL

A number of studies have investigated the effects of oxidant stress on cardiac electrophysiology and action potential configuration. Most have reported a transient action potential prolongation followed by shortening (Barrington *et al.*, 1988; Hayashi *et al.*, 1989; Tarr and Valenzeno, 1989; Beresewicz and Horackova, 1991; Shattock *et al.*, 1991) (see Fig. 4.5). However, a monophasic shortening of the action potential during oxidant stress, with no transient prolongation, has also been described (Pallandi *et al.*, 1987; Goldhaber *et al.*, 1989). A consistent observation in virtually all studies of oxidant stress, however, is the gradual appearance of after-depolarizations. These diastolic oscillations in membrane potential soon exceed the threshold for excitation and ventricular muscle preparations, that would normally be quiescent unless stimulated, show automaticity (Pallandi *et al.*, 1987; Barrington *et al.*, 1988; Hayashi *et al.*, 1989; Matsuura and Shattock, 1991a, b). It is this automaticity that is presumed to underlie the arrhythmias induced in isolated hearts by oxidant stress (Matsuura and Shattock, 1991a).

3.2 EFFECTS OF OXIDANT STRESS ON MEMBRANE CURRENTS

In order to investigate which membrane currents underlie the changes in the action potential and the arrhythmogenic oscillatory after-depolarizations, we have

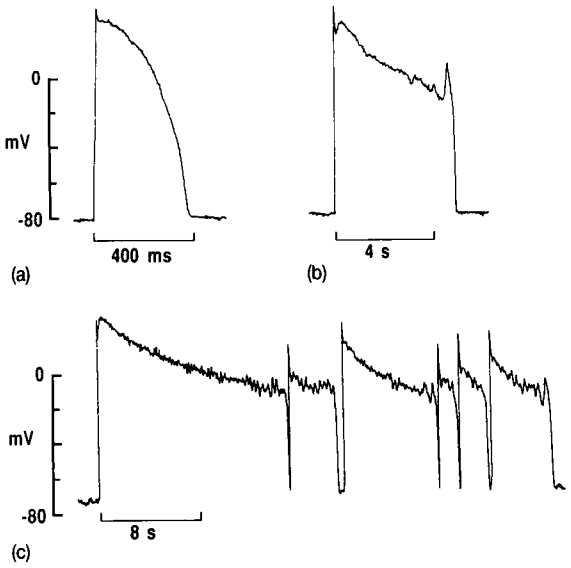

Figure 4.5 Influence of oxidant stress on action potentials recorded in an isolated rabbit ventricular myocyte. (a) Control action potential. (b) Action potential recorded 3 min after exposure to oxidant stress induced by the photoactivation of rose bengal (50 nM). (c) Spontaneous and repetitive action potential discharges induced 6.5 min after exposure to rose bengal. Action potentials were recorded via a 2.5 MΩ suction electrode and a current-clamp amplifier. The cell was stimulated at 0.1 Hz with a 2 ms suprathreshold current pulse and, when the cell showed automaticity (after 6 min), stimulation was stopped. Redrawn from Matsuura and Shattock (1991b).

exposed voltage-clamped isolated myocytes to oxidant stress (see Fig. 4.6). Using a protocol designed to investigate the calcium inward current, three significant oxidant stress-induced changes were observed: (1) a decrease in the peak Ca current; (2) a change in the steady-state holding current, and (3) the gradual appearance of oscillatory transient inward currents on repolarization (Matsuura and Shattock, 1991a). These changes were progressive, developed over a similar time-course to the changes in the action potential and were dependent on the extent of oxidant stress imposed. We have since undertaken a detailed study of the effects of oxidant stress on each of these current systems (Shattock *et al.*, 1990; Matsuura and Shattock, 1991a, 1991b).

3.2.1 Calcium and Sodium Inward Currents

In experiments using oxidant stress induced by the photoactivation of rose bengal (10 nM), a 75% decline in the calcium inward current was observed after 10 min, with only a slight acceleration in the inactivation kinetics of the current (Shattock *et al.*, 1990). However, this decline in the calcium inward current appears to occur secondary to an oxidant stress-induced calcium overload and not as a consequence of a direct effect on the L-type calcium channel. When this calcium overload was prevented, by removing the trans-sarcolemmal sodium gradient, oxidant stress induced by the photoactivation of rose bengal did not affect the calcium inward current (Shattock *et al.*, 1990; Matsuura *et al.*, 1991). Other studies, using alternative methods of generating oxidant stress, have similarly reported the relative insensitivity of the calcium inward current to oxidant stress. Bhatnager *et al.* (1990) demonstrated no effect on calcium inward current in frog ventricular cells exposed to *t*-butylhydroperoxide (tBOOH). Similarly, Beresewicz and Horackova (1991), using guinea-pig myocytes exposed to H_2O_2, reported that the L-type Ca channels were essentially unaltered under their experimental conditions. These observations were all made in studies in which the exposure to oxidant stress was minimized and the lack of effect on the calcium channel was observed over a time-course when other membrane currents were changing (Bhatnagar *et al.*, 1990; Shattock *et al.*, 1990; Beresewicz and Horackova, 1991; Matsuura *et al.*, 1991). In contrast, Coetzee and Opie (1988, 1992) have reported changes in the kinetics of the L-type calcium current after 20–30 min exposure to xanthine/xanthine oxidase such that the net integrated calcium influx is increased by oxidant stress. In these experiments, in which cells were dialysed with 5 mM EGTA, the lack of change in the reversal potential of the calcium current indicated that these changes may occur without any significant increase in intracellular calcium. The consensus in the literature, however, is that the Ca channel is relatively insensitive to oxidant stress in comparison with other channels and that any changes that are induced may occur late in the time-course of cellular injury.

The studies of Bhatnager *et al.* (1990) and Beresewicz and Horackova (1991) also report a significant and important increase in the inward movement of Na through the TTX-sensitive Na channel in cells exposed to oxidant stress. It is likely that this increased inward current may play a role in prolonging the action potential and in loading the cell with sodium. Both of these effects would combine to create a situation that would tend to load the cell with calcium through alteration in the activity of the Na/Ca exchange mechanism (Matsuura *et al.*, 1991).

3.2.2 Steady-state Background Currents

As with the effects of oxidant stress on the calcium channel, part of the change in the steady-state background current could also be attributed to an indirect effect secondary to the elevation of intracellular calcium (Matsuura and Shattock, 1991b). However, oxidant stress also exerted a direct effect on the inward rectifying potassium current (I_{K1}). The combination of an inhibition of I_{K1} and the activation of a calcium-dependent current are likely to contribute to the prolongation of the action potential duration and the increased susceptibility

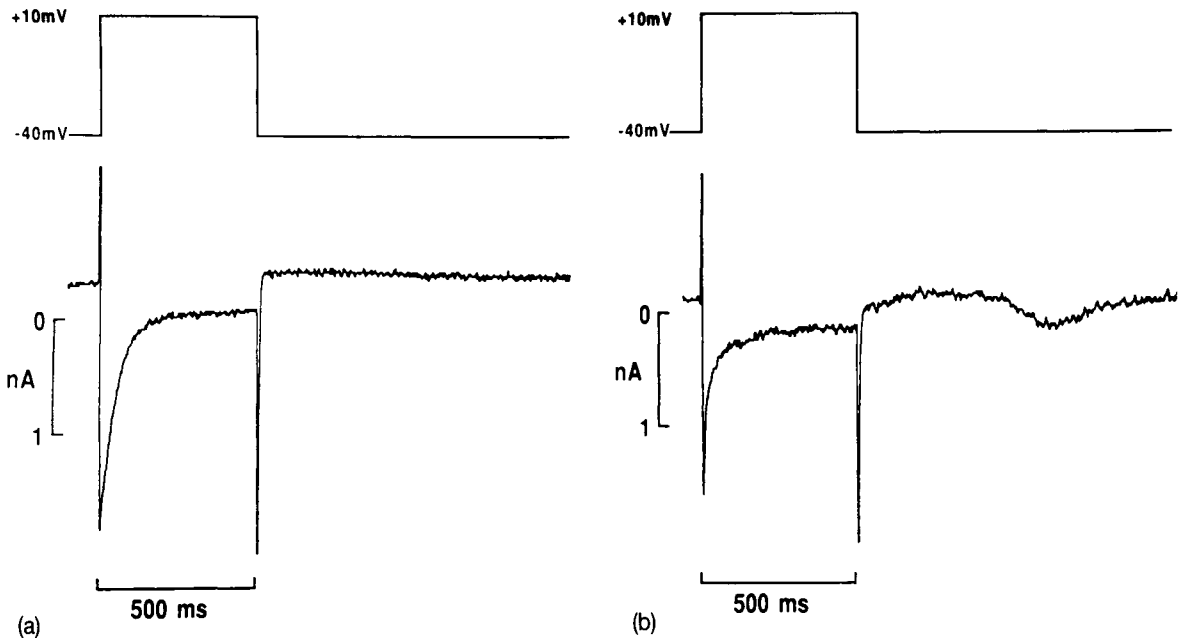

Figure 4.6 Effects of oxidant stress on membrane currents recorded from a voltage-clamped isolated guinea-pig myocyte. (a) Currents recorded under control conditions during a 500 ms voltage step to +10 mV from a holding potential of −40 mV. Note the rapidly activating calcium inward current. (b) Currents recorded during a similar voltage protocol 5 min after exposure to photoactivated rose bengal (50 nM). Note the reduction in peak calcium inward current, the inward shift in holding current, and the induction of an oscillatory transient inward current on repolarization after the clamp step. Reproduced with permission from Matsuura and Shattock (1991a).

to arrhythmogenic transient inward currents observed in other studies (Matsuura and Shattock, 1991a, b).

3.2.3 Transient Inward Current

A consistent observation in all studies of oxidant stress and cellular electrophysiology is the induction of oscillations in membrane potential that lead eventually to automaticity (Pallandi *et al.*, 1987; Barrington *et al.*, 1988; Hayashi *et al.*, 1989; Matsuura and Shattock, 1991a, 1991b). These oscillations in membrane potential are inhibited by agents that prevent sarcoplasmic reticulum (s.r.) calcium release, such as caffeine (Shattock *et al.*, 1991) or ryanodine (unpublished observations), and are easier to induce in species in which excitation–contraction coupling is relatively s.r. dependent (i.e. sensitivity of rat > rabbit ≫ frog) (Shattock *et al.*, 1991). We have examined these oscillations in membrane potential in more detail in isolated rabbit ventricular myocytes and investigated the underlying membrane currents (Matsuura and Shattock, 1991a). From these studies, it is clear that there is a progressive appearance of an arrhythmogenic oscillatory transient inward current that is activated on repolarization from voltage–clamp steps. Similar transient inward currents have been described in a wide range of experimental conditions that result in cellular calcium overload such as high external calcium (Kass *et al.*, 1978), low external potassium (Fedida *et al.*, 1987), or sodium pump inhibitors (for review, see Eisner and Lederer, 1985). The electrogenic process underlying this current has been attributed to either the Na/Ca exchange mechanism (Karagueuzian and Katzung, 1982) or the calcium-activated non-selective cation channel (Kass *et al.*, 1978). Both of these current systems can be activated by elevations in intracellular calcium and can oscillate when calcium is cyclically released and taken up again by the s.r. The oscillatory current activated by oxidant stress is carried predominantly by the electrogenic Na/Ca exchange mechanism (Matsuura and Shattock, 1991a).

Transient inward currents similar to those observed during oxidant stress have been observed during reoxygenation of previously hypoxic myocytes (Benndorf *et al.*, 1991). These reoxygenation-induced currents were also dependent on Na/Ca exchange and showed virtually identical characteristics to those induced by oxidant stress. It therefore seems likely that these arrhythmogenic currents induced by reoxygenation are induced by oxidant stress and are likely to contribute to the genesis of reperfusion-induced arrhythmias in whole hearts.

3.3 OXIDANT STRESS AND CALCIUM OVERLOAD

Many of the effects of oxidant stress on the current systems described above may occur secondary to a cellular

calcium overload. Elevation of intracellular calcium can induce changes in the calcium inward current (Lee *et al.*, 1985), background currents (Vandeberg, 1987) and the activation of oscillatory transient inward currents (Kass *et al.*, 1978). Evidence from a number of studies suggests that oxidant stress may significantly elevate intracellular calcium (Burton *et al.*, 1990; Vandeplassche *et al.*, 1990; Shattock *et al.*, 1991). Figure 4.7 is reproduced from Burton *et al.* (1990) and shows intracellular calcium measured in cultured cardiac myocytes exposed to a xanthine oxidase-based radical-generating system. Free radicals induce a cessation of spontaneous activity and associated calcium transients in these cultured cells and a subsequent increase in intracellular calcium. It is likely, therefore, that at least a part of the changes in the current systems described above may be attributable to elevation of intracellular calcium.

If some of the electrophysiological effects of oxidant stress occur secondary to an elevation in intracellular calcium, it is important to consider the possible factors that may underlie the initial elevation of calcium. In the simplest analysis, elevation of cytosolic calcium may be due to: (1) redistribution of intracellular calcium stores; (2) increased calcium influx; or (3) decreased calcium efflux.

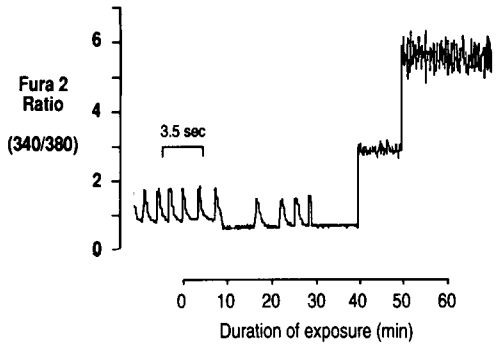

Figure 4.7 Changes in intracellular calcium in cultured rat ventricular myocytes exposed to oxidant stress. Calcium was measured using the fluorescent probe Fura-2. The ratio of the Fura-2 fluorescence measured at 340 and 380 nm excitation is shown and this is proportional to the intracellular calcium concentration. The fast-speed traces shown (note the 3.5 s time-scale) were recorded after various durations of oxidant stress. Myocytes under control conditions (before $t = 0$) show spontaneous calcium transients. These transients decreased in frequency with oxidant stress until cells failed to show spontaneous activity but continued to maintain a low intracellular calcium. Eventually, after 40–50 min of oxidant stress, intracellular calcium increased. These changes were irreversible when cells were reperfused with control buffer at the end of the protocol. Redrawn with permission from Burton *et al.* (1990).

3.3.1 Redistribution of Intracellular Calcium Stores?

The appearance of after-contractions, after-depolarizations and transient inward currents in cells exposed to oxidant stress suggests that the s.r. cyclically releases and takes up calcium. Oscillatory calcium release can be induced in undamaged s.r. simply by loading the cell with calcium and there is therefore no need to invoke a mechanism in which the s.r. is itself directly damaged by oxidant stress. However, we have recently investigated the effects of oxidant stress on calcium release channels isolated from sheep heart s.r. and reconstituted into lipid bilayers (Holmberg *et al.*, 1991). In these studies, rose bengal-induced oxidant stress caused an increase in channel opening and eventually an irreversible loss of channel function. In addition, oxidant stress can inhibit s.r. CaATPase and hence limit calcium reaccumulation into the s.r. (Kaneko *et al.*, 1989). However, even in the presence of caffeine, when s.r. function should be inhibited, cells still show evidence of profound cellular calcium overload (Shattock *et al.*, 1991). This suggests that, although the s.r. may contribute to the *expression* of cellular calcium overload, the primary event causing this overload may occur at the cell surface membrane. Thus, it seems likely that two factors may be important in mediating the effects of oxidant stress on s.r. function. First, a cellular calcium overload, mediated by changes in the calcium-regulating properties of the surface membrane, is in itself likely to cause spontaneous s.r. calcium release and arrhythmias. Second, s.r. calcium release (Holmberg *et al.*, 1991) and reuptake (Kaneko *et al.*, 1989) may be affected directly by oxidant stress.

Recent studies by Crompton *et al.* have shown that oxidant stress may open a Ca-sensitive, non-selective pore in the inner mitochondrial membrane that is blocked by cyclosporin A (Crompton, 1990; Crompton and Costi, 1990). This pore opening results in massive mitochondrial swelling, dissipation of the transmembrane proton gradient and disruption of mitochondrial energy production (Crompton *et al.*, 1992). Since mitochondria may play a role as a slow, high-capacity cytosolic calcium buffer (Isenberg *et al.*, 1993), disruption of mitochondrial function may also contribute to calcium overload and cell injury.

3.3.2 Increased Cellular Calcium Influx?

Coetzee and Opie (1988, 1992) have suggested that the cellular calcium overload induced by oxidant stress is mediated by an increase in calcium influx through the L-type calcium channel. However, a number of other studies, using different radical-generating systems, experimental conditions and species, have described no significant effects on the calcium channel over the period of exposure necessary to induce cellular calcium overload (Bhatnagar *et al.*, 1990; Shattock *et al.*, 1990; Beresewicz

and Horackova, 1991; Matsuura *et al.*, 1991). At present, therefore, it is unclear what contribution calcium influx via this route may make to cellular calcium overload during oxidant stress.

Some insight into the possible route of calcium entry into the cell was gained in a series of experiments undertaken to investigate the effects of oxidant stress on the calcium inward current in the absence of intracellular and extracellular sodium (Shattock *et al.*, 1990; Matsuura *et al.*, 1991). In these experiments, the transarcolemmal sodium gradient was removed by dialysing cells with sodium-free solutions via the patch pipette and bathing cells in sodium-free Tyrode's solution. Under these conditions, cells showed no evidence of an oxidant stress-induced cellular calcium overload and no changes in the calcium inward current. These results suggest that the Na/Ca exchange may contribute significantly to calcium overload and that the calcium inward current may be unaffected by oxidant stress.

The importance of the Na/Ca exchange in mediating oxidant stress-induced cellular calcium overload has also been suggested by a number of other studies (Bhatnagar *et al.*, 1990; Beresewicz and Horackova, 1991). Inward calcium transport by the exchanger may also be facilitated by two other important consequences of oxidant stress: (1) the prolongation of the action potential (Barrington *et al.*, 1988; Hayashi *et al.*, 1989; Tarr and Valenzeno, 1989; Beresewicz and Horackova, 1991; Shattock *et al.*, 1991; Barrington *et al.*, 1992); and (2) intracellular sodium loading following increased sodium influx (Bhatnagar *et al.*, 1990) and inhibition of the Na/K pump (Shattock and Matsuura, 1993).

3.3.3 Decreased Cellular Calcium Efflux?

The major sarcolemmal mechanism responsible for the removal of intracellular calcium is Na/Ca exchange. This exchange process is capable of rapidly removing significant quantities of calcium (Barry and Bridge, 1993) and its perturbation may result in cellular calcium overload (Bers, 1991). As indicated in the previous section, oxidant stress may create conditions that may both promote calcium influx via this route and also reduce calcium extrusion. In addition, we have directly measured the Na/Ca exchange current in isolated cells and shown it to be inhibited by oxidant stress (Ichikawa *et al.*, 1992). This latter observation, however, is not supported by three other studies, two in isolated sarcolemmal vesicles and one in intact cells, which report that Na/Ca exchange may be *stimulated* by oxidant stress (Reeves *et al.*, 1986; Shi *et al.*, 1989; Goldhaber and Weiss, 1993). The precise effects of oxidant stress on the Na/Ca exchanger are therefore unclear. However, the dependence of the cellular calcium overload on the sodium gradient implies that the exchanger may still play an essential role in cellular calcium loading. This may occur irrespective of whether its activity is up- or down-regulated by the direct effects of oxidant stress on the exchanger protein itself.

In addition to the Na/Ca exchange mechanism, the sarcolemma also contains a CaATPase that removes calcium from the cytosol. This calcium pump plays only a small role in the extrusion of calcium from the cell during normal activity (Bers, 1991; Barry and Bridge, 1993). However, Kaneko *et al.* (1989), have shown that the activity of the CaATPase may be inhibited by free radicals and thus the ability of the cell to extrude calcium in the presence of a maintained oxidant stress may be further compromised (Kaneko *et al.*, 1989).

3.3.4 Na/K Pump Activity and its Role in Cellular Calcium Homeostasis

The sarcolemmal Na/K pump plays an important, although indirect role in the regulation of cellular calcium homeostasis. The transmembrane Na gradient is maintained by the activity of the Na/K pump and the thermodynamic energy of this gradient in turn drives the Na/Ca exchange mechanism (Sheu and Fozzard, 1982; Barry and Bridge, 1993). Thus, the intracellular Ca concentration is closely related to intracellular Na and the activity of the Na/K pump (Bers and Ellis, 1982).

A sustained inhibition of the Na/K pump following a period of oxidant stress would be expected to raise intracellular sodium and favour calcium influx via the Na/Ca exchanger. Ischaemia and reperfusion-induced oxidant stress, therefore, may result in a loss of Na/K pump activity, an effect that may involve free-radical-mediated changes in cellular thiol status.

Preliminary experiments in Langendorff perfused rat hearts suggest a role for sulphydryl-mediated control of

Table 4.1 Effect of selected thiols, disulphides, amino acids and antioxidants on the time to the onset and the time to reach maximal ischaemic contracture in isolated perfused rat hearts. Hearts were perfused for a control period of 10 min at the end of which global low-flow (10% of control) ischaemia was initiated. The interventions described above were included in the perfusion fluid 5 min prior to the onset and throughout the ischaemic period. The data are shown as means ± standard errors of the means ($n = 6$)

Drug	*Concentration (mM)*	*Onset (min)*	*Maximal (min)*
Controls		7.6 ± 0.2	15.3 ± 0.2
GSH	1	10.1 ± 0.3*	18.1 ± 0.5*
Cysteine	1	10.1 ± 0.2*	19.8 ± 0.95*
Glycine	1	7.4 ± 0.3	15.6 ± 0.5
Glutamate	1	7.3 ± 0.2	15.5 ± 0.4
GSSG	1	6.0 ± 0.2*	13.5 ± 1.0*
Cystine	1	6.1 ± 0.3*	12.9 ± 0.5*
Penicillamine	1	9.8 ± 0.8*	18.6 ± 2.3*
Dithiothreitol	1	10.2 ± 1.6*	18.6 ± 1.5*
SOD/catalase/ mannitol	10 U/ml/300 U/ ml/50mM	10.1 ± 0.2*	18.2 ± 0.2*

* $P = 0.05$, cf. controls in the absence of any intervention.

the Na/K pump exposed to oxidant stress. In these experiments, glutathione (1 mM) delayed the onset of an ischaemic contracture (Haddock *et al.*, 1989) and reduced reperfusion-induced arrhythmias (Woodward and Zakaria, 1985) – both of which may be mediated by disturbances in intracellular calcium homeostasis (see Table 4.1).

4. *Oxidant Stress, Redox State and Protein Function*

The major contributor to radical-induced myocardial injury appears to involve a disturbance of intracellular ion levels. Ionic homeostasis is normally maintained by the activity of membrane-bound proteins, however, many studies have invoked lipid peroxidation as the most likely mechanism underlying these ionic perturbations (Kellogg and Fridovich, 1975; Kramer *et al.*, 1984; Kukreja *et al.*, 1988; Nakaya *et al.*, 1987). Peroxidation of membrane lipids would undoubtedly be expected to occur under severe and prolonged oxidant stress, and this may ultimately modify membrane fluidity and cell function. However, there is a growing body of evidence to suggest that the activity of several key ion-translocating proteins (such as the sarcolemmal Na/Ca exchanger, the Na/K pump and the calcium release channel of the s.r.) may be modulated *prior* to any evidence of lipid peroxidation (Daly *et al.*, 1984; Nishioka *et al.*, 1984; Reeves *et al.*, 1986; Matsuoka *et al.*, 1990; Furukawa *et al.*, 1991; Lue and Boyden, 1992). Oxidant stress may alter protein redox state with great rapidity, resulting in the activation or inactivation of these ion-translocating processes. This may be manifest as a loss of ionic homeostasis (Knopf *et al.*, 1990), myocardial stunning (Bolli, 1990), abnormal vascular reactivity (Jackson *et al.*, 1986), contractile failure (Webb *et al.*, 1983) or arrhythmogenesis (Bernier *et al.*, 1986; Hearse *et al.*, 1989).

4.1 THIOL STATUS AND PROTEIN ACTIVITY

Both the secondary and tertiary structures of proteins are dependent on the number and location of free sulphydryl groups (-SH) (conferred by cysteine residues) and intramolecular disulphides (-SS-). The formation of intramolecular disulphide bonds between adjacent -SH groups or their oxidation to intermolecular mixed thiols by their interaction with low molecular weight thiol compounds, such as reduced glutathione, can modify protein structure and thereby regulate enzyme activity (Ziegler, 1985; Shi *et al.*, 1993). These intermolecular disulphide bridges may be readily reduced and may provide a switching mechanism that is sensitive to cellular redox or glutathione status (Williford *et al.*, 1990). Conversely, intramolecular disulphides are not reduced so freely and their formation may lead to the permanent inactivation of proteins and enzymes (Williford *et al.*, 1990). Indeed, it has been suggested that this type of oxidation may tag proteins for subsequent degradation thereby requiring *de novo* synthesis of the new protein (Ziegler, 1985). Under oxidant stress, the formation of *reversibly* oxidized proteins, as opposed to these irreversibly oxidized species, may allow the heart to salvage native proteins during reperfusion and the restoration of cellular redox state.

Several enzyme systems have been characterized with respect to their modulation by thiol-disulphide exchange. These include phosphofructokinase, hexokinase, glutathione reductase, glucose-6-phosphatase, adenylate cyclase, guanylate cyclase and cAMP-dependent protein kinase (Brigelius, 1985; Ziegler, 1985; Miller *et al.*, 1990). Glutathione reductase can go through a cycle of activation and inactivation within a few milliseconds by switching pairs of protein thiol groups between the dithiol and disulphide state. This enzyme, therefore, provides an illustration of the rapidity with which cellular redox state can modulate protein function (Trimm *et al.*, 1986).

The major determinant of myocardial redox status is the glutathione content of the heart (Griffith and Meister, 1979). Therefore, fluctuations in myocardial glutathione status may exert a regulatory role in cellular metabolism in a comparable manner to the phosphorylation and dephosphorylation of proteins and enzymes.

4.1.2 Influence of Glutathione Status on Sarcolemmal Protein Function

Under pathological conditions, the oxidation of protein thiols results in the impairment of transmembrane metabolite and ion transport, a loss of enzyme activity and disturbances in contractile function of the heart (Jones, 1985). Depression in cardiac thiol content (achieved using thiol-oxidizing agents such as GSSG and diamide) has been shown to have a profound effect on intropic state, a response that is antagonized by ruthenium red, an inhibitor of mitochondrial calcium transport (Kosower, 1970; Gailis and Nguyen, 1975; Caparrotta *et al.*, 1983). If thiol–disulphide exchange is involved in the modulation of ion-translocating protein activity in cardiac tissue, then this may help to explain the relatively rapid molecular events that initiate reperfusion arrhythmias, contractile failure and vascular injury. The Na/Ca exchange mechanism in sarcolemmal vesicles has been shown to be stimulated 10-fold by combinations of reduced and oxidized glutathione, which suggests that the conformation and activity of the Na/Ca exchanger protein may be altered by the oxidation of protein sulphydryl groups (Reeves *et al.*, 1986). The Na/Ca exchange may, therefore, be vulnerable to ischaemia and reperfusion-induced oxidant stress via an associated increase in the synthesis of superoxide, hydrogen peroxide and oxidized glutathione. Furthermore, at this

time the myocardium may be sodium loaded and, therefore, susceptible to calcium gain via this mechanism (Sheu and Fozzard, 1982).

The cardiac Na/K ATPase is considered to be the catalytic component of the Na/K pump and is essential for the normal regulation of intracellular sodium and potassium (Jorgensen, 1982). The thermodynamic energy of the Na gradient maintained by Na/K pump activity is then exploited by other ion exchange or co-transport systems that play an important role in controlling other intracellular ions such as Ca^{2+}, Cl^- and pH_i (Mullins, 1981). Therefore, the modification of the structure, function and activity of the Na/K pump during periods of ischaemia/reperfusion-induced oxidant stress may represent a potential mechanism by which free radicals may profoundly disrupt cellular ionic homeostasis, and precipitate electrical and mechanical dysfunction. The Na/K pump may also represent a therapeutic target for free-radical scavengers and antioxidants, such as glutathione, thereby maintaining cellular ionic homeostasis and reducing the incidence of reperfusion-induced arrhythmias.

4.2 OXIDANT STRESS AND NA/K PUMP FUNCTION

4.2.1 Na/K Pump Structure and Regulatory Role of Sulphydryl Groups

The Na/K ATPase has been extensively purified and characterized, and consists of a catalytic α subunit of around 95 kDa and a glycoprotein β subunit of approximately 45 kDa (Skou, 1992). The functional transporter exists as a dimer with each monomer consisting of an α and β subunit. Hiatt *et al.* (1984) have suggested that the non-catalytic β subunit may be involved in the correct insertion of the α subunit into the lipid bilayer and, therefore, it is conceivable that a modification of the β subunit structure may be reflected by changes in the catalytic activity of the α subunit. Therefore, in studies involving the manipulation of tissue glutathione levels, alterations of intracellular redox state may have an effect on substrate binding at an extracellular site on this ion-translocating protein.

The protein sulphydryl and disulphide content of the native cardiac Na/K ATPase protein has been studied extensively (Kawamura and Nagano, 1984). Most studies indicate 12–17 free sulphydryl groups on each catalytic α subunit of the Na/K ATPase dimer (Harris and Stahl, 1980). In addition, there are three disulphide bridges present in each glycoprotein β subunit that are present in the hydrophobic, membrane-spanning regions of the enzyme, and are thought to contribute to sodium and potassium translocation. Kawamura and Nagano (1984) demonstrated that there may be a single disulphide in the β subunit that may be protected from reduction by relatively high concentrations of sodium and/or potassium. Potassium is known to compete with ouabain for an extracellular binding site and, therefore, it has been postulated that the free thiol and disulphide status of the Na/K ATPase may be responsible, at least in part, for its physiological regulation. Kirley (1990) demonstrated that Na/K ATPase activity and ouabain binding are reduced in parallel with the reduction of a single disulphide bond in the β subunit. This effect was prevented by relatively high concentrations of sodium and potassium (Kawamura *et al.*, 1985). The consensus appears to be that the reduction of disulphides or the oxidation of protein thiols may cause a change in the tertiary structure of the α and/or the β subunit of the Na/K ATPase (Kirley, 1990). This may result in steric interference with cation binding sites or a modulation of the interaction between the two subunits of the protein. One obvious set of circumstances that may promote this scenario may be alterations in myocardial glutathione status during oxidant stress outlined previously.

4.2.2 Free Radicals and Na/K Pump Function

The direct interaction of oxygen free radicals with sarcolemmal proteins and the subsequent alterations of protein function have been reported by several groups. Kramer *et al.* (1984) demonstrated the *in vitro* inhibition of Na/K ATPase activity in crude sarcolemmal homogenates in response to exogenous free-radical-generating systems. It is difficult to establish whether this type of experiment demonstrates the selective interaction of free radicals with sarcolemmal proteins, such as the Na/K ATPase, or a more generalized oxidant-induced damage to a multitude of cellular constituents. The reactive nature of free radicals makes it likely that a degree of non-specific interactions occur with a diverse array of cellular components. However, there is some evidence that suggests that, under sustained oxidant stress, the Na/K pump is a primary target for radical-mediated modification of protein or enzyme function. Xie *et al.* (1990) demonstrated, using the xanthine/xanthine oxidase system to impose an oxidant stress, a reduction in Na/K ATPase activity prior to any quantifiable changes in the activity of other sarcolemmal ion translocators. Further indirect evidence for a role of free radicals in the modulation of Na/K pump function has been provided by Kim and Akera (1987). In guinea-pig hearts subjected to ischaemia/reperfusion, they demonstrated a reduction in Na/K ATPase activity – an effect that could be prevented, at least in part, by selected free-radical scavengers and antioxidants such as superoxide dismutase, catalase, allopurinol, α-tocopherol, histidine, vitamin E and dimethylsulphoxide (see Fig. 4.8).

4.3 GLUTATHIONE AND NA/K PUMP FUNCTION

The direct effect of both reduced and oxidized glutathione on the activity of an isolated bovine ventricular

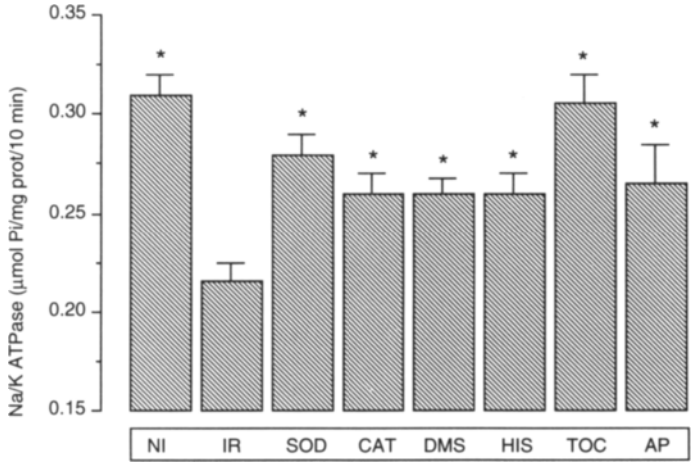

Figure 4.8 Reduction of Na/K ATPase activity in isolated guinea-pig hearts subjected to ischaemia/reperfusion and its prevention by various agents: control non-ischaemic hearts (NI); guinea-pig hearts subjected to global ischaemia for 2 h and subsequently reperfused for 1 h (IR). In other preparations, superoxide dismutase (SOD) 100 U/ml, catalase (CAT) 150 U/ml, dimethylsulphoxide (DMS) 50 mM, histidine (HIS) 10 mM, vitamin E (TOC) 50 μg/ml, or allopurinol (AP) 1 mM were added to the perfusion fluid 5 min before the onset of ischaemia and throughout the reperfusion period. Data are presented as the mean of 6–13 experiments and the bars represent the standard errors of the means. Asterisks indicate $P < 0.05$, cf. value observed with ischaemia/reperfusion alone. Redrawn with permission from Kim and Akera (1987).

Na/K ATPase has been investigated recently. These studies were undertaken to investigate the direct modulatory effects of glutathione on Na/K ATPase activity *in the absence of other factors present during ischaemia and reperfusion*.

The possible role of cellular glutathione status in the controlling sarcolemmal protein activity has been addressed by studying the effect of GSH, GSSG and several other thiol and disulphide compounds on Na/K ATPase activity using: (1) an isolated bovine ventricular Na/K ATPase preparation; (2) crude sarcolemmal preparations (biochemical studies); (c) Langendorff-perfused isolated hearts (cytochemical studies); and (4) isolated ventricular myocytes (electrophysiological studies).

4.3.1 Isolated Na/K ATPase Studies

Although it is widely accepted that ischaemia/reperfusion-induced oxidant stress is associated with a reduction of Na/K ATPase activity, it is difficult to determine which features of this process are responsible for this effect. A classical approach to this type of problem has been to determine the effect of the application of selected metabolites or agents on the activity of the enzyme of interest, an approach that has been exploited for the sarcolemmal Na/K ATPase and glutathione (Haddock *et al.*, 1990). The application of GSH (0.1–1.0 mM) induces a concentration-dependent increase in the activity of a bovine isolated ventricular Na/K ATPase preparation (determined by the ouabain-sensitive hydrolysis of ATP to release inorganic phosphate). In the presence of 1 mM GSH there was a 38% stimulation of activity compared to untreated control samples (see Fig. 4.9). In contrast, in the same preparation, the application of GSSG (0.1–1.0 mM) resulted in a concentration-dependent reduction of Na/K ATPase activity such that, in the presence of 1 mM GSSG, there was a 30% reduction of activity compared to untreated control samples.

If the cardiac redox state (reflected by fluctuations in the ratio of GSH and GSSG) is important in the regulation of the function of some enzymes, the manipulation of this ratio may result in parallel changes in enzyme activity. Thus, a reduction of Na/K ATPase activity associated with the application of 1 mM GSSG has been shown to be reversed and completely overcome, in a concentration-dependent manner, by the subsequent addition of GSH (0.1–1.0 mM) (Haddock *et al.*, 1991) (see Fig. 4.10).

The biological activity associated with reduced glutathione is assumed to be endowed by its cysteine residue, the only amino acid to contain a free sulphydryl group. This has been tested by investigating the effects of glutamate and glycine on Na/K ATPase activity (the other constituent amino acid of glutathione) and has been compared with the effect of cysteine. Neither 1 mM glutamate nor glycine had any significant effect on Na/K ATPase activity, whereas 1 mM cysteine was as potent as GSH in stimulating the enzyme. This suggests that the free thiol component of GSH, endowed by its cysteine residue, may be responsible for the stimulatory action of GSH. Other thiol-containing compounds, such as D,L-penicillamine, reduced dithiothreitol and the ACE inhibitor captopril, have been tested in the same model. All of these compounds mimicked, to varying degrees, the

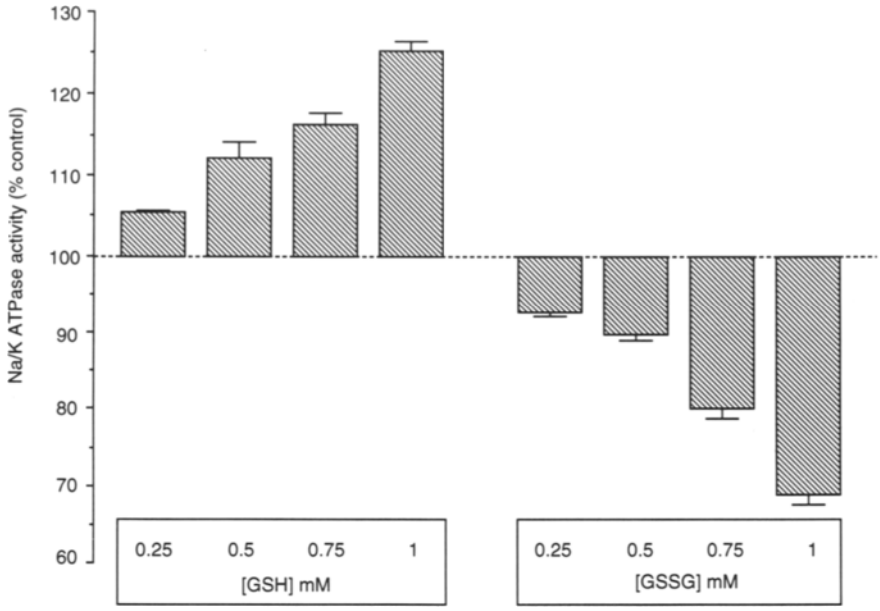

Figure 4.9 Effect of reduced glutathione (GSH) (0.25–1.0 mM) and oxidized glutathione (GSSG) (0.25–1.0 mM) on ouabain-sensitive Na/K ATPase activity. An isolated Na/K ATPase preparation was prepared from fresh bovine ventricular tissue. Na/K ATPase activity was determined and quantified by the ouabain-sensitive hydrolysis of ATP to yield inorganic phosphate. The rate of inorganic phosphate production was compared prior to and following the addition of either GSH or GSSG to the incubation mixture. The data are presented as means ± standard errors of the means ($n = 6$).

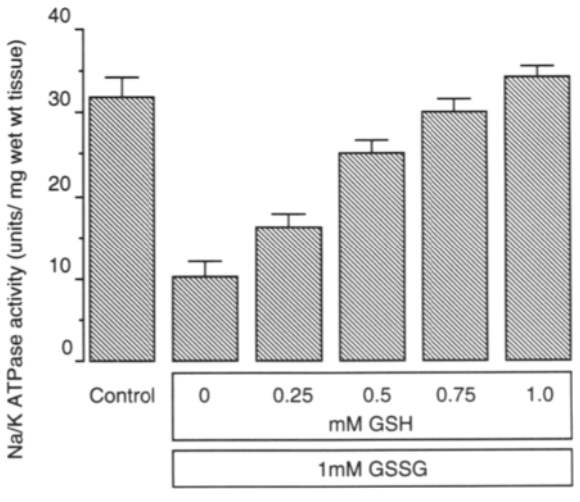

Figure 4.10 Effect of reduced glutathione (GSH) (0–1.0 mM) on Na/K ATPase inhibition associated with the addition of oxidized glutathione (GSSG) (1 mM). Experiments were performed using an isolated bovine ventricular Na/K ATPase preparation. Na/K ATPase activity was quantified by the ouabain-sensitive hydrolysis of ATP to yield inorganic phosphate. The data are presented as means ± standard errors of the means ($n = 6$).

stimulatory effect of GSH, suggesting that it is the free thiol of glutathione that may be important for its effects in this system (Haddock *et al.*, 1991).

Additional studies have also demonstrated that the application of other disulphides, such as oxidized dithiothreitol and cystine, produce similar effects to GSSG over an identical concentration range (Haddock *et al.*, 1991). Therefore the Na/K ATPase activity may be modified by disulphides derived from a variety of species.

These data demonstrate that both GSH and GSSG have profound effects on Na/K ATPase activity and may act in concert to modify enzyme activity during oxidant stress. However, it should be recognized that the steric conformation of an isolated enzyme preparation in a chemically buffered solution may be considerably different to the native enzyme located in a dynamic lipid bilayer. For this reason, these investigations have been extended to include a variety of preparations in which the Na/K pump is in its native environment.

4.3.2 Cytochemical Analysis of Na/K Pump Function in Langendorff-perfused Isolated Hearts: Effect of Glutathione

The cytochemical quantification of ouabain-sensitive Na/K ATPase activity in 10 μm thick whole heart slices,

prepared from reperfused tissue, avoids the problem of tissue disruption after the completion of the previous experimental protocol. This approach has enabled changes in ventricular Na/K ATPase activity following 60 min of ischaemia and 10 min reperfusion (in the presence and absence of GSH) to be quantified. In control, non-ischaemic hearts, Na/K ATPase activity was 18.2 ± 1.1 units, which was reduced to 12.1 ± 0.5 units following ischaemia and reperfusion. When 1 mM GSH was perfused through the coronary vasculature during the pre-ischaemic and reperfusion periods, this loss of Na/K ATPase was abolished (see Fig. 4.11), thus supporting the hypothesis that glutathione maintains protein thiol groups in a reduced state and protects sarcolemmal protein function (Haddock, 1991; Haddock *et al.*, 1991).

4.3.3 Biochemical Analysis of Na/K ATPase Activity in Sarcolemmal Preparations

Whilst experimentally it is relatively easy to investigate the effect of the exogenous application of GSH and GSSG on cardiac Na/K ATPase activity, one further approach that has been exploited in many aspects of oxidant-induced cell injury has been the depletion of cellular glutathione levels. The hypothesized importance of GSH in the cell's antioxidant armoury would be expected to be reflected in an increased susceptibility to oxidant stress-induced tissue damage and cell death following GSH depletion.

The glutathione content of an intact animal can be rapidly and reproducibly diminished by up to 95% within 30 min by pretreating them with a single intraperitoneal injection of diethylmaleate (DEM) (Boyland and Chausseaud, 1967). In adult guinea pigs such a dosing regime has been utilized to establish a dose-dependent relationship between DEM and tissue GSH levels (see Fig. 4.12a); DEM does not alter tissue GSSG levels *per se* (Haddock and Hearse, 1993). In identical tissue, ouabain-sensitive Na/K ATPase activity was expressed

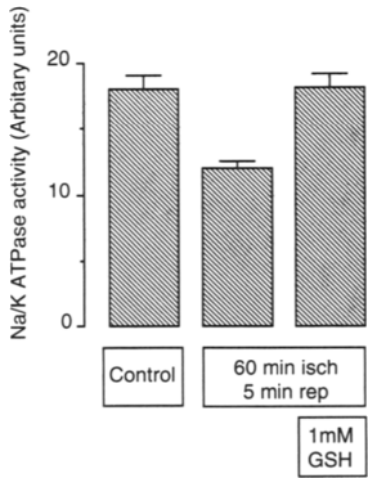

Figure 4.11 Effect of glutathione on the reduction of myocardial Na/K ATPase activity associated with ischaemia and reperfusion. Isolated rat hearts were perfused in the Langendorff mode with oxygenated Tyrode's solution for a control period of 10 min. This was immediately followed by a 60 min period of global, stop-flow ischaemia and 5 min subsequent reperfusion. Glutathione (GSH) (1 mM) was added to the perfusion fluid 5 min prior to the onset of ischaemia and throughout the reperfusion period. The data are presented as means ± standard errors of the means ($n = 6$).

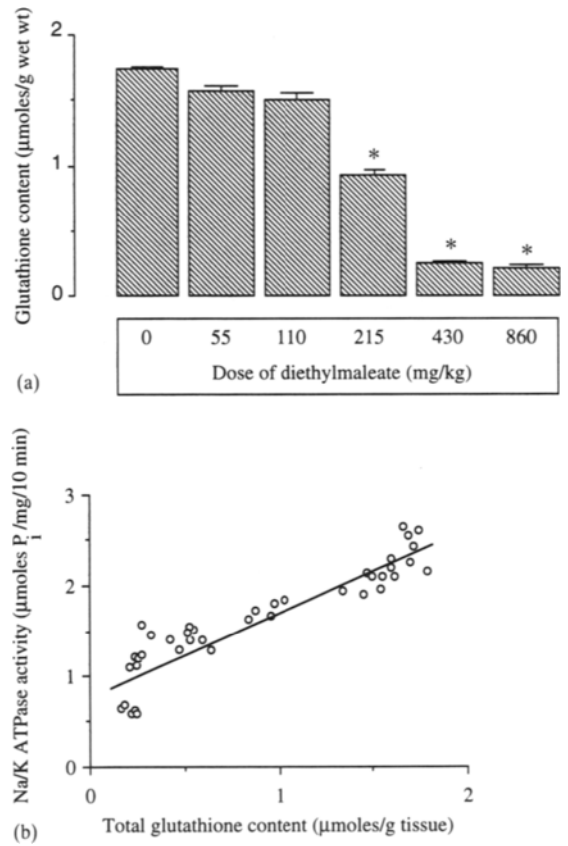

Figure 4.12 (a) Effect of diethylmaleate pretreatment on cardiac glutathione content. Male adult guinea pigs were injected with diethylmaleate (DEM) (0–860 mg/kg, i.p.) 30 min prior to study. Ventricular total glutathione content was determined in perchloric acid extracts. The data are presented as mean ± standard errors of the means ($n = 8$ hearts/group). Asterisks indicate $P < 0.05$, cf. 0 mg/kg control group. (b) The relationship between ventricular glutathione content and ouabain-sensitive Na/K ATPase activity. Guinea pigs were pretreated with DEM as described above. Ventricular homogenates were prepared and Na/K ATPase activity quantified as the ouabain-sensitive hydrolysis of ATP. Each data point represents an individual experiment.

against tissue GSH level. These experiments suggested a linear relationship between tissue GSH levels and Na/K ATPase activity (see Fig. 4.12b).

In contrast to experiments involving the chemical conjugation and depletion of GSH, organic hydroperoxides, such as hydrogen peroxide and tBOOH, may be used effectively to induce a rapid and significant rise in tissue GSSG (and concomitant fall in GSH levels) through the induction of glutathione peroxidase (Haddock *et al.*, 1990). In combination with mercaptosuccinate (a potent inhibitor of glutathione reductase), this effect may be potentiated. The incubation of sarcolemmal homogenates with 10 μM tBOOH results in a time-dependent loss of GSH and rise in GSSG levels (reaching a maximum within 20 min). During this time-course a concomitant depression in Na/K ATPase activity has been characterized. This effect was potentiated in the presence of 100 μM mercaptosuccinate (unpublished observations). These data suggest that changes in glutathione status (decrease in GSH and increase in GSSG content) may be one facet of oxidant stress responsible for a loss of Na/K ATPase activity.

4.3.4 Whole-cell Voltage–Clamp Recordings of Na/K Pump Current: Effects of Cellular Glutathione Manipulation

The advent of the whole-cell voltage–clamp technique has enabled the measurement of the activity of numerous electrogenic ion transporters and ion channels in both cardiac and smooth muscle cells. The electrogenicity of the Na/K pump is endowed by the fact that, for each complete enzymatic cycle of the ATPase, three intracellular sodium ions are exchanged for two intracellular potassium ions, resulting in a net outward charge movement of +1. Although Na/K ATPase assays, both in homogenates and in tissue sections, can provide important information, they have a number of limitations as discussed earlier. A number of these can be overcome by measuring Na/K pump current ($I_{Na/K}$) using voltage–clamp techniques in intact isolated cardiac myocytes (Gadsby *et al.*, 1985). In addition, this technique permits the internal and external environment of the cell to be controlled allowing experiments designed to investigate the effects of both intracellular and extracellular thiol manipulation on Na/K pump activity to be undertaken. Clearly, using crude sarcolemmal and isolated enzyme preparations these types of experiments are not feasible. Furthermore, because of its electrogenicity, effects on the voltage-dependence of the Na/K pump can also be determined.

This technique has been used to investigate the effect of the photoactivation of rose bengal on $I_{Na/K}$ in isolated rabbit ventricular myocytes (Shattock and Matsuura, 1993). Using this procedure, 5 min exposure to illuminated rose bengal reduced $I_{Na/K}$ to 60% of control at 0 mV and to 75% of control at −75 mV. In the absence of extracellular potassium, no active ionic currents remain (all channels, pumps and exchangers being blocked) and the remaining current is a reflection of the passive resistance of the cell membrane. Following oxidant stress induced by the photoactivation of rose bengal, the passive membrane resistance was not altered, suggesting that, under these circumstances, oxidant stress does not cause non-specific membrane damage. This important observation adds to other data indicating that oxidant stress can significantly influence the function of membrane-bound proteins without causing significant alterations in the structure and permeability of the lipid bilayer *per se*. In these experiments no determinations of cellular glutathione content were made.

Preliminary data, in which extracellular and intracellular glutathione status were altered in the absence of a free-radical-induced oxidant stress have demonstrated a similar effect on $I_{Na/K}$. In these experiments the extracellular concentration of GSH was altered by varying the levels in the extracellular perfusate. In contrast, the intracellular GSH content was controlled by the inclusion of the required concentration of GSH in the patch pipette. After 5 min exposure to GSH there was an approximately 20% increase in $I_{Na/K}$ at 0 mV. Conversely, in a separate group of cells, 5 min after the application of GSSG there was an approximately 15% decrease in $I_{Na/K}$ (Haddock, 1991).

The effect of the indirect pharmacological manipulation of cellular glutathione status on $I_{Na/K}$ has also been studied using the same model. Guinea-pig ventricular myocytes exposed to 50 μM tBOOH showed a similar profile of glutathione content modification to that previously described in experiments using sarcolemmal homogenates. Five minutes after the addition of tBOOH, GSH levels fell from a control value of 0.2 ± 0.04 to 0.05 ± 0.01 nmol/1×10^6 cells. This was paralleled by an increase in GSSG from undetectable levels to 0.25 ± 0.03 nmol/1×10^6 cells. Over the same time period there was a reduction in holding current (at a holding potential of −30 mV) from 1.1 ± 0.1 to 0.24 ± 0.02 pA/pF. The holding current in zero extracellular potassium remained unchanged following the exposure to tBOOH (-1.2 ± 0.07 and -1.1 ± 0.05 pA/pF, respectively) suggesting that $I_{Na/K}$ was decreased. This was confirmed by an inward shift of the current–voltage relationship of $I_{Na/K}$ at all potentials tested.

The effect of cellular GSH depletion on $I_{Na/K}$ has also been studied using this model. Guinea-pig ventricular myocytes prepared from DEM-treated animals have been used to determine the effect of glutathione depletion on $I_{Na/K}$. Myocytes prepared from DEM-treated animals showed a similar profile of glutathione content modification to that previously described in experiments using sarcolemmal homogenates. GSH levels in DEM-treated were reduced from a control value of 0.2 ± 0.04 to 0.02 ± 0.01 nmol/1×10^6 cells. $I_{Na/K}$ at 0 mV was reduced from a control value of 1.1 ± 0.12 to

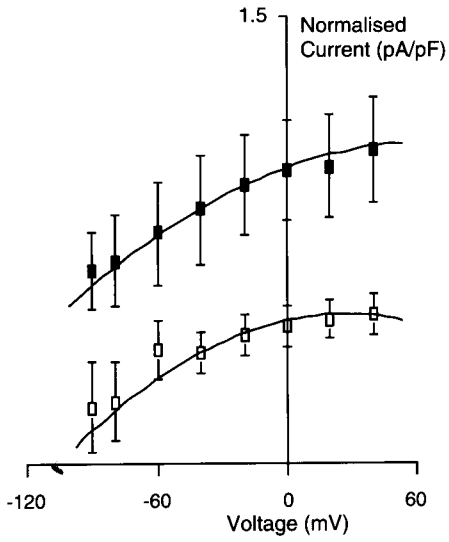

Figure 4.13 Effect of glutathione depletion on normalized Na/K pump current in isolated guinea-pig ventricular myocytes. Adult male guinea pigs were injected with diethylmaleate (DEM) (0.8 ml/kg, i.p.) 30 min prior to study. Isolated ventricular myocytes were prepared from control and DEM-treated animals using standard enzymatic techniques. Cells were held at a membrane potential of −30 mV. Current–voltage relationships were constructed using negative-going ramps (+40 to −90 mV). Membrane current density was expressed in terms of cell capacitance measured at the start of each experiment using a fast (1.8 V/s) ramp protocol. ■, Control untreated animals; □, DEM-treated animals. The bars represent the standard errors of the means (n = 8 cells per group).

0.45 ± 0.09 pA/pF. This was confirmed by an inward shift of the current–voltage relationship of $I_{Na/K}$ at all potentials tested (see Fig. 4.13).

5. *Molecular Mechanism of Oxidant Stress-induced Modifications of Protein Function*

If cellular redox state, determined by the glutathione status of the heart, plays a role in the modulation of ion transporter activity in cardiac tissue, it is important to identify possible mechanisms by which these effects are mediated. Protein S-thiolation is a process that was originally used to describe the formation of adducts of proteins with low molecular thiols such as glutathione (Miller *et al.*, 1990). In view of the significant alterations of cardiac glutathione status (GSH and GSSG) and ion-transporter activity during oxidant stress, the process of S-thiolation may be responsible for modifications of protein structure and function.

Cellular proteins with reactive sulphydryl groups are particularly prone to modification during oxidant stress (Ziegler, 1985). The concentration of these reactive sites may equal the concentration of glutathione in many cell types. These protein sulphydryls react preferentially with the large intracellular pool of glutathione to form mixed disulphides. The large number of proteins that can be S-thiolated and their distribution in all cell types suggests that members of this protein family may significantly contribute to cellular antioxidant defences. In addition, the S-thiolation of specific proteins may provide a form of metabolic control by increasing the activity of key enzymes such as those of the pentose phosphate shunt during oxidant stress (Zimmer, 1992).

S-Thiolation of proteins may occur by two main processes as shown in Fig. 4.14. The first relies on an increase in GSSG levels while the second method depends on free radical production and the GSH concentration (Miller *et al.*, 1990). Therefore, it is clear that a significant increase in tissue GSSG levels is not an absolute prerequisite for S-thiolation to occur.

During ischaemia and reperfusion-induced oxidant stress, there is both a burst of free-radical production (see Fig. 4.1) and a parallel alteration of cardiac glutathione content (Ferrari *et al.*, 1989) (i.e. a fall in GSH and a rise in GSSG levels). These are ideal conditions for the formation of S-thiolated adducts of ion-translocating proteins, such as the sarcolemmal Na/K pump. S-thiolation of the Na/K ATPase may therefore underlie the depression of isolated Na/K ATPase activity in response to the exogenous application of millimolar concentration of GSSG. This type of protein modification is reversible upon the addition of a suitable reducing agent (such as dithiothreitol). A reversal of the GSSG-induced inhibition of Na/K ATPase activity by the subsequent application of GSH may suggest that the Na/K pump is thiolated under oxidant stress and associated alterations in the glutathione status of the myocardium.

6. *Summary and Conclusions*

Many studies now suggest that oxidant stress, during the early moments of reperfusion, may contribute to both contractile and electrical dysfunction in the heart. The decreased contractile function may persist for many hours or days and this myocardial stunning, and the ionic derrangements underlying the ventricular arrhythmias, may have profound clinical significance. It is clear that derrangements in cellular ion movements ultimately underlie reperfusion arrhythmias. Studies of the cellular basis of oxidant stress-induced arrhythmias indicate that it is disturbances in the activity of key ion-translocating proteins, rather than lipid peroxidation, that mediate these effects. Evidence now suggests that, although some of these proteins may be relatively insensitive to oxidant stress (i.e. the L-type Ca channel), others react either by increasing their ion-translocating activity (i.e. the s.r. Ca

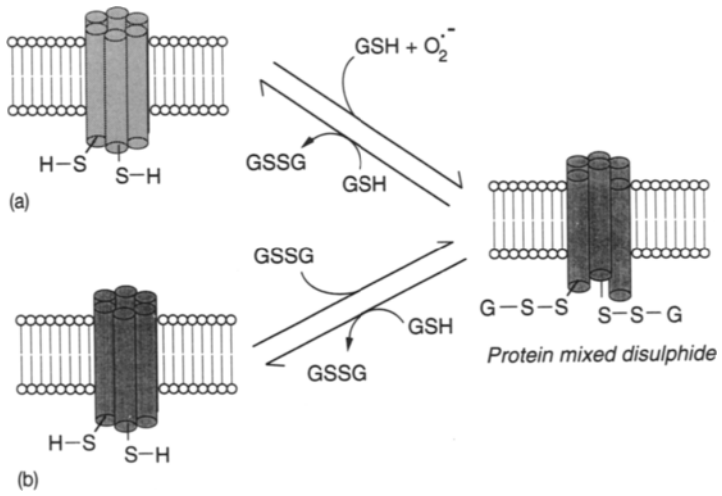

Figure 4.14 Diagrammatic representation of (a) oxy-radical-mediated S-thiolation and (b) thiol/disulphide-initiated S-thiolation of protein sulphydryl groups. Under both circumstances mixed disulphides are formed between glutathione and protein thiols located on the ion-translocator protein resulting in an alteration of protein structure and function. Both of these mechanisms are completely reversible by the addition of a suitable reducing agent, such as reduced glutathione, returning the protein to its native form.

release channel or the Na channel) or by decreasing their activity (i.e. the Na/K pump). Cellular calcium overload is an important factor in the expression of oxidant injury and this may be mediated by alterations in the activity of the Na/Ca exchanger and the Na/K pump. We have reviewed some recent evidence from our own work, and that of others, which has focused on the Na/K pump. These studies implicate the modification of key sulphydryl groups on the Na/K ATPase protein in the effects of oxidant stress. Controlling sulphydryl groups on ion-translocating proteins may thus modulate the activity of the proteins and respond to changes in cellular redox state. Such redox-induced changes in the capacity of the cell to regulate its intracellular ionic environment may play a significant role in mediating reperfusion-induced injury and may provide an important target for potential therapeutic interventions.

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5. *Free Radicals in Central Nervous System Diseases*

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1. *Evidence for the Existence of Free Radicals in the Brain*

The existence of free radicals in the brain and in other biological systems is not a question remaining to be answered. It is well known that oxygen radicals or H_2O_2 and lipid hydroperoxides are produced in the normal metabolic pathways for catecholamines (Marker *et al.*, 1981), arachidonic acid (Yamamoto, 1983) and phagocytosis (Fantone and Ward, 1982). Obviously, if there were no need to be concerned about free-radical attack, the brain and other tissues would not have evolved the multiple defence mechanisms against radical damage and lipid peroxidation. The enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase, and the radical scavengers, ascorbate, α-tocopherol, β-carotene and glutathione are relatively abundant in living tissues. Lack of any one of these protective mechanisms can result in pathological conditions. The important question that remains to be addressed here is not whether free radicals are present, but rather where are they present in amounts sufficient to overwhelm the defence mechanisms and cause cell death in various central nervous system (CNS) pathological conditions.

The brain is only about 2% of the total body mass, yet it consumes approximately 20% of all the inspired oxygen. This fact alone would make it particularly vulnerable to oxidative stress resulting from the generation of reactive oxygen species (ROS) unless it were unusually rich in antioxidant defence mechanisms. No evidence exists, however, that the CNS has an abundance of antioxidant defence mechanisms that exceeds that found in other body tissues. Neurons appear to be highly vulnerable to reduced free-radical defences. For example, Murphy *et al.* (1989) demonstrated that blockade of cystine uptake (precursor to glutathione) was lethal to a neuronal cell line. Primary cultures of rat hippocampal neurons were also shown to degenerate in medium with lowered cystine concentration. Blockade of cystine uptake by glutamate was also shown to produce toxicity in Huntington's disease fibroblasts in culture (May and Gray, 1985).

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In spite of the high vulnerability of the brain to oxygen radical attack, oxygen free-radical reactions and oxidative damage are in most cases held in check by antioxidant defence mechanisms under basal conditions. Pathological conditions of the central nervous system exist, however, where excessive amounts of oxygen free radicals are produced that impair defence mechanisms and result in serious brain damage. Only in recent years have increases in free radicals been measured in the brain under pathological conditions. The difficulty of detection of radicals has been mainly due to the short life span of a radical and the problem of sampling them from brain tissue without generating artefacts. It was extremely difficult, if not impossible, to observe superoxide and hydroxyl free radicals directly in the brain because in most cases the instantaneous concentration of these species was extremely low and their rate of reaction was essentially equivalent to the rate of production. Recently, these difficulties have been overcome by using techniques such as free-radical spin trapping and salicylate trapping of the hydroxyl free radical. In the technique of spin trapping, a spin-trap reagent is used to react with the radical to form a stable free radical. The reaction product builds up and can be analysed by using electron paramagnetic resonance methods. In the salicylate trapping method, salicylate reacts with hydroxyl free radical and yields 2,3- and 2,5-dihydroxybenzoic acid (DHBA) products. These products can be detected by high-performance liquid chromatography (HPLC) methodology. In addition to using radical traps, oxygen free-radical reactions with endogenous molecules have been used as evidence for the presence of radicals. These include products of lipid peroxidation, products of protein oxidation and products from nucleic acid oxidative damage.

Through the utilization of the above techniques, evidence was obtained for increased radical production in cerebral ischaemia/reperfusion models. In Mongolian gerbils subjected to 10 min of global ischaemia followed by 2 h of reperfusion, Oliver *et al.* (1990) reported the formation of a *N-tert*-butyl-α-phenylnitrone-(PBN)-dependent nitroxide radical in the lipid fraction of the ischaemia/reperfusion-lesioned brains. This study indicated that free-radical fluxes increased during the reperfusion phase of the ischaemia-lesioned gerbil brain. Sakamoto *et al.* (1991) produced forebrain ischaemia in the rat by bilateral occlusion of the common carotid arteries combined with haemorrhagic hypotension. They homogenized the cerebral cortex in the presence of the spin-trap reagent PBN. Spin adducts were detected using electron spin resonance (e.s.r.) spectroscopy. After 20 min of ischaemia followed by 5 min of reperfusion, there was an abrupt burst of free radical formation. When the cortex was homogenized with a high concentration of SOD, the intensity of the spectrum was decreased, indicating that at least some of the radical formed was superoxide. Using the salicylate trapping method, Cao *et al.* (1988) measured the production of hydroxyl free radicals during the reperfusion stage in gerbils that were subjected to global forebrain ischaemia. Chan and Fishman (1980) determined that the brain is capable of producing superoxide radicals in response to free fatty-acid-induced brain swelling. Sun *et al.* (1993) reported that human astrocytoma cells were capable of generating reactive oxygen species when subjected to various toxins such as iodoacetate or *t*-butyl-hydroperoxide. The addition of these substances caused a rapid and immediate accumulation of intracellular reactive oxygen species. Superoxide anion production has been observed in another brain cell type, the microglia, by Tanaka *et al.* (1993) and Sotomatsu *et al.* (1993). Another radical that is known to exist in the brain and can be measured is nitric oxide. The literature on nitric oxide generation in the brain has become so vast that no attempt will be made here to review it. Radicals can also be produced during nitric oxide synthesis. Pou *et al.* (1992) observed, using a spin-trapping technique combined with e.s.r. spectroscopy, that brain nitric oxide synthetase generates superoxide in a calcium/calmodulin-dependent manner. In an early study by Imaizumi *et al.* (1984), chemiluminescence was used to examine the effects of cerebral hypoxia on free radical reactions in brain tissue lipid constituents. The results of their study suggest that free-radical reactions occurred in the hypoxic and post-hypoxic brain. Nelson *et al.* (1992) measured superoxide production as the SOD inhibitable portion of nitro blue tetrazolium reduction after cerebral ischaemia/reperfusion in anaesthetized cats equipped with cranial windows. Significant superoxide production was found in the early reperfusion period and continued for more than 1 h after ischaemia. Superoxide could not be detected in control animals not subjected to ischaemia.

Brain unsaturated fatty acids are highly vulnerable substrates for radical attack, and measurements of lipid peroxidation have been used as evidence for the presence of radicals. Several investigators have reported studies where they measured increases in products of lipid peroxidation during the reperfusion phase after global forebrain ischaemia (Chan and Fishman, 1980; Nayini *et al.*, 1985; Bromont *et al.*, 1989; Oliver *et al.*, 1990; Sakamoto *et al.*, 1991). Oliver *et al.* (1990) reported that ischaemia/reperfusion also induced oxidative damage to proteins in gerbil brain. They reported there was a 35% decrease in the activity of glutamine synthetase, an enzyme that is quite sensitive to oxidative damage. Finally, merely breathing 100% oxygen after global brain ischaemia in gerbils was reported to result in increased lipid peroxidation and increased mortality (Mickel *et al.*, 1987).

Taking into account all of the abovementioned studies, one is compelled by the evidence to conclude that, at least under conditions of ischaemia/reperfusion, the brain certainly has the capacity to generate reactive oxygen species and that these reactive oxygen species are likely to play an important role in the pathology that results from

brain ischaemia. Moreover, it appears that under basal physiological conditions, the free radicals that are generated are probably inactivated to a great extent by the endogenous free-radical defence mechanisms. As we shall see later, however, many years may pass before pathology from slow progessive free radical damage becomes evident.

2. *Sources of Free Radicals*

If a search were undertaken for a tissue that is a good substrate for free-radical reactions, it would be difficult to find one more suitable than the brain. Compared to other organs, there are some unique features of the brain that render it highly susceptible to radical attack. Many of these features can be attributed to compositional characteristics and metabolic conditions of the brain that favour ROS formation. The brain has a high rate of oxygen consumption, it is totally dependent on oxidative metabolism of glucose as an energy source, has a high rate of blood flow, has a high concentration of unsaturated fatty acids, has a high concentration of neurotransmitters capable of undergoing autooxidation, and has neurons with long processes, some several centimetres over which high ionic gradients must be maintained for normal function. These characteristics can predispose the brain to radical attack. We will now examine some of these characteristics in more detail.

2.1 RADICAL PRODUCTION DURING NORMAL METABOLISM

Free radicals are produced in small amounts as unwanted by-products of normal cellular metabolic processes. For example, the one-electron reduction of a molecule of oxygen yields superoxide, a potentially destructive radical. The mitochondrial electron transport system is designed to add four electrons to a molecule of oxygen, reducing it to water and avoiding the reactive superoxide species. Cytochrome oxidase and other proteins that reduce oxygen have been designed not to release superoxide. However, leaks in the mitochondrial electron transport chain allow oxygen to accept single electrons forming superoxide radical. Radicals are also formed as intermediates in the electron transport chain. In the transfer of electrons towards cytochrome oxidase, ubiquione is reduced to a free-radical semiquione intermediate. Free radicals can be put to use in the mechanisms and control of enzymatic reactions such as ribonucleoside diphosphate reductase, cytochrome P450 and cyclooxygenase; alternatively, radical species may be products of enzyme catalysed reactions. For example, a stable organic free radical is part of the active site of ribonucleoside diphosphate reductase. This enzyme is involved in reduction of ribonucleoside diphosphate to deoxyribonucleoside diphosphate. This is an important step in DNA synthesis. Coenzyme B_{12} is involved in catalysing intramolecular migration involving hydrogen. The role of B_{12} in these intramolecular migrations is to serve as a source of free radicals for the abstraction of hydrogen atoms. Therefore, free radicals can serve as necessary components in normal physiological processes as well as be unwanted by-products of the electron transport chain. When considering the high-energy demands of neuronal tissue, it seems likely that greater amounts of reactive oxygen species can potentially be produced in the brain than in other tissues of the body with lower metabolic demands.

2.2 CATECHOLAMINES

One potential source of free radicals that exists in the brain but which is not present to a significant extent in most peripheral tissues is catecholamines. Of particular concern is dopamine, the most easily oxidizable catecholamine. Catecholamines are normally enzymatically oxidized by monoamine oxidase resulting in the formation of hydrogen peroxide (Marker *et al.*, 1981). Dopamine can also be oxidized by molecular oxygen and, for every molecule of dopamine oxidized, two superoxide anion radicals are produced. Catecholamines can reduce transition metals so that they can participate in Fenton or Haber–Weiss style reactions generating the reactive hydroxyl radical from hydrogen peroxide (Lovstad, 1984). Dopamine can also react with hydroxyl radicals to produce a dopaminergic neurotoxin, 6-hydroxydopamine (Slivka and Cohen, 1985). Thus, several ways exist through which catecholamines could contribute to the formation of toxic free radicals.

2.3 TRANSITION METALS AND FREE-RADICAL FORMATION

Low molecular weight forms of iron or copper can actively catalyse oxygen-radical and lipid-peroxidation reactions. Under normal circumstances, low molecular weight forms of redox-active iron and copper in the brain are maintained, as in other tissues, at extremely low or non-existent levels. Most of the work regarding transition metal contributions to free-radical formation has been done with iron. Because of the sequesteration of iron, it is normally not available to catalyse free-radical reactions; however, under conditions such as cerebral ischaemia or haemorrhage resulting from mechanical trauma, low molecular weight forms of iron become available. Extracellularly, the iron transport protein transferrin and intracellularly the iron storage protein, ferritin, tightly bind iron in the non-catalytic Fe^{3+} form. At neutral pH, both ferritin and transferrin have a high affinity for iron. However, this protein-bound iron will dissociate at low pH. Intracellular and extracellular pH drops markedly during ischaemia (Vonhanweh *et al.*, 1986). Normally, glucose is metabolized to pyruvate and

NADH, which enters the Krebs cycle. However, during cerebral ischaemia, metabolism becomes anaerobic, which results in a precipitous decrease in tissue pH to below 6.2 (Smith *et al.*, 1986; Vonhanweh *et al.*, 1986). Tissue acidosis can now promote iron-catalysed free-radical reactions via the decompartmentalization of protein-bound iron (Rehncrona *et al.*, 1989). Superoxide anion radical also has the ability to increase the low molecular weight iron pool by releasing iron from ferritin reductively (Thomas *et al.*, 1985). Low molecular weight iron species have been detected in the brain in response to cardiac arrest. The increase in iron coincided with an increase in malondialdehyde (MDA) and conjugated dienes during the recirculation period (Krause *et al.*, 1985; Nayini *et al.*, 1985).

Ascorbate can also reduce iron from the ferric form to the ferrous form thereby potentiating damage during reperfusion by generating reactive oxygen intermediates. Ascorbate normally protects tissues against free-radical attack, however, conditions can exist under which ascorbate can participate in the generation of free-radical species. Under ischaemic conditions, ascorbate can interact with transition metals such as iron or copper to produce the highly damaging hydroxyl free radical. In the presence of hydrogen peroxide, ascorbate reduces iron salts and gives rise to a superoxide independent form of hydroxyl radical (Rowley and Halliwell, 1983a). A similar interaction has been shown to occur between copper and ascorbate (Rowley and Halliwell, 1983b).

Haemoglobin contains another source of iron that could contribute to the generation of reactive oxygen species. Haemoglobin itself has been reported to stimulate lipid peroxidation (Sadrzadeh *et al.*, 1984). Hydrogen peroxide or lipid hydroperoxide can release iron from haemoglobin (Gutteridge, 1986). Thus, the cells that supply tissue with oxygen may also contribute to ischaemic damage. Low oxygen tension has been demonstrated to enhance the rate of superoxide leakage from erythrocytes (Levy *et al.*, 1988). This may be due to enhanced auto-oxidation of haemoglobin, which is know to occur at low oxygen tensions. A low level of perfusion in ischaemic regions could enhance this process because most of the ischaemic area in focal stroke receives some perfusion by blood, however, at a greatly reduced level. Haem that disassociates from extracellular haemoglobin is likely to insert into the hydrophobic domain of the CNS membranes, break down and release free iron. The iron can cause extensive peroxidation of CNS polyunsaturated fatty acid and inhibition of crucial membrane transport enzymes such as sodium/potassium ATPase. It is interesting that these oxidative events caused by free iron are evidently dependent upon CNS-derived ascorbic acid, one of the body's main defence mechanisms against radicals (Sadrzadeh and Eaton, 1992). It therefore appears that other than radicals generated as by-products of normal metabolism, the contribution to radical generation by iron likely occurs under conditions of ischaemia or haemorrhage due to brain trauma or haemorrhagic stroke.

2.4 GENERATION OF OXYGEN RADICALS FROM LIPIDS

Prostaglandins and leukotrienes are formed from arachidonic acid, which is released from phospholipids by the action of phospholipase A_2. During the normal metabolism of arachidonic acid, fatty-acid cyclooxygenase gives rise to superoxide via an enzymatic mechanism, which involves the formation of the radicals of NAD or NADP (Kukreja *et al.*, 1986). Free radicals are also involved in the lipoxygenase pathway of eicosanoid metabolism. In conditions such as cerebral ischaemia, the generation of oxygen radicals from arachidonic acid metabolism may be intensified. During ischaemia, brain concentrations of free arachidonic acid and other unsaturated fatty acids rise to high levels (Guadet and Levine, 1979; Edgar *et al.*, 1982). During reperfusion after ischaemia, the enzymes of arachidonic acid cascade would be particularly active because of an abundance of their substrate, free arachidonic acid, which would have been released from membrane phospholipids during ischaemia, and with reperfusion, oxygen would also be available. The phospholipid component of cellular membranes of the brain is a highly vulnerable target due to the susceptibility of its unsaturated fatty-acid side chains to free-radical attack, ultimately forming lipid hydroperoxides. Any free radical that has sufficient reactivity to extract a hydrogen atom from the reactive methylene group of an unsaturated fatty acid can induce lipid peroxidation. Many species of radicals may be involved. During the process of lipid peroxidation, the lipid radical that is formed then takes up oxygen to form the peroxyl radical. Peroxyl radicals can combine with each other or they can attack membrane proteins. They are also capable of abstracting hydrogen from adjacent fatty-acid side chains in a membrane to propagate the chain reaction of lipid peroxidation. Thus a single radical abstracting a hydrogen from a lipid can result in the conversion of vast numbers of fatty-acid side chains into lipid monohydroperoxides. The propagation phase can be repeated many times. The lipid hydroperoxides are reasonably stable molecules, however, under conditions of cerebral ischaemia or cerebral haemorrhage, they are able to contribute to the formation of additional free radicals. The decomposition of lipid hydroperoxides is catalysed by transition metals or haem protein complexes (O'Brien, 1969; Labeque and Marnett, 1988).

The generation of radicals from lipids appear to be dependent on the abstraction of hydrogen by other radicals. Consistent with this idea is the observation that either lipid peroxidation or anoxia can cause a release of free arachidonic acid from culture cells, and this release can be blocked by antioxidants (Braughler *et al.*, 1985, 1988).

2.5 FREE RADICAL GENERATION BY PHAGOCYTIC CELLS

Under basal conditions, phagocytic cells probably make little or no contribution toward the generation of free radicals in the brain. However, under pathological conditions, free radical generation by phagocytic cells may be significant. Neutrophils, macrophages and microglia when activated have a capacity to release a variety of products such as proteolytic enzymes, arachidonic acid metabolites, and reactive oxygen species including superoxide radical, hydrogen peroxide, hydroxyl radical and hypochlorous acid (Fantone and Ward, 1982; McCord, 1987). There is a significant literature associating myocardial infarct size resulting from reperfusion and the amount of neutrophil infiltration (Romson *et al.*, 1983; Lucchesi and Mullane, 1986; Lucchesi *et al.*, 1989), but there is less evidence to associate neutrophil contribution to brain damage. Granulocytes labelled with indium III were shown to accumulate in regions of ischaemic brain in a dog model of focal ischaemia during the first 4 h of the post-ischaemic phase, thus implicating granulocyte participation in the acute phase of ischaemic brain injury and early reperfusion (Hallenbeck *et al.*, 1986). Studies using the anti-neutrophil monoclonal antibody RP3 in the rat middle cerebral artery occlusion/reperfusion model of focal stroke resulted in a significant decrease in brain oedema as compared to saline-treated rats (Shiga *et al.*, 1991). The synthesis of superoxide anion radicals via the NADPH oxidase of leucocytes could result in cellular damage by exposing tissue to high local concentrations of this reactive oxygen species (McCord, 1987). Animals rendered granulocytopenic or leucopenic in different models of ischaemic stroke showed a significantly better outcome than controls (Dutka *et al.*, 1989; Schurer *et al.*, 1990; Vasthare *et al.*, 1990). Grau and Berger (1992) reported that granulocytes taken from stroke victims had significantly greater adhesion to laminin and fibronectin, and also showed greater superoxide production after stimulation by phorbol ester.

Microglial cells respond to many pathological situations. They carry phenotypic markers of monocytes, which is indicative of their role in the monocyte phagocytic system. Amoeboid microglia appear to contribute to neuronal death in primary neuronal cultures and after ischaemia (Giulian and Robertson, 1990; Thery *et al.*, 1991). Amoeboid microglia begin to accumulate about 24 h after an ischaemic episode (Giulian and Robertson, 1990). Activated microglia can be detected as early as 20 min after reperfusion in a model of global ischaemia (Morioka *et al.*, 1991). Activated microglia can produce free radicals as well as other cytotoxins. Recent reports demonstrated that microglia can produce superoxide (Banati *et al.*, 1993) and superoxide production can be stimulated by adding phorbol myristate acetate to cultures of microglia or by adding aluminium ions (Sotomatsu *et al.*, 1993; Tanaka *et al.*, 1993). Activated microglia can also produce nitric oxide which itself is a free radical. Nitric oxide in reactions with superoxide produces the toxic hydroxyl radical (Beckman *et al.*, 1990). Nitric oxide has also been found to be produced by neurons. Neurons releasing nitric oxide innervate the cerebral vasculature. In this case nitric oxide assumes the role of endothelium-derived relaxing factor (EDRF) and is responsible for cerebral vascular dilation. While it is accepted that macrophages produce nitric oxide as one of the toxins to kill invading pathogens, the role of nitric oxide in brain pathology is at the present time unclear.

3. *Defences against Free Radical Attack*

The free-radical defence mechanisms utilized by the brain are similar to those found in other tissues. The enzymes SOD, catalase, glutathione peroxidase, and the typical radical scavengers, ascorbate, vitamin E and vitamin A are present in the brain, as they are in peripheral tissues. However, the brain may actually be slightly deficient in some of these defence mechanisms when compared to the amounts present in other tissues.

4. *Evidence for a Role for Free Radicals in CNS Diseases*

4.1 CEREBRAL ISCHAEMIA

At the present time it is difficult to single out any one factor that could be held ultimately responsible for cell death after cerebral ischaemia. Recent studies, however, have provided us with sufficient evidence to conclude that free radical damage is at least one component in a chain of events that leads to cell death in ischaemia/reperfusion injury. As noted earlier in this review, much of the evidence for free radicals in the brain and the sources of free radicals come from studies in animals subjected to cerebral ischaemia. Perhaps the best evidence for a role for free radicals or reactive oxygen species in cerebral ischaemia is derived from studies that demonstrate protective effects of antioxidants. Antioxidants and inhibitors of lipid peroxidation have been shown to have profound protective effects in models of cerebral ischaemia. Details of some of these studies will be mentioned later. Several reviews have been written on the role of oxygen radicals in cerebral ischaemia (Braughler and Hall, 1989; Hall and Braughler, 1989; Kontos, 1989; Floyd, 1990; Nelson *et al.*, 1992; Panetta and Clemens, 1993).

4.2 PARKINSON'S DISEASE

Evidence has accumulated recently suggesting that free radicals may be involved in the genesis of Parkinson's disease. As noted earlier, dopamine is susceptible to autooxidation and, in the process, quinones are generated as well as the superoxide anion. Graham (1984) was the first to propose that molecular pathogenesis of Parkinson's disease was due to catecholamine toxicity. He proposed that Parkinson's disease was due to cytotoxicity from products of catecholamine oxidation including superoxide anion, hydroxyl radical and hydrogen peroxide. Ogawa *et al.* (1993) showed that levodopa scavenged both superoxide and hydroxyl radicals; however, brain homogenates preincubated with levodopa scavenged little hydroxyl radical, which suggested levodopa can antagonize the intrinsic scavenging activity. Furthermore, e.s.r. spectrometry show that oxidation of levodopa produced levodopa radicals, i.e. quinone radicals. In addition, in rats pretreated with 6-hydroxydopamine, the administration of levodopa produced a marked increase in thiobarbituric acid-reactive substances. The results suggest that the most commonly used drug to treat Parkinson's disease, levodopa, may actually accelerate the disease process. The toxic effects of free radicals on dopamine neurons has been linked to the selective accumulation of iron, a known catalyst of free-radical formation, in the zona compacta of the substantial nigra. Ben-Shackar *et al.* (1992) demonstrated that the iron chelator, desferroxamine, prevented lesions of the nigrostriatal dopamine system induced by 6-hydroxydopamine. They also found that intranigral injection of 50 μg of iron resulted in a substantial selective decrease of striatal dopamine and produced impaired dopamine-related responses.

The beneficial effect of deprenyl in Parkinson's disease was suggested to be in part due to its effect on increasing the levels of SOD activity in several brain regions (Carrillo *et al.*, 1993). Deprenyl is known to inhibit monoamine oxidase type B, which results in a reduction in hydrogen peroxide formation by blockade of the oxidative deamination of dopamine. That is believed to be the major mechanism of action of this drug in inhibiting the progression of Parkinson's disease.

In Parkinson's disease, a case for oxidative stress as a causative agent in the disease process can be made as a consequence of increased formation of reactive oxygen species. This could be attributed to age-related decreases in free-radical scavenger systems. Experimentally, the synthetic neurotoxin, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is capable of inducing Parkinson's disease in humans and other primates by conversion to a toxic free radical by monoamine oxidase type B. MPTP is unlikely to be involved in normal disease pathology; however, it does point out the susceptibility of the dopaminergic system to free-radical attack. Although free-radical attack on the dopaminergic neuronal system due to the presence of a potentially deadly combination of catecholamines and iron is an attractive hypothesis to explain the cause of Parkinson's disease, definitive proof of radical participation in the disease pathogenesis is still lacking.

4.3 FREE RADICALS AND ALZHEIMER'S DISEASE AND DOWN'S SYNDROME

Rather scanty evidence exists for the participation of free radicals in Alzheimer's disease and Down's syndrome. However, more recently, reports have appeared that suggest possible free-radical involvement in the pathogenesis of these two conditions. Zemlan *et al.* (1989) reported that the activity of the free-radical scavenging enzyme, SOD, was significantly increased in fibroblast cell lines derived from familial Alzheimer's and Down's patients. They hypothesized that the elevation in SOD activity observed in the Alzheimer patients supports the theory that paired helical filaments are formed by free-radical hydroxylation of proline residues. They further suggested that SOD levels might also be increased in the brains of Alzheimer's and Down's patients, and that the increase in SOD may reflect an enhanced generation of free radicals.

On the other hand, SOD could potentiate the formation of free radicals if there were a deficiency of catalase by making more hydrogen peroxide available for conversion to the toxic hydroxyl free radical. Thus, overexpression of SOD has a potential for producing toxic effects from free-radical generation. Little evidence is available, however, that SOD is overexpressed in the brains of Alzheimer's victims but SOD overexpression is known to occur in Down's syndrome. Consistent with the idea that high levels of SOD could be toxic are the findings of Ceballos-Picot *et al.* (1991) where they developed transgenic mice that expressed high levels of human copper–zinc SOD. Expression of the human transgene resulted in increased copper–zinc SOD activity predominantly in the brain. The amount of thiobarbituric acid-reactive material was significantly higher in transgenic brains compared to controls, strongly suggesting an increased level of lipid peroxidation *in vivo*. They suggested that the SOD gene dosage effect could play a role in the pathogenesis of Down's syndrome. Since levels of catalase in the CNS are much lower than SOD (Volcier and Crino, 1990), the increase in lipid peroxidation may be due to higher levels of hydrogen peroxide generated by the SOD. In the mouse, trisomy of chromosome 16 is the counterpart of trisomy of chromosome 21 in the human, which results in Down's syndrome. Colton *et al.* (1990) reported enhanced production of superoxide anion by microglia from trisomy-16 mice. These findings provide further evidence that increased production of SOD can result in enhanced

production of oxygen radicals. This may be due to an imbalance that is set up between SOD and catalase as previously mentioned.

In studies in Alzheimer's brain, *in vitro* induction of lipid peroxidation by iron is more intense than in control cortical samples (Andorn *et al.*, 1990; Subbarao *et al.*, 1990; McIntosh *et al.*, 1991). The 21-aminosteroid U-74500A has been shown to effectively inhibit iron-induced lipid peroxidation in Alzheimer's brain samples (Subbarao *et al.*, 1990).

Despite the indications for involvement of free radicals in Alzheimer's disease and Down's syndrome pathogenesis summarized above, more evidence is needed to establish a role for free-radical mechanisms in these disease processes. If free radicals can be demonstrated to play a role in the pathogenesis of Alzheimer's disease and Down's syndrome, then this would set the stage for chronic therapy with antioxidants in these disease states.

4.4 AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a degenerative disorder of motor neurons in the cortex, brain stem and spinal cord. It is late in onset, progressive and ultimately fatal, typically within 5 years. About 10% of the cases are familial and are inherited as an autosomal dominant trait. Rosen *et al.* (1993) demonstrated convincingly that a familial variant of ALS is linked to defects in the SOD gene. Based upon the findings of Rosen *et al.* (1993), McNamara and Fridovich (1993) advanced the hypothesis that excitotoxicity through activation of non-NMDA, glutamate receptors may play a role in pathogenesis of ALS. Excitotoxicity through the non-NMDA receptors depolarizes the membrane and causes calcium entry through voltage-gated calcium channels. The increased intracellular calcium activates a protease, which converts xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase becomes a cellular source of cytotoxic superoxide radicals. Persistent production of superoxide radicals may result in eventual motor neuron death as seen in ALS. More work will be needed to confirm or refute this hypothesis. Although the defects in the SOD gene that encodes the cytosolic form of the enzyme has been shown to occur in only 10% of the ALS patients, this does not mean oxygen radicals are not involved in the other 90% of ALS patients. Further studies directed at determining why the motor neuron might be a unique target for destruction in this disease will be important.

4.5 FREE RADICALS AND NORMAL AGEING

During the ageing process, the increased levels of an "age pigment", lipofuscin, which results from lipid peroxidation, is convincing evidence of accumulative free-radical damage. Lipofuscin accumulates in neurons of the CNS (Sohal and Wolfe, 1986). The free-radical theory of ageing suggests that small but persistent defects in protection against free radicals produces progressive tissue damage (Harman, 1988). A significant correlation was established between the ratio of copper/zinc SOD activity to metabolic rate and maximum life-span potential in several mammalian species (Cutler, 1982). Animals with higher SOD activity appeared to live longer.

The content of oxidized proteins in cells increases during ageing. Oxidized proteins as measured by protein carbonyl content increases with age in gerbil brain and human postmortem brain tissue (Carney *et al.*, 1991; Smith *et al.*, 1991). Glutamine synthetase is an important enzyme involved in the inactivation of glutamate by converting it to glutamine. If glutamine synthetase becomes deficient, glutamate may build up and could potentially have neurotoxic effects. Carney *et al.* (1991) and Smith *et al.* (1991) have observed age-related declines in glutamine synthetase activity in gerbil brain and in human brain tissue, respectively. Consistent with the regional distribution of pathological changes observed in Alzheimer's disease, Smith *et al.* (1992) have observed that the rate of protein carbonyl accumulation increases with age and the rate of increase in the frontal pole is twice that in the occipital pole. This pattern is also seen in Alzheimer's disease and parallels the neuropathological distribution of senile plaques and neurofibrillary tangles.

Another factor contributing to increased radical formation during ageing is increased mitochondrial electron leakage in the face of marked alterations of the cerebral glutathione antioxidant system (Benzi *et al.*, 1988, 1989). Gomi *et al.* (1993) reported that ageing retards spin clearance of nitroxide radicals from mouse brain and suggested that ageing may be related to a reduction in antioxidant capacity. Increased oxidative stress therefore appears to be a feature of the ageing brain. The challenge of the future will be to determine how increased oxidative stress may be linked to the multiple alterations in the physiology of the ageing brain.

5. *Protective Effects of Antioxidants*

The most extensive evidence that supports a role for free radicals in pathological conditions of the brain is provided by studies on experimental models of cerebral ischaemia/reperfusion. Although a burst of free-radical production occurs during the reperfusion phase after temporary cerebral ischaemia, the contribution of this radical burst to brain cell death can not be directly quantified. Perhaps the best way to quantify the contribution of free radicals to brain damage after ischaemia/reperfusion is to assess damage after treatment with free-radical scavengers or antioxidants. Numerous studies have been reported where free-radical scavengers/antioxidants have been used to try to ameliorate brain

damage in experimental models of stroke and global cerebral ischaemia. Most, but not all, studies have demonstrated neuroprotective effects against ischaemia/reperfusion damage. Examples of some agents that showed neuroprotective effects are phenyl-*t*-butylnitrone (Yu *et al.*, 1992), polyethylene glycol-conjugated SOD and catalase (Beckman *et al.*, 1989), liposome-entrapped SOD (Imaizumi *et al.*, 1990), tirilazad mesylate (Hall *et al.*, 1988), dimethylthiourea (Martz *et al.*, 1989) and the antioxidant, LY178002 (Clemens *et al.*, 1991).

We recently began studies in our laboratory on a new antioxidant LY231617 (Clemens *et al.*, 1993). We evaluated it in a model of temporary middle cerebral occlusion (MCAO) in the spontaneously hypertensive rat. In this study the rats were anaesthetized with halothane and allowed to breath a mixture of halothane in nitrogen mixed with 30% oxygen. The MCA was exposed surgically and clamped at the level of the rhinal fissure. The clamp was left in place for 2 h. After 2 h of MCAO, the rats were allowed to wake from the anaesthesia and were housed in single cages. One hour before clamp removal, LY231617 was given i.v. at a dose of 10 mg/kg over a period of 5–7 min followed by a constant infusion of 5.0 mg/kg per hour for 24 h.

Brains were removed 24 h after MCAO, serially sectioned and stained with cresyl violet. Infarct volume was determined by image analysis. Table 5.1 shows that treatment with LY231617 resulted in a highly significant reduction in infarct volume. The results of this experiment are in agreement with previous work demonstrating that antioxidants can reduce brain damage from ischaemia/reperfusion and add support to the hypothesis that free radicals/reactive oxygen species contribute to the cascade of destruction that leads to cell death after cerebral ischaemia.

6. *Summary*

Free radicals are produced in the brain during the course of normal metabolism and are known to be involved in several enzymatic reactions. In fact, some enzymes exist with free radicals at the active site. It seems reasonable that the high oxygen consumption by the brain would render the brain more susceptible than other organs to oxidative radical attack. In experimental models of cerebral ischaemia, which are accompanied by reperfusion, a burst in radical production has been measured during the reperfusion phase. The question that remains is, what is the pathological significance of this increase in radical formation after cerebral ischaemia. How does it contribute to the ensuing neuronal damage? This question has been partly answered by studies where free-radical scavengers/antioxidants were given to animals subjected to cerebral ischaemia. These studies demonstrated that free-radical scavengers/antioxidants have neuroprotective effects. The role of free radicals in the pathophysiology of other CNS diseases is less clear and more speculative. Evidence is continuously mounting, however, that free radicals may be involved in the pathophysiology of several adult-onset chronic neurodegenerative diseases. Thus, the role of free radicals in diseases of the brain will continue to be a fertile area for future research.

Table 5.1 Effects of compound 231617 on infarct volume in the middle cerebral occlusion model of focal stroke

Treatment[a]	*Number of rats*	*Infarct volume (mm^3)*
Vehicle	15	128 ± 17
LY231617[b]	10	74 ± 8
		($P = 0.008$)

[a] Treatment was initiated 1 h after MCAO.
[b] Compound LY231617 was given at a dose of 10 mg/kg i.v. over a period of 5–7 min beginning 1 h after MCAO. Reperfusion occurred at 2 h after MCAO and at that time the rats received 5.0 mg/kg i.v. for 24 h. Mean arterial blood pressure (MABP), heart rate, arterial blood pH, pO_2 and pCO_2 were measured. Body temperature was regulated at 36–37°C for the entire 24 h period.

7. *References*

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6. Renal Transplantation and Ischaemia–Reperfusion Injury

Colin J. Green

1. The Clinical Problem

Over 4000 patients present each year in the UK with end-stage renal failure. Since the clinical advent of cyclosporin in 1978 (Calne *et al.*, 1978), renal transplantation (RTx) has become the treatment of choice for these patients and is superior in terms of survival, quality of life and financial cost to the community to haemodialysis and ambulatory peritoneal dialysis in all but a few cases. However, full exploitation of RTx is severely limited by the availability of suitable donor organs. In the event only 1500–1600 RTx are performed each year in the UK. Unless and until the hyperacute rejection process associated with xenotransplantation of organs into man from another species such as pigs is overcome, this problem is unlikely to be resolved. It is essential therefore to make use of every possible organ that becomes available from non-HIV-positive donors from any part of the globe. In effect this means minimizing the damage caused by: (1) ischaemia during the harvesting operation; (2) cold ischaemia during storage and transplantation to an electively prepared and tissue-matched recipient; and (3) ischaemia–reperfusion injury immediately after engraftment and restoration of oxygenated blood to the kidney.

2. Renal Ischaemia

Proximal tubule cells are exquisitely sensitive to vascular disturbances and acute tubular necrosis (ATN) can occur naturally in areas of poor perfusion resulting from falling blood pressure, or vasospasm of renal vessels or arterioles. In other words hypoxia associated with partial ischaemia can cause severe damage. It is not then surprising that anoxia associated with iatrogenic, surgically induced total ischaemia produces irreversible damage within a short time unless steps are taken to prevent it.

Iatrogenic warm ischaemia (WI) may be induced when: (1) the renal vessels are clamped *in situ* for exploratory or reconstructive surgery of the kidney; (2) when the organ is removed from the donor, reconstructed *in vivo* and then replanted orthotopically; and (3) when a kidney is harvested from a donor for transplantation.

Reduction in temperature to above 0°C is the common denominator to all techniques for inhibiting warm ischaemic damage. However, hypothermia alone can only delay the onset of irreversible damage and not prevent it. Furthermore, cold can itself be damaging. Kidneys for transplantation are in clinical practice cooled to 0°–10°C either after they have been removed from

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the donor or whilst the donor is cooled by whole body cooling. Hence a kidney is likely to be subject to warm (37°C), hypothermic (5–30°C) and cold (0–5°C) ischaemia between the donor's death and the restoration of blood after transplantation.

3. *Current Methods of Renal Preservation*

In almost all transplantation centres, donor kidneys are initially flushed free of blood by infusing a cold electrolyte solution via the renal artery and allowing this to run away via the renal vein. The kidney is then immersed in the same cold solution. The organs are thereafter stored at 0–4°C in this solution surrounded by ice to inhibit metabolism as completely as possible or they are continuously perfused at 5–10°C to provide for some limited metabolism and the removal of waste products. Simple infusion using a variety of solutions including a high potassium high osmolar solution (Collins *et al.*, 1969), hypertonic citrate (Ross *et al.*, 1976) and Wisconsin solution (Belzer and Southard, 1988) allows up to 48 h of safe cold ischaemic time. However, since immediate primary function is the aim and we know that even relatively minor ischaemic damage can compound the nephrotoxicity of cyclosporin and other modern immunosuppressive drugs, and is therefore likely to be significant in long-term survival of the organ, it is probably true to say that kidneys can only really be safely stored for up to 27 h.

Proponents of continuous hypothermic perfusion using colloidal solutions such as cryoprecipitated plasma (Belzer *et al.*, 1972) claim that it provides better immediate life-sustaining function after transplantation. This is probably true and it offers more potential for storage periods of 4 days or more. However, it is technically more difficult to maintain sterility over such long periods, and its expense and complexity makes it dependent on the availability of very skilled staff.

The formulation of these successful asanguinous solutions has been based on the perceived need to prevent leakage of intracellular ions into the extracellular compartment in exchange for sodium and water, and to provide some buffering capacity. Hence, they have been rendered hypertonic with high-potassium low-sodium concentrations (as compared to extracellular fluid) and hyperosmolar with relatively impermeant species such as glucose, sucrose, mannitol, raffinose or lactobionate (Collins *et al.*, 1969; Ross *et al.*, 1976; Belzer and Southard, 1988). In addition to mannitol, which enters cells slowly and prevents cells swelling, the hypertonic citrate solution (Ross *et al.*, 1976) contains sufficient citrate to sequester free calcium in the extracellular fluid before it enters cells down membrane gradients, where the ATPase pumps have been switched off through ischaemia and hypothermia.

However, although high potassium concentration was thought essential in early experimental work (Acquatella *et al.*, 1972), Fuller and Pegg (1976) reported that potassium concentrations much higher than normal plasma levels led to poor renal function. A number of more recent studies have claimed that solutions containing high concentrations of sodium ions were equally or even more effective than those with high potassium (Moen *et al.*, 1989; Sumimoto *et al.*, 1989; Marshall *et al.*, 1991).

Other pharmacological agents used in preservation solutions have included diuretics such as frusemide and mannitol, although their supposed mechanisms of protection are not thought to be mediated by their diuretic effects. Mannitol is osmotically active and functions by decreasing swelling of cells during preservation, preventing compression of renal vasculature and thus preventing the "no reflow" phenomenon upon reperfusion (Flores *et al.*, 1972; Jamieson, 1974). Frusemide is thought to be beneficial by preventing the vasospasm triggered by handling during the harvest operation (Panijayanond *et al.*, 1973). Agents such as chlorpromazine (Bilde and Dahlager, 1977) and methylprednisolone have been claimed to stabilize membranes by phospholipase inhibition, calcium chelation and increased intralysosomal pH (Lokkegaard *et al.*, 1971). However, more recent experimental evidence that a simple phosphate-buffered sucrose solution provides at least as good functional results after storage for 72 h and transplantation casts doubt on much of the rationale behind the formulation of earlier solutions (Lam *et al.*, 1989).

4. *Presumed Mechanisms of Ischaemic Damage*

In simple normothermic ischaemia, many pathological changes only become visible after restoration of the blood supply. The events that have taken place during warm ischaemia are therefore difficult to define. Mitochondrial electron transport is inhibited and mitochondria swell so that ATP cannot be generated. Adenine nucleotides are catabolized through AMP and ADP to hypoxanthine (Buhl and Jorgensen, 1975). As ATP levels fall, membrane pumps are inactivated and internal homeostasis between intracellular compartments is lost. Ions diffuse down electrical and chemical gradients. Potassium is lost to the extracellular space whilst calcium, sodium and chloride (accompanied by water) enter the cell because energy-dependent pumps, which normally exclude them, fail. Hence, cytosolic concentrations of these ions rise and the net influx of water produces cellular swelling and hydropic degeneration. Reduced pyridine nucleotides (NADH, NADPH) accumulate because they cannot be processed by mitochrondrial electron transfer. The altered intracellular environment allows lysosomal disruption and release of autolytic enzymes into the cells.

Toxic metabolites such as lactic acid and denatured proteins accumulate. The pH falls with accumulation of H^+ ions (Green and Pegg, 1979).

The damage observed macroscopically upon reperfusion of badly damaged kidneys suggests not surprisingly that the vascular bed is a prime site for these intracellular lesions. An outflow block after initial reflow when the blood supply is restored to the organ followed by acute renal failure is commonly observed (Summers and Jamieson, 1975). Endothelial cell (EC) swelling, breaks in EC intercellular junctions, protein leaks through these pores leading to extracellular oedema and loss of erythrocyte deformability (Weed *et al.*, 1969) probably together conspire toward sludging and rouleaux formation such that the microcirculation becomes blocked. This is not thought to involve coagulation mediated by blood coagulatory factors at least initially; the microcirculation is simply slowed or even blocked until eventually cells distal to occluded vessels are subjected to secondary warm ischaemia. Hence, at its simplest level, ischaemia–reperfusion injury is manifestly obvious to the surgeon.

Depending on species, the length of time that kidneys can tolerate normothermic ischaemia without irreversible and lethal damage varies from 60 min to 90 min. In any event, in man a kidney rendered ischaemic for 60 min at 37°C is severely compromised and may take many days or even weeks to recover. Since most enzymes decrease their activity nearly two-fold for every 10°C depression in their temperature and it has been shown that the metabolic rate of a kidney falls to about 5% of normal at a temperature of 5°C (Levy, 1959; Green and Pegg, 1979), it is not surprising that hypothermia is used to prevent ischaemic damage. However, little is known about the spatial development of damaging cascades resulting from low temperature *per se* even though clearly there is temporal inhibition of ischaemic damage. Again, from gross observation of post-storage, post-transplantation events, it appears that the microcirculation is most severely damaged during hypothermic ischaemia (Jacobsson *et al.*, 1988). It has been suggested that ATPases of EC are particularly sensitive to cold such that EC swelling is mainly responsible for poor reflow during reperfusion. Again after storage, damage is manifest only on reperfusion (except for very subtle intracellular changes observed by transmission electron microscopy even before reperfusion). Since every attempt to prevent the storage damage syndrome has had only limited success, it clearly has a multifaceted aetiology, which may or may not lend itself to pharmacological and therapeutic manipulation. During the past two decades, strenuous efforts have been made to identify the causes of ischaemia–reperfusion injury in renal transplantation but with only limited success. Particular attention has been paid to: (1) oxidative stress and the role of oxygen-derived free radicals (ODFRs); (2) the role of catalytic iron and loss of homeostasis linked to falling pH; (3) the role of disrupted intracellular calcium homeostasis; (4) changes in the physiochemical balance of saturated polyunsaturated fatty acids in phospholipid bilayers; (5) changes in the cyclooxygenase cascade and release of prostaglandins; (6) the role of leukotrienes; (7) the possible importance of induced nitric oxide synthase; and (8) interference with intracellular signalling and secondary messengers such as phosphotidylinositols.

5. *Oxidative Stress*

Oxidative stress caused either directly or indirectly by overproduction of free radicals (ODFRs) and failure to scavenge them due to the normal mechanisms being overwhelmed or dislocated during cold storage of ischaemic tissue has been implicated in reperfusion damage of ischaemic kidneys, and in the storage damage syndrome (Bosco and Schweizer, 1988; Paller *et al.*, 1984; Koyama *et al.*, 1985; Laurent and Ardaillou, 1986; Ratych *et al.*, 1986). Most attention has to date been paid to the peroxidation of polyunsaturated fatty acids (Wills, 1969) but there is a developing interest in ODFR attack on proteins and DNA.

In early attempts to elucidate the role of ODFRs in warm and cold renal ischaemia, we utilized a well-characterized rabbit model but limited our studies to lipid peroxidation. Markers such as diene conjugates, thiobarbituric acid-reactive products (TBARs) and Schiff bases were assayed in homogenates prepared from kidneys, which had been subjected to warm or cold ischaemia. A clear-cut and highly significant increase in these markers occurred in a time-dependent mode during incubation at 37°C *in vitro* (Green *et al.*, 1986c). In the cold-storage studies (Green *et al.*, 1986a, 1986b), rabbit kidneys were either flushed and stored for 24 h in isotonic saline known to be damaging even for periods as short as 8 h, or were flushed and stored for periods up to 72 h with the hypertonic citrate (HCA) solution formulated by Ross and his co-workers in 1976 (Ross *et al.*, 1976). The temperature throughout the storage period was 0–2°C. Survival studies in which rabbits have been bilaterally nephrectomized and then autografted with kidneys stored with HCA in this way have resulted in 100% survival after 24 h of cold storage, 50% after 48 h, 30% after 72 h and no survival after 96 h. Monitoring of renal function in these studies revealed a close correlation between length of cold ischaemia (CI) time and physiological dysfunction. The data from the lipid peroxidation studies showed a strong correlation between increased formation of *in vitro* lipid peroxidative markers with increased length of cold storage and thence with poorer renal function after transplantation *in vivo*. Reperfusion of the ischaemic kidneys with oxygenated blood after release of clamps on the renal vessels generally led to further increases in the formation of markers of lipid peroxidation. When mannitol, uric acid or the

iron-chelator desferrioxamine (DFX) were added to the flush and storage solutions to see if adding free-radical scavengers might be beneficial, there was significant inhibition of *in vitro* lipid peroxidation providing yet more indirect evidence that ODFRs were implicated (Green *et al.*, 1986b). It must be emphasized, however, that these assays *in vitro* after homogenization can only provide circumstantial evidence for the role of ODFRs and are themselves subject to artefactual error however well controlled one endeavours to make them. Our attempts to obtain more direct evidence of ODFR production using electron spin resonance (e.s.r.) after inserting the spin-trap probe α-phenyl-*t*-butyl nitrone (PBN) into the solution have so far proved unsuccessful for a number of technical reasons (unpublished data).

From those early studies, we concluded that a period of ischaemia primed kidneys for reperfusion injury and rendered them in some way more susceptible to ODFR attack. Given the weakness of *in vitro* assessment *after* a 1 h period of incubation at 37°C, it seemed reasonable to claim that the data revealed an increase in *susceptibility* to reperfusion injury. The mechanisms of this damage became the subject of much speculation. One possibility is that during ischaemia, the level of antioxidant defences is reduced. Another is that enzymes such as xanthine dehydrogenase alter during ischaemia so that ODFR production is enhanced following reperfusion; however, whether the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase during ischaemia, possibly as a result of calcium-dependent proteolysis, is as important as when first proposed (Roy *et al.*, 1983) is a matter of debate, but the theory goes that xanthine oxidase is likely to generate ODFRs from incoming molecular O_2 and from hypoxanthine accumulated during ischaemia via catabolism of ATP. A more persuasive argument can be made for the idea that mitochondrial injury during ischaemia allows reduced components of the electron-transfer chain to accumulate, resulting in increased leakage of single electrons to O_2 hence increased production of O_2^- during reoxygenation. Perhaps most important to the endothelial cells lining the renal microvasculature, damaged ischaemic tissue may release chemotactic factors that attract polymorphonucleocytes in sufficient numbers to damage cells locally by ODFRs generated during the respiratory burst (Granger *et al.*, 1989). Finally, because it is known that membrane-dependent ionic shuttles are disturbed and intracellular pH falls during ischaemia, it seemed sensible to investigate in some detail the way metal ion fluxes (particularly iron and calcium) are disturbed and how this relates to other potential mechanisms of damage.

6. *Delocalization of Iron*

The importance of iron in ischaemia–reperfusion injury through generation of ODFRs via the Haber–Weiss reaction (Halliwell, 1978) and the ability of iron-centred species to catalyse hydrogen atom abstraction from polyunsaturated acids and thus initiate lipid peroxidation (Minotti and Aust, 1987), as well as the ability of iron salts to degrade lipid hydroperoxides to reactive peroxy and alkoxy radicals, and propagate a chain reaction (Halliwell and Gutteridge, 1984) led us to study the role of iron in stored rabbit kidneys.

Since the high molecular weight iron chelator DFX is known (Keberle, 1964) to bind iron with such high affinity (10^{31}) that the metal ion is prevented from catalysing peroxidative reactions (Gutteridge *et al.*, 1979), it was decided to evaluate this agent in the rabbit kidney model both as an investigative tool and perhaps as a preventative therapy against storage damage. It was found that i.v. administration of DFX to rabbits 15 min before reperfusion of kidneys, which had been subjected to 60 or 120 min of normothermic ischaemia *in situ*, significantly inhibited rises in markers of lipid peroxidation ($P < 0.001$) (Green *et al.*, 1986c). Later, it was found that DFX was very effective at reducing oxidative damage in rabbit kidneys, which had been hypothermically (0–2°C) stored either for 24 h after flush with isotonic saline (Green *et al.*, 1986b) or for periods up to 72 h after flush with HCA (Gower *et al.*, 1989a). It was most effective if administered i.v. to the rabbits both before the kidneys were harvested for storage and before reperfusion when they were autotransplanted. However, when homogenates of cortex were compared with those of the medulla, rises in markers of lipid peroxidation were inhibited to different degrees by DFX; the agent was markedly more effective in the cortex than in the medulla and from this data we hypothesized that perhaps the high levels of cyclooxygenase known to be present in renal medulla (Robak and Sobanska, 1976) were more important in lipid peroxidation in that region than iron-catalysed mechanisms. Experiments carried out to test this idea showed that administration of the cyclooxygenase inhibitor indomethacin indeed inhibited oxidative stress in the medulla but not in the cortex, and further studies in which DFX and indomethacin were administered concurrently effectively inhibited peroxidation in both cortex and medulla (Gower *et al.*, 1989a). In a separate study to evaluate a novel synthetic hexadentate iron chelator (CP130) in ischaemic rabbit kidneys and compare it with DFX, it was found that CP130 provided better protection against overt morphological damage but no significant protection in terms of post-transplant function (Gower *et al.*, 1993).

Although these data provided indirect evidence of iron involvement in ischaemia–reperfusion injury in kidneys and the combined administration of DFX and indomethacin had proved beneficial in actual survival experiments (Gower *et al.*, 1989a), we still felt frustrated by our inability to generate more direct evidence. At that time, information was just emerging that a small pool of intracellular iron was available in catalytic form as chelates

with low molecular weight (LMW) species such as ATP, citrate and glycine (Bakkeren *et al.*, 1985; Mulligan *et al.*, 1986). These LMW chelates are thought to be important physiological vehicles for iron in transit from extracellular transferrin or haemoglobin to intracellular ferritin where the iron is stored "safely" as ferric hydroxides (Aisen and Listowsky, 1980). We hypothesized that a low pH during ischaemia would not only release "free" iron from ferritin but increase the amount of catalytic iron available in these LMW chelates. Hence, an assay of "chelatable iron" might provide more direct evidence in subsequent experiments. We therefore set about developing an accurate HPLC assay in which a ratio was measured between DFX-bound iron and its parent compound ferrioxamine (FX). A ratio of the area of FX peak to area of DFX peak was found to provide an accurate determination of DFX-available (DFX-A) iron levels in homogenates of kidneys after ischaemia and reperfusion (Gower *et al.*, 1989b). Subsequent experiments showed that measurable levels of DFX-A iron were present in both cortex and medulla of fresh control rabbits prior to ischaemia. These levels increased two-fold in both regions after 1 h of normothermic ischaemia *in situ* or 24 h of hypothermic storage in HCA. These levels rose still further in kidneys stored in HCA for up to 72 h. The differences between groups stored for 24 h and longer periods were much more marked after reperfusion (Gower *et al.*, 1989c). After 24 h of cold ischaemia, the levels of DFX-A iron decreased immediately upon reperfusion and returned to control levels within 5 min. In contrast, following 48 h of storage, DFX-A iron levels remained elevated in both cortex and medulla during the first 5 min of reperfusion and only returned to control levels after 30 min. Analysis of *total* iron content in these kidneys by atomic absorption spectroscopy revealed that these levels remained constant, suggesting that ischaemia leads to redistribution of intracellular iron to forms more easily chelated by DFX (Healing *et al.*, 1990). Whether or not more DFX-A iron means that more iron is available for catalysing lipid peroxidation can only be speculative but it must be a possibility. Certainly our data showing that DFX was more effective in cortex after ischaemia (Gower *et al.*, 1989a) and data here indicating that increased DFX-A were more marked in cortex during ischaemia suggests a correlation.

The release of iron from intracellular ferritin stores is thought to involve the reduction of Fe^{3+} to Fe^{2+} (Funk *et al.*, 1985) and one would expect this reduction to be facilitated by the low oxygen tension, increased levels of reducing species and the low pH shown by nuclear magnetic resonance (NMR) to be as low as 6.9 after only 6 h of cold storage (Fuller *et al.*, 1988). Exogenous redox-active quinones such as adriamycin have been shown to catalyse lipid peroxidation in the presence of ferritin under hypoxic conditions (Vile and Winterbourne, 1988), and lipid peroxidation is stimulated in microsomes in the presence of purified ferritin and flavin mononucleotides during aerobic incubation following a period of hypoxia (Goddard *et al.*, 1986).

The increased release of iron into the DFX-A pool on reperfusion of our rabbit kidneys after longer periods of cold storage may be the result of iron release from ferritin by reducing agents accompanying incoming oxygen. Superoxide anions produced during reperfusion have been shown in studies elsewhere to release iron from ferritin *in vitro* (Biemond *et al.*, 1988; Monteiro and Winterbourne, 1988). Perhaps, too, the persistent high levels of DFX-A iron during reperfusion could be due to impairment of uptake of intracellular iron species into "safe" sites.

We now believe that redistribution of iron to DFX-accessible forms occurs as an early event during ischaemia itself. These LMW iron complexes are then readily accessible to catalayse ODFR generation on reperfusion with blood and molecular oxygen.

7. *Loss of Calcium Homeostasis*

The importance of calcium homeostasis is well established in maintaining normal renal function (Evered and Whelan, 1986). Calcium ions play a role in regulation of cell metabolism through activation of many enzymes and maintenance of normal blood flow through controlled production of eicosanoids. In normal physiological conditions, energy-dependent pumps remove calcium from cells and sequester excess cytosolic calcium into intracellular organelles and calcium can only enter cells through specific voltage or receptor-operated channels. The gradient between low (10^{-7} M) cytosolic levels and high extracellular concentrations (10^{-3} M) is thought to be controlled by receptor-mediated turnover of phosphatidylinositols involving a GTP-binding protein and a specific phospholipase C (Berridge, 1984).

It is not surprising therefore, since calcium pumps are energy dependent, that marked rises in cytosolic calcium are a consistent feature of kidneys subjected to ischaemia and reperfusion (Naylor *et al.*, 1988), and that function may be irreversibly damaged if these rises continue apace (Farber, 1981). Studies using ^{31}P-NMR spectroscopy carried out by our group (Fuller *et al.*, 1988) have revealed that ATP and ADP peaks virtually disappear after 4 h of hypothermic ischaemia even when the kidneys have been flushed with the most effective preservation solutions available and this degradation of nucleotides is accompanied by rises in cytosolic calcium (Fuller *et al.*, 1988). However, whether altered intracellular calcium homeostasis is important in initiating ischaemia–reperfusion injury or is involved during a pathological cascade, or whether the measured rises in cytosolic calcium are merely the result of many other events has never been adequately resolved even though many workers have claimed that it is intimately involved in the aetiology of this syndrome (Cheung *et al.*, 1986; Opie, 1989).

We have carried out a series of experiments in the rabbit renal autograft model to address these questions. Prior to periods of cold ischaemia, kidneys were flushed with storage solutions containing different agents, which either affect calcium fluxes or interfere with calcium-dependent enzymes (Cotterill *et al.*, 1989a). Oxidative damage was assessed by indices of lipid peroxidation in *in vitro* incubated homogenates of cortex and medulla. The results were interesting. Blockage of voltage-operated calcium channels by including verapamil reduced the extent of oxidative damage to low levels in both cortex and medulla of kidneys flushed with isotonic saline and stored for 24 h, but to our surprise had no apparent effect after storage for 72 h following flush with the HCA solution. Deliberate elevation of extracellular calcium levels by addition of $CaCl_2$ to the storage solution increased oxidative damage *only* when added to the isotonic saline flush solution and made no difference when added to HCA. From this evidence, it was concluded that influx of extracellular calcium through voltage-operated channels was a significant mediator of oxidative damage to organs stored in isotonic saline. We hypothesized that the apparent paradox when kidneys were stored in HCA, even though for far longer and presumably more damaging periods, might be accounted for by the excess of citrate (55 mM) in HCA; it seemed likely that the citrate would chelate excess calcium and the carefully balanced ion concentrations in HCA may have protected voltage-operated channels during the ischaemic period. Indeed, it is now believed that one main reason why HCA is so effective a preservation solution in clinical transplantation is that it is so effective in chelating extracellular calcium (Cotterill *et al.*, 1989b).

Further studies were then designed to investigate the potentially damaging role of redistribution of intracellular calcium during ischaemia. When A23187, an ionophore which permeabilizes both plasma and intracellular membranes to calcium, was added to the flush solution, post-ischaemic rates of peroxidation were significantly raised above the high peroxidation levels already produced by cold ischaemia alone both in saline and HCA-stored kidneys (Cotterill *et al.*, 1989a). Addition of ruthenium red, a polysaccharide dye that inhibits mitochrondrial calcium uptake, also potentiated oxidative damage to stored kidneys, regardless of which solution was used for the initial pre-ischaemic flush. Taken together, these results suggested that, even in the absence of extracellular calcium, redistribution of the metal takes place within cells during ischaemia and contributes in some way to increased peroxidation of cellular lipids on reoxygenation.

Increased levels of cytosolic calcium could potentiate ischaemia–reperfusion injury in several ways. For example, conversion of xanthine dehydrogenase to xanthine oxidase may be catalysed by a calcium-dependent protease (McCord, 1985). However, because it has been so difficult to demonstrate the presence of xanthine dehydrogenase at all in some tissues where lipid peroxidation is taking place, the importance of this conversion is not very convincing. We therefore set up experiments to address this question using allopurinol as a potent xanthine oxidase inhibitor. In one set of experiments, the agent effectively inhibited lipid peroxidation of rabbit kidneys subjected to a lethal period (120 min) of warm ischaemia (Cotterill *et al.*, 1989b). Similarly, addition of allopurinol to the isotonic saline flush solution prevented the increase in lipid peroxidation markers following storage in the presence of the calcium ionophore A23187 (Cotterill *et al.*, 1989b). It is just possible that allopurinol could have conferred benefit during reperfusion by preventing irreversible loss of purine nucleotides through inhibition of nucleotide dephosphorylating enzymes but its ability to inhibit xanthine oxidase activity is more likely (Cotterill *et al.*, 1989b). Another possible cause of damage through calcium is that calcium overload of mitochondria causes damage during reperfusion (Arnold *et al.*, 1985), thence to increased leakage of single electrons from the electron transport chain on to O_2, thus increasing O_2^- production. A further link between raised intracellular calcium concentration and increased oxidative stress is the involvement of calcium-dependent phospholipases that hydrolyse membrane phospholipids from which free fatty acids (FFA) are released and lysophosphatide residues are left behind in the membrane. In experiments to test this possibility, we found that addition of dibucaine, a specific inhibitor of phospholipase A_2, to the storage solution significantly protected against oxidative stress following different periods of cold ischaemia (Cotterill *et al.*, 1989b).

More evidence for phospholipase activation in rabbit kidneys during cold storage was obtained by analysing FFAs in freeze–clamped renal tissue by gas liquid chromatography (Cotterill *et al.*, 1989c). Levels of unsaturated FFAs ($C_{18:1}$, $C_{18:2}$, $C_{20:4}$) rose significantly in kidneys stored for 72 h after flush with HCA whereas the levels of saturated FFAs ($C_{16:0}$, $C_{18:0}$) did not change. The elevated levels of free arachidonic acid ($C_{20:4}$) after 72 h of cold ischaemia may be particularly important as release of this FFA from membranes is the rate-limiting step in the formation of prostaglandins (Isakson *et al.*, 1978) and any imbalance in production of these vasoactive eicosanoids might well lead to the "no reflow" phenomenon (Schlondorff and Ardaillon, 1986). Others have shown that after ischaemia there is a decrease in levels of prostacyclin and elevation of thromboxanes with overall imbalance in function toward vasoconstriction and platelet aggregation (Lelcuk *et al.*, 1985; Schmitz *et al.*, 1985). The release of free arachidonic acid during cold ischaemia would tie in with our earlier data, which revealed an increased rate of indomethacin-inhibitable peroxidation via the cyclooxygenase pathway observed in the medulla of stored rabbit kidneys (Gower *et al.*, 1989a). Increased phospholipase activity may result in the formation of lipoxygenase products such as

leukotrienes, which are powerful mediators of vascular constriction and have been implicated in vascular injury (Lefer, 1985).

In a study to assess this possibility, we conducted a series of experiments in the rabbit renal autograft model using a novel 5-lipoxygenase inhibitor (BWB70C). This agent had already been shown (Salmon and Garland, 1991) *in vitro* to be a free-radical scavenger, iron chelator and a specific inhibitor of 5-lipoxygenase (binding to the prosthetic iron of that enzyme and quenching the peroxy radical). In particular, synthesis of leukotriene B_4 (LTB_4) is inhibited with an IC_{50} of 1.0 μM (Salmon and Garland, 1991). This could have important beneficial consequences therapeutically, since LTB_4 is a powerful chemotactic agent and increases vascular permeability (Lefer, 1985) so the agent could be expected to protect kidneys from inflammatory damage and ODFR attack upon reperfusion. It was found that treatment with BWB70C indeed resulted in significant inhibition of LTB_4 synthesis but this did not appear to influence vascular permeability in this model nor did it improve post-transplant function or morphological appearance at postmortem (unpublished data).

Storage of kidneys in the presence of dibucaine or A23187 revealed the existence of a good correlation between the extent of FFA accumulation and the rate of post-ischaemic lipid peroxidation. Unfortunately, the experiments described above are too crude to tell us anything about the spatial and temporal sequence of events involving calcium, phospholipase activity, free-radical generation and lipid peroxidation in ischaemia–reperfusion injury. Nevertheless, we have postulated a possible sequence of events derived from the data of our own and others' experiments. Early redistribution of calcium concentrations due to rapid depletion of energy stores leads to the activation of phospholipases. The released unsaturated FFAs unprotected by endogenous, membrane-bound antioxidants such as vitamin E then provide an easy target for free-radical attack upon reoxygenation. The resulting peroxy radicals can then initiate peroxidation of membranes directly or they may break down to relatively stable hydroperoxides that can diffuse to other sites inside cells and stimulate lipid peroxidation through interaction with catalytic iron complexes, which generate reactive lipid radicals (Halliwell and Gutteridge, 1984). Meanwhile the build up of residual lysophosphatides left behind in membranes when unsaturated FFAs escape the normal phospholipid bilayer configuration, alters membrane fluidity and permeability (Weltzem, 1979) and renders them even more likely to free-radical attack (Ungemach, 1985). Peroxidation of membrane lipids resulting in further loss of FFAs from the membranes leads to increased lysophosphatide levels (Ungemach, 1985) accompanied by increased membrane rigidity (Demopoulos *et al.*, 1980). As phospholipase A_2 activity has been shown to increase in rigid membranes (Momchilova *et al.*, 1986), it is reasonable to assume that elevated levels of lipid peroxidation upon reoxygenation could lead on to further increase in phospholipase activity. This damaging cycle of events would then become self-generating. As membranes become extensively damaged they would become permeabilized to calcium so that cytosolic calcium concentrations rise still further. Oxidative damage has already been shown to inhibit plasma-membrane calcium-extruding systems (Nicotera *et al.*, 1985) and to destroy the ability of intracellular organelles to sequester calcium (Bellomo *et al.*, 1985). It is not difficult to imagine this sequence resulting in irreversible damage.

8. *Second Messengers*

Another possibility that we have investigated in our rabbit kidneys is that ischaemia followed by reoxygenation may affect the cleavage of membrane-bound phosphatidylinositols (PIP_2). This second messenger system involves the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes calcium from intracellular stores and DAG stimulates phosphorylation of protein kinase C (PKC), a process which requires phospholipids and calcium for maximum activity (Berridge, 1984). In kidneys, PIP_2 hydrolysis triggered by activation of α-adrenoreceptors evokes several responses including increased sodium reabsorption (Hesse and Johns, 1984), prostanoid production and vasoconstriction (Cooper and Malik, 1985), gluconeogenesis (Kessar and Saggerson, 1980) and inhibition of renin releases (Matsumura *et al.*, 1985).

In our experiments, cortical slices were incubated *in vitro* at 37°C either under an atmosphere of 95% O_2:5% CO_2 (as controls) or gassed with and incubated under N_2 to exclude O_2. After 120 min all slices were then oxygenated and incubated anaerobically for a further 30 min. The formation of lipid peroxidative markers increased during the first 60 min of incubation in the presence of O_2 but then formed a plateau for the remaining time investigated. Lipid peroxidation was also evident in the hypoxic slices but proceeded at a slower rate than in the oxygenated samples, and then also levelled off after 60 min. Reoxygenation following 120 min of hypoxia resulted in a significant ($P < 0.001$) increase in rate of lipid peroxidation, which was not observed in the slices gassed with N_2 or when slices incubated in the presence of O_2 for 120 min were regassed (Cotterill *et al.*, 1993). This *in vitro* system seemed therefore to mimic closely the increase in ODFR-mediated oxidative stress we had observed in earlier experiments with whole rabbit kidneys subjected to ischaemia–reperfusion (Gower *et al.*, 1989a).

In further experiments to determine the rate of PIP_2 hydrolysis in these rabbit kidney slices, radiolabel was

incorporated into the membrane-bound phosphatidylinositol pool by incubating the slices in the presence of myo-[2-^{3}H]inositol for 60 min at 37°C. These slices were then repeatedly washed and incubated under aerobic or hypoxic conditions for 120 min followed by oxygenation. Aliquots of slices were taken every 30 min and the hydrolysis products of PIP_2 [IP_3 and its derivative metabolites inositol biphosphate (IP_2) and inositol monophosphate (IP_1)] were analysed by high-performance liquid chromatography (HPLC) with scintillation counting (Irvine *et al.*, 1985). There was no change in the rate of PIP_2 breakdown in the hypoxic slices during the 120 min incubation period compared with control slices incubated under aerobic conditions whether in the presence of calcium or in calcium-free medium containing EGTA. However, immediately upon reoxygenation of hypoxic slices incubated in the presence of calcium, PIP_2 breakdown increased rapidly at highly significant levels ($P < 0.001$) and was maintained over the remaining 30 min of aerobic incubation. No increase in PIP_2 breakdown was observed when slices incubated in the presence of calcium and oxygen for 120 min were regassed with 95% O_2: 5% CO_2, nor did reoxygenation of hypoxic slices in calcium-free media containing EGTA alter the rate of PIP_2 breakdown. No significant changes were seen in slices incubated in the presence of O_2 and ethylene glycol bis (β-aminoethyl ether) *N,N,N,N'*-tetraacetic acid (EGTA) (Cotterill *et al.*, 1993).

These findings clearly demonstrated that hydrolysis of phosphatidylinositols to secondary messenger products is activated very rapidly upon reoxygenation of renal tissue after a period of hypoxia. Inhibition of this effect by EGTA strongly suggests that calcium is involved at some stage in this sequence of events. Changes in PIP_2 breakdown were not observed during hypoxia itself.

What might be the consequences of PIP_2 cleavage in ischaemia–reperfusion injury in kidneys? There are several possibilities. One of the products of lipid peroxidation, 4-hydroxynonenal, has been shown to stimulate adenylate cyclase, guanylate cyclase and PIP_2 breakdown *in vitro* (Dianzani *et al.*, 1989). It is possible that increased levels of aldehydic products of lipid peroxidation produced during reperfusion stimulate increased PIP_2 hydrolysis. The alterations in membrane configuration described earlier (Ungemach, 1985) may then affect the interaction of phospholipase C (PLC) with membrane-bound regulatory components or make it more accessible to PIP_2. Perhaps rapid changes in cytosolic calcium concentrations could also provoke an enhanced PLC response. Rapid hydrolysis of PIP_2 on reoxygenation following ischaemia might then result in deregulation of receptor-mediated function via this intracellular secondary messenger system and the resultant loss of balance in eicosanoid production would be expected to contribute to vascular dysfunction.

9. *Respiratory Chain Dysfunction*

The well-known fact that in irreversibly damaged cells, respiratory control is lost and is accompanied by oxidation of cytochromes a and a_3, as well as NADH (Taegtmeyer *et al.*, 1985), was originally thought to be due to substrate deficiency (Chance and Williams, 1955) but may be due to an enzymatic defect resulting in an inability to metabolize NADH-linked substrates (Pelican *et al.*, 1987). It seems likely therefore that return of function is dependent on preservation of mitochondrial membrane integrity, and the structure and activities of respiratory chain (RC) complexes I–IV (Chance and Williams, 1955).

In recent studies on perfused rats hearts (Veitch *et al.*, 1992), it was found that differences in the sensitivity of complexes I–IV to ischaemic damage were dependent upon the duration of ischaemia and the presence of oxygen. The demonstration that complex 1 is a major defective site dependent upon isolation of mitochondria from homogenates of the tissue by *in vitro* methods seemed important to us. We therefore decided to attempt to make noninvasive measurements of mitochondrial function soon after reperfusion in transplanted rabbit kidneys by surface fluorescence (for mitochondrial NADH levels) and near infra-red spectroscopy (NIRS) for the redox state of cytaa$_3$.

It was found that in autografted unstored kidneys, there was a significant ($P < 0.001$) change in the redox state of cytaa$_3$ in all kidneys on reperfusion and this correlated with increased NADH oxidation, good organ viability and minimal oedema on histological examination. In contrast, kidneys autografted after 72 h of cold storage showed no significant change in cytaa$_3$ compared to baseline levels and this correlated with poor long-term organ viability, slower NADH oxidation and severe cortical oedema.

Because we had noted severe congestion of the renal medulla with erythrocytes in all kidneys stored for 72 h or more, we suspected that much of the cortical ATN that ensued might be attributable to secondary ischaemia rather than direct oxidative injury. However, when we correlated high-resolution barium sulphate angiography for intra-renal distribution of blood flow with long-term viability, morphological damage and respiratory chain (RC) responses, the results in which the RC response was both slower and of lesser magnitude after prolonged ischaemia and transplantation suggested that poor perfusion was unlikely to account for mitochondrial dysfunction in the superficial cortex. We concluded that renal ischaemia–reperfusion injury can occur rapidly and directly with or without medullary congestion and secondary ischaemia (Thorniley *et al.*, 1994).

10. *Conclusions*

It is clear that no single biochemical event is responsible

for the deterioration of kidneys subjected to ischaemia and reperfusion. It is therefore difficult to design pharmacological strategies that might protect these organs. The investigations reviewed in this chapter suggest that oxidative damage following renal ischaemia can be significantly inhibited by many different agents, which indicates that a complex interaction of a number of factors is responsible for post-ischaemic damage. It is not possible to say at what stage or in what combination these become irreversible. Thus, upon reperfusion, ATP may be regenerated and provide energy for ionic pumps including those responsible for calcium, potassium, sodium and magnesium homeostasis. Similarly, delocalized intracellular iron appears to be rapidly sequestered upon reoxygenation after a short ischaemic period. However, these early events seem to be crucial in priming the kidney for subsequent reperfusion damage and the longer the period of ischaemia the more important they become.

Upon reperfusion, a burst of O_2^- production from incoming O_2 would react with increased levels of catalytic iron to yield more reactive radical species. Damage to cellular components would follow including peroxidation of lipids in membranes already compromised by increased calcium-dependent phospholipase activity. The resulting loss of integrity of the plasma membrane and intracellular organelles would cause further imbalances in intracellular ionic homeostasis. A self-perpetuating cycle could then become established until cellular structures were so perturbed as to become irreversibly damaged.

Disturbances in the vasculature also contribute to post-ischaemic organ failure. These can result from biochemical changes in the vessel wall and trapped ischaemic blood, and through interactions between these components and incoming fresh blood during reperfusion. Imbalances in eicosanoid production due to calcium-dependent accumulation of free arachidonic acid, production of inflammatory mediators such as leukotrienes, release of chemotactic substances with subsequent adhesion, and activation of polymorphonucleocytes and derangement of receptor-mediated functions such as the phosphatidyl inositol secondary messenger system may each play a part in the vascular bed.

Evidence for the involvement of a number of biochemical mechanisms during ischaemia–reperfusion injury is supported by the ability of many different pharmacological agents to afford at least some protection to kidneys subjected to ischaemic insult. These include iron chelators, free-radical scavengers, including superoxide dismutase and catalase, allopurinol, calcium antagonists and prostacyclin analogues. The very multiplicity of biochemical derangements makes it extremely unlikely that any one agent will be particularly effective. The most we can hope for, at least in the forseeable future, is that administration of agents systemically to the patient or infused through the organ will delay the onset of irreversible damage. It is most likely that a combination of agents will prove successful.

Maintenance of the kidneys in optimum condition during *in situ* or bench surgery and the need for longer periods of cold storage of kidneys for transplantation are both increasingly important in the surgical management of patients in renal failure. Hypothermic storage in special solutions with improved ionic composition has already yielded benefits. Further advances will be likely to involve a combined pharmacological strategy including infusing kidneys with solutions rendered hyperosmolar (310–420 mosmol/l) with relatively inert and impermeable molecules such as mannitol, raffinose, trehalose, or sucrose; solutions containing impermeant anions such as sulphate or phosphate and buffering systems, which are effective at low temperatures in static situations; solutions containing antioxidants (in the widest sense) such as DFX, superoxide dismutase, catalase, allopurinol or verapamil; and treating the patients with agents such as DFX, verapamil, indomethacin, prostacyclin, mannitol, dextrans, anti-platelet aggregating agents and vasodilating agents. It may prove necessary to give several agents together or it may be better to give them in a logical sequence. Retarding biochemical changes such as altered intracellular iron and calcium homeostasis, and preventing the loss of antioxidant protection are most likely to make a significant contribution in this field.

11. References

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7. A Vascular Basis for Free Radical Involvement in Inflammatory Joint Disease

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Immunopharmacology of Free Radical Species
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1. *Introduction*

Human chronic inflammatory diseases are characterized by populations of cells with altered regulation and function. A large body of evidence suggests that many of these cellular abnormalities may be linked to an increase in the production of free radicals and/or deficiencies of antioxidant defence systems. Oxygen free radicals attack cell structures, altering their function, and are cytotoxic. They have therefore been implicated in the pathogenesis of rheumatoid arthritis as well as many other human diseases (Halliwell, 1991).

Although atherosclerosis and rheumatoid arthritis (RA) are distinct disease states, both disorders are chronic inflammatory conditions and may have common mechanisms of disease perpetuation. At sites of inflammation, such as the arterial intima undergoing atherogenesis or the rheumatoid joint, oxygen radicals, in the presence of transition-metal ions, may initiate the peroxidation of low-density lipoprotein (LDL) to produce oxidatively modified LDL (ox-LDL). Ox-LDL has several pro-inflammatory properties and may contribute to the formation of arterial lesions (Steinberg *et al.*, 1989). Increased levels of lipid peroxidation products have been detected in inflammatory synovial fluid (Rowley *et al.*, 1984; Winyard *et al.*, 1987a; Merry *et al.*, 1991; Selley *et al.*, 1992; detailed below), but the potential pro-inflammatory role of ox-LDL in the rheumatoid joint has not been considered. We hypothesize that the oxidation of LDL within the inflamed rheumatoid joint plays a pro-inflammatory role just as ox-LDL has the identical capacity within the arterial intima in atherosclerosis.

This chapter addresses: (1) the mechanisms, antioxidant defences and consequences in relation to free-radical production in the inflamed rheumatoid joint; (2) lipid abnormalities in RA; (3) the potential contribution of ox-LDL to RA (the role of ox-LDL in coronary heart disease is discussed in Chapters 2 and 3 and will not be fully discussed here); and (4) the therapeutic aspects of chain-breaking antioxidant interventions in RA.

2. *Mechanisms, Antioxidant Defences and Consequences of Free-radical Production in the Rheumatoid Joint*

Molecular oxygen (O_2) is essential for aerobic life, yet may be toxic when supplied in excess concentrations. In biological systems, O_2 is a major source of injurious free radicals and other reactive oxygen species. They are generated continuously *in vivo* at chronically inflamed sites. It is a well-established view that the arthritic joint is a site of oxidative stress, where the overproduction of free radicals exceeds inherent antioxidant capacity, causing damage at the molecular, cellular and tissue levels (Halliwell, 1991). A broad range of oxidized biomolecules have been detected in extracellular fluids in patients with RA (Winyard *et al.*, 1992). These altered biomolecules are markers of free-radical production and some may play a crucial role in disease perpetuation. Phagocytosis, the formation of nitric oxide and cycles of hypoxic reperfusion injury are mechanisms that generate free radicals within the inflamed rheumatoid joint and are discussed below.

2.1 MECHANISMS OF FREE-RADICAL FORMATION IN THE RHEUMATOID JOINT

2.1.1 Phagocytosis

A significant increase in phagocytic cell populations within synovial fluid and tissue [polymorphonuclear neutrophils (PMNs) and macrophages, respectively] is characteristic of the inflamed arthritic joint. The function of these cells is the neutralization of potentially harmful pathogens. Phagocytic cells engulf foreign particles into a plasma membrane vesicle and produce reactive oxygen metabolites (ROMs), which are subsequently released into the phagocytic vacuole and are responsible for antigen destruction. Activated leucocytes are a substantial source of the superoxide radical ($O_2^{\cdot-}$), the hydroxyl radical ($^{\cdot}OH$) and hypochlorous acid (HOCl) when they undergo a respiratory burst in response to opsonized particles, aggregated immunoglobulin G (IgG), complement or interleukin-1 (IL-1) (Green *et al.*, 1979). The respiratory burst, the accelerated uptake of O_2 by phagocytic cells, occurs simultaneously with glucose consumption and is due to the activation of the membrane-associated enzyme complex, NADPH oxidase. NADPH is oxidized in the cytoplasm to form $NADP^+$ and, therefore, stimulated leucocytes increase their NADPH production via the pentose phosphate pathway. NADPH oxidase catalyses the reduction of O_2 to $O_2^{\cdot-}$, and the lethal reaction products of $O_2^{\cdot-}$ are partly responsible for the removal and destruction of bacteria (Babior and Woodman, 1990). It has been calculated that 38% of respiratory-burst

O_2 is consumed by NADPH and 90% of this is released extracellularly as $O_2^{\cdot -}$ and H_2O_2 (Huber, 1980).

Myeloperoxidase (MPO) is an enzyme derived from the azurophilic granules of PMNs and monocytes. Azurophilic granules fuse with the phagocytic vesicle and release MPO, which, in the presence of H_2O_2 and Cl^-, catalyses the formation of hypochlorous acid (HOCl). This ROM will oxidize several bacterial cell wall components to destroy the invading pathogen (Babior, 1978). The extracellular secretion of MPO during phagocytosis allows it to catalyse HOCl formation in the presence H_2O_2. The effect of extracellular HOCl secretion is variable: it inactivates α_1-antitrypsin, activates collagenase and gelatinase (Desrochers and Weiss, 1988), and combines with plasma albumin to become non-toxic (Halliwell and Gutteridge, 1990).

2.1.2 Formation of Nitric Oxide

The family of nitric oxide synthases remove the terminal guanidino nitrogen atom oxidatively from L-arginine to form nitric oxide (NO) and citrulline, where O_2 acts as an electron acceptor (Ignarro, 1990). Citrulline can be recycled enzymatically to arginine in some cell types to perpetuate NO synthesis. The free radical NO has been shown be an important factor in the regulation of vasomotor tone, initially by the demonstration of its role as the endothelium-derived relaxation factor (EDRF) and subsequently by the observation of constitutive NO-synthase enzymes in numerous cell types (e.g. endothelium, platelets, chondrocytes) and tissues (e.g. synovium, adrenal glands, brain; Moncada *et al.*, 1988). Vascular endothelial cell-derived NO causes relaxation of the surrounding smooth muscle cells to induce vasodilatation. Cell populations containing the constitutive forms of NO synthase rapidly and transiently produce small amounts of NO in response to an increase in intracellular Ca^{2+}.

NO is also generated from arginine by macrophages, PMNs and lymphocytes, indicating a possible role in inflammatory disorders (Stefanovic-Racic *et al.*, 1993). Leucocyte formation of NO occurs via a calcium-independent cytokine-inducible mechanism, the expression of which results in the delayed and prolonged release of NO in amounts exceeding those from the constitutive pathway. Formation of NO is essential for the cytotoxicity of activated macrophages against tumour cells and protozoa.

The early stages of bone pathology in rheumatoid disease manifest as periarticular osteoporosis and juxta-articular bone erosion. Osteoclast overactivity is the predominant influence in such bone erosion and NO has a direct inhibitory effect on osteoclastic bone resorption (MacIntyre *et al.*, 1991). Endothelial cells, present in abundance and in close proximity to the osteoclast may therefore play a role in down-regulating osteoclast activity through the production of NO. Since the osteoclast is of macrophage lineage, it is likely to be capable of producing NO, giving rise to the possibility of a feedback control loop for osteoclast activity.

NO spontaneously oxidizes to produce inactive nitrite and nitrate under aqueous aerobic conditions. The measurement of nitrite in serum and synovial fluid from patients with RA and osteoarthritis (OA) as well as a normal control group has been reported (Farrell *et al.*, 1992). Serum nitrite concentrations in RA and OA patients were significantly higher than controls. In both disease groups, synovial fluid nitrite was significantly higher than serum nitrite, suggesting NO synthesis in the synovium. Additionally, RA serum and synovial fluid nitrite levels were significantly elevated compared with OA, suggesting a role for NO as an inflammatory mediator in rheumatic diseases.

Chin *et al.* (1992) have suggested that oxidized LDL and high-density lipoprotein (HDL) inactivate endothelial cell-derived NO. NO inactivation was due to the oxidized lipids within the lipoprotein particles and was thought to be explained by a chemical reaction between the lipoproteins and NO. Other investigators have shown that relaxation of vascular smooth muscle by acetylcholine or bradykinin (endothelium-dependent vasodilators) is inhibited by LDL (Andrews *et al.*, 1987). The role of NO in the modification of LDL is discussed in full detail in Chapter 2.

2.1.3 Hypoxic–Reperfusion Injury

It has been proposed that PMNs and endothelial cells may initiate lipid peroxidation and other injurious sequelae by the production of ROM within the inflamed joint cavity during repeated cycles of hypoxic reperfusion-induced oxidative stress (Blake *et al.*, 1985, 1989; Henderson *et al.*, 1993). In 1970, Jason and Dixon demonstrated that the normal joint has a subatmospheric pressure at rest as well as during exercise (Jason and Dixon, 1970a, 1970b). Inflamed joints, however, were shown to have an elevated resting intra-articular pressure (1 atm), which increased above the local capillary perfusion pressure when exercised. The rheumatoid synovial cavity is both hypoxic and acidotic, and the oxygen tension is further lowered upon exercise. This is because, during exercise of the inflamed joint, the intra-articular pressure is transmitted directly to the synovial membrane vasculature, producing occlusion of the superficial synovial capillary bed and ischaemia. Subsequently, transient pO_2 levels in synovial fluid sharply decline (Unsworth *et al.*, 1988). Joint effusions with a low pO_2 are associated with high synovial lactate and low pH, suggesting that the inflamed synovium is under sufficient hypoxic stress to require anaerobic glycolysis (Richman *et al.*, 1981).

The mechanism for the production of $O_2^{\cdot -}$ in ischaemic tissue appears to involve changes in purine metabolism within ischaemic cells. Sublethal hypoxia decelerates mitochondrial oxidative phosphorylation, rendering the production of ATP dependent upon the

anaerobic and relatively inefficient mechanisms of glycolysis. This results in raised concentrations of adenosine metabolites, including xanthine and hypoxanthine, which are substrates for the xanthine dehydrogenase system. Xanthine dehydrogenase is a cytosolic metalloenzyme, which catalyses the oxidation of either hypoxanthine or xanthine to their respective metabolites, xanthine or uric acid, in the presence of O_2. Under physiological conditions, xanthine dehydrogenase utilizes NAD^+ as an electron acceptor in these reactions. However, in ischaemic conditions, the enzyme is converted from the dehydrogenase form to an oxidase form, where O_2 is utilized as the electron acceptor (Henderson *et al.*, 1993). On reperfusion, the formation of $O_2^{\cdot -}$ and uric acid results. One mechanism for enzyme conversion may involve the action of a Ca^{2+}-dependent protease activated during the ischaemia-induced influx of Ca^{2+} ions into the cell.

Deprived of their substrate in severe or prolonged hypoxia, some ATPase-driven systems, including ion pumps, may become impaired. Further, with the decrease in the availability of O_2 as its terminal electron acceptor, the mitochondrial transport chain becomes increasingly unable to accept reducing equivalents from cellular metabolic processes. Hence the intracellular pH falls, subjecting the cell as a whole to a reductive stress and favouring those enzyme systems with acid pH optima.

Reperfusion of the synovial membrane occurs when exercise is stopped and O_2 is subsequently reintroduced to the tissue. O_2 is a substrate required for xanthine oxidase activity and $O_2^{\cdot -}$ is generated. Therefore, repeated cycles of rest–exercise–rest in the inflamed joint may provide a continuous flux of destructive ROM.

Immunohistochemical studies carried out in our laboratories have demonstrated the presence of xanthine oxidase in synovial endothelial cells (Stevens *et al.*, 1991). As expected, the activity of this enzyme per unit weight of tissue is generally higher in synovia taken from RA patients due to their increased vascularity (Allen *et al.*, 1987). In addition, it has also been shown that rheumatoid synoviocytes contain increased levels of iron-saturated ferritin (Morris *et al.*, 1986). Xanthine oxidase (but not dehydrogenase) is able to mobilize iron from ferritin, supplying the necessary transition metal catalyst for the Haber–Weiss reaction and promoting $^{\cdot}OH$ formation (Biemond *et al.*, 1986).

Despite their short half-lives, it is possible to detect free radicals in biological tissues by the addition of non-radicals such as nitrones or nitroso compounds, which act as spin traps by forming relatively stable free radicals on reaction with the endogenous radical species. Utilizing the technique of electron spin resonance (e.s.r.) spectroscopy, we have demonstrated ROM generation by human rheumatoid synovium when subjected to cycles of hypoxia/normoxia *in vitro*. Using 3,5-dibromo-4-nitroso-benzenesulphonate (DBNBS) as a spin trap, a characteristic three-line signal was formed when synovial tissue explants were rendered transiently hypoxic and then reoxygenated. The three-line signal has been assigned to a one-electron peroxidatic oxidation of DBNBS (Nazhat *et al.*, 1990). This flux of radical species may have the effect of perpetuating synovial inflammation by initiating the peroxidation of LDL as well as other biomolecules, thereby accelerating joint damage.

2.2 ANTIOXIDANT DEFENCES AGAINST FREE-RADICAL DAMAGE IN THE HUMAN JOINT

The efficient removal of $O_2^{\cdot -}$ and H_2O_2 will diminish $^{\cdot}OH$ formation and therefore antioxidant defence systems have evolved to limit their accumulation. Enzymic and low molecular weight antioxidants exist to scavenge free radicals as self-protection mechanisms. Some proteins exhibit antioxidant properties because they chelate transition-metal catalysts. The significance of antioxidants in relation to inflammatory joint disease is discussed below.

2.2.1 Enzymic Antioxidants

2.2.1.1 Superoxide Dismutases

Superoxide dismutases (SODs) are a family of cytosolic metalloenzymes that specifically remove $O_2^{\cdot -}$ (reviewed by Omar *et al.*, 1992). SOD distribution within the body is ubiquitous, being found in erythrocytes as well as most organs and cell types. Three distinct mammalian SOD forms exist: CuZnSOD, MnSOD and extracellular SOD (EC-SOD). Their amino-acid sequences differ as well as the transition metals at their active sites. Rheumatoid synovial fluid contains low levels of SOD activity and hence little protection from ROM generated by infiltrating PMNs (Blake *et al.*, 1981). Furthermore, leucocytes from patients with RA are deficient in MnSOD, which might promote the extracellular leakage of $O_2^{\cdot -}$ (Pasquier *et al.*, 1984).

EC-SOD is a copper–zinc enzyme located on endothelial cell surfaces. It is believed that EC-SOD binds to the vasculature via specific glycosaminoglycans – probably heparan sulphate on the endothelium. The association of EC-SOD with endothelial cell surfaces may indicate a cell-specific protective role. Eighty per cent of SOD activity in control, noninflamed synovial fluid is due to EC-SOD and its concentration is decreased by 50% in RA fluid (Marklund *et al.*, 1986).

2.2.1.2 Catalase

Intracellular H_2O_2 is catalytically removed by catalase. The enzyme contains Fe(III) at its active site and is found in the cytosol of erythrocytes as well as the mitochondria and peroxisomes of most other cells. The concentration of catalase in rheumatoid synovial fluid is extremely low and may only be present as a result of erythrocyte lysis.

This decreased level of catalase does not offer sufficient protection against the extracellular generation of radicals within the inflamed joint (Blake *et al.*, 1981).

2.2.1.3 *Glutathione and Glutathione Peroxidase*

Selenium-containing glutathione peroxidase is essential for the reduction of H_2O_2 to H_2O and the removal of lipid hydroperoxides after cleavage from membranes. The substrate for glutathione peroxidase is reduced glutathione (GSH), a scavenger of $^{\cdot}OH$ and singlet oxygen. GSH peroxidase catalyses the oxidation of GSH in which two GSH molecules are oxidized at the expense of one H_2O_2 molecule. Subsequently, thiol groups of cysteine on each GSH molecule lose their hydrogen atoms to form a disulphide bond. Oxidized glutathione (GSSG) is reduced by glutathione reductase at the expense of NADPH. The *in vitro* exposure of mouse peritoneal macrophages to ox-LDL results in an initial depletion of GSH due to oxidative stress (Darley-Usmar *et al.*, 1991). GSH depleting agents, such as bromobenzene, promote lipid peroxidation and severe cellular damage (Comporti, 1987).

2.2.2 Low Molecular Weight Antioxidants

2.2.2.1 *α-Tocopherol*

The major lipid-soluble antioxidant primarily associated with lipid membranes is α-tocopherol (vitamin E). Circulating α-tocopherol is carried by chylomicrons, LDL and HDL and also has extracellular antioxidant capacities. As a chain-breaking antioxidant, it short circuits the propagation phase of lipid peroxidation because the peroxyl radical will react with α-tocopherol more rapidly than a polyunsaturated fatty acid (Burton and Traber, 1990). The resulting α-tocopheryl radical reacts with a second peroxyl radical to form an inactive, nonradical complex. *In vitro*, ascorbate regenerates the tocopheryl radical into its native non-radical form (Burton and Traber, 1990).

The importance of vitamin E for maintenance of lipid integrity *in vivo* is emphasized by the fact that it is the only major lipid-soluble chain-breaking antioxidant found within plasma, red cells and tissue cells. Esterbauer *et al.* (1991) have shown that the oxidation resistance of LDL increases proportionately with α-tocopherol concentration. In patients with RA, synovial fluid concentrations of α-tocopherol are significantly lower relative to paired serum samples (Fairburn *et al.*, 1992). The low level of vitamin E within the inflamed joint implies it is being consumed via its role in terminating lipid peroxidation and this will be discussed further in Section 3.3.

In contrast, Bowry and Stocker (1993) have recently proposed that α-tocopherol may act as a pro-oxidant within the LDL particle *in vitro*. Their studies have indicated that tocopherol-mediated LDL oxidation may take place when water-soluble alkyperoxyl radicals react with tocopheryl radicals in the absence of agents that regenerate the tocopheryl radical into a non-radical species (for example, ascorbate). Under these conditions, lipid peroxidation may be accelerated by an increase in the level of vitamin E within LDL. However, LDL oxidation via the α-tocopherol radical is inhibited by plasma and therefore is unlikely to take place *in vivo*.

2.2.2.2 *β-Carotene*

The orange colour of freshly isolated human plasma LDL is due to inherent carotenoids, the most abundant being β-carotene (Oncley *et al.*, 1950). Carotenoids enhance the immune response, inhibit mutagenesis, reduce nuclear damage and offer tissue protection against neoplastic events (Bendich and Olson, 1989). β-Carotene is classified as a protective antioxidant because it is extremely efficient at quenching singlet O_2. Additionally, under conditions of low pO_2, β-carotene functions as a radical-trapping antioxidant (Burton *et al.*, 1985) and inhibits lipid peroxidation (Jialal *et al.*, 1991). The β-carotene content of LDL isolated from rheumatoid synovial fluid is low when compared to matched plasma LDL, as determined by visible absorption spectroscopy (M.L. Kus, unpublished observations). This may be a result of an O_2-scavenging mechanism active under repeated cycles of hypoxic–reperfusion injury where synovial fluid pO_2 levels are low. The role of β-carotene as an antioxidant in LDL will be discussed in Section 3.3.

2.2.2.3 *Ascorbate*

Ascorbate has multiple antioxidant capacities and may be the most important water-soluble defence against free-radical damage in human plasma. At millimolar concentrations, ascorbate scavenges $O_2^{\cdot -}$, $^{\cdot}OH$ and HOCl (Blake *et al.*, 1983). The latter reaction protects plasma lipids against degradation by activated PMNs.

It has long been recognized that ascorbate levels are low in patients with RA (Lunec and Blake, 1985) and ascorbate is predominantly found in the dehydro form. The presence of increased dehydroascorbate has been suggested to indicate its rapid oxidation by stimulated PMNs (Halliwell and Gutteridge, 1990). When ascorbate concentrations are lower than about 20 μmol/l, as can occur in rheumatoid synovial fluid, the Fe(III) reducing effects of ascorbate outweigh its radical-scavenging effects. Ascorbate then causes increased $^{\cdot}OH$ formation and promotes lipid peroxidation (Blake *et al.*, 1981).

There is evidence from a number of *in vitro* studies that the vitamin E peroxyl radical formed during fatty-acid degradation may be converted to vitamin E plus non-radical through the actions of vitamin C (Burton *et al.*, 1985). RA patients have reduced serum ascorbate levels (Situnayake *et al.*, 1991) and potentially a reduced capacity for the regeneration of vitamin E. *In vitro* studies suggest that vitamin E becomes a pro-oxidant when ascorbate levels are low (Bowry and Stocker, 1993).

2.2.2.4 *Urate*

The metabolism of adenine and purine-based

compounds via xanthine oxidase yields urate, a chelator of copper and iron (reviewed by Becker, 1993). Urate scavenges peroxy and alkoxy radicals, singlet oxygen, HOCl and protects DNA from radical damage. Uric acid appears to be essential in preventing the oxidation of ascorbate in human blood by forming stable complexes with transition metal ions (Davies *et al.*, 1986). Production of urate by the endothelium preserves the capacity of these cells to mediate vasomotor tone. Urate prevents the oxidative inactivation of endothelial cell-derived cyclooxygenase and stimulates the production of prostacyclin (a vasodilator) and thromboxane (a vasoconstrictor). This implies a relationship between the site of urate formation and the requirement for a powerful radical scavenger. Scavenging of $^{\cdot}OH$ by uric acid results in its oxidation to allantoin, and increased levels of allantoin have been detected in serum and synovial fluid of rheumatoid patients (Grootveld and Halliwell, 1987). *In vitro*, urate inhibits copper-induced LDL oxidation in a dose-dependent manner (Esterbauer *et al.*, 1990).

2.2.3 Transition-metal Chelators

2.2.3.1 Transferrin and Lactoferrin

Proteins that bind transition-metal catalysts (for example, copper and iron ions) are critical in preventing adverse free-radical reactions in extracellular fluids (reviewed in Halliwell and Gutteridge, 1990). Transferrin is an 80 kD glycoprotein, with two Fe(III) binding sites for transport and delivery of iron to cells. The binding constant, K, for the binding of iron ions to transferrin is high (10^{20} at physiological pH) and only 20–30% of these sites are loaded. This effectively keeps the concentration of free circulating iron at nil. Iron ions bound to transferrin will not promote $^{\cdot}OH$ formation or lipid peroxidation as the iron is only released under acidic conditions (Halliwell and Gutteridge, 1985). It has been shown that the intravenous infusion of iron dextran into rheumatoid patients results in saturation of transferrin iron-binding capacity and the appearance of catalytic iron. These changes closely correspond to exacerbations in rheumatoid synovitis (Winyard *et al.*, 1987a).

Lactoferrin resembles transferrin in terms of molecular weight, amino-acid sequence homology and number of Fe(III) binding sites. Lactoferrin is released from activated PMNs upon degranulation and may play a role in myelopoiesis, primary antibody response, lymphocyte proliferation, cytokine production and complement activation.

2.2.3.2 Caeruloplasmin

Caeruloplasmin (Cp) is an acute phase glycoprotein with a copper transport function. At least 90% of total plasma copper is bound to Cp with the remaining 10% associated with albumin, histidine and small peptides. Lipid peroxidation requires the presence of trace amounts of transition metals and the copper-containing active site of Cp endows it with antioxidant capacity (further discussed in Section 4.1.2). Cp is a powerful inhibitor of iron and copper-dependent O_2 free-radical reactions (Winyard and Blake, 1989). The ferroxidase activity of Cp by which Fe(II) is oxidized to Fe(III), inhibits Fe(II) catalysed reactions such as lipid peroxidation and the Fenton reaction (Winyard *et al.*, 1984). Additionally, the copper-binding activity of Cp effectively removes "free copper" to prevent lipid peroxidation.

Several groups have suggested that the pool of non-Cp-bound ("free") copper, capable of catalysing oxidative reactions, is elevated in rheumatoid extracellular fluids (Niedermeier, 1965; Gutteridge, 1986). Some of the earlier reports of relatively high concentrations of "free" copper in rheumatoid synovial fluids may be attributable to a storage-induced artefact, since Cp may readily lose its copper during storage-induced oxidation/proteolysis (Winyard *et al.*, 1989). Gutteridge (1986) used the "phenanthroline assay" to detect successfully a species of copper capable of catalysing free-radical reactions in stored rheumatoid synovial fluid. However, "phenanthroline" copper was not detected in fresh synovial fluid (Winyard *et al.*, 1987b). The latter result might mean that "free" copper is not present in fresh synovial fluid. On the other hand, it is also conceivable that a substance(s) present in fresh synovial fluid interferes in the "phenanthroline assay", thereby preventing the detection of "phenanthroline" copper.

2.3 SOME BIOLOGICAL CONSEQUENCES OF ROM IN THE RHEUMATOID JOINT

The complex cascades that comprise the inflammatory reaction are designed primarily to limit tissue damage and prevent or inhibit infection. ROMs play a critical role in both these beneficial processes. However, high level fluxes of toxic free radicals are capable of causing damage to diverse biomolecules, including lipids, proteins, DNA and carbohydrates (discussed below).

In addition, it is becoming increasingly apparent that low-level fluxes of ROM may have cell-signalling roles. The action of NO has been described above, whilst the ROM-mediated control of the transcription nuclear factor (NF-κB) is detailed below.

2.3.1 Lipid Peroxidation and the Oxidative Modification of LDL

Lipid peroxidation is a radical-mediated chain reaction resulting in the degradation of polyunsaturated fatty acids (PUFAs) that contain more than two covalent carbon–carbon double bonds (reviewed by Esterbauer *et al.*, 1992). One of the major carriers of plasma lipids is LDL, a spherical molecule with a molecular weight of 2.5×10^6. A single LDL particle contains 1300 PUFA molecules (2700 total fatty-acid molecules) and is

therefore an immediate target of radical (most likely $^{\cdot}OH$) attack. Of course, the fatty-acid constituents of cell membranes also undergo peroxidation reactions.

Three distinct chronological phases characterize the process of LDL oxidation *in vitro*: lag, propagation and termination. During the lag phase, endogenous chain-breaking antioxidants that protect PUFAs from oxidation are consumed as a result of scavenging free-radical initiators. After complete antioxidant depletion, lipid peroxidation enters the propagation phase, where PUFAs are oxidized to form lipid hydroperoxides. Subsequently, these lipid hydroperoxides attack neighbouring PUFAs (reactions intercepted by chain-breaking antioxidants) and the cycle is repeated until total fatty-acid deterioration results. The final, termination, phase occurs when the PUFA substrate is used up. The lag, propagation and termination phases of lipid peroxidation can be followed spectrophotometrically because conjugated dienes (peroxidized fatty acids) strongly absorb at 234 nm (Esterbauer *et al.*, 1990).

Cytotoxic aldehydes form as a consequence of lipid hydroperoxide degradation. *In vitro*, malondialdehyde (MDA) is produced in greatest concentration followed by 4-hydroxynonenal, although experimental conditions influence the proportions of the different aldehydes that are produced (Esterbauer *et al.*, 1990). MDA and 4-hydroxynonenal are hydrophilic and are released from LDL into the aqueous surroundings. *In vitro* studies have shown that incubation of LDL with MDA renders the LDL particle immunogenic (Esterbauer *et al.*, 1992). Hydroxynonenal is biologically active and can cause severe cell dysfunction both biochemically and genetically. Hydroxynonenal is chemotactic for PMNs at picomolar concentrations, inhibits cell proliferation and is mutagenic (Esterbauer *et al.*, 1988).

In contrast to MDA and hydroxynonenal, other aldehyde products of lipid peroxidation are hydrophobic and remain closely associated with LDL to accumulate to millimolar concentrations. Aldehydes at these elevated levels react with the protein portion of the LDL molecule, apolipoprotein B (apoB). Accumulated aldehydes bind the free amino groups from lysine residues in addition to other functional groups (–OH, –SH) on the apoB polypeptide. Consequently, the protein takes on a net negative charge and complete structural rearrangement results in the formation of ox-LDL. ox-LDL is no longer recognized by the LDL receptor, and has several pro-inflammatory properties (discussed below).

Evidence that oxidized lipids play a role in the pathogenesis of RA comes from studies demonstrating the presence of lipid products arising from radical attack in rheumatoid synovial fluid. This is consistent with oxidation reactions occurring locally in the joint. Lipid peroxidation products that react with thiobarbituric acid (TBARs) have been detected in rheumatoid knee-joint synovial fluid (Rowley *et al.*, 1984). In addition, the concentrations of TBARs and iron salts capable of driving the Fenton reaction correlated with disease activity. 4-Hydroxynonenal has been identified by mass spectrometry in rheumatoid serum and synovial fluid, and levels appeared to correlate with disease status (Selley *et al.*, 1992).

One technique to assess lipid peroxidation utilizes second-derivative spectrophotometric analysis of cyclohexane or ethanol-reconstituted extracts to determine the *cis–trans* and *trans–trans* isomeric forms of conjugated dienes and oxodiene species within synovial fluid. Using this method, a rise in the synovial fluid concentration of conjugated oxodienes, hydroxydienes and hydroperoxydienes was found to follow joint exercise (Merry *et al.*, 1991).

Single-pulse proton nuclear magnetic resonance (NMR) spectroscopy has been used to demonstrate that the mean chain length of synovial fluid triglycerides is shorter than that in corresponding sera (Naughton *et al.*, 1993). Further resonances attributable to the CH_3 groups of ketone bodies, predominantly 3-D-hydroxybutyrate, were also found in all synovial fluid samples studied. These data indicate an elevated utilization of fats for energy, despite the overall hypoxic environment of the synovial tissue (Merry *et al.*, 1991; Henderson *et al.*, 1993).

2.3.2 IgG Denaturation

Rheumatoid factor (RF) is an antibody, usually IgM, directed against "self" IgG, suggesting it has acquired antigenicity by some alteration. A majority of patients with RA are seropositive for RF. IgG exposure to free-radical generating systems *in vitro* results in aggregation and denaturation of the protein (Lunec *et al.*, 1985). The constituent IgG amino acids cysteine, tryptophan, tyrosine and lysine are particularly susceptible to free-radical attack and their oxidation results in autofluorescent (excitation 360 nm and emission 454 nm), monomeric and polymeric IgG forms. Fluorescent IgG will react with RF and these antigen–antibody complexes stimulate $O_2^{\cdot-}$ release from PMNs. Furthermore, ROM-altered IgG exhibits an increased ability to bind the first component of the complement cascade (Swaak *et al.*, 1989). Studies performed on rheumatoid serum and synovial fluid suggest that identical immune complexes can form *in vivo* to enhance and perpetuate PMN-mediated free-radical production within the inflamed joint (Henderson *et al.*, 1993). Furthermore, levels of autofluorescent IgG and albumin increase in rheumatoid synovial fluid following an exercise cycle (Blake *et al.*, 1989). Whether ROM damage to IgG triggers rheumatoid factor (RF) production has not yet been established.

2.3.3 Inactivation of α_1-Antitrypsin

Activated PMNs exert many pro-inflammatory effects by extracellular secretion of elastase, which alters vascular

permeability and mediates cytotoxicity. Elastase is a serine protease capable of digesting a broad range of connective tissue components including elastin, collagen, fibronectin and proteoglycans. *In vivo*, PMN elastase activity is regulated by α_1-antitrypsin, an acute-phase protein and serine protease inhibitor. Elastase inhibition is caused by the rapid formation of a stable 1 : 1 complex with α_1-antitrypsin.

In inflammatory conditions, activated PMNs may proteolytically (by release of lysosomal enzymes) and oxidatively (by release of HOCl) inactivate α_1-antitrypsin. Studies of synovial fluid samples from patients with RA showed that α_1-antitrypsin was both cleaved and oxidized, resulting in inactivation (Chidwick *et al.*, 1991, 1994). Free-radical attack on α_1-antitrypsin and its subsequent inactivation may contribute to the destruction of joint tissues in arthritis due to the imbalance between elastase and its inhibitors.

Activated PMNs also secrete collagenase, which is a metalloproteinase. *In vitro* studies suggest that a contribution to the α_1-antitrypsin inactivation observed in biological fluids might be made by collagenase (Desrochers and Weiss, 1988). The activation of collagenase is dependent on MPO-catalysed HOCl generation (Desrochers and Weiss, 1988). In addition, connective tissue metalloproteinases, such as stromelysin and endothelial cell collagenase, are capable of catalysing the proteolytic cleavage of α_1-antitrypsin (Winyard *et al.*, 1991; Zhang *et al.*, 1994). These enzymes cleave at the exposed loop of α_1-antitrypsin, which contains the reactive centre. Fragments of α_1-antitrypsin of molecular weights consistent with such cleavage have been detected by Western blotting in rheumatoid synovial fluid (Zhang *et al.*, 1990).

Although the role of ROM in the activation of metalloproteinases is unclear, it is possible that hypoxic–reperfusion-induced production of ROM could lead to increased metalloproteinase activity, resulting in the inactivation of α_1-antitrypsin. Thus, α_1-antitrypsin inactivation might be either the direct or indirect result of ROM formation. To test this hypothesis, we recently studied the molecular form (by Western blotting) and the elastase inhibitory activity of α_1-antitrypsin in synovial fluid samples sequentially aspirated after patients had undertaken a basic walking exercise. Exercised patients, but not those rested, showed a significant though temporary decrease in the specific elastase inhibitory activity of α_1-antitrypsin. Western blotting did not suggest α_1-antitrypsin cleavage, implying that the inactivation was not proteolytic during the exercise period (Zhang *et al.*, 1993).

2.3.4 DNA Damage

The hydroxylation of single- and double-stranded DNA are changes characteristic of free-radical attack (reviewed by Winyard *et al.*, 1992). An important mechanism is site-specific $^{\cdot}OH$ generation, catalysed by iron bound to cellular DNA. The hydroxyl radical will react with the DNA nucleoside deoxyguanosine to produce 8-hydroxydeoxyguanosine, which is mutagenic, a potential carcinogen and may initiate autoimmunity (Winyard *et al.*, 1992). Lymphocyte DNA from patients with autoimmune diseases contains significantly higher levels of 8-hydroxydeoxyguanosine in comparison with healthy adults (Bashir *et al.*, 1993; Harris *et al.*, 1994). Additionally, spontaneously increased unwinding of DNA (a measure of DNA strand breaks) has been found in the blood mononuclear cells from patients with RA when compared with healthy controls (Bhusate *et al.*, 1992).

2.3.5 Fragmentation of Hyaluronic Acid

In inflamed synovial fluid, the repeating disaccharide hyaluronic acid is fragmented with a corresponding reduction in its viscosity and increase in the concentration of dialysable hyaluronate saccharide monomers. Using high-resolution proton Hahn spin-echo suppressed NMR spectroscopy, several intermediates and end-products of oxidative attack on purified hyaluronic acid have been defined *in vitro* (Grootveld *et al.*, 1991). Identical products have been found to be elevated within the synovial fluid of rheumatoid patients. Again, following a simple exercise schedule, oligosaccharide fragments are rapidly generated within the synovial fluid and then cleared or further degraded (Henderson *et al.*, 1991).

2.3.6 NF-$\varkappa$B Activation

NF-$\varkappa$B, originally defined as the enhancer of kappa light-chain expression in B lymphocytes, is a heterodimeric protein that can rapidly activate several genes associated with the inflammatory process (reviewed by Schreck *et al.*, 1992). The DNA binding, nuclear form, of NF-$\varkappa$B is a heterodimer composed of one Rel-A (65 kD) and one p50 (50 kD) subunit. However, both subunits can form homodimers that also have DNA-binding activity. The inactive form of NF-$\varkappa$B in non-stimulated cells is localized to the cytoplasm of resting cells, and is bound to its inhibitor I$\varkappa$B.

Upon stimulation of cells by a wide range of agents, including H_2O_2, tumour necrosis factor-α (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), phorbol myristate acetate (PMA), viruses, lipopolysaccharide (LPS) and calcium ionophores, NF-$\varkappa$B dissociates from the NF-$\varkappa$B–I$\varkappa$B complex and the Rel-A nuclear location sequence (NLS) is unmasked. NF-$\varkappa$B migrates to the cell's nucleus, where it controls gene expression. The mechanism of NF-$\varkappa$B–I$\varkappa$B dissociation is unclear, but several antioxidants (including vitamin E and pyrrolidinedithiocarbamate) have been shown to inhibit NF-$\varkappa$B activation, implying free-radical involvement.

NF-$\varkappa$B activates genes encoding MHC class I antigens, IL-1, TNFα, IL-6, the interleukin-2 (IL-2) receptor, some acute-phase proteins and the adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1) (Iademarco

et al., 1992) and endothelial leucocyte adhesion molecule-1 (ELAM-1) (Montgomery *et al.*, 1991). *In vitro* studies demonstrate that TNFα and IL-1 can activate NF-κB and their excess production, induced by NF-κB activation, may perpetuate the cycle.

In collaboration with PA Baeuerle (Freiburg), active NF-κB has been detected immunohistochemically in rheumatoid synovium by using a polyclonal antibody directed against the Rel-A NLS subunit of NF-κB (M.L. Kus, unpublished observations). The antibody employed in these studies was considered to be "activity specific" because IκB sterically masks the NLS sequence. NF-κB activation by ROM in the rheumatoid joint may orchestrate some of the chronic inflammatory processes characteristic of this disease. It is plausible that IL-1 and TNFα generated in the inflamed synovium as well as the up-regulated expression of adhesion molecules may be under the influence of NF-κB.

NF-κB activation has been linked with atherosclerosis (Andalibi *et al.*, 1993; Liao *et al.*, 1993). Mice that were maintained on an atherogenic diet, which resulted in ox-LDL accumulation in the liver and arteries, showed NF-κB activation in hepatic tissues. Furthermore, inflammatory gene up-regulation corresponded to the concentration of accumulated lipid peroxides as well as genetic susceptibility to fatty-streak development.

3. *Lipid Abnormalities in Inflammatory Joint Disease*

Lipid abnormalities are associated with a wide spectrum of pathological conditions. In particular, recent advances have been made in understanding the role of lipoproteins in coronary heart disease (Davies and Woolf, 1993). However, included within the spectrum of diseases associated with perturbations of lipid metabolism are a number of musculoskeletal problems including inflammatory polyarthritis, tenosynovitis, osteoporosis and bone cysts.

3.1 HYPERCHOLESTEROLAEMIA AND INFLAMMATORY ARTHRITIS

Familial hypercholesterolaemia is characterized by a significant elevation in plasma LDL concentration. The basic metabolic defect appears to be abnormal LDL receptor function, arising from mutations in the LDL receptor gene. Several receptor mutations have been identified and hypercholesterolaemia severity as well as the age of onset of ischaemic heart disease has recently been demonstrated to vary according to the type of LDL receptor gene defect (Moorjani *et al.*, 1993).

Approximately half of the male heterozygote population for familial hypercholesterolaemia develop ischaemic heart disease by the age of 50 and the homozygote subgroup may develop ischaemic heart disease by the second or third decades of life. Approximately 40% of patients with familial hypercholesterolaemia manifest musculoskeletal complications, including inflammatory arthritis (Mathon *et al.*, 1985). Furthermore, in some cases of familial hypercholesterolaemia, articular symptoms disappear when serum cholesterol levels are reduced (McDonagh *et al.*, 1993).

The pathogenic mechanisms responsible for the association of musculoskeletal abnormalities with hypercholesterolaemia remain obscure. However, it has been hypothesized that the deposition and modification of lipoprotein components within and around the joint of patients with hyperlipidaemia-associated arthropathy results in a pro-inflammatory response, giving rise to articular disorders (Prete *et al.*, 1993).

3.2 LIPOPROTEIN (a) AND RHEUMATOID ARTHRITIS

Lipoprotein (a) [LP(a)] is distinguished from the related LDL particle by the addition of a large glycoprotein, apolipoprotein (a) (Scanu and Fless, 1990). This molecule is an independent atherogenic factor that accounts for much of the previous unattributable risk for cardiovascular disease. LP(a) has been detected in arterial lesions and experimental evidence confirms that elevated plasma concentrations constitute a major risk factor for atherosclerosis. LP(a) has recently been shown to bind to macrophages via a specific high-affinity receptor and promotes the transformation of macrophages into foam cells, particularly after it has been oxidized (Zioncheck *et al.*, 1991). In addition, LP(a) promotes smooth muscle cell proliferation (Grainger *et al.*, 1993).

The plasma concentration of LP(a) has been measured in patients with RA and significantly increased levels have been reported (Rantapaa-Dahlqvist *et al.*, 1991). Whilst the plasma concentration of cholesterol is lower in patients with RA than in normal controls the concentration of LP(a) is increased, whether or not the concentration is corrected for total lipids. Rantapaa-Dahlqvist *et al.* (1991) suggest that LP(a) may be an important cause of coronary heart disease in patients with RA.

3.3 DEPLETION OF LIPID-ASSOCIATED ANTIOXIDANTS IN THE RHEUMATOID JOINT

Depletion of the antioxidant capacity of LDL is an early event in the oxidation process. The main antioxidant in LDL is α-tocopherol, with smaller quantities of β-carotene and γ-tocopherol also present. The importance of antioxidants in inhibiting the oxidative modification of LDL is suggested by human and animal studies on the prevention of atherosclerosis. Preliminary reports

from long-term clinical trials indicate that dietary supplementation with vitamin E might decrease the risk of coronary heart disease in women (Stampfer *et al.*, 1993) and men (Rimm *et al.*, 1993).

Esterbauer *et al.* (1991) have demonstrated that β-carotene becomes an effective antioxidant after the depletion of vitamin E. Our studies of LDL isolated from matched rheumatoid serum and synovial fluid demonstrate a depletion of β-carotene (Section 2.2.2.2). Oncley *et al.* (1952) stated that the progressive changes in the absorption spectra of LDL were correlated with the auto-oxidation of constituent fatty acids, the auto-oxidation being the most likely cause of carotenoid degradation. The observation that β-carotene levels in synovial fluid LDL are lower than those of matched plasma LDL (Section 2.2.2) is interesting in that β-carotene functions as the most effective antioxidant under conditions of low pO_2 (Burton and Traber, 1990). As discussed above (Section 2.1.3), the rheumatoid joint is both hypoxic and acidotic. We have also found that the concentration of vitamin E is markedly diminished in synovial fluid from inflamed joints when compared to matched plasma samples (Fairburn *et al.*, 1992). This difference could not be accounted for by the lower concentrations of lipids and lipoproteins within synovial fluid. The low levels of both vitamin E and β-carotene in rheumatoid synovial fluid are consistent with the consumption of lipid-soluble antioxidants within the arthritic joint due to their role in terminating the process of lipid peroxidation (Fairburn *et al.*, 1992).

4. *Formation of ox-LDL in the Inflamed Human Joint*

It has been pointed out that some of the histopathological changes in rheumatoid synovitis are similar to those noted adjacent to atherosclerotic plaques. For example, accumulation of mononuclear cells at the extraluminal surface of endothelial cells in the adventitia and the deposition of immune complexes have been reported in both diseases (Rothschild and Masi, 1982).

It is unlikely that the damaging effects of ox-LDL are relevant only to the walls of blood vessels and there is no reason to suppose they are confined to one disease. The initial histopathological sign of coronary heart disease is the appearance of the fatty streak on the luminal surface of arteries. Fatty streaks are composed of aggregated macrophages that have taken up ox-LDL via the scavenger receptor. Recently, we have detected such foam cells in the rheumatoid synovium (Section 5.5).

4.1 POSSIBLE MECHANISMS OF LDL OXIDATION IN THE RA JOINT

The peroxidation of LDL occurs after depletion of the molecule's inherent antioxidants. Other characteristics of LDL incubated with cells and/or transition metal ions include increases in density, free cholesterol, lysolecithin content and negative charge. A number of mechanisms have been proposed for the modification of LDL. However, their relative importance *in vivo* is unclear. Human endothelial cells, PMNs, monocytes, macrophages, smooth muscle cells (Steinberg *et al.*, 1989) and lymphocytes (Lamb *et al.*, 1992) oxidatively modify LDL via their extracellular release of ROM. Additionally, LDL modification can be mediated by transition-metal ions in the absence of cells. The complex biological mechanisms in which cells/transition-metal catalysts interact with LDL to produce a modified form are obscure, although most investigators agree that modification must take place extravascularly due to the abundance of protective plasma antioxidants in the circulation. Proposed mechanisms of lipid peroxidation in relation to atherosclerosis are discussed in Chapters 2 and 3, and will not be repeated in detail here.

4.1.1 15-Lipoxygenase and Phospholipase A_2

Mechanisms of lipid peroxidation that have been implicated in atherosclerosis may be pertinent to RA. Cellular lipoxygenase enzymes may promote LDL modification by inserting hydroperoxide groups into unsaturated fatty-acid side chains of the LDL complex (Yla-Herttuala *et al.*, 1990). 15-Lipoxygenase has been implicated as an initiator of LDL oxidation (Cathcart *et al.*, 1991) whilst 5-lipoxygenase does not appear to be involved (Jessup *et al.*, 1991). Products of activated lipoxygenase enzymes within inflammatory synovial fluid suggest that this pathway could be activated in RA (Costello *et al.*, 1992).

During the oxidative modification of LDL, lecithin is converted to lysolecithin, catalysed by a phospholipase A_2 (PLA_2) activity thought to be an intrinsic component of the LDL molecule (Parthasarathy and Barnett, 1990). Inhibitors of PLA_2 block the formation of lysolecithin, the propagation of lipid peroxides and ox-LDL production. It has been argued that, during endothelial cell-induced LDL modification, PLA_2 releases lipid peroxides, thus favouring further free-radical-mediated lipid peroxidation (Parthasarathy *et al.*, 1985). Serum PLA_2 activity of patients with RA correlates with both clinical and laboratory measures of disease activity (Pruzanski *et al.*, 1988). It would be of interest to determine whether the elevated levels of PLA_2 activity detected in rheumatoid synovial fluid are associated with synovial fluid LDL.

4.1.2 Degradation of Caeruloplasmin

Copper salts such as $CuSO_4$ are potent catalysts of the oxidative modification of LDL *in vitro* (Esterbauer *et al.*, 1990), although more than 95% of the copper in human serum is bound to caeruloplasmin. Cp is an acute-phase protein and a potent inhibitor of lipid peroxidation, but is susceptible to both proteolytic and oxidative attack with the consequent release of catalytic copper ions capable of inducing lipid peroxidation (Winyard and

Blake, 1989; Winyard *et al.*, 1989). We suggest that within the inflamed rheumatoid joint (or the artery wall in atherogenesis), the production of ROM and proteases by endothelial cells and/or macrophages may cause the release of copper ions from Cp (see Section 2.2.3.2). It has been reported that Cp is cleaved faster in serum from patients with inflammatory diseases when compared to normal serum (Laurell, 1985). The oxidative modification of LDL by Cp-derived copper ions may explain the observation that increased serum cholesterol values are associated with accelerated atherosclerotic progression in men with high serum copper concentrations (Salonen *et al.*, 1991).

5. *Potential Pro-inflammatory Influences of ox-LDL in RA*

The LDL particle, which has been oxidatively modified by the mechanisms described above, is no longer recognized by the classic LDL receptor and is taken up by the macrophage scavenger receptor. Importantly, ox-LDL also exhibits a variety of pro-inflammatory activities, as described below.

5.1 CYTOKINE EXPRESSION

The interactions of vascular endothelial cells and monocytes are crucial in the progression of atherosclerosis, and several mechanisms incriminating ox-LDL have been proposed. The *in vitro* studies of Rajavashisth *et al.* (1990) have shown that minimally modified LDL (mmLDL; prepared by prolonged storage of LDL or mild iron ion oxidation, but still recognized by the LDL receptor) induces the expression of granulocyte–macrophage colony-stimulating factor (GM-CSF), monocyte/macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF) mRNA and protein in human aortic endothelial cells. These growth factors influence the differentiation, proliferation, migration and metabolism of macrophages/granulocytes. Further investigations demonstrated that mmLDL (Berliner *et al.*, 1990) and ox-LDL (Navab *et al.*, 1991) induced the expression of endothelial and smooth muscle cell monocyte chemotactic protein-1 (MCP-1) mRNA and protein. MCP-1 increases monocyte adhesion and migration and its up-regulation by ox-LDL is inhibited by β-carotene and α-tocopherol (Navab *et al.*, 1991). Finally, it has been reported that monocytes containing intracellular ox-LDL increase their expression of TNFα (Barath *et al.*, 1990) and IL-1β (Akeson *et al.*, 1991) *in vitro*.

Rheumatoid synovial cells produce a broad range of cytokines (Brennan *et al.*, 1991). The formation of IL-1α, IL-1β, GM-CSF, G-CSF, TNFα and other cytokines may be under the direct influence of ox-LDL and/or NF-κB (Section 2.3.6). Additionally, patients with RA have increased concentrations of MCP-1 in their serum and synovial fluid when compared to patients with non-inflammatory degenerative joint disease (Koch *et al.*, 1992). Macrophages in the RA synovial lining constitutively express MCP-1, unlike normal circulating monocytes.

5.2 EXPRESSION OF ADHESION MOLECULES

Oxidatively modified LDL up-regulates the surface expression of VCAM-1 and intracellular adhesion molecule-1 (ICAM-1) in cultured endothelial cells, promoting the interactions between both cell types (Kume *et al.*, 1992). This may play a pivotal role in the development of atherosclerosis by promoting the penetration of circulating monocytes into the subendothelial space whilst inhibiting the mobility of resident macrophages. It has been previously demonstrated that ICAM-1, E-selectin, and VCAM-1 are up-regulated in the microvasculature of rheumatoid but not control synovium (Corkill *et al.*, 1991; Koch *et al.*, 1991). The association between ox-LDL and increased expression of adhesion molecules in the inflamed synovium has yet to be studied.

5.3 CYTOTOXICITY

There is a long-standing hypothesis that the microvasculature plays a pathological role in forms of chronic inflammatory polyarthritis, particularly RA (Rothschild and Masi, 1982). One of the proposed mechanisms of vascular damage in connective tissue disease is the direct action of a cytotoxic serum factor inducing endothelial cell damage. Blake *et al.* (1985) have suggested that the vascular abnormalities associated with RA may be linked to oxidized lipoproteins because they are cytotoxic to endothelial cells.

5.4 ANTI-LIPOPROTEIN ANTIBODIES IN RA

Antibodies to very low density lipoprotein (VLDL) and LDL have been detected in the serum of patients with RA, but not control groups (Lazarevic *et al.*, 1993). In these studies, 38% of patients with active RA tested positive for anti-VLDL/LDL antibodies whilst these autoantibodies were not detected in patients with psoriatic arthritis, osteoarthritis or healthy subjects. Lipoproteins were found in the dissociated components of circulating immune complexes in the serum of 30% of the RA patients. It was concluded that dyslipoproteinaemia in some RA patients may be due to an autoimmune component of the disease.

Autoantibodies to cardiolipin, which cross-react with

ox-LDL, have been detected in patients with another autoimmune disease, systemic lupus erythematosus (SLE) (Vaarala *et al.*, 1993). There are structural similarities between the target antigen for anti-cardiolipin antibodies and LDL. Cardiolipin consists of apolipoprotein H and phospholipids, whilst ApoB and a mixed phospholipid monolayer are components of LDL; therefore, antibody cross-reaction with ox-LDL is not completely unexpected. The elevated incidence of atherosclerosis in SLE patients is well recognized (Vaarala *et al.*, 1993) and an immune response to ox-LDL may be responsible for the vascular complications associated with SLE.

5.5 FOAM-CELL FORMATION

In atherosclerosis, ox-LDL is taken up ultimately by macrophages and smooth muscle cells in the arterial intima. Once loaded with lipid, these cells have a foamy appearance when examined histologically. The accumulation of these so-called "foam cells" in the artery wall leads to the formation of "fatty streaks", which can lead to atheromatous plaque formation and consequent coronary heart disease.

Recently, we carried out immunohistochemical studies using an antibody directed against ox-LDL to demonstrate the presence of foam cells and fatty streaks around blood vessels within the subintimal lining of rheumatoid synovial tissue (Winyard *et al.*, 1993). Collections of positively stained macrophages, arranged in a linear fashion and with the morphological characteristics of foam cells – that is, fatty streaks – were identified. In addition, scattered, positively stained foam cells were present in association with fibrin deposits. The positive staining in all rheumatoid patients, but not in traumatic knee injury (control) patients, suggests that atherosclerosis and RA have analogous pathological features.

6. *Chain-breaking Antioxidants as Therapeutic Interventions in Human Inflammatory Diseases – A Cautionary Note*

The association of free-radical damage with many disease states is well documented, and a wide range of natural and synthetic antioxidants have been proposed for therapeutic use. Vitamin E, in its capacity as a chain-breaking antioxidant, has been used as a therapeutic agent for the treatment of RA (Blankenthorn and Clewing, 1993). Whilst these studies have provided encouraging preliminary data, placebo-controlled double-blind studies are needed to further assess the value in treating RA patients with vitamin E.

Studies have demonstrated reduced serum concentrations of vitamin E in patients with RA (Honkanen *et al.*, 1989; Situnayake *et al.*, 1991). No correlation between disease activity and serum vitamin E concentrations was found, but it was suggested that such patients might suffer a reduced antioxidant capacity. However, it is conceivable that a decreased serum antioxidant status is a primary event in the evolution of RA. Recent studies (Heliovaara *et al.*, 1994) have demonstrated that lowered levels of vitamin E, β-carotene and selenium (required for glutathione peroxidase) together may be a risk factor for subsequent development of RA.

The crucial role of ox-LDL formation in atherogenesis is now well established. Clinical trials of the anti-atherogenic effects of antioxidants such as vitamin E are in progress but, of necessity, these trials require large study populations, with the associated high costs, and the clinical outcome will be unclear for many years (Rimm *et al.*, 1993; Stampfer *et al.*, 1993). The inflamed rheumatoid joint may serve as a model for the study of inflammatory mechanisms thought to operate at focal sites of oxidative stress, including the arterial intima undergoing plaque formation. Although the local formation of ox-LDL in the rheumatoid joint probably does not predispose RA patients to coronary heart disease (presumably because plasma LDL remains unmodified), lipid-soluble antioxidants may have anti-inflammatory activity in RA, as well as anti-atherogenic properties.

Although there is evidence that free-radical damage contributes to many pathological conditions, the value of dietary nutrients with antioxidant properties in disease therapy has not been proven (Steinberg, 1993). Effective antioxidant treatment must ensure the compound reaches its target in sufficient concentrations to offer protection against free-radical damage (Halliwell *et al.*, 1992). One animal study has indicated that modest dietary treatment with vitamin E preserves endothelium-dependent vasodilatation function, whilst higher doses were associated with endothelial cell dysfunction and arterial intimal proliferation (Keaney *et al.*, 1994). The Alpha-Tocopherol, β-Carotene Cancer Prevention Group (1994) has investigated the association of antioxidant vitamin supplementation with cancer risk over a 5–8 year period. No evidence of a beneficial effect of vitamin E was found and those who received β-carotene were found to have an increased incidence of lung cancer over those who did not receive β-carotene.

The effect of prolonged antioxidant therapy in relation to normal physiological processes (for example, redox cycling, cell–cell signalling, transcription factor activation) must be assessed. It is conceivable that the overload of one antioxidant by dietary supplementation (for example, α-tocopherol) may shift the levels of other antioxidants (for example, by decreasing ascorbate and β-carotene concentrations), with unknown consequences. To assess the potential for lipid-soluble antioxidant treatment in inflammatory diseases such as RA, further investigations into these questions will be needed.

7. References

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8. Reactive Oxygen Species in Skin Inflammation

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1. Introduction

The skin provides a protective barrier over the body surface. As such it is continuously exposed to injury by mechanical, infectious and chemical means. Such insults can cause an inflammatory response, which is usually acute and short term, but may become chronic if physiological regulation of humoral and cellular response fails. The normal cellular response leads to migration of phagocytic leucocytes – macrophages, and polymorphonuclear leucocytes (PMNs) – and lymphocytes into the damaged site. During PMN phagocytosis in normal individuals, cells undergo a "respiratory burst" and consume increased amounts of oxygen. Such activation upregulates NADPH production via the hexose monophosphate shunt and generates superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$) and hypochlorous acid (HOCl). Such reactive oxygen species (ROS) are capable of damaging cell membranes and a wide variety of other biomolecules. The H_2O_2 produced is derived from $O_2^{\cdot -}$ by a process called dismutation and is a substrate for myeloperoxidase-mediated production of HOCl. In the presence of catalytic iron, the highly reactive $^{\cdot}OH$ radical is produced, which peroxidizes polyunsaturated fatty acids (PUFAs) in cell membranes. This is a particularly significant process in the skin that contains a very high concentration of cell-membrane PUFAs (Camp, 1988). The major effect of lipid peroxidation is to decrease membrane fluidity with consequent loss of receptor integrity. Also, lipid peroxidation products, particularly aldehyde derivatives, are released and these have the ability to inhibit protein synthesis, block macrophage action and cause changes in chemotactic responses. Later in this chapter the role of phagocyte-derived ROS and the major importance of lipid peroxidation in skin inflammatory reactions will be discussed in detail. Phagocytic cells are not, however, the only known sources of ROS in the skin since fibroblasts also produce $O_2^{\cdot -}$ via NADPH oxidase (Emmendorffer *et al.*, 1993). Psoriatic epidermis (Kizaki *et al.*, 1977) and burned skin (Till *et al.*, 1989) contain much increased levels of xanthine oxidase activity, an enzyme capable of generating $O_2^{\cdot -}$, which has been detected histochemically in endothelial cells (Jarasch *et al.*, 1986). The radical, nitric oxide (NO) originally known as endothelium-derived relaxing factor, is a potent vasodilator, which is also cytotoxic to tumour cells, parasitic fungi, protozoa, helminths and Mycobacteria (Moncada and Higgs, 1991). NO is known to be produced locally in cutaneous microvasculature (Hughes *et al.*, 1990) and influences oedema formation in skin inflammation.

ROS also have effects on gene activation, which is highly relevant to skin inflammation. They rapidly induce the immediate early genes c-fos, c-myc and c-jun, possibly through the induction of DNA strand breaks (Amstad *et al.*, 1992). These genes encode transcription factors, which have major roles in the induction of cell growth, differentiation and development. Basset-Séguin

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et al. (1990) have shown that c-fos is highly expressed in normal adult skin, most transcripts being found in the basal epidermal layers where proliferation occurs.There have been a number of studies of proto-oncogene expression in psoriatic skin with highly variable results. Mordovtsev *et al.* (1988) found an increase in c-fos expression, whereas Elder *et al.* (1990) reported a decrease. This result was confirmed by Basset-Séguin *et al.* (1991), who also showed a decrease in c-jun. These results suggest that c-fos and c-jun do not play a role in the control of keratinocyte proliferation *in vivo*, though they may be involved in differentiation of these cells. It has already been shown that very low doses of hydrogen peroxide can stimulate division of both fibroblasts and endothelial cells *in vitro* (Murrell *et al.*, 1990), a process likely to accelerate both fibrosis and wound healing. This process can also take place by the action of lipid peroxidation products on fibroblasts inducing collagen gene expression and consequently collagen synthesis (Chojkier *et al.*, 1989). However, it can readily be appreciated that the overproduction of radicals, which occurs during inflammation, has the potential to cause excessive tissue proliferation by overactivation of oncogenes. ROS have also been implicated in the activation of nuclear factor $\varkappa$B (NF-$\varkappa$B) (Baeuerle and Baltimore, 1991; Schreck *et al.*, 1991). This factor is important in inflammation as it controls cytokine genes, such as interleukin-2 (IL-2), tumour necrosis factor-α (TNFα), IL-2 receptor and also MHC class 1. As yet, there have been no direct published studies on NF-$\varkappa$B in the skin.

2. *Antioxidant Defence Mechanisms*

ROS are produced constantly by cells *in vivo* as a normal physiological event. There are, therefore, cellular antioxidant defence systems that protect against any toxic effects of ROS and also specific repair mechanisms, which prevent the accumulation of damaged molecules (Halliwell and Gutteridge, 1989). For instance, $O_2^{\cdot -}$ is converted into O_2 and H_2O_2 by the enzyme superoxide dismutase (SOD) but other enzymes, whose function tends to overlap, are required for the removal of H_2O_2, which is potentially highly toxic since it diffuses rapidly over a considerable area and crosses cell membranes. Such enzymes destroying H_2O_2 are glutathione peroxidase (GSHPx) and catalase (CAT) (Halliwell and Gutteridge, 1989). The skin also contains important non-enzyme antioxidants including glutathione, tocopherols and ascorbic acid. Tocopherols, for example, inhibit the lipid peroxidation process by scavenging intermediate radicals such as lipid peroxyls (Burton and Ingold, 1989), by donating a single hydrogen atom. The tocopheryl radical thus generated is probably then removed by reaction with ascorbate (Burton and Ingold, 1989). The major antioxidant properties of ascorbate are, however, related to its rapid reaction with peroxy radicals and other ROS resulting in a semidehydroascorbate radical, which is enzymatically reduced back to ascorbate by dehydroascorbate reductases (at the expense of reduced glutathione) or NADH-semidehydroascorbate reductase (at the expense of NADH).

Glutathione (GSH) is a tripeptide that makes up some 90% of non-thiol protein intracellularly, an important component of the antioxidant system in most tissues including the skin (Vessey, 1993). GSH reacts directly with ROS in a chain-breaking reaction to form oxidized glutathione (GSSG), which is regenerated to GSH by the catalytic activity of glutathione reductase. This reaction requires NADPH, which is generated by glucose-6-phosphate dehydrogenase and the cytoplasmic enzyme isocitrate dehydrogenase (Reed, 1986). It inhibits melanin formation in the skin (Seiji *et al.*, 1969) and studies in the mouse have shown that GSH tissue content declines with increasing age (Hazelton and Lang, 1980). GSH is particularly important in the skin, as it inhibits ROS-induced lipid peroxidation both enzymatically and non-enzymatically (Reed and Beatty, 1980). Studies have shown that depletion of cellular GSH leads to cell damage, a view strengthened by the finding that the hepatotoxicity and cardiotoxicity of some drugs is directly related to their ability to reduce cellular GSH (Hinson, 1980; Kaplowitz, 1980; Olson *et al.*, 1980; Williamson *et al.*, 1982). Under normal circumstances, intracellular GSH is maintained at a fixed level, which is specific for individual cell and tissue types. It is possible, however, to manipulate GSH content by inhibiting γ-glutamylcysteine synthetase activity using buthionine sulphoximine. This leads to a slow depletion over 8–24 hours (Dethmers and Meister,1981). In contrast, compounds such as 2-cyclohexen-1-one, 1-chloro-2,4-dinitrobenzene and diethyl maleate, which inhibit GSH by conjugation of GSH transferase, act rapidly and show a 90% GSH depletion in 10–120 min (Novogrodosky *et al.*, 1979; Parker *et al.*, 1980; Fishman *et al.*, 1981; Reed *et al.*, 1983). Such information is essential to our understanding of *in vivo* events.

Potentially the most toxic of all the ROS, the $^{\cdot}OH$ radical, lacks a specific enzyme-based defence system and is generated following reduction of ferric and cupric ions by $O_2^{\cdot -}$. The resultant ferrous ions react with H_2O_2 to produce the $^{\cdot}OH$ radical, which, although unstable, is highly reactive within 1–5 molecular diameters of its site of formation (Lunec and Blake, 1990). Protection from this process is achieved by compartmentalizing the iron intracellularly in the form of ferritin. Extracellularly the iron is bound to transferrin and lactoferrin, and intravascularly to haemoglobin. Ferritin is a complex macromolecule consisting of 24 apoprotein subunits surrounding an iron oxyhydroxide core, which also contains some phosphate. The ferritin molecule can, therefore, exist in varying degrees of iron saturation up to 4000 ferric (Fe^{3+}) atoms (Worwood, 1990). Iron enters ferritin in the ferrous (Fe^{2+}) form, which is oxidized by

the protein shell to Fe^{3+} and sequestered in the core. For iron to become catalytic it must be released from the core by a single electron reduction to Fe^{2+}. This is likely to be an important event in the skin inflammation process as will be discussed later in relation to lipid peroxidation. Transferrin is an iron-transport protein in the plasma consisting of a single-chain glycoprotein (75–78 kDa) with the polypeptide chain folded to form two globular domains, each with one binding site for a single Fe^{3+}. It is synthesized mainly in the liver and to a lesser extent in the brain, muscle and spleen (Weinberg, 1989). The transferrin receptor is increased in the skin, testis, pancreas and liver, which are susceptible to increased iron deposition (Gatter *et al.*, 1983). Lactoferrin closely resembles transferrin but has a higher binding capacity for Fe^{3+} at acidic pH. It is present in neutrophils and various secretions and is released from activated phagocytic cells. Normally, both transferrin and lactoferrin are only partially iron loaded and are unlikely to catalyse $^{\cdot}OH$ radical formation *in vivo* (Halliwell and Gutteridge, 1985). One study has shown that potentially catalytic iron mobilized from ferritin by $O_2^{\cdot-}$ is transferred to apotransferrin and apolactoferrin actively inhibiting lipid peroxidation (Monteiro and Winterbourne, 1988). A major difference between transferrin and lactoferrin, which may be important in the acidotic conditions of the skin, is that lactoferrin retains iron down to pH = 4.0. Transferrin releases iron at a pH of 5.6 and may release iron ions to catalyse ROS reactions in a low pH environment. Some of this iron will, however, be bound to lactoferrin, providing a protective mechanism (Halliwell and Gutteridge, 1985). The ability of these proteins to promote $^{\cdot}OH$ in the presence of $O_2^{\cdot-}$, H_2O_2 and H_2O_2 with ascorbate, has been studied at different pH values, the conclusion being that iron bound in this way *in vivo* is essentially inactive in accelerating oxidative damage at physiological pH in the absence of some form of chelation (Aruoma and Halliwell, 1987).

The skin and eyes are unique in being highly vascular organs exposed to high levels of oxygen and light, the former being essential to ROS genesis and reactivity, and the latter a potent inducer of ROS through ultraviolet stimulation. We may expect, therefore, that the skin surface will have a well-developed oxidant defence system in comparison to deeper layers and this appears to be the case. In studies of hairless mice, Shindo *et al.* (1993) have shown that catalase, glutathione peroxidase and glutathione reductase are higher in the epidermis than the dermis by 49%, 86% and 74%, respectively, when expressed in units per gram of tissue. SOD did not show this difference being slightly (but not significantly) lower in terms of activity per gram of tissue and significantly lower ($P < 0.001$) in terms of activity per milligram of protein. The lipophilic antioxidant α-tocopherol was significantly higher ($P < 0.05$) in the epidermis, as was the hydrophilic antioxidant dehydroascorbate ($P < 0.05$). These results agree with the previous studies of Connor and Wheeler (1987) for glutathione and related enzymes, also in the skin of mice, and of Wheeler *et al.* (1986).

Interestingly, the SOD levels in mouse skin measured by Shindo *et al.* (1993) are the opposite of those in human skin, which has a 20% greater activity in the epidermis than dermis (Kim and Lee, 1987). Whether this reflects technical differences in measurement of enzyme activity or a real difference between these species is currently unknown. The human dermis is, however, much thicker than that of the mouse and human skin also has varying degrees of pigmentation, which may affect the antioxidant levels. Yohn *et al.* (1992), in a comprehensive study of cultured human cutaneous fibroblasts, keratinocytes and melanocytes, showed an antioxidant enzyme content hierarchy amongst the cell types studied. Fibroblasts had more peroxidase, GSHPx, CAT and SOD than did keratinocytes ($P < 0.05$), which had more of these defence enzymes than melanocytes ($P < 0.05$). There was, however, no difference in enzyme content between Caucasian and Black subjects. It is not known why such a hierarchy exists but there is likely to be an interactive relationship between enzymes in the various cell types. For instance, higher peroxidase content will be necessary to remove greater levels of H_2O_2 (Hodgson and Friedovich, 1975), and both CAT (Kono and Friedovich, 1982; Shimizu *et al.*, 1984) and GSHPx (Blum and Friedovich, 1985) are inactivated by $O_2^{\cdot-}$. Levels of $O_2^{\cdot-}$ and H_2O_2 are important as they are the "substrates" for iron-catalysed production of $^{\cdot}OH$ and consequent membrane peroxidation. Yohn *et al.* (1992) suggest, therefore, that cells ensure survival by having matched activities of SOD and peroxidases working together to remove $O_2^{\cdot-}$ and H_2O_2. Cells with unbalanced antioxidant enzyme levels, for example, high SOD and low peroxidase activities are prone to oxidant damage as demonstrated by Seto *et al.* (1990) in their study of *Drosophila* flies. They showed that transgenic flies expressing high levels of SOD were more readily killed by the superoxide-generating pesticide paraquat than were the wild-type flies, presumably due to rapid production of H_2O_2 by dismutation and insufficient levels of peroxidase defence. Similar results have been shown in SOD-overproducing mammalian cells (Elroy-Stein *et al.*, 1986). Since skin fibroblasts, keratinocytes and melanocytes are grown in different culture media, it may be argued that differences in antioxidant capability between these cells may merely reflect the *in vitro* conditions. Preliminary studies by Yohn *et al.* (1992) suggest that, although culture conditions do have an effect on antioxidant levels in fibroblasts, keratinocytes and melanocytes, the hierarchy of enzyme activity between the cells always remains the same. CAT was always present in highest levels in all these cell types.

Of all the epidermal cells studied *in vitro* with respect to antioxidant status, perhaps keratinocytes are the most important. These cells differentiate as they move upwards through the epidermis and there is evidence that, in

animals at least, keratinocyte antioxidant enzyme levels change when this occurs. Reiners *et al.* (1988) demonstrated an increase in murine keratinocyte CAT levels with increasing stages of differentiation, and the same group (Reiners and Rupp, 1989) have also shown that conversion of xanthine dehydrogenase to oxidase occurs during keratinocyte differentiation and that CAT levels are also dependent on this phenomenon. This is an important finding as $O_2^{\cdot -}$ can be produced from this enzyme system under conditions of hypoxia, i.e. following burn injury. This will be discussed in detail in Section 6 of this chapter. It is not yet known whether human keratinocytes similarly increase their antioxidant enzymes during the differentiation process. Human melanocytes have the lowest levels of antioxidants and are consequently exquisitely sensitive to H_2O_2-induced toxicity (Yohn *et al.*, 1992). The consensus of opinion is that this reflects the commitment of melanocytes to the production of melanin, an energy-hungry process that leaves little available for defence-enzyme production. Melanin is, itself, a ROS scavenger (Sealy *et al.*, 1980; Ranadive and Menon, 1986), and readily participates in oxidation and reduction reactions (van Woert 1967; Gan *et al.*, 1977; Geremia *et al.*, 1984). It is possible, therefore, that melanocytes use these properties of melanin to protect themselves from oxidative stress, although this process would seem inefficient as melanin is stored intracellularly in membrane-bound vesicles. This inefficiency would be particularly evident during epidermal inflammation, where the oxidative insult would be directed against the cell membrane.

Under normal physiological conditions, therefore, antioxidant defences in the skin are able to modulate free-radical production. The initiation of an inflammatory event has the potential for increasing ROS production to such an extent that defence systems are overwhelmed and tissue damage occurs. This event results in the production of even more toxic oxidants and the development of overt disease requiring treatment. Section 4 of this chapter will describe the role of ROS in skin inflammation.

3. *Catalytic Iron and Its Sources*

In the presence of transition metals, usually iron in biological systems, H_2O_2 is converted to $^{\cdot}OH$, which is capable of initiating lipid peroxidation in cell membranes. In practice, the iron must be in a "free" form for this to occur and biological systems protect themselves by maintaining iron in a complexed form. Such complexes are formed in association with phosphate esters (as in ATP, ADP, GTP), carboxylic acids (citrate) or proteins (ferritin, transferrin, lactoferrin) (Jacobs, 1977). The total amount of iron in an average man is 4–5 g, with about 1 mg absorbed by the gut mucosa and the same amount excreted daily. The exact amount of iron cycling through the body at a given time is, however, closely dependent on individual metabolic requirements (May and Williams, 1980). The skin is a significant site of iron turnover, 0.24–0.6 mg being lost daily by epithelial-cell desquamation and consequent loss of associated glands, and also by secretion of sweat (Green *et al.*, 1968). The amount of iron lost by both the epidermis and dermis is greatly increased in iron- overload syndromes such as haemochromatosis (Weintraub *et al.*, 1965) and transfusional haemosiderosis (Zeimer *et al.*, 1978). Iron loss is also increased in pathological skin diseases such as psoriasis (Molin and Webster, 1973). In iron-overload syndromes the presence of the oxyhydroxide of iron, haemosiderin, increases. It is present in intracellular and extracellular granules as a brown pigment, particularly in areas of old haemorrhage or blood extravasation. Haemosiderin iron is similar to the iron-storage protein ferritin from which it is derived, but is chemically heterogeneous being associated with carbohydrate, haem and lipid. Haemosiderin is insoluble in aqueous media and is generally found in membrane-bound vesicles or siderosomes (Richter, 1978). In comparison to ferritin, haemosiderin is a relatively poor donator of iron to $^{\cdot}OH$ generation and, consequently, conversion of ferritin to the more inert complex is biologically advantageous as it decreases the ability of iron to promote free-radical reactions (O'Connell *et al.*, 1986). Partially saturated ferritin does not provide catalytic iron for $^{\cdot}OH$-radical production *in vivo*. Mobilization does occur in the presence of reducing agents by a process that is dependent on the presence of stimulated neutrophils. These are present in quantity at sites of skin inflammation and release $O_2^{\cdot -}$, which enters the iron core, a process prevented by the addition of SOD, but not CAT or dimethyl sulphoxide (DMSO) (Biemond *et al.*, 1984). The authors were of the opinion that their studies ruled out H_2O_2 or $^{\cdot}OH$ as the active reductants. As already mentioned, $O_2^{\cdot -}$ can be generated by xanthine oxidase in conditions of partial ischaemia in the skin and this also has the ability to release iron (Biemond *et al.*, 1988). Bolann and Ulvik (1990), however, disagreed with these findings showing that not only is $O_2^{\cdot -}$ a poor releaser of iron from ferritin, but that ferritin itself is able to increase $O_2^{\cdot -}$ dismutation to H_2O_2. The authors did not, however, measure this product directly. In the presence of ferritin, ascorbate is able to stimulate lipid peroxidation, suggesting that ascorbate can mobilize redox active iron. At acid pH (pH = 4.5) iron is released spontaneously from haemosiderin but ascorbate is a necessary adjunct to haemosiderin-dependent lipid peroxidation at pH = 7.4 (O'Connell *et al.*, 1985). It is also likely that iron will be released from ferritin degraded by proteolytic action within the autophagic vacuoles of macrophages during the inflammatory process (Sakaida *et al.*, 1990).

Haemoglobin-derived haem iron has multiple pro-inflammatory effects resulting from its ability to initiate decomposition of lipid hydroperoxides from PUFAs. In

the presence of ascorbic acid *in vitro*, haemoglobin and myoglobin scavenge H_2O_2, preventing $^{\cdot}OH$ generation, rather than being activated by H_2O_2 themselves to initiate toxic oxidative reactions (Harel and Kanner, 1989). In the absence of reducing agents, generation of $^{\cdot}OH$ may therefore occur via iron-catalysed Fenton-type reactions. Oxyhaemoglobin may react with low H_2O_2 levels in inflamed tissues to form a reactive radical species whose exact nature is uncertain (Puppo and Halliwell, 1988). It is also known that simple iron chelates exist as short-lived intermediates constituting a transit pool of low molecular mass redox-active iron complexes. These appear to be in equilibrium with other iron-containing compounds whose exact chemical composition is unclear, but may represent iron ions attached to phosphate esters such as ATP, ADP or GTP, as well as to organic acids, DNA and to the polar head groups of membrane lipids (Jacobs, 1977). There is an increased skin parenchymal iron content, and reduced ability to regulate iron flow in and out of cells in haemodialysis patients (Friedlander *et al.*, 1988) and in patients with haemochromatosis (Cavill *et al.*, 1972). This could result in local production of $^{\cdot}OH$ and consequent tissue damage.

4. *Ultraviolet-induced Skin Inflammation*

Recently, both direct and indirect evidence has been presented implicating ROS in inflammatory skin disease, this group having demonstrated the pro-inflammatory nature of H_2O_2 in rat skin *in vivo* (Trenam *et al.*, 1991, 1992). Perhaps the most well-known situation is the production of ROS by skin exposure to ionizing or ultraviolet irradiation (UVR) under aerobic conditions (Black, 1987). UVR increases vascular permeability leading to leakage, sunburn cell formation, disorganization and desquamation of the epidermis, and leucocyte infiltration. There is alteration in lysosome structure and associated enzymes, cell proliferation, and increased melanin production associated with increases in melanocyte size and number. These changes are related to the fact that UVR excites ground-state O_2, resulting in the production of a number of ROS. These have not been identified with absolute certainty but are likely to include $O_2^{\cdot -}$ (Cunningham *et al.*, 1985), which has the ability to release catalytic "free" iron from ferritin (Biemond *et al.*, 1984, 1988), singlet oxygen ($^1O_2$), H_2O_2 and $^{\cdot}OH$, which ultimately induces lipid peroxidation (Halliwell and Gutteridge, 1984). Punnonen *et al.* (1991a) have shown that keratinocytes exposed to UVR are able to produce lipid peroxidation very rapidly. As we have already mentioned, such mechanisms are dependent on the antioxidant status of the skin cells and Moysan *et al.* (1993) have shown that in ultraviolet A (UVA)-irradiated (180 kJ/m^2) cultures of human skin fibroblasts, levels of thiobarbituric acid reactive substances (TBARs, an index of lipid peroxidation) correlate positively with SOD but negatively with CAT activity. They found no correlation with reduced glutathione (GSH) or GSHPx activity, and concluded that $O_2^{\cdot -}$ is generated by UVA. CAT was, however, strongly inhibited and GSHPx unchanged. The loss of CAT was an early response, with a 50% inhibition after exposure to only 60 kJ/m^2. Their results also suggested that H_2O_2 generated by $O_2^{\cdot -}$ dismutation by SOD is not completely removed by CAT and that UVA-induced lipid peroxidation was dependent on a balance between SOD and CAT activities within the cell. CAT is known to be inactivated by light in hepatocytes (Chen and Packer, 1979) and fibroblasts (Vuillaume *et al.*, 1986) *in vitro*.

Studies have also been made on the effect of UVB irradiation in hairless mice, a single exposure of 300 mJ/cm^2 on freshly excised skin producing a significant fall in glutathione reductase and CAT activity immediately post-irradiation. There was no change in GSHPx or SOD, but glutathione levels decreased in association with an increase in glutathione disulphide. Perhaps not surprisingly, there was also a decrease in the photo-oxidation susceptible antioxidants α-tocopherol, ubiquinol 9 and ubiquinone 9 (Fuchs *et al.*, 1989). Miyachi *et al.* (1987), using a higher single dose of UVB (1 J/cm^2) showed a significant decrease in SOD activity at 24 h and 48 h post-irradiation, which returned to normal after 72 h. They also showed that SOD levels remained unchanged during the first 3 h post-irradiation, confirmed by the Fuchs *et al.* (1989) study. This lack of change in SOD levels is interesting as it is associated with an absence of lipid peroxidation products post-irradiation (Fuchs *et al.*, 1989). Other studies (Moysan *et al.*, 1993), have shown a direct correlation between raised SOD levels and increased amounts of lipid peroxidation products in cultured cells. Similar effects of UVB have been demonstrated *in vitro* using cultured keratinocytes, both Punnonen *et al.* (1991a, 1991b) and Yohn *et al.* (1992) showing induction of lipid peroxidation, and reduction of SOD and CAT activity. A similar reduction in SOD was found (Hashimoto *et al.*, 1991) in pig epidermis. It would be expected, however, that major inflammatory effects induced by ultraviolet (UV)-generated ROS would be initiated at the skin surface and it will be remembered that earlier in this chapter we described that higher antioxidant levels occurred in the epidermis than in the dermis. Following UVA and UVB irradiation of hairless mice to simulate sunlight, Shindo *et al.* (1993) showed a significant decrease in CAT and SOD in both epidermis and dermis. α-Tocopherol, ubiquinol 9, ubiquinone 9, ascorbic acid, dehydro-ascorbic acid and reduced glutathione decreased in both epidermis and dermis by 26–93%. The reduction in total ascorbate and catalase was very much greater in the epidermis. ROS formation resulting from UV irradiation has been demonstrated in isolated whole human skin (Norrins,

1962; Pathak and Stratton, 1968) and in skin homogenates (Nishi *et al.*, 1991; Ogura *et al.*, 1991), although direct measurement of such *in vivo* events is technically very difficult. As can be seen from the discussion so far, most authors have opted for indirect assessment of ROS activity by looking at defence enzyme levels *ex vivo* or *in vivo*, which are easier to measure.

4.1 ROLE OF PHAGOCYTES

UVR activates phospholipase A_2 (PLA_2) in cultured mammalian cells including skin (De Leo *et al.*, 1988; Hanson and De Leo, 1990). The two pertinant hydrolysis products of PLA_2 activity in mammalian cells are lysophosphatidylcholine and arachidonic acid (AA). These two products have the ability to activate cytosolic protein kinase C (PKC), which activates the NADPH-oxidase (McPhail *et al.*, 1984; Oishi *et al.*, 1988) system in phagocyte cell membranes releasing superoxide (Watson *et al.*, 1991). We can speculate therefore that infiltrating skin phagocytes represent an alternative source of ROS in UVR skin inflammation. Human neutrophils exposed to UVR *in vitro* release superoxide and preferentially release secondary granules, exemplified by increased lysozyme release. These reactions are dependent on intact cell membrane-associated oxidative metabolism and are mediated by the UVB component of UVR, rather than the UVA. Neutrophils also release AA from membrane phospholipids presumably as a result of the activation of PLA_2, as the effect is prevented by the PLA_2 inhibitor 4-*p*-bromophenyl bromide (Savage *et al.*, 1993).

5. *Lipid Peroxidation*

We have already stressed the potential importance of lipid-rich membranes in the skin as potential targets for ROS-induced damage and ageing of human skin is morphologically identical to changes found by peroxidative processes (Serri *et al.*, 1977). The involvement of AA metabolites in skin disease, and in particular psoriasis, has been the subject of much recent interest. Studies have included topical and intradermal administrations of AA metabolites, and assay of such products in clinical specimens. Results show that concentration of AA, 12-hydroxy-eicosatetraenoic acid (12-HETE), PG and leukotrienes are increased in psoriatic lesions (Hammarstrom *et al.*, 1975; Camp *et al.*, 1983; Brain *et al.*, 1984; Duell *et al.*, 1988) and also that full-thickness epidermis from normal and diseased skin has the enzymatic capacity to convert AA to some of the same metabolites (Hammarstrom *et al.*, 1975, 1979; Camp *et al.*, 1983; Brain *et al.*, 1984; Ziboh *et al.*, 1984; Duell *et al.*, 1988). The biological effect of both 12-HETE and leukotrienes was confirmed by both topical application and intradermal injection, which caused epidermal inflammation and proliferation (Soter *et al.*, 1983; Chan *et al.*., 1985). The precise mechanism by which 12-HETE, the major product of AA metabolism, is derived in the epidermis is currently uncertain but has been thought to be the result of arachidonate lipoxygenase activity. A recent paper has shown that the 12-HETE derived from psoriatic lesions is not the 12-S-hydroxy enantiomer as would be expected for 12-lipoxygenase breakdown. Instead the predominant isomer is the 12-R (Woollard, 1986), suggesting the possibility that ROS-activated cytochrome P-450 monooxygenases in the inflamed epidermis may be the major active enzymes, since they are known to produce the 12-R isomer of HETE rather than the 12-S (Capdevila *et al.*, 1986). The human epidermis certainly contains active membrane-bound monooxygenase(s), which are able to generate 12-HETE from AA (Holtzman *et al.*, 1989) and these are O_2-dependent mechanisms inhibited by carbon monoxide, which also require NADPH. The role of the NADPH–cytochrome P450 flavoenzymes in membrane lipid peroxidation was established by Pederson *et al.* (1973).

Much work has been directed towards identifying the ROS that initiate endogenous NADPH-stimulated lipid peroxidation. Fong *et al.* (1973), and Koster and Slee (1980) suggest that the $^{\cdot}OH$ radical, produced by iron-catalysed reduction of H_2O_2, is induced in the NADPH-cytochrome P450 reductase ADP-Fe^{3+}-dependent peroxidation of microsomal lipids (Lai and Piette, 1978). Such a role for $^{\cdot}OH$ has, however, been questioned by Kornbrust and Mavis (1980). Tien and Aust (1982) concluded that both NADPH–cytochrome P450-dependent (microsomal) and xanthine oxidase/ADP/Fe^{3+} promote lipid peroxidation by reducing ADP-chelated iron either via NADPH–cytochrome P450 reductase or $O_2^{\cdot -}$. The mechanism of $O_2^{\cdot -}$ cytotoxicity is the so-called "site-specific" iron-dependent Haber–Weiss reaction. High levels of 12-S HETE are formed by normal human epidermal cells in comparison to the 12-R formed in psoriatic skin. The 12-R HETE isomer is, interestingly, a much more potent chemoattractant for neutrophils than the 12-S (Cunningham and Woollard, 1987; Evans *et al.*, 1987). Infiltration of neutrophils is an early event in the pathogenesis of psoriatic lesions, which precedes the characteristic epidermal changes (Pinkus and Mehergan, 1966; Fogh *et al.*, 1989). As well as being potent sources of ROS, activated neutrophils are sources of leukotriene B_4 (LTB_4) and other AA derivatives such as 5-HETE. LTB_4 is produced from leukotriene A_4 (LTA_4) possibly by epidermal cells, which contain the necessary epoxide hydrolase (Sola *et al.*, 1992). The combination of neutrophils and epidermal cells may, therefore, contribute to the increased amount of LTB_4 in psoriatic lesions and other inflammatory dermatoses.

Skin inflammation is invariably associated with itching and lesions tend to be traumatized by scratching. This causes bleeding into tissues leading to haemoglobin

release from erythrocytes. Exposure of haemoglobin to H_2O_2 generated from neutrophils or xanthine oxidase (*vide infra*) in inflamed lesions causes degradation and release of both catalytic iron ions (Puppo and Halliwell, 1988) and toxic free haem (Gutteridge, 1986; Gutteridge and Smith, 1988; Balla *et al.*, 1991; Kim and Sevanian, 1991). Exposure of haemoglobin to H_2O_2 probably also generates a haem-ferryl species capable of stimulating lipid peroxidation. All these events are likely to increase the inflammatory response.

6. *Xanthine Oxidase*

The purine catabolism enzymes xanthine oxidase (XO) and xanthine dehydrogenase (XD) convert hypoxanthine to xanthine, and finally to uric acid. $O_2^{\cdot -}$ is produced in both conversion steps and XO was the first biological source of $O_2^{\cdot -}$ to be described (McCord and Fridovich, 1968). The precursor form of XO is the NAD-dependent XD (D-form) which is converted to the XO (O-form), which is oxygen dependent. The conversion process involves thiol groups and proteolysis (Stirpe and Della Corte, 1969). Following addition of xanthine to XO, the enzyme generates $O_2^{\cdot -}$ and H_2O_2 in amounts dependent on pH, O_2 concentration and substrate (xanthine) concentration (Fridovich, 1970; Porras *et al.*, 1981). In general terms, however, decreasing the steady state of reduction of the enzyme (increasing O_2 or lowering xanthine levels) causes the generation of $O_2^{\cdot -}$, whilst increasing it favours H_2O_2 generation. These general principles are well illustrated by hypoxic/reperfusion events. Following ischaemia, XD can be converted to XO *in situ* under the influence of a Ca^{2+}-dependent protease. The XO does not transfer electrons to NAD^+ but, to O_2 instead, producing $O_2^{\cdot -}$. When reperfusion occurs, O_2 as the electron acceptor and high levels of hypoxanthine produce a flux of $O_2^{\cdot -}$, which can be converted spontaneously to H_2O_2 or via the action of SOD. XO is also able to mobilize iron from ferritin by the action of $O_2^{\cdot -}$, and this iron catalyses the formation of $^{\cdot}OH$ from H_2O_2 and $O_2^{\cdot -}$ with the potential for initiating lipid peroxidation.

In mouse models of skin inflammation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), there is a close association between elevated XO activity in the epidermis and hyperplasia (Pence and Reiners, 1987). This association is also seen in psoriasis patients (Eisen and Seegmiller, 1961; Zimmer and Demis, 1966; Kizaki *et al.*, 1977). In the study by Kizaki *et al.* (1977), the epidermis was increased about five-fold in comparison to normal. It is not known whether XO-derived ROS have any role in psoriatic epidermal hyperproliferation but low levels of hydrogen peroxide added to the culture medium are well known to induce skin fibroblast proliferation *in vitro*, an effect that is greatest at low passage numbers (Murrell *et al.*, 1990). The generation of H_2O_2 by hydrocolloid wound-management materials has a similar effect on fibroblasts *in vitro* (Chung *et al.*, 1993). Also, H_2O_2 is known to accumulate in skin treated with hyperproliferative agents (Perchellet *et al.*, 1988). Interestingly, although significant hyperplasia is present in the skin of hairless mice following UVR, neither XD or XO are induced (Pence and Naylor, 1990). In the rat, XO also has an important role in thermal injury to the skin, which is closely linked to inflammatory changes. The primary event is a 25% reduction in skin blood flow followed by vascular damage and oedema, which reach their peak some 60 min post-injury. This effect is neutrophil independent (Alexander *et al.*, 1984). There are marked increases in plasma XO levels as early as 15 min and excision of the damaged skin largely prevents this, suggesting a skin origin for the XO. Also, the skin permeability changes are greatly attenuated by treatment with CAT, SOD and iron chelators suggesting an important role for ROS in the development of vascular injury (Ward and Till, 1990). Permeability changes are also negated by the use of XO inhibitors such as allopurinol and lodoxamide tromethamine, suggesting that XO is the source of ROS which induce vascular injury, and that the actual damage is probably caused by $^{\cdot}OH$ (Till *et al.*, 1989), which is able to initiate lipid peroxidation.

Peroxidation products are extractable from burned skin and from distant organs, particularly the lung (Nishigaki *et al.*, 1980; Till *et al.*, 1985; Demling *et al.*, 1987). Under such circumstances, vascular injury is perhaps not surprising as the enzyme is present in quantity in vascular endothelium (Jarasch *et al.*, 1986), although the mechanism of activation, conversion of XD to XO, is not well understood. A likely possibility is linked to hypoxia induced by a drop in blood flow immediately after burn injury, promoting the conversion of XD to XO with the production of $O_2^{\cdot -}$ and H_2O_2, as described earlier in this section. Also, it is known that XO-derived ROS cause complement activation in rats following thermal injury (Oldham *et al.*, 1988). One such product, complement fragment 5a (C5A), reacts directly with endothelial cells to cause intracellular conversion of XD to XO (Friedl *et al.*, 1989). C5a can also cause skin mast-cell and basophil degranulation and histamine release. Histamine and its metabolites methyl-histamine, methyl-imidazole-4-acetic acid and imidazole-4-acetic acid can act directly on XO, and increase its catalytic activity (Friedl *et al.*, 1989). Blockers of histamine release such as cromolyn completely inhibit skin oedema as measured by leakage of radiolabelled albumin and increase in plasma XO in thermally injured rats (Friedl *et al.*, 1989).

7. *Nitric Oxide*

The radical nitric oxide (NO) has been shown to be a labile humoral substance causing vasodilatation by

relaxing vascular smooth muscle via guanylate cyclase (Moncada *et al.*, 1989). NO is formed by macrophages stimulated by interleukin-1 (IL-1) (Hibbs, 1991), and mediators of inflammation such as LTB_4 induce the formation and release of NO by neutrophils (Rimele *et al.*, 1988; Sturm *et al.*, 1989). NO is therefore present at sites of inflammation and it is highly likely to have an important role in the inflammatory process. NO, which is relatively unstable in aerobic conditions, is generated in an NADPH-dependent process from L-arginine by NO-synthase (NOS), which appears to have at least three subfamilies. The two main types of NOS are the Ca^{2+}-dependent constitutive form (c-NOS), present in, and controlling the vascular tone of endothelial cells (Moncada and Higgs 1991; Nathan and Hibbs, 1991), and the inducible form (i-NOS), which is Ca^{2+}-independent and present in vascular smooth muscle, and to a lesser extent in endothelial cells (Moncada and Higgs, 1991; Nathan and Hibbs, 1991).

The sunburn reaction to UVR is largely the result of increased blood flow to the skin, induced by an unknown mechanism. The reaction is attenuated by non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids (Hughes *et al.*, 1992) in man suggesting that prostaglandins have some role. Like NSAIDs, corticosteroids are able to inhibit i-NOS (Di Rosa *et al.*, 1990; Radomski *et al.*, 1990; Nathan and Hibbs, 1991), and it is therefore likely that both cyclooxygenase and NO play a role in UV-induced increases in skin blood flow. This role of NO was demonstrated by Demiconstantinos *et al.*(1992) in cultured endothelial cells and by Warren *et al.* (1993) who showed that the NOS inhibitors L-nitroarginine methyl ester (L-NAME) and L-nitromonomethyl-arginine (L-NMMA) significantly reduced the delayed UV-induced vasodilation response. NO release by i-NOS is induced by an increasing list of pro-inflammatory agents, IL-1, TNF and endotoxin (Moncada and Higgs, 1991; Nathan and Hibbs, 1991). Using primary cultures of human keratinocytes and a mouse keratinocyte cell line, Hecke *et al.* (1992) demonstrated production of both NO and H_2O_2 following challenge with TNFα, γ-interferon and lipopolysaccharide (LPS). They showed that these mediators inhibited keratinocyte growth, an effect reversed by NOS inhibition, and also that epidermal growth factor was a potent agonist of NO and ROS production in cultured keratinocytes.

There have been recent studies on the importance of NO in modulating skin blood flow in both normal animals and in inflammatory models. Khan *et al.* (1993), using laser-Doppler techniques, showed that the NOS inhibitor L-NAME inhibited rabbit ear blood flow. It was possible to do this chronically for up to 2 weeks using implanted osmotic pumps. Pons *et al.* (1993) also used laser Doppler to show that the vasodilator effect of LPS in rabbit skin, which mimics the effect of Gram-negative bacteria, was likely to involve both i-NOS and IL-1. We have already discussed the damaging effects of neutrophils in the skin, and Mulligan *et al.* (1991, 1992) used an immune complex deposition model of neutrophil accumulation in rabbit skin to show that a range of L-arginine analogues, *N*-iminoethyl-L-ornithine, L-NMMA, NG-nitro-L-arginine and L-NAME prevented vascular damage by inhibition of NO. The protective effect was most pronounced with *N*-iminoethyl-L-ornithine, but was not associated with a reduction in neutrophil accumulation. The protective effect is reversed and damage enhanced in the presence of L-arginine (but not D-arginine). The studies of Ialenti *et al.* (1992) show that inhibition of NO synthesis by L-NAME and L-NMMA not only inhibits increased blood flow at a site of inflammation but also oedema, at least in acute models of carageenin-induced rat skin inflammation. Dependent on the time-course of the inflammatory response, it is likely that NO is generated by either c-NOS or i-NOS. Cutaneous hyperaemia in rat skin neurogenic inflammation induced by mustard oil is also NO dependent but the associated vascular leakage is not (Lippe *et al.*, 1993a, 1993b). Interestingly, the opposite effect was noted using substance P-induced inflammatory skin oedema in rats (Hughes *et al.*, 1990). Here, co-administration of L-NAME inhibited oedema, an effect reversed by L-arginine. At the time of writing, there are no studies on the role of NO in human skin disease but in such a rapidly developing field, it will only be a matter of time before this is investigated.

8. References

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9. Free Radicals, Inflammation and Eye Diseases

M. Goss-Sampson, A.J. Vivian *and* F.J. Kelly

1. Introduction

The eye is a highly specialized organ, designed to provide the brain with visual images of the surrounding environment. This is achieved by producing an optical image on light-sensitive cells within the retina, which is transmitted to the visual cortex by electrical signals. The process is initially dependent on photons entering the eye and being focused on the retina by a transparent lens. The structure of the eye is shown in Fig. 9.1. As light passes through the eye to the retina it traverses, in turn, the cornea, the aqueous humour, the crystalline lens and the vitreous humour before falling on the retina.

The reception, transference and absorbance of incident radiation by the eye in this manner means that it is exposed daily to potentially harmful radiation. Ultraviolet (UV) light is harmful, although both visible light and near UV (300–400 nm) are important sources of reactive oxygen species, in particular singlet oxygen (Delmelle, 1978; Shevedona *et al.*, 1983). Additional sources of intraocular oxidants include those released from activated neutrophils and macrophages during episodes of inflammation. Furthermore, the retinal pigment epithelial monolayer, which lies beneath the neural retina, has been shown to produce superoxide radicals during phagocytosis (Dorey *et al.*, 1989). Basic research over the past decade has highlighted our awareness of the role of oxygen free radicals in various pathological processes, and their impact on the eye and its various specialized structures has received considerable attention.

It is clear that normal cell function depends on the balance between the production of oxygen free radicals and the presence of adequate antioxidant defences. Ocular structures normally contain a wide range of antioxidants, such as vitamin E, vitamin C, superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT). Antioxidants neutralize free radicals by both enzymatic and non-enzymatic means, thereby reducing or preventing tissue injury. In addition there are a number of metal-binding molecules that maintain the pools of free metal ions at low concentrations, thus preventing their participation in free-radical generation. A wide range of these different types of antioxidants are found distributed in several compartments of the eye.

Each structure within the eye is highly specialized and hence susceptible in different ways to oxidative damage. For example, the lens absorbs increasing amounts of UV light with increasing age (Lerman, 1984) and this is associated with an increased incidence of cataract. Cataract, uveitis and retinal degeneration are responsible for about one-third of all causes of blindness and each is thought to involve free-radical-induced damage. Because of this diversity of susceptibility (and presumably antioxidant protection), this review will consider each of the main components of the eye separately in respect to its

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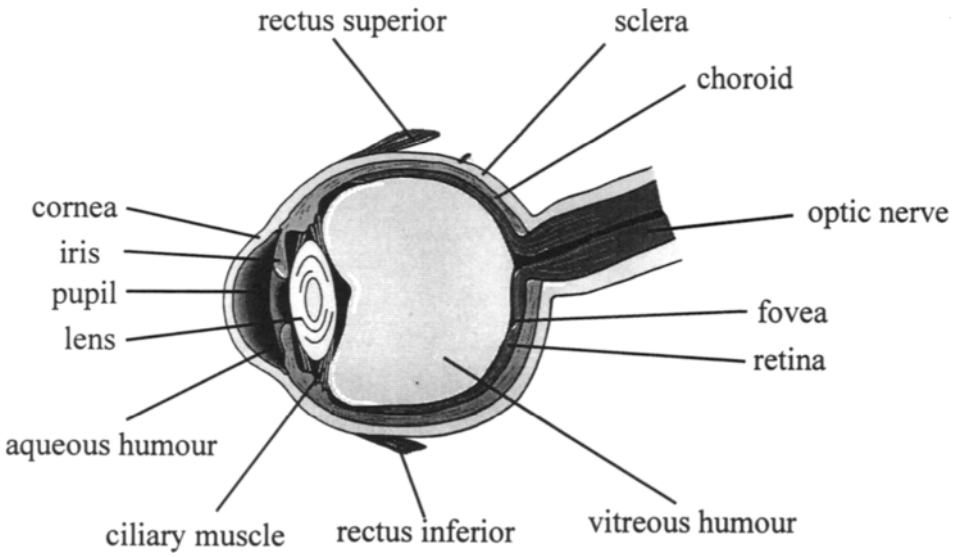

Figure 9.1 Vertical section through the human eye to show its structure.

unique problems and the potential free-radical basis of those problems.

2. *Cornea*

The cornea is the first structure of the eye to be in contact with incident light. It is composed of five distinct layers lying parallel to its surface: the outer epithelium, which is continuous with the epithelial layers of the conjunctiva; the epithelial basal lamina; the keratocyte-containing stroma, which is a collagen structure arranged so that it is transparent; Descemet's membrane; and, finally, the endothelium adjacent to the aqueous humour.

Normal cornea is composed of water (78%), collagen (12–15%), proteoglycans (1–3%), soluble proteins, glycoproteins and lipids. Electrolytes and low molecular weight solutes are also present. Transport of sodium and chloride ions across the epithelium into the tear film and across the endothelium into the aqueous is important for maintaining its hydration (Baum *et al.*, 1984). Since the cornea is avascular (except at the corneoscler limbus), the tear film provides (atmospheric) oxygen, which penetrates the epithelial layer at 3.5–4.0 $\mu l/cm^2$. The principal energy substrate is glucose, which is provided by the aqueous humour. Glycogen stores are used only during periods of oxygen deprivation or in cases of trauma (Friend, 1987). A proportion of the glucose is metabolized by the pentose phosphate pathway in order to generate NADPH for maintaining glutathione (GSH) and ascorbate in their reduced states. This reducing environment is important for the protection of the cornea from potential damage by hydrogen peroxide and free radicals.

As well as being avascular, the cornea also lacks lymphatic drainage, except when new blood vessels have grown into a previously normal cornea. Antigens and other substances that enter from the blood stream must do so from the limbus and diffuse into the cornea. Inflammatory cells also enter by this route and many types of corneal inflammation involve such cell traffic (Friedbender and Dvorak, 1977). The cornea is a target in many inflammatory disorders of the eye. For example, epithelial keratitis, Trantas' dots (eosinophil accumulation) and Wesslely rings (antigen:antibody precipitation) all feature corneal inflammation.

Since the cornea derives its energy from the breakdown of glycogen and glucose, it requires oxygen, which it derives from the atmosphere, the tears and from the aqueous humour. A high concentration of lactate is built up, which is eventually removed by passage into the aqueous humour.

Injury (either physical or chemical) to the corneal endothelial cells has a marked effect on occular function as these cells are responsible for maintaining the thickness and clarity of the cornea, yet they cannot be replaced if damaged. Immunohistochemical studies have revealed that enzymatic antioxidant defences, SOD, CAT and GSHPx, are similarly distributed in the corneal epithelium and endothelium (Rao *et al.*, 1985; Attala *et al.*, 1987, 1988). Other antioxidants include ascorbate, carotenoids and vitamin E (Heath, 1962).

In recent years considerable attention has focused on the mechanisms of oxidative damage to the cornea. In a study to determine the interaction of ascorbate, hydrogen peroxide and oxygen in the eye, Riley *et al.* (1986) found that ascorbate promoted corneal swelling when isolated corneas were perfused with glucose-deficient medium. This was due to the rapid oxidation of

ascorbate yielding hydrogen peroxide. Since oxidation of ascorbate in the aqueous humour is limited by the presence of glutathione, the concentration of hydrogen peroxide is dependent on the concentrations of ascorbate and glutathione. In reality the picture is likely to be even more complex with other antioxidants/oxidants also contributing. In a subsequent study, Hull and Green (1989) demonstrated that corneal endothelial cells were susceptible to damage from hydrogen peroxide. Interestingly, enzymatically generated superoxide anion produced endothelial damage, which could not be blocked with SOD but could be blocked by catalase. This indicates that hydrogen peroxide rather than superoxide is the toxic agent involved. In addition, these authors showed that young animals were better able to withstand oxidative stress to the eye than older animals. They, therefore, proposed a link with CAT, as the effects of oxidative stress in young animals could be made comparable to those in older animals by depletion of CAT with 3-aminotriazole.

The GSH reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) also promotes corneal swelling in the isolated cornea. The addition of GSH prevents the action of BCNU as the cornea needs a constant supply of NADPH for maintaining adequate concentrations of reduced glutathione for the detoxification of hydrogen peroxide. It has been shown that hydrogen peroxide and BCNU primarily affect the permeability of the endothelial cells rather than the active processes transporting sodium and chloride ions across the membrane (Riley, 1985).

Other studies have focused on corneal collagen. *In vitro* studies of collagen aggregation and degradation by Chance *et al.* (1991) suggested that collagen cross-linking may be a prerequisite for degradation. Cross-linking in this study was due to the hydroxyl radical, which they suggested was consistent with age-dependent increases in insoluble collagen.

Exposure to UVB radiation has a profound effect on the corneal endothelium. Following exposure to UV radiation, the cornea swells, the extent and duration of which is directly related to the magnitude and duration of the exposure (Riley *et al.*, 1987). At very high *in vivo* exposures, these authors reported a decrease in ascorbate concentration and an increase in protein content, which they suggested resulted from a breakdown of the blood–aqueous barrier. They concluded that UV radiation may cause or promote changes in the endothelium associated with ageing.

The cornea is avascular and is thus immunologically privileged to some extent. Inflammatory cells and antibodies can only enter the cornea from the corneoscleral limbus or from the aqueous. However, if inflammation occurs, the almost inevitable result is opacification of the cornea with visual deterioration, which is frequently permanent.

The most potent inducers of corneal inflammation are infection and trauma (Plate 1). Infective agents include bacteria (especially secondary to contact lens wear), viruses (the most destructive being herpes simplex and zoster) and chlamydia (the cause of trachoma, a leading cause of blindness worldwide). All these infections can lead to corneal opacification following the influx of inflammatory cells and the release of inflammatory mediators. The most destructive corneal inflammation follows chemical or thermal burns and much research into the underlying mechanisms involved in corneal inflammation has used the alkali-injured rabbit cornea as a model (Levinson *et al.*, 1976). Superoxide radical-scavenging agents, in particular ascorbate, applied topically to recently alkali-injured rabbit eyes have been shown to limit corneal damage and prevent corneal perforation (Pfister and Patterson, 1980; Nirankari *et al.*, 1981). Pfister and colleagues (1982) found that ascorbate concentration in aqueous was reduced after alkali injury. Reim (1992) who studied alkali injuries in human cornea, considered that ischaemia contributed significantly to early damage, and resulted in nutrient deficiency and accumulation of toxic metabolities in the damaged cornea. He reported that the initial response to injury was a polymorphonuclear (PMN) infiltration into the cornea following the liberation of histamine, prostaglandins, angiotensin and leukotrienes from damaged cells. After 10 days, lymphocytes and macrophages invade the necrotic tissue. The success of topically applied citrate in decreasing the number of corneal perforations following alkali damage is probably due to inhibition of PMNs (Pfister *et al.*, 1988).

3. *Aqueous Humour*

Aqueous humour occupies the anterior and posterior chambers of the eye between the cornea and the anterior surface of the lens. It is a transparent colourless fluid with a unique composition of electrolytes, proteins, biologically active substances and low molecular weight solutes such as glucose, reduced glutathione and ascorbate. These compounds are derived principally from the plasma by passive diffusion and from the non-pigmented ciliary epithelium by active secretion involving plasma membrane bound Na^+/K^+-ATPase (Cole, 1984). Metabolites also enter the aqueous from surrounding tissues such as corneal endothelium, lens and vitreous, which, in turn, utilize the nutrients present. For example, the lens and the cornea both depend on the aqueous for their glucose requirements.

The intraocular pressure depends primarily on the rate of secretion of aqueous humour. The most notable constitutional difference between aqueous humour and blood plasma lies in their protein contents. Protein concentration in the aqueous is 5–15 mg/100 ml, and that of the plasma is 1000-fold greater (6–7 g/100 ml). However, all the plasma proteins are present in aqueous humour – albeit at very low concentrations, therefore it is

widely accepted that aqueous humour is derived from plasma. The concentration of ascorbate in the aqueous humour is approximately 20-fold higher (1.1 mol/l) than that of plasma (0.05 mmol/l) and is predominantly transported into the aqueous from the ciliary epithelium in its reduced form (Spector and Garner, 1981). In 1947, Kinsey showed that when the plasma concentration of ascorbic acid was raised to 3 mg/100 ml, the concentration of the aqueous also rose until a level of 50 mg/ml was reached. Raising the plasma concentration beyond the saturation level caused no further rise in the concentration in the aqueous. According to Linner (1952) the ability of the eye to maintain this concentration of ascorbic acid depends critically on the blood flow. Unilateral carotid ligation caused a 17% fall in ascorbic acid concentration on the ligatured side.

Ascorbate is known to act as a water-soluble antioxidant, reacting rapidly with superoxide, hydroxyl and peroxyl radicals. However, reduced ascorbate can react non-enzymatically with molecular oxygen to produce dehydroascorbate and hydrogen peroxide. Also, ascorbate in the presence of light, hydrogen peroxide and riboflavin, or transition metals (e.g. Fe^{2+}, Cu^{2+}), can give rise to hydroxyl radicals (Delaye and Tardieu, 1983; Ueno *et al.*, 1987). These phenomena may also be important in oxidative damage to the lens and subsequent cataract formation.

Hydrogen peroxide is present in normal aqueous (approximately 30 μmol/l) whilst mean concentrations of around 70 μmol/l have been reported in aqueous from patients with cataracts, supporting a role for oxidative damage in the formation of cataracts (Spector and Garner, 1981). Diamine oxidase is one of the few enzymes to have been detected in bovine aqueous humour (albeit in trace quantities). It has been suggested that the hydrogen peroxide present in aqueous may be the product of the oxidative deamination of diamine substrates. This hypothesis is still unproven, since diamine oxidase substrates have not been identified in aqueous humour.

GSHPx, CAT and SOD, which normally protect cells from free-radical damage have not been detected in aqueous humour. It has therefore been suggested that damage by free radicals and hydrogen peroxide to the anterior segment is prevented by a non-enzymatic extracellular oxidoreduction system involving a constant supply of reduced glutathione to the aqueous fluid from the ciliary epithelium, cornea and lens (Riley, 1983).

4. *Lens*

The lens is an avascular transparent tissue enveloped in the lens capsule, a basement membrane composed of type IV collagen (Harding and Crabbe, 1984). The single layer of epithelial cells under the anterior portion of the capsule divide throughout life, accumulate crystallin proteins, and synthesize membrane lipids and proteins to mature into lens fibre cells. A mature lens fibre is a lipid bilayer plasma membrane containing proteins (crystallins and cytoskeletal proteins), polyribosomes (which may still be able to make protein as a repair mechanism), water, amino acids, low molecular weight solutes (including urea and ascorbic acid) and ions (Na^+, K^+, Ca^{2+}). When fully differentiated the lens fibres lose their nuclei, mitochondria and other cytoplasmic elements. The continued presence of these structures would lead to the absorption and scattering of light (Berman, 1991).

The major structural protein components of the lens are the crystallins whose function is to maintain transparency and refractive power. They constitute about 90% of the soluble proteins in the lens and consist of two superfamilies α and βγ (βHβL and γ) with molecular masses of between 900 and 21 kD (Table 9.1).

Small angle X-ray-scattering studies and light-scattering studies of lens extracts show that the transparency of the lens is the result of the short-range spatial order of lens proteins (Delaye and Tardieu, 1983). The molecular structure of the individual crystallins is well ordered even though the overall pattern in the lens may appear chaotic.

Morphologically, the lens is arranged so that dedifferentiated or disorientated lens fibres are not eliminated but are pushed to the core (Spector, 1984,). Damage to the cell-fibre membranes or to the proteins they contain is irreversible. The lens is therefore dependent on the peripheral cells of the epithelial layer for protection against insult.

Cataract, defined as a partial or total opacity of the lens, is a major cause of visual impairment in man, often with serious personal, social and economic consequences (Fig. 9.2). The surgical management and visual rehabilitation of adult onset cataract is, however, relatively straightforward. The management of cataract in young children is a more difficult problem owing to its deprivational effects upon the visual system during critical periods of the child's early development (Cole, 1984). This often leads to irreversible impairment of visual development. It is therefore important to determine the aetiological or risk factors that predispose to the development of cataracts in

Table 9.1 Characteristics of mammalian crystallins

Property	α	*βHβL*	γ
Molecular mass	600–900 kD	50–200 kD	21 kD
Molecular form	Aggregate	Aggregate	Monomer
Polypeptide composition (primary gene products)	20 kD (αA2: αB2) αA1: αB1	20–30 kD	21 kD
Thiol content	Low	High	High
Isoelectric points	pH 4.8–5.0	pH 5.7–7.0	pH 7.1–8.1
Electrophoretic mobility (to anode at pH 8–9)	High	Medium	Low

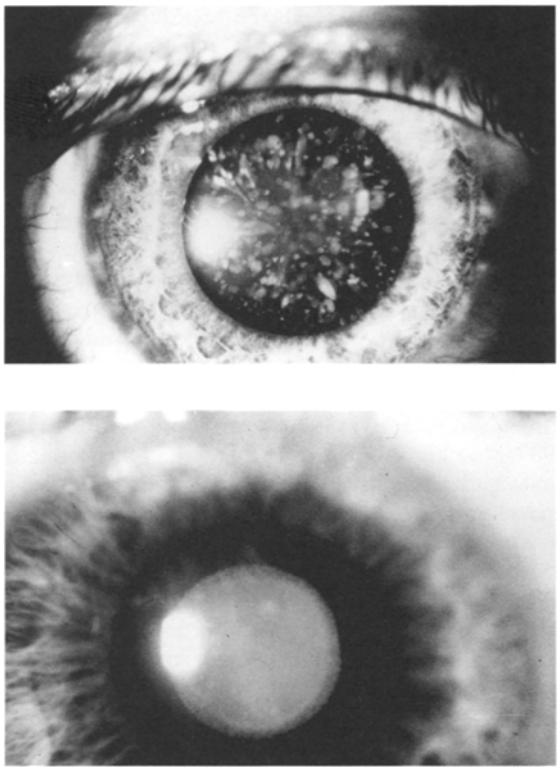

Figure 9.2 Multiple lens opacities, or partial cataract (top) and total cataract.

children, and to apply this knowledge to avoid or delay the onset of cataract and subsequent visual deprivation.

In recent years, damage to the lens by reactive oxygen species have been implicated in the aetiology of cataract formation (Augusteyn, 1981; Harding, 1981; Balzas and Delinger, 1984). This type of damage is normally prevented by cellular antioxidant defences such as SOD, GSHPx, vitamin E and ascorbate (Wayner *et al.*, 1987). Nevertheless, the potential for generating damaging concentrations of free radicals in and around the lens is considerable. For example, the lens is constantly exposed to light, which it absorbs in the near-UV region (300–400 nm); this has been shown to cause oxidative damage directly and through photosensitizers (Green *et al.*, 1984; Spector, 1985). The principal molecules in the lens that absorb light in this UV region are free or bound tryptophan, tyrosine and phenylalanine, of which tryptophan absorbs over 95% of the incident energy. *In vitro* experiments using lens homogenates have shown that, in the presence of UV radiation and oxygen, oxidative cleavage of tryptophan to *N*-formylkynurenine occurs. *N*-formylkynurenine as well as 3-hydroxynurenine, another oxidation product of tryptophan, have been shown to act as photosensitizers in the generation of singlet oxygen and are able to mediate in the photopolymerization of lens protein (Dillon, 1984). Hydrogen peroxide, a potentially toxic substance, is also produced metabolically within the lens (Riley, 1983).

The human lens is rich in ascorbate, which is required for normal collagen synthesis and acts as a water-soluble antioxidant, reacting rapidly with superoxide, hydroxyl and peroxyl radicals. However, ascorbic acid can undergo auto-oxidation and, at certain concentrations, can form hydroxyl radicals with hydrogen peroxide in the presence of light and riboflavin as described above (Delaye and Tardieu, 1983; Ueno *et al.*, 1987).

The peripheral epithelial cell layer and lens fibre membranes make up only 3–5% of the net weight of the human lens, but are the major region of metabolic activity. They are also an important first line of defence for protecting lens fibres and associated crystallins from oxidative damage. The hydroxyl radical is potentially the most damaging free radical *in vivo* and reacts predominantly with cell-membrane lipids. Oxidation of the membrane fatty acids produces lipid peroxide radicals, which are also highly reactive. Thus a chain reaction develops. At present vitamin E (α-tocopherol) is the only known lipid-soluble chain-breaking antioxidant *in vivo*. Its major role appears to be the protection of lipid peroxidation by scavenging lipid peroxyl radicals thereby breaking the chain reaction (Wayner *et al.*, 1987).

Studies using lens cultures in the presence of free-radical-producing systems have shown formation of cataract *in vitro* with a concomitant increase in indices of lipid peroxidation and changes in Na^+/K^+-ATPase activity leading to an ionic imbalance within the lens fibres (Garner *et al.*, 1983). Changes in monovalent and divalent cation concentrations, ATP levels and water content have all been reported in cataractous lenses (Riley, 1983; Balzas and Delinger, 1984; Deussen and Pau, 1989). How lipid peroxidation initiates these changes is not yet fully understood; however, the mechanism is likely to be either direct oxidation of membrane proteins or oxidation of the membrane lipids, which may affect membrane fluidity and protein function (Richter, 1987). The earliest abnormalities in the Nakano mouse, which has an early onset cataract with nuclear lens opacities occurring about 24 days after birth, is reduced Na^+/K^+-ATPase activity. The osmotic changes probably lead to reduced amino-acid uptake and impaired synthesis of lens proteins.

Primary and secondary products, and end-products of lipid peroxidation have all been shown to accumulate in senile cataracts (Babizhayev, 1989b; Simonelli *et al.*, 1989). Accumulation of these compounds in the lenticular epithelial membranes is a possible cause of damage preceding cataract formation. In senile cataracts there is also extensive oxidation of protein methionine and cysteine in both the membrane and cytosol components (Garner and Spector, 1980), while in aged "normal" lenses a lesser extent of oxidation was confined to the membrane. The authors therefore suggested that oxidation of membrane components was a precataract state.

If attacked by free radicals, lens proteins become cross-linked, change their shape and solubility and lose their transparency (Balzas and Delinger, 1984). The $\beta\gamma$ crystallins have a high thiol content and are most susceptible to oxidative modifications. *In vitro* oxidative modification of lens crystallins by hydrogen peroxide in the presence of chelated iron leads to covalent cross-linking of polypeptides and formation of high molecular weight insoluble protein aggregates. These modified protein aggregates are similar to those found in senile cataracts and are further evidence in support of the suggestion that free radicals are involved in cataract formation (Zigler *et al.*, 1989). The high molecular weight disulphide-linked aggregates contain a 43 kD polypeptide. Immunofluorescent studies have shown this polypeptide to be located at the lenticular plasma membrane suggesting that the formation of the high molecular weight aggregates is initiated in the lens membrane.

There have been some studies that suggest that reduced glutathione in the lens has the important function of protecting protein thiol groups from free-radical damage, and therefore preventing crystallin cross-linking and aggregation. Using a model of X-ray-induced cataract, it has been shown that one of the earliest effects of radiation was the decrease in reduced glutathione concentrations; this was quickly followed by loss of protein thiol groups, protein aggregation and subsequent formation of opacities 2 months after irradiation (Reddy and Giblin, 1984). With the accumulation of insoluble protein aggregates, there is also a concomitant increase in lens "browning" and fluorescence. It is thought that photoactivated tryptophan residues and reactive free radicals may be involved in this process. These same photoactivated species have also been shown to be involved in the inactivation of lens enzymes including Na/K-ATPase, Mg-ATPase, glutathione reductase, catalase, xanthine oxidase and cytochrome oxidase (Zigman, 1985).

Studies by Martensson *et al.* (1989) have shown a close relationship between lens glutathione concentrations and cataract formation. Specifically, they have found that treatment of newborn rats and mice with buthionine sulphoximine, an inhibitor of glutathione synthesis, leads to the rapid development of cataracts, which can be prevented by treatment with glutathione monoester but not by glutathione. The cataracts are associated with glutathione deficiency in the lens epithelium, which undergoes severe degeneration. Further investigation of this phenomenon has revealed that deficiency of glutathione leads to a marked decrease in the concentration of lens ascorbic acid (Martensson and Meister, 1991). More importantly, provision of ascorbic acid to glutathione-depleted newborn rats partially protected against cataract formation.

From the evidence, it can be hypothesized that oxidation initiated at the membrane leads to peroxidation of membrane lipids and proteins with subsequent changes in the structure and permeability of the membrane. In the presence of weakened membrane "defences" and further lipid peroxidation, oxidative cross-linking and aggregation of the crystallins occurs which results in lens opacity.

Evidence that oxidative damage may be involved in cataract formation *in vivo* has come from a number of studies in animals and human beings. Cataracts have been induced experimentally in animals by methods such as radiation (Ross *et al.*, 1983), diabetes mellitus (Abraham *et al.*, 1989), systemic methyl prednisolone, aminotriazole and hyperbaric oxygen (Varma *et al.*, 1984), all of which have been shown to increase indices of free-radical activity and lipid peroxidation. Treatment with antioxidants such as vitamin E (Richter, 1987), ascorbate (Varma and Richards, 1988), butylated hydroxytoluene (Ansari and Srivastava, 1990) and carnosine (Babizhayev, 1989a) has been shown to be effective in preventing or limiting cataract formation. In a canine model of senile cataract, in which increased lipid peroxidation was demonstrated, antioxidant therapy partially restored the transparency of the lens (Boldyrev *et al.*, 1987). It has also been shown that the posterior subcapsular cataracts that develop in the RCS rat appear to be initiated by oxidative products of unsaturated fatty acids in the retina, which leak into the vitreous humour and attack the posterior surface of the lens (Spector, 1984). This mechanism may be similar to that in human cataracts associated with retinal dystrophies, a view strengthened by the observation that the opacities associated with retinitis pigmentosa and Usher syndrome in humans are first apparent posteriorly in the lens.

Patients in which oxidative damage may be an important aetiological factor cataract formation include those with Down's syndrome, since there is now evidence that they have increased indices of free-radical activity and lipid peroxidation. It has been suggested that this is due to the increased levels of Cu/Zn-SOD (carried on chromosome 21) generating increased concentrations of hydrogen peroxide (Bras *et al.*, 1989). In the presence of superoxide radicals these produce highly reactive hydroxyl radicals.

Patients with both type I (juvenile onset) and type II (non-insulin-dependent) diabetes frequently develop cataract, retinal damage, atherosclerosis and neuropathies. An important feature of these complications appears to be oxidative stress. Diabetic patients have been reported to have increased indices of lipid peroxidation and ascorbate utilization in conjunction with reduced concentrations of lens and erythrocyte reduced glutathione and platelet vitamin E, all of which are suggestive of an ongoing oxidative process. It has been shown in experimentally induced diabetes in animals, using streptozotocin or alloxan, that these agents produce high concentrations of reactive peroxides in the islets of Langerhans, which are susceptible to damage since their levels of glutathione peroxidase are low.

It has been suggested that sugars such as glucose, which is present in high concentrations in diabetic patients, can

produce oxidizing substances and free radicals directly. In the presence of transition metals, monosaccharides can oxidize to produce hydroxyl and superoxide radicals as well as hydrogen peroxide and toxic carbonyls, which act with amino groups of proteins. The hydroxyl radicals formed can then react on other monosaccharide molecules to form hydroxyalkyl radicals,which decompose to yield more carbonyls (Fig. 9.3).

There have been many epidemiological studies assessing the factors contributing to cataract formation. There is now increasing evidence that antioxidant supplementation may reduce the risk of cataract and, conversely, that the risk of cataract associated with low antioxidant status is increased. For example, a recent Canadian study (Knekt *et al.*, 1992) showed that patients with cataract were less likely to take antioxidant (vitamins E and C) supplements than normal control subjects. Non-users of vitamin E had a 2.5-fold and non-users of vitamin C a 4-fold increase in risk of cataract. In another study it was found that individuals with low serum vitamin E and β-carotene concentrations had a 2.6-fold risk of cataract compared to those with higher levels. Poor diet and exposure to light have been shown to be implicated in the early onset of "senile" cataract in regions of India. Exposure to UV light and diets with a low content of natural antioxidants such as vitamins C and E appear to be major factors.

5. *Vitreous Humour*

The vitreous is a transparent extracellular matrix occupying the space between the posterior lens and the retina and, in the majority of vertebrate species, constitutes the major part of the volume of the eye. Embryologically it can be considered as the basement membrane of the retina. It provides a mechanical support for surrounding tissues and acts as a shock absorber by virtue of its viscoelastic properties (Balzas and Delinger, 1984). Vitreous consists mainly of water (98%) and colloids (0.1%) with ions and low molecular weight solutes making up the remainder. It is not fully developed at birth, and changes in both volume and chemical composition occur postnatally.

The mature vitreous contains a class of mononuclear phagocytic cells called hyalocytes (Balzas and Delinger, 1984). These cells are generally embedded in the vitreous humour 20–50 μM away from the basal lamina, forming a single layer of scattered cells. In the developing eye they are located more centrally and are capable of synthesizing the main solid constituents of the vitreous gel.

Hyaluronic acid and collagen are the two main structural components. The collagen is similar to that found in cartilage, consisting of three alpha-chains and known as type II collagen, although subtle differences are now recognized (Swann and Constable, 1972; Swann, 1980).

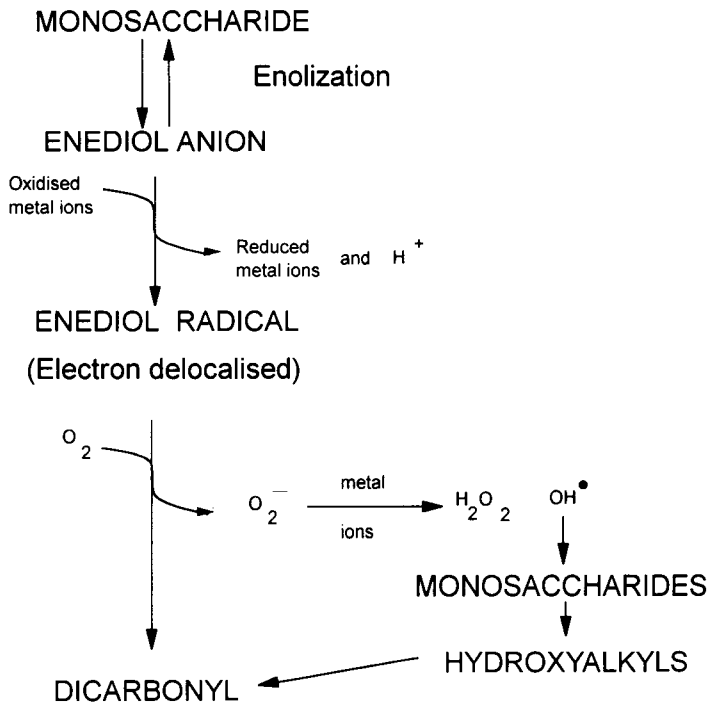

Figure 9.3 The proposed involvement of free radical and transition metals in the formation of dicarbonyls from monosaccharides.

Random collagen fibres provide an insoluble gel-like primary network and polyanionic hyaluronic acid a secondary network, which confers the viscoelastic property and stabilizes the collagen network (Balzas and Delinger, 1984).

Increased vitreous fluidity, synchysis, is known to be associated with ageing in the normal human eye and occurs in some pathological conditions (Green *et al.*, 1984). It is thought that this is due to alterations in the composition or conformation of hyaluronic acid and its interaction with the collagen network. Hyaluronic acid is known to be attacked and depolymerized by superoxide-generating systems, such as light, causing loss of viscosity (Ueno *et al.*, 1987). This is due to the superoxide-dependent formation of hydroxyl radicals in the presence of transition metals (e.g. Fe^{2+}). Many experiments have been carried out whereby iron salts or fragments have been introduced into the vitreous of a variety of animal models, and increases in indices of oxidative damage have been correlated with structural and physiological degeneration of the visual system.

Under normal conditions, reactions involving iron are limited by the presence of an iron-binding protein similar to transferrin in the vitreous. Iron when bound to transferrin has a much reduced capacity for stimulating free-radical reactions compared to low molecular mass iron complexes. However, severe visual impairment is common in patients with intraocular iron overload, e.g. vitreous haemorrhage or those whose eyes have been penetrated by iron objects, and sometimes this leads to blindness. The pathological process in these cases is thought to be due to the generation of hydroxyl radicals within the vitreous and their effects on the vitreous itself and on surrounding structures such as the retina and lens.

6. *Retina*

The retina comprises two principal components, the non-neural retinal pigment epithelium and the neural retina. The retinal pigment epithelium is an essential component of the visual system both structurally and functionally. It is important for the turnover and phagocytosis of photoreceptor outer segments, the metabolism of retinoids, the exchange of nutrients between the photoreceptors, and the choroidal blood vessels and the maintenance of an efficient outer blood–retinal barrier.

The neural retina is organized into nine distinct layers but can be considered as two functional regions, the photoreceptor "light-detecting" and the inner "signal-processing" layers. The highly specialized photoreceptor layer is composed of two cell types: the rod cells, which provide scotopic (dim light/night) vision and the cone cells, which provide photopic vision, specialized for colour, and require a much higher degree of illumination than rod cells in order to function. In the human eye the retina has in the region of 120 million rods and six million cones. The density of the cone cells is highest in the centre around the fovea, whereas the rod cells are at their densest in the peripheral retina. There are no rods in the fovea centralis. The blood supply to the photoreceptors is provided by the choroidal circulation and accounts for approximately 75% of the oxygen consumed by the neural retina. The inner retinal layers, which require a much lower oxygen consumption, are supplied by the retinal circulation.

The membranes of the rod outer segment of the retina contain 40–50 mol% of the highly unsaturated fatty acid docosahexaenoic acid (C22:6ω3–DHA). This is the highest concentration found in any human tissue. DHA appears to be conserved in the retina and may be recycled by phagosomes in the retinal pigment epithelium, or released by the retinal pigment epithelium into the bloodstream for recycling through the liver and plasma. These retinal membranes also have a high phospholipid content (80–90 mol%) and a low cholesterol content (8–9 mol%), which, together with the high DHA concentration, account for their high fluidity. The significance of the unusual lipid composition of the retina is not yet fully understood but it has been suggested that it may be important for phototransduction. The light-sensitive pigment rhodopsin is embedded in a regular array within the lipid double layer of the membrane discs in the outer segments (Fig. 9.4). Rhodopsin changes its conformation rapidly when activated by a photon of light, causing it to move laterally and rotationally in the membranes of the rod outer segment. Thus a high degree of membrane fluidity would facilitate this dynamic behaviour.

Arachidonic acid, also present in the retinal outer segment membrane, is derived from dietary ω-6 series, and is the precursor of prostaglandins and thromboxanes. DHA, on the other hand, is derived from dietary ω-3 series and cannot take part in the synthesis of arachidonic acid pathway products (Kelly, 1991). Fatty acids provide an important store of energy in the body but, more importantly, they play a leading role in the structure and function of membranes, acting as substrates for the production of biologically active mediators, the eicosanoids, n-6 fatty acids and n-3 fatty acids, of which linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3) are the respective parent compounds that give rise to the different families of prostaglandins and leukotrienes (Fig. 9.5). Prostaglandins influence many physiological functions including platelet function, vascular tone and inflammation. Leukotrienes also are involved in inflammation.

It is now widely appreciated that polyunsaturated fatty acids (PUFAs) are highly susceptible to oxidative damage. Indeed, the process of lipid peroxidation was broadly defined as the "oxidative deterioration of polyunsaturated lipids" by Tappel (1979). The presence of a double

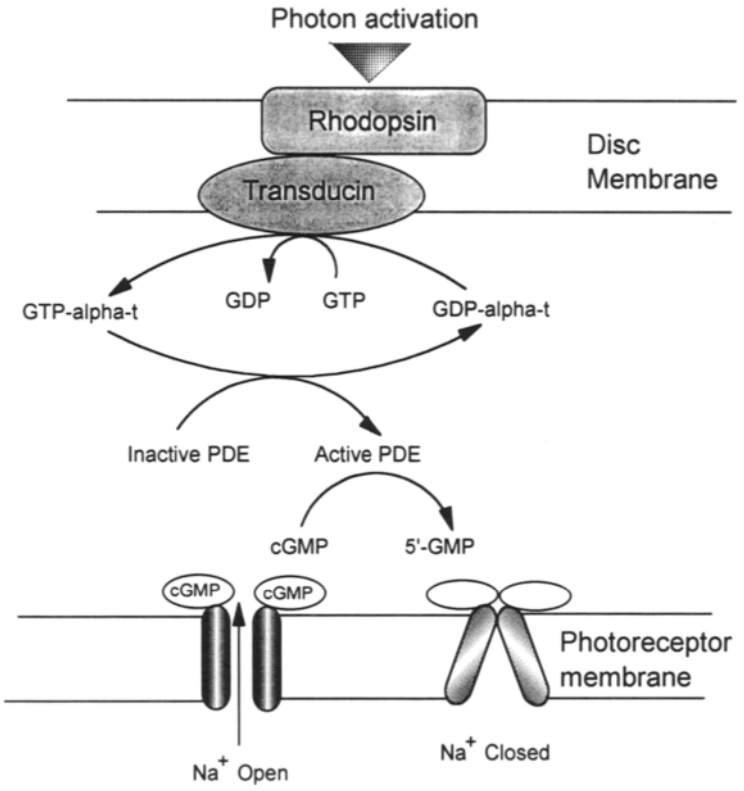

Figure 9.4 A diagrammatic scheme of the role of rhodopsin in phototransduction Photon-activated rhodopsin undergoes a conformational change causing GTP to be exchanged for GDP on the alpha subunit (alpha-t) of the G protein, transducin. The alpha-t subunit then dissociates itself from the tranducin and activates phosphodiesterase (PDE), which hydrolyses cGMP. As the concentrations of cGMP falls, cGMP is dissociated from the Na^+ channels, causing them to close, thus resulting in the hyperpolarizaion of the photoreceptor plasma membrane and transmission of the visual impulse.

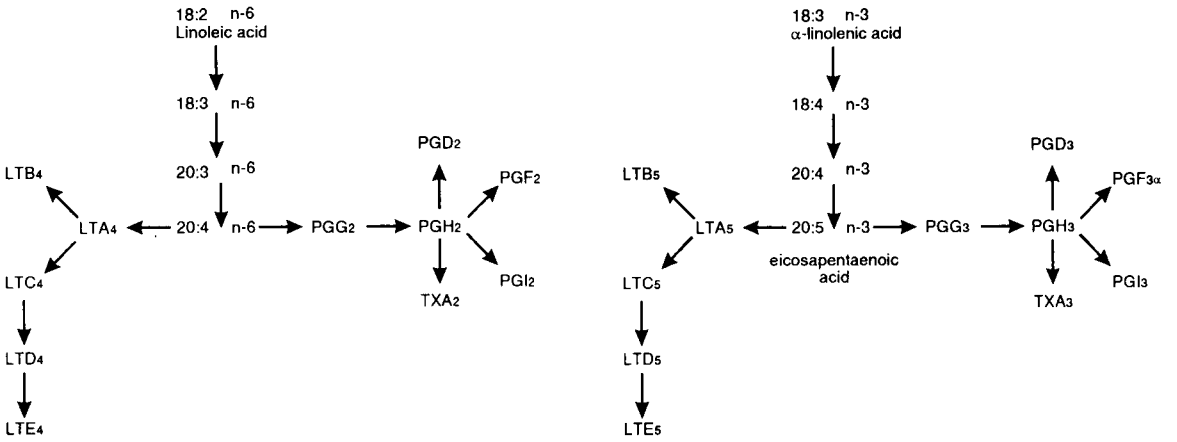

Figure 9.5 Prostaglandins (PG) and leukotrienes (LT) derived from arachidonic (*n* - 6) and eicosapentaenoic acid (*n* - 3). TX, thromboxanes.

bond in an unsaturated lipid leaves it more susceptible to attack by radicals; it weakens the C–H bonds on the carbon atom adjacent to the double bond, so easing the removal of the hydrogen atom. It was initially considered that all PUFAs would peroxidize at similar rates, even though early chemical experiments on autoxidation of free fatty acids frequently showed that the rate of autooxidation was dependent on the number of double bonds in the molecule. It has since been shown that ω-3 fatty acids are peroxidized at a much faster rate than fatty acids containing four double bonds (e.g. arachidonate), and that peroxidation studied in cellular membranes is mainly the peroxidation of ω-3 fatty acids containing five or six double bonds (Hammer and Willis, 1979).

The photoreceptor outer segment membranes are potentially very susceptible to oxidative damage by virtue of their high concentration of PUFAs. In normal conditions the retina is protected from oxidative damage, since glutathione enzymes capable of reducing hydrogen peroxide and lipid hydroperoxides are present in the rod outer segment, the site of peroxide formation. Under oxidative stress, such as constant illumination, both glutathione peroxidase and glutathione-S-transferase activities have been shown to be elevated (Penn *et al.*, 1987). Moreover, SOD and CAT have both been localized to the photoreceptor inner segments. Vitamin E (α-tocopherol) is also present in the rod outer segment; it is the only well-recognized chain-breaking lipid-soluble antioxidant *in vivo*, and appears to have an important role in the maintenance of the structure and function of the photoreceptor membrane.

The potential for free-radical damage in the retina, particularly the rod outer segment, is high since it is exposed to light daily and, with the abundant mitochondria photoreceptor cells have a high demand for oxygen, hence there is a constant flux of oxygen from the choriocapillaries across the photoreceptor membranes. The retina, in fact, consumes 5–10 times more oxygen per milligram than any other tissue tested. Retinal damage due to lipid peroxidation has been demonstrated following chronic/severe exposure to light, oxidative stress and antioxidant deficiency.

Oxidative stress in the form of iron toxicity is a serious clinical problem. Following penetration of the eye by an iron-containing foreign body, extensive retinal degeneration leads to loss of vision (Plate 2). In an acute model of iron toxicity in the frog eye, there is loss of phototransduction as determined by the electroretinogram, degeneration of the photoreceptor layers with sparing of all the other retinal layers, a decrease in the concentration of DHA and an increase in lipid hydroperoxide levels (Anderson *et al.*, 1984).

The longitudinal effects of experimental vitamin E deficiency on visual function in the rat have been studied by Goss-Sampson *et al.* (1992). After 12 months of deficiency, visual function as assessed by electroretinography was absent or grossly abnormal. This was associated with undetectable concentrations of vitamin E, a 90% loss of DHA from the retina and changes in retinal membrane fluidity. It appears that a deficiency of vitamin E will render the long-chain PUFAs including DHA susceptible to peroxidative degradation, leading to changes in retinal membrane structure and function.

Patients with abetalipoproteinaemia, a rare inborn disorder of lipoprotein metabolism, are totally deficient in vitamin E from birth and, if untreated, invariably develop a characteristic pigmentary retinopathy similar to that seen in retinitis pigmentosa and peroxisomal disorders. The same retinopathy has been observed in other patients with severe and chronic vitamin E deficiency. Aggressive vitamin E replacement therapy in all these patients has been shown either to prevent, to halt the progression of, or in some cases, to improve the characteristic visual abnormalities (Muller and Lloyd, 1982).

The sensitivity of the retina to light allows damage to occur either through the direct effects of radiation on the photoreceptors or by thermal injury. Both forms of injury produce retinal inflammation, which aggravates the damage. Wolken (1966) was the first to describe the disorganization of the structure of the retinal rod outer segment upon exposure to light. This was subsequently shown to be associated with a decrease in unsaturated lipid content and an increase in lipid peroxidation (Wiegland *et al.*, 1983). Photic injury was also associated with increased concentrations of reduced ascorbate in the retina (Organisciak *et al.*, 1985). Interestingly, vitamin C, but not vitamin E, was found to protect against photic damage.

6.1 RETINOPATHY OF PREMATURITY

Retinopathy of prematurity, first described by Terry (1942), was initially observed in low birthweight infants treated with high concentrations of oxygen at a time when the available technology did not allow for the assessment of tissue oxygenation. Since the dangers were unappreciated, many thousands of babies lost their eyesight before the association between treatment and retinal damage was fully appreciated (Silverman, 1980). After the identification of oxygen in the aetiology of retinopathy of prematurity, its use in the treatment of preterm babies was greatly reduced by modifying ventilation parameters, resulting in a dramatic decrease in the incidence and severity of the retinopathy. With the advent of blood gas monitoring in nurseries in the mid-1960s, the use of oxygen could be tailored more effectively to the needs of the individual infant. The incidence is again rising, however, as very low birthweight infants, born as early as 23 weeks gestation are surviving after prolonged supportive therapy.

The process of retinal vascularization is unique. Before the fourth month of gestation, there are no blood vessels in the human retina, the inner layers being nourished by the embryonic system of hyaloid vessels in the vitreous.

At about four months' gestation, mesenchymal cells emanating from the central hyaloid vessel at the optic disc invade the inner layers of the retina. These endothelial complexes develop into capillaries as vascularization proceeds anteriorly in all directions towards the ora serrata from the optic nerve. As this progresses, so the embryonic hyaloid vessels in the vitreous undergo regression. These retinal vessels do not, however, reach the most anterior portion of the retina until 8 months' gestation and the anterior temporal retinal periphery, farthest removed from the optic nerve, is not vascularized until about full term (Flower and Patz, 1971).

After controlling for birthweight or gestational age, prolonged administration of oxygen is still the variable most closely associated with the development of retinopathy of prematurity (ROP) (Majima, 1977; Shahinian and Malachowski, 1978; Gunn *et al.*, 1980; Campbell *et al.*, 1983). It has been suggested that hyperoxic exposure leads to the formation of endothelial precursor (spindle) cells with excessive junctional gaps; this in turn effectively halts the normal growth of retinal vessels growth (Kretzer *et al.*, 1984). On return of the infant to air breathing, the remaining retinal vessels that have not been permanently occluded rapidly proliferate. These new vessels lack structural integrity and haemorrhage is common. Not surprisingly, a number of other risk factors have been reported (Table 9.2).

The incidence and severity of ROP both rise with decreasing birthweight and gestational age. Fielder *et al.* (1992) have shown that the incidence of ROP in infants of < 1700 g birthweight is about 50%, although only 5% had severe disease. Severe ROP results in vitreous haemorrhage from new vessel formation on the immature retina, retinal traction and eventually retinal detachment (Fig. 9.6). In the most severe cases, retinal detachment is complete, resulting in total blindness. Treatment with cryotherapy or laser has halved the incidence of retinal detachment.

In addition to the well-established risk factors in the development of ROP, recent research has investigated the possibility that light may damage the immature retina (Robinson and Fielder, 1990). Light-induced free-radical oxidation of rod outer segment membrane lipids has been proposed as a mechanism for retinal damage following light exposure in ROP (Slater and Riley, 1970). Vitamin

Table 9.2 Risk factors for retinopathy of prematurity

Low birthweight (< 1000 g)
Prematurity (< 31 weeks gestation)
Supplemental oxygen
Hypoxia
Hypercapnia
Indomethacin
Acidosis
Light

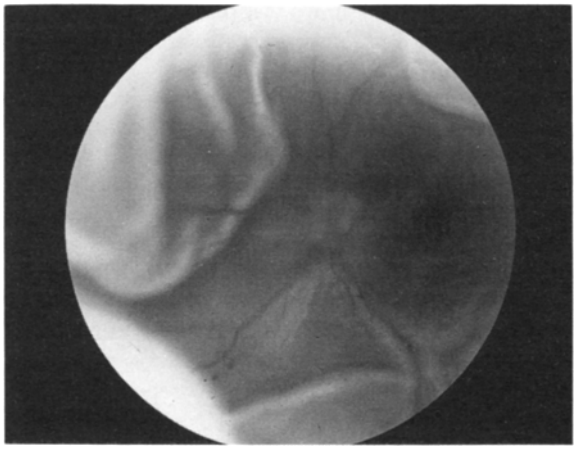

Figure 9.6 Retinal detachment associated with retinopathy of prematurity.

C is thought to aid vitamin E regeneration and its level in the preterm retina is 50% greater than adult levels (Nielsen *et al.*, 1988). The level of vitamin E in immature retina is, however, only about 10% of adult levels. Preterm infants are also deficient in selenium (Mantagos *et al.*, 1989). This trace element is a cofactor for both glutathione-S-transferase and glutathione peroxidase, enzymes both involved in preventing autoxidative damage.

Most of the studies investigating the pathophysiology of ROP have used newborn kittens and puppies. In these species, the eyes are relatively immature even in the normal full-term animal and the stage of retinal vascularization at birth is comparable to that observed in the human fetus at approximately 6 months' gestation (Patz, 1969). Numerous animal experiments have shown that the sensitivity to oxygen damage is directly proportional to the degree of immaturity of the retinal vessels (Ashton *et al.*, 1953; Ashton and Cook, 1954; Flower and Patz, 1971). In this respect it should be noted that the human infant born 12–16 weeks prematurely has part of the nasal retina incompletely vascularized and a large proportion of the anterior temporal retina completely unvascularized. Animal experiments have also shown that, when the retina is fully vascularized, the oxygen is no longer damaging. It therefore seems that incomplete vascularization of the retina and hyperoxic exposure are fundamental to the development of ROP.

Ashton and colleagues (1953) were the first to show that vasoconstriction occurs within the first few minutes after exposure to oxygen. The diameter of the retinal blood vessels is reduced by approximately a half, before returning to its original level. Continued exposure to oxygen results in progressive but gradual vasoconstriction over the next 4–6 hours. If the animal is removed to air at this point, the vasoconstriction is reversible but, if hyperoxic exposure is maintained, the vasoconstriction is sustained and the retinal vessels close completely. Animal

experiments have also shown that the response to oxygen is dose related, and the degree of vascular closure is directly proportional to the concentration of oxygen and the duration of exposure, and inversely proportional to the degree of development of the retinal vessels at the time of exposure.

It is known that the endothelial cells lining the capillary bed are markedly damaged during hyperoxic exposure. However, it is not at present known whether this damage is due to free-radical-induced injury of the endothelial cells or whether endothelial cells are simply responding to the diminished blood flow produced by the severe vasoconstriction. The theoretical basis for free-radical involvement is sound and it has been proposed that low oxygen tensions (hypoxia) followed by periods of reoxygenation are the most likely explanation for the disorder (Kelly, 1993).

As soon as it was appreciated that oxygen toxicity was somehow involved in retrolental fibroplasia, antioxidant administration was empirically investigated in both animal models and babies. In 1949, Owens and Owens reported a protective effect of vitamin E; unfortunately this could not be substantiated in subsequent controlled trials. Phelps and Rosenbaum (1977) investigated whether vitamin E supplementation would influence oxygen-induced retinopathy in kittens and found it to be beneficial in reducing the severity of the lesions. Nevertheless, vitamin E has not yet been used with much success in preterm babies.

7. *Uveitis*

As already mentioned, the retina is particularly susceptible to damage from activated inflammatory cells. Uveitis is inflammation of the uveal tract (iris, ciliary body and choroid). It may be associated with infection or trauma, but is most common in association with autoimmune disorders (such as juvenile chronic arthritis, Behçet's disease, ankylosing spondylitis) or idiopathic conditions such as sarcoidosis. Visual prognosis is worst in those patients with severe intraocular inflammation resulting from injury to retinal and uveal tissue in the form of cystoid macular oedema and increased vascular permeability (Dinning, 1981; Smith and Nozik, 1989). Chemical mediators of inflammation are derived from either plasma proteins, such as complement and fibrinolytic substances, or from inflammatory cells in the form of various lymphokines. Lipoxygenase and cyclooxygenase products of the arachidonic acid cascade (see above) have been shown to play an important role in ocular oedema and inflammation (Rao *et al.*, 1987a). Although B- and T-lymphocytes initiate the process, it is polymorphonuclear leucocytes and monocytes that are subsequently attracted to the area of injury or irritation, which are the important parts of the host defence response (Fig. 9.7). When activated, these cells produce and release

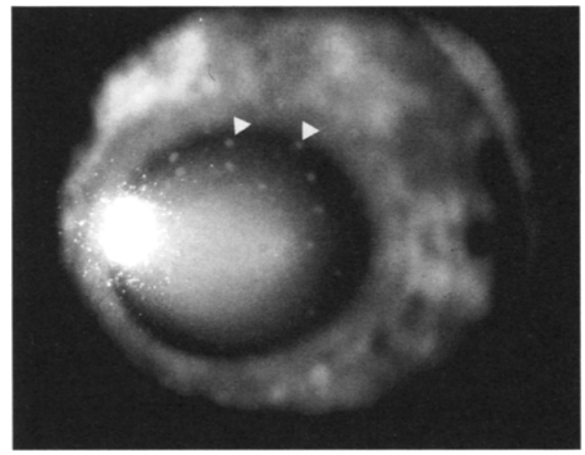

Figure 9.7 Mild anterior uveitis. Collections of macrophages (keratic precipitates) can be seen on the endothelial surface of the cornea (arrowheads).

deleterious products such as superoxide and hydrogen peroxide, which in turn form highly reactive hydroxyl radicals (Maestro *et al.*, 1980) that are thought to be largely responsible for the destruction of tissue in acute inflammation (Southorn and Powis, 1988). If properly regulated and the cause of the irritation removed or the injury repaired, the inflammatory response is turned off. Often, however, this appears not to be the case, and the inflammation persists and may actually contribute to the ocular injury, possibly in part due to the fact that the co-ordinating signals are not specific and lead to non-specific reactions.

It is widely appreciated that the retina contains an abundance of PUFAs that can undergo extensive oxidation following free-radical attack. Rao and colleagues in their various publications have reported the occurrence of lipid peroxidation in retinal S-antigen-induced uveitis and have shown that peroxidation can be suppressed by antioxidants (SOD and CAT) with marked reduction of uveal inflammation. This response coincided with a better-preserved retina and retinal pigment epithelium. These findings led Rao and colleagues to suggest that reactive oxygen metabolites (presumably from invading inflammatory cells) play a major role in both the direct dysfunction of ocular tissue and the amplification of the inflammatory process in experimental uveitis.

A number of different animal models of uveitis have been developed) including that induced by organ-specific ocular antigens such as retinal S-antigen, rhodopsin and lens protein (Wacker *et al.*, 1977; Rao *et al.*, 1979). Other models are based on the injection of proteins foreign to the host, such as intravitreal injections of albumin or γ-globulin (Zimmerman and Silverstein, 1959; Kaplan *et al.*, 1979). More recently, a third group of models has been developed based on the injection of inflammatory mediators such as interleukins-1 and 2, and tumour necrosis factor (Bhattacherjee and Henderson, 1987;

Rosenbaum *et al.*, 1988; Samples *et al.*, 1989). Rao (1990) has suggested that the primary inflammatory response in all these models based on intravitreal injection is an acute vitritis rather than uveitis.

Control of these inflammatory events by drugs is now forming an important area of investigation. Glucocorticoid and non-steroidal agents have been used extensively, but with relatively little success (Jaanus, 1984; Polansky and Weinreb, 1984). Although efficient in reducing recurrence of chronic disease states such as uveitis, associated side effects usually preclude prolonged administration. In an attempt to offset this disadvantage, research has focused on the potential benefit of free-radical scavengers and a wide range of antioxidants have been considered, including sodium benzoate (Rao *et al.*, 1987b), vitamin E (Tripathi and Tripathi, 1984; Tanaka *et al.*, 1989), dimethyl sulphoxide (Coles *et al.*, 1986; Skrypuch *et al.*, 1987), desferroxamine (Rao *et al.*, 1986), ascorbic acid (Pirie *et al.*, 1970; Williams and Paterson, 1986; Varma and Richards, 1988), CAT and glutathione peroxidase (Guy *et al.*, 1989a, b). All of these agents have been shown to provide some benefit in animal models and this is thought to be due to the elimination of phagocyte-generated oxygen metabolites, although probably other factors are also involved. For example, antioxidants can alter chemotaxis, antibody formation and T-cell proliferation (Bowern *et al.*, 1984).

8. *Acknowledgement*

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9. *References*

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10. Free Radicals in Gastrointestinal and Hepatic Disease

Nicola J. Simmonds

1. Introduction

Free radicals are involved in the pathophysiology of many gastrointestinal and hepatic diseases (Arthur, 1988; Braganza, 1989; Rozga, 1989; Verspaget *et al.*, 1991; Babbs, 1992; Harris *et al.*, 1992; Stark and Szurszewski, 1992; Van der Vliet and Bast, 1992). Reactive oxygen metabolites (ROM) may be formed by activated inflammatory cells, during the metabolism of xenobiotics or from xanthine oxidase during ischaemia–reperfusion injury (Blake *et al.*, 1987; Cross *et al.*, 1987; Grisham and Granger, 1988; Braganza, 1989). Recent evidence relating to the role of nitric oxide suggests that side effects of this free radical, at least, may be beneficial rather than harmful (Stark and Szurszewski, 1992).

The purpose of this chapter is to examine, in some detail, the evidence that abnormal metabolism of free radicals is of pathogenic importance in a number of diseases. In order to do this, the contribution of free radicals to the inflammatory or pathological process will be

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Table 10.1 Criteria for the evaluation of the pathogenic role of free radicals in inflammatory diseases

1	Free radicals should be present in increased quantities at the site of injury.
2	Exogenous application of free radicals should reproduce or enhance the changes observed.
3	Agents that prevent the formation or release, or that block the effects of free radicals should prevent or ameliorate the changes observed.

examined using criteria similar to those set out by Halliwell *et al.* (1992) (Table 10.1). This evidence will be discussed in relation to anatomical site, except for that relating to the mechanisms of carcinogenesis and its possible prevention by antioxidant treatment, which will be discussed as a topic in its own right. Throughout the discussions below it must be remembered that the measurement of reactive oxygen metabolites is difficult; many of the methods used are either indirect, relying on "trapping" or measurement of the end-products of oxidative damage, or relatively non-specific. Hence, rigorous proof that free radicals are important in the pathogenesis of disease is often lacking.

2. *Involvement of Free Radicals in the Inflammatory Response*

2.1 STOMACH AND DUODENUM

2.1.1 Increased Production of Free Radicals

Direct evidence of increased production of free radicals comes from chemiluminescence studies on antral and duodenal biopsies (Davies *et al.*, 1991, 1992a). Increased production of chemiluminescence by duodenal biopsies was related to the degree of inflammation assessed both macroscopically and microscopically. In the antrum, chemiluminescence was increased in patients with *Helicobacter pylori* compared to those without, even in the presence of comparable degrees of inflammation and neutrophil infiltration. In both sites, studies using the inhibitors superoxide dismutase (SOD), catalase, dimethyl sulphoxide (DMSO) and sodium azide confirmed that the increased chemiluminescence observed was due to the production of free radicals and suggested that neutrophils were the main source of these (Davies *et al.*, 1992a, b). Gastric mucosal cells in culture exposed to ethanol produce superoxide (as measured by the SOD-inhibitable reduction of cytochrome c). The amount of superoxide produced correlates with the degree of cell damage (Mutoh *et al.*, 1990a).

Helicobacter pylori is associated with chronic gastritis, peptic ulceration and possibly involved in the pathogenesis of gastric carcinoma (Correa and Ruiz, 1992; Dixon, 1992; Sobala and Axon, 1992; Wyatt, 1992). Whilst many pathogenic mechanisms have been postulated in *Helicobacter pylori* infection (Rathbone and Heatley, 1992), there is now good evidence that *Helicobacter pylori* is chemotactic for human neutrophils and monocytes (Kozol *et al.*, 1991; Mai *et al.*, 1991; Nielsen and Andersen, 1992a) and also activates phagocyte oxidative metabolism (Mooney *et al.*, 1991; Davies *et al.*, 1992c; Nielsen and Andersen, 1992b). That *Helicobacter pylori* is chemotactic for human neutrophils whereas *Helicobacter mustelae*, which is not associated with neutrophil infiltration in the ferret, is not (Kozol *et al.*, 1991), suggests that this factor may be important in determining the neutrophil infiltration and subsequent activation in the *H. pylori*-associated gastritis in humans.

Indirect evidence for increased production of free radicals in human peptic ulcer disease comes from studies of circulating antioxidants (Jankowski *et al.*, 1991) and studies demonstrating decreases in gastric juice ascorbic acid in association with *H. pylori* infection (Rathbone *et al.*, 1989; Sobala *et al.*, 1991a). In addition, the ascorbic acid in such patients is predominantly in its oxidized form. Eradication of *Helicobacter pylori* results in an increase in gastric juice ascorbic acid concentrations (Sobala *et al.*, 1992). Kishi *et al.* (1990) have demonstrated a decrease in the levels of SOD activity in the margins of peptic ulcers, followed by increases in activity in healing ulcers. This may reflect the induction of SOD in response to oxidative stress. Such findings are consistent with decreases in levels of copper–zinc SOD in the gastric mucosa in the rat model of diethyldithiocarbamate-induced gastric ulceration (Ogino *et al.*, 1990) and with the demonstration of induction of manganese SOD in rat epithelial cell lines by lipopolysaccharide (LPS), tumour necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) (Valentine and Nick, 1992).

Studies in animals have also provided some indirect evidence of increased free-radical production. Yoshikawa *et al.* (1991), Takeuchi *et al.* (1991a) and Itoh *et al.* (1992) have shown an increase in measures of lipid peroxidation in the gastric mucosa of rat models of gastric ulceration, induced by ischaemia–reperfusion (I/R), indomethacin or by LPS. However, Pihan *et al.* (1987) failed to show increases in lipid peroxidation in rat gastric mucosal damage induced by ethanol or aspirin. Shaw *et al.* (1990) have shown that a large dose of ethanol administered to rats *in vivo* decreases gastric GSH. Similarly, 80% ethanol sprayed on to human gastric mucosa at endoscopy caused significant decreases in GSH and in total sulphydryl levels (Loguercio *et al.*, 1990). Changes in the mucosal levels of antioxidant enzymes in response to acute gastric mucosal injury in rats treated with ethanol and hydrochloric acid, particularly when considered in relation to the timing of the mucosal damage, suggest that these may be secondary to the damage (Mozsik *et al.*, 1991). Hence decreases in antioxidants do not necessarily implicate free radicals in the production of mucosal damage.

2.1.2 Production of Mucosal Damage

Cultured gastric mucosal cells have been used to demonstrate cytotoxicity (as measured using ^{51}Cr release) due to nascent hydrogen peroxide or to ROM generated by glucose/glucose oxidase (Hiraishi *et al.*, 1990) or by xanthine/xanthine oxidase (Hiraishi *et al.*, 1987; Kobayashi *et al.*, 1991). Manoeuvres that decrease intracellular reduced glutathione (GSH) increase ROM-induced damage (Hiraishi *et al.*, 1990). Indomethacin irradiated with hydroxyl radicals caused significantly more gastric mucosal damage in rats than an equivalent dose of non-irradiated indomethacin (Del Soldato *et al.*, 1985). Local infusion of *S*-nitroso-*N*-acetyl-penicillamine (which spontaneously generates NO) in a rat model of gastric mucosal injury was protective in low doses, but produced haemorrhagic damage at higher doses (Whittle and Lopez-Belmonte, 1992). L-Nitromonomethyl arginine (L-NMMA), an inhibitor of NO production, increases gastric mucosal damage by ethanol in the uraemic rat, but not in control rats (Quintero and Guth, 1992). In rats pretreated with capsaicin, L-NMMA increases damage due to acid saline and indomethacin (Whittle, 1990). Inhibition of NO formation also appears to exacerbate damage due to I/R (Iwata *et al.*, 1992), and to prevent the protective effects of antacids and sucralphate (Konturek *et al.*, 1992a, 1992b; Lambrecht *et al.*, 1992). Hence, although in high doses NO may be harmful, NO appears to be involved in the regulation of vascular tone in the gastric microcirculation and to mediate some of the protective effects of antacids, sucralphate and mild irritants through its effects on blood flow (Pique *et al.*, 1990; Konturek *et al.*, 1992b; Lambrecht and Peskar, 1992; Quintero and Guth, 1992).

2.1.3 Effects of Antioxidants

2.1.3.1 Cell Culture

Ethanol-induced damage to cultured gastric mucosal cells can be decreased by SOD, catalase, desferrioxamine and DMSO (Mutoh *et al.*, 1990a). Cells cultured in the presence of *N*-acetyl-cysteine have an increased content of intracellular GSH, which correlated with decreased cytolysis on exposure to ethanol (Mutoh *et al.*, 1990b). Endogenous glutathione appears to be important in protecting cultured cells against oxidant stress (Hiraishi *et al.*, 1990). Nitecapone protects cultured human gastric cells against the effects of *tert*-butyl hydroperoxide and scavenges peroxyl radicals. It has been suggested that it may exert its gastroprotective action by an antioxidant mechanism (Nissinen *et al.*, 1991).

2.1.3.2 Animal Models

A number of studies have demonstrated protection against ethanol-induced gastric mucosal damage with several antioxidants, including SOD, catalase, allopurinol, DMSO, thiourea, dimethylthiourea, propylgallate and sulphydryl-containing substances, such as cysteine and methionine methyl sulphonium (Mizui *et al.*, 1987; Pihan *et al.*, 1987; Szeleyni and Brune, 1988; Smith *et al.*, 1991; Salim, 1990a, 1992a, 1992b). Similarly, the antiperoxidative drugs butylated hydroxytoluene, quercetin and quinacrine, also prevent ethanol-induced gastric damage in rats (Mizui *et al.*, 1987). However, studies by Kvietys *et al.* (1990) failed to demonstrate any protection from ethanol-induced gastric damage in rats by SOD, catalase or sodium benzoate. In their hands, neutrophils appeared to mediate the ethanol-induced damage by mechanisms not involving ROM. This is supported by the protective effect of neutrophil depletion. Szeleyni and Brune (1988) also failed to show a protective effect of catalase, glutathione peroxidase, caeruloplasmin, α-tocopherol and β-carotene. A similar paradox emerges from the studies of Robert *et al.* (1984) in which protection from ethanol-induced damage appeared to correlate with depletion of endogenous mucosal GSH. Furthermore, 5-aminosalicylic acid (5-ASA), which is known to have significant antioxidant activity (Yamada *et al.*, 1990), does not protect against ethanol-induced injury of the gastric mucosa (Saibil *et al.*, 1990).

Gastric mucosal injury induced by non-steroidal anti-inflammatory drugs such as aspirin and indomethacin has also been extensively studied, again with somewhat conflicting results. Several studies have shown a protective effect of SOD, catalase, hydroxyurea and desferrioxamine (Takeuchi *et al.*, 1991a; Vaananen *et al.*, 1991; Naito *et al.*, 1992). Del Soldato *et al.* (1985) also found aminopyrine, thiourea and its derivative, MK 447, and SAZ to be protective. Allopurinol has been shown to be both protective (Takeuchi *et al.*, 1991a) and ineffective (Vaananen *et al.*, 1991).

Ischaemia–reperfusion injury is thought to be one of the mechanisms by which the gastric mucosa is damaged. Again the results from different studies are not entirely consistent. SOD and allopurinol appear to be protective in all studies (Itoh and Guth, 1985; Perry *et al.*, 1986; Von Ritter *et al.*, 1986; Smith *et al.*, 1987; Kitahora and Guth, 1991; Andrews *et al.*, 1992). DMSO was effective in some studies (Perry *et al.*, 1986; Salim, 1992c) but not in others (Itoh and Guth, 1985). Desferrioxamine and a sodium tungstate diet, which inactivates xanthine oxidase (XO), were also found to protect the gastric mucosa against damage (Smith *et al.*, 1987; Andrews *et al.*, 1992). However, that ischaemia alone can cause significant mucosal damage, which cannot be prevented by any of the agents mentioned above (Itoh *et al.*, 1986; Andrews *et al.*, 1992), suggests that ROMs are not solely responsible for the damage observed.

Diethyldithiocarbamate can cause both gastric and duodenal ulceration in the rat. Such damage can be prevented by SOD, catalase, allopurinol and glutathione (Oka *et al.*, 1990a; Takeuchi *et al.*, 1991b). Rebamipide, a novel anti-ulcer compound, also inhibits damage and has been shown to inhibit chemiluminescence production by activated neutrophils, suggesting its anti-ulcer

activity may be by an antioxidant mechanism (Ogino *et al.*, 1992).

Studies on stress-induced gastric mucosal injury have also produced conflicting results. Tarnasky *et al.* (1990) were unable to show any benefit from a sodium tungstate diet, allopurinol or DMSO in a cold-water immersion restraint model in the rat. Similarly, although treatment with vitamin E decreased gastric lipid peroxidation in rats, it had no effect on the gastric ulceration induced by immobilization (Armario *et al.*, 1990). Hirota *et al.* (1989, 1990) have demonstrated inhibition of gastric injury induced by water-immersion restraint by increasing plasma GSH and also by a long-acting SOD derivative.

Salim (1992d) has performed several studies using reserpine to produce a chronic model of peptic ulceration in rats. Administration of allopurinol, DMSO, cysteine or methionine-*S*-methylsulphonium chloride protected against injury. In addition allopurinol and DMSO were found to stimulate healing in this model. In an acute model of duodenal ulceration induced by pentagastrin and carbachol allopurinol, DMSO, cysteine or methionine-*S*-methylsulphonium chloride all protected against injury.

2.1.3.3 Humans

Studies of the effects of antioxidants in human gastroduodenal disease are as yet relatively few. Loguercio *et al.* (1993) have shown that glutathione, given parenterally, prevents ethanol-induced gastric mucosal damage and also depletion of sulphydryl compounds. Salim (1990b) has also studied the effect of sulphydryl-containing agents (cysteine and methionine methyl sulphonium chloride) and found them to be beneficial in the treatment of gastric haemorrhage caused by non-steroidal anti-inflammatory drugs, and in the stimulation of healing in duodenal ulceration and protection against recurrence (Salim, 1992e). Allopurinol and DMSO have also been shown to be effective in the prevention of stress-induced gastric mucosal injury (Salim, 1989), in preventing relapse of duodenal ulcer, and in improving both healing and relapse rates in smokers with peptic ulcer disease (Salim, 1992f). In addition, it is perhaps of interest that cimetidine, ranitidine and omeprazole have all been shown to inhibit neutrophil superoxide production (Zimmerman and Millard, 1989; Wandall, 1992). However, this effect requires concentrations much higher than those found at pharmacological doses and so is likely to be irrelevant in terms of the mechanism of ulcer healing.

2.1.4 Conclusions

There is now a considerable body of evidence to suggest that ROMs are involved in the pathophysiology of gastroduodenal damage. However, we await confirmation that antioxidants, such as allopurinol, are beneficial in human peptic ulcer disease. Salim (1990c) found allopurinol to be superior to cimetidine in the prevention of relapse in duodenal ulcer. Confirmation of such results would not only support the hypothesis that ROMs are important but would also revolutionize the treatment of peptic ulceration.

2.2 SMALL BOWEL

2.2.1 Ischaemia–Reperfusion

The evidence relating to the role of free radicals in I/R injury has been reviewed recently (Zimmerman and Granger, 1992). In summary, hypoxic stress results in the accumulation of hypoxanthine from ATP and also in the conversion of NAD-reducing xanthine dehydrogenase to the free radical producing xanthine oxidase. When the intestine is reperfused XO-derived oxidants initiate the production and release of proinflammatory agents, which lead to neutrophil infiltration and activation. The neutrophils then mediate tissue injury through further free-radical production and through the release of proteases. The evidence supporting such an hypothesis is summarized below.

2.2.1.1 Increased Production of Free Radicals

Parks *et al.* (1988) have shown that ischaemia results in an increase in the proportion of XO as a percentage of total xanthine oxidoreductase activity from approximately 19% to 46% after 2 h. Although the rate of conversion appeared much slower than previously estimated, their calculations indicate that such concentrations of XO could produce sufficient free radicals to account for the rapid tissue damage occurring within 10 min of reperfusion. Direct evidence of increased production of free radicals with I/R comes from studies using sodium salicylate as a trap for the hydroxyl radical in rat intestine (Udassin *et al.*, 1991), electron spin resonance (e.s.r.) in the cat small intestine (Nilsson *et al.*, 1989) and low-level chemiluminescence in rat jejunum (Roldan *et al.*, 1989). Lipid peroxidation products are significantly increased after reperfusion of the ischaemic intestine (Younes *et al.*, 1987). The decrease in levels of GSH and SOD found in feline ileum in association with reperfusion is indirect evidence of increased free-radical production (Grisham *et al.*, 1986).

2.2.1.2 Production of Mucosal Damage

Several studies have demonstrated the production of mucosal damage and/or increased mucosal permeability by hydrogen peroxide, the hydroxyl radical, or by installation of xanthine or glucose oxidase (Grøgaard *et al.*, 1982; Parks *et al.*, 1984; Joubert-Smith *et al.*, 1991; Kohen *et al.*, 1992).

2.2.1.3 Effects of Antioxidants

Allopurinol, which inhibits XO and scavenges superoxide, prevents the increase in free-radical production

associated with reperfusion (Nilsson *et al.*, 1989; Roldan *et al.*, 1989). A number of antioxidants (allopurinol, pterin aldehyde, SOD, catalase, DMSO) have been shown to ameliorate I/R-induced changes in mucosal and vascular permeability, and tissue damage in animal models (Parks *et al.*, 1982, 1983; Parks and Granger, 1983; Granger *et al.*, 1986; Deitch *et al.*, 1988; Filez *et al.*, 1990; Kohen *et al.*, 1992). Similarly, a tungsten-supplemented diet, which inactivates XO, reduces I/R injury (Deitch *et al.*, 1988; Pitt *et al.*, 1991). Neutrophil infiltration following I/R can be reduced by allopurinol, SOD, catalase, desferrioxamine or dimethylthiourea, but not by DMSO (Grisham *et al.*, 1986; Zimmerman *et al.*, 1990). Prevention of neutrophil infiltration with allopurinol or SOD, or using antibodies directed against leucocyte adhesion molecules prevents the changes in permeability, and indices of oxidant stress and tissue damage (Grisham *et al.*, 1986; Hernanadez *et al.*, 1987; Schoenberg *et al.*, 1991a; Kubes *et al.*, 1992). Furthermore, recent studies have demonstrated the presence of an intestinal peroxidase that can be found in both mucin granules and in the mucus gel (Sibley *et al.*, 1991). This generates species toxic to bacteria and intestinal epithelial cells in the presence of hydrogen peroxide. Ischaemia–reperfusion results in a marked secretory response from intestinal goblet cells and hence release of intestinal peroxidase, which may contribute to the pathogenesis of tissue damage. Certainly, the installation of catalase, which removes its substrate hydrogen peroxide, prevents tissue damage (Joubert-Smith *et al.*, 1991). However, this will also prevent the formation of other cytotoxic species such as the hydroxyl radical. Further evidence that antioxidants prevent against the development of I/R injury comes from studies that show that previous ischaemic injury in the small intestine of rats results in prevention of further damage on subsequent ischaemic challenge in association with measured increases in mucosal glutathione peroxidase and catalase (Osborne *et al.*, 1992).

2.2.1.4 Conclusions

There is now a substantial body of evidence to support a role for free radicals in the pathogenesis of I/R injury in the small bowel, at least in animal models. The relevance of this to human disease processes and their possible treatment by antioxidants is less clear. It has been postulated that Crohn's disease results from a granulomatous vasculitis (Wakefield *et al.*, 1991). If vasculitis, and hence ischaemia, are important in determining the progress of Crohn's disease, then specific antioxidants might be expected to be beneficial. As yet, the only trial to date has been an open study of SOD (Emerit *et al.*, 1989). Whilst 23 of 26 patients had a good initial response and 16 patients remained in remission long term, the trial was complicated by the use of other drugs such as metronidazole and desferrioxamine. A good controlled trial of a XO inhibitor or other antioxidants is still awaited. Conceivably, such drugs might also find a place in the treatment of other forms of mesenteric ischaemia and also in the realms of small bowel transplantation.

2.2.2 Necrotizing Enterocolitis

This is a potentially fatal condition of the newborn associated with prematurity and thought to be related to decreased perfusion of the intestine (Kleinhaus *et al.*, 1992). Although there is no data relating to free-radical activity in humans, several animal models have been developed (Parks *et al.*, 1983). Pitt *et al.* (1991) showed that more severe injury occurred in the proximal small intestine, where levels of XO were highest. SOD has been reported to be protective in a rat model (Dalsing *et al.*, 1983) and more recently a tungsten-supplemented diet was also found to be of benefit (Pitt *et al.*, 1991). However, whether such treatment could modify the human disease, which usually develops within 10 days of birth, remains to be seen.

2.2.3 Radiation Enteritis

Radiation-induced damage is thought to be a consequence of free-radical generation and has many similarities with the changes produced by intestinal ischaemia (Parks *et al.*, 1983). Irradiation results in the production of the hydroxyl radical from water (Halliwell and Gutteridge, 1989) and radiation-induced gut damage can be attenuated using hydroxyl radical scavengers (Grzelinska *et al.*, 1982) or α-tocopherol (Empey *et al.*, 1992). Acetylsalicylic acid, which may have antioxidant activity, can also decrease radiation enteritis in humans (Ludgate, 1985). However, a recent study was unable to show any benefit from 5-ASA in the prevention of radiation enteritis (Baughan *et al.*, 1993).

2.3 COLON

2.3.1 Inflammatory Bowel Disease

2.3.1.1 Increased Production of Free Radicals

2.3.1.1.1 Direct evidence of increased production of free radicals

2.3.1.1.1.1 Animal models Chemiluminescence has been used to demonstrate increased mucosal production of reactive oxygen metabolites by the inflamed colorectal mucosa in both rat acetic acid colitis and mitomycin-C-induced colitis (Keshavarzian *et al.*, 1992a, 1992b). Addition of a variety of inhibitors or scavengers (SOD, catalase, azide or indomethacin) all decreased the observed chemiluminescence, consistent with the hypothesis that neutrophils are the source of the chemiluminescence in the inflamed mucosa. In addition, Boughton-Smith *et al.* (1992a) have demonstrated increased levels of NO synthase activity in the inflamed colon of the rat trinitrobenzene sulphonic acid model of colitis. Plasma levels of nitrate and nitrite are increased during chronic colonic inflammation induced by bacterial cell wall polymers in rats (Yamada *et al.*, 1992).

2.3.1.1.1.2 Human studies Increased mucosal production of ROM in relation to disease activity has been demonstrated using colorectal mucosal biopsies (Keshavarzian *et al.*, 1992c; Simmonds *et al.*, 1992). *In vitro* studies using inhibitors suggest that most of the increased production is from inflammatory cells, but possibly with a small contribution from XO (Simmonds *et al.*, 1992). Some activation of XO in the intestinal mucosa of patients with inflammatory bowel disease (IBD) might be expected in the light of evidence showing that xanthine dehydrogenase can be converted to the oxidase by activated neutrophils and by a variety of inflammatory mediators (Friedl *et al.*, 1989; Phon *et al.*, 1989). That sufficient ROMs are produced to cause mucosal damage is perhaps indicated by the findings of increased thiobarbituric-acid reactive substances in the colorectal mucosa of patients with ulcerative colitis (UC) in relation to disease activity (Ahnfelt-Rønne *et al.*, 1990). Low levels of mucosal trypsin inhibitor (Playford *et al.*, 1990), SOD, metallothionein (Mulder *et al.*, 1990), glutathione (Inauen *et al.*, 1988) and circulating SOD and glutathione peroxidase (Verspaget *et al.*, 1988; Burdelski *et al.*, 1990), and increased levels of circulating leucocyte elastase (Adeyemi *et al.*, 1985) and colorectal mucosal collagenase (Sturzaker and Hawley, 1975) may also reflect the activity of ROMs in patients with active IBD. Increased NO synthase activity has also been demonstrated in the supernatants from homogenates of inflamed human colon from patients with UC (Boughton-Smith *et al.*, 1992b). This may account for the mucosal hyperaemia and reduced colonic smooth muscle tone in UC. Rectal dialysates from patients with active, but not quiescent, colitis contain enhanced levels of nitrite (Roediger *et al.*, 1986). Although the source of this was not shown in the original study, subsequent evidence implicates NO derived from inflammatory cells (Roediger *et al.*, 1990).

2.3.1.1.2 Indirect evidence of increased production of free radicals

2.3.1.1.2.1 ROM production by isolated intestinal phagocytes Phagocytes isolated from the inflamed mucosa of patients with UC, Crohn's disease and appendicitis produce significantly more luminol-dependent chemiluminescence when stimulated by phorbol myristate acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and latex beads than those isolated from non-inflamed or control mucosa (Williams, 1989). However, there were no differences in the basal levels of chemiluminescence in any of the cells assayed, which makes extrapolation of these findings to the *in vivo* situation difficult. It is also of interest that the results in appendicitis were similar to those found in patients with active IBD, suggesting that the findings were related to an acute inflammatory response rather than to IBD *per se*. Similarly, Mahida *et al.* (1989) found that an increased proportion of intestinal macrophages isolated from inflamed bowel in UC and Crohn's disease were able to undergo a respiratory burst (as assessed by the reduction of nitroblue tetrazolium to formazan) in response to stimulation with PMA and zymosan. Superoxide production, as assessed by reduction of cytochrome c, is enhanced in macrophages isolated from inflamed bowel compared to that of circulating monocytes (Verspaget and Beeken, 1985). This suggests that either local factors produced at the site of inflammation modify the response of phagocytic cells or that primed cells accumulate at the site of inflammation. It should be noted that inevitably the cells under investigation had been subjected to various isolation procedures, which might mean that the results obtained are not necessarily applicable to the *in vivo* situation. In addition, the yield of phagocytic cells obtained by Williams (1989) was greater in specimens from patients with active UC or appendicitis than from controls or patients with Crohn's disease, and so may reflect a different subpopulation of cells. All three studies have looked at the oxidizing capacity of stimulated cells, but have either not looked at or not been able to demonstrate any differences using unstimulated cells.

2.3.1.1.2.2 ROM production by peripheral blood monocytes Production of ROM by peripheral blood monocytes in response to a variety of stimuli is increased in patients with active IBD (Table 10.2), suggesting that such cells may respond to local stimulants within the gut more readily than in normal subjects or those with quiescent disease, and so may play a role in perpetuating the inflammatory response. Recent studies have suggested that peripheral blood monocytes in Crohn's disease may be primed by the bacterial cell wall products LPS and peptidoglycanpolysaccharide (Muraki *et al.*, 1992).

2.3.1.1.2.3 ROM production by peripheral blood neutrophils The histology of the gut in Crohn's disease bears some similarities to that occurring in chronic granulomatous disease (Ament and Ochs, 1973). On the basis of this, it was suggested that neutrophil dysfunction may also occur in Crohn's disease. The results of numerous studies are summarized in Table 10.3. From these it can be seen that, whilst some authors have been able to demonstrate a decrease in oxidative metabolism, particularly in those with active IBD, others have demonstrated an increase. Anton *et al.* (1989) found that the increase in neutrophil chemiluminescence in response to FMLP in Crohn's disease correlated well with an increase in the number of FMLP receptors in these patients. Kelleher *et al.* (1990) have suggested that these differences might be related to the method of cell separation used. However, even when this is taken into consideration, there are still some discrepancies and it seems likely that a defect in neutrophil oxidative metabolism is not a major underlying pathological abnormality, but reflects the underlying inflammatory response and individual differences in disease activity, extent and treatment. It also underlines the fact that events in the peripheral blood do not necessarily reflect those occurring in the gut wall. The cells in the circulation may represent those that

Table 10.2 Oxidative metabolism of monocytes in inflammatory bowel disease

Author (year)	Assay used	Separation procedure	Stimulus	Crohn's disease		Ulcerative colitis	
				Active	Inactive	Active	Inactive
Baldassano *et al.* (1991)	Cyt. c	SDC	FMLP	↑	—	—	—
Kayaba *et al.* (1991)	CL	1-step	mIgG2a-SRBC	↑	↔	—	—
Kitahora *et al.* (1988)	CL	2-step	PMA	↑		—	—
			Op. zymosan	↑		—	—
Okabe *et al.* (1986)	CL	1-step	Op. zymosan	↑		—	—
Suematsu *et al.* (1987a)	CL	2-step	PMA	↑		↑	↔
Williams *et al.* (1990)	CL	1-step	FMLP	↑	↔	↑	↔
			PMA	↑	↔	↑	↔
			Latex beads	↑		↑	↔

Cyt. C ferricytochrome c; CL, chemiluminescence; SDC, serial density centrifugation; FMLP, formyl-methionyl-leucyl-phenylalanine; mIgG2a-SRBC, murine monoclonal anti-trinitrobenzene IgG2a antibody conjugated with trinitrobenzene-sulphonated sheep red blood cells; PMA, phorbal-12-myristate-13-acetate; Op. zymosan, opsonized zymosan. Results are given according to the disease and the disease activity where specified in the original papers or according to the disease studied. ↑, increased activity compared to controls; ↔, no difference in activity from controls; —, no data. Where symbols lie between the columns for active/inactive this signifies that a mixed group of patients was studied.

have not yet been activated or recruited to the site of inflammation.

2.3.1.1.2.4 Antioxidant defences in IBD Plasma levels of vitamins A, C and E, β-carotene and serum zinc were found to be decreased in patients with active colitis, whilst serum copper was increased (Fernandez-Banares *et al.*, 1989, 1990). Studies on peripheral blood have demonstrated decreases in SOD in neutrophils from patients with IBD (Verspaget *et al.*, 1988; Pippenger *et al.*, 1991) and in glutathione peroxidase in monocytes from patients with active Crohn's disease (Munck *et al.*, 1991). The colon is relatively deficient in mucosal levels of oxidant defence mechanisms when compared to the liver (Grisham *et al.*, 1990a) and these are further depleted in patients with active IBD. Low levels of SOD, metallothionein (an ubiquitous metal-binding protein, which regulates zinc and copper metabolism, but which also has hydroxyl radical-scavenging properties and can protect against ROM-mediated damage in a variety of systems) and glutathione have been found in the mucosa of patients with active IBD (Inauen *et al.*, 1988; Mulder *et al.*, 1991). However, whether these changes are primary or secondary remains to be established.

2.3.1.2 Production of Mucosal Damage

2.3.1.2.1 Cell culture Stimulated neutrophils are known to be cytotoxic to cells *in vitro* (Dull *et al.*, 1987; Dallegri *et al.*, 1990; Grisham *et al.*, 1990b). Several *in vitro* systems have been used to demonstrate oxidative damage to intestinal cells. Xanthine/XO increased ^{51}Cr release and decreased [^{3}H]thymidine uptake by IEC-18 small intestinal epithelial cell monolayers in a dose-dependent manner (Ma *et al.*, 1991). Rat enterocytes show decreased trypan blue exclusion and increased protein release when incubated with neutrophils stimulated by PMA, or with XO, or with leucine and amino acid oxidase (Baker and Campbell, 1991). Similarly, CaCo cells show decreasing trypan blue exclusion with increasing concentrations of XO or amino acid oxidase (Baker and Baker, 1990). Kawabe *et al.* (1992) have shown that ROMs generated by the xanthine/XO system *in vitro* are cytotoxic to cultured rabbit colonic epithelial cells.

2.3.1.2.2 Animal models ROMs are important in some animal models of IBD. Colitis can also be induced in rats by intrarectal installation of a ROM initiator [2,2′-azobis(2-amidinopropane) dihydrochloride] (Tamai *et al.*, 1992). Trinitrobenzene sulphonic acid may be metabolized to yield O_2^- and H_2O_2, and produces an acute colitis in rats when administered in ethanol intrarectally (Grisham *et al.*, 1991). Millimolar concentrations of stable oxidants (H_2O_2, OCl^- and monochloramine) cause a dose-dependent increase in mucosal permeability in animal models and are toxic to intestinal epithelial cells (Grisham *et al.*, 1990b). Monochloramine at micromolar concentrations, comparable to those found in inflamed tissue *in vivo*, stimulates rat colonic secretion of prostaglandin E_2 and chloride, and results in morphological changes in mucosal depth and crypt architecture (Tamai *et al.*, 1991). Nitric oxide also stimulates anion secretion and inhibits sodium chloride reabsorption in the rat colon (Wilson *et al.*, 1992). Carrageenan-induced colitis in guinea pigs is associated with increased tissues levels of XO, and decreased levels of SOD and catalase (Song *et al.*, 1992). Furthermore, colitis can be produced in animals by agents known to attract and stimulate neutrophils. FMLP instilled into the colon can cause an acute colitis, similar in histological appearance to human UC, in rabbits (LeDuc and Nast, 1990), rats and mice (Chester *et al.*, 1985). Similarly

Table 10.3 Oxidative metabolism of neutrophils in inflammatory bowel disease

Author (year)	Assay used	Separation procedure	Stimulus	Crohn's disease		Ulcerative disease	
				Active	Inactive	Active	Inactive
Anton *et al.* (1989)	CL	3-step	FMLP	↑		↔	
Curran *et al.* (1991)	Cyt. c	SDC	PMA	↓	↓	—	—
			PMA2	↓	↓	—	—
			C. albicans	↔	↔	—	—
Faden and Rossi (1985)	CL	2-step	Op. zymosan	↑			
Kayaba *et al.* (1991)	CL	1-step	mIgG2a-SRBC	↑	↔	—	—
Kelleher *et al.* (1990)	CL	1-step	Op. zymosan	↓			
		2-step	Op. zymosan	↑			
Kitahora *et al.* (1988)	CL	2-step	PMA	↔		—	—
			Op. zymosan	↔		—	—
Okabe *et al.* (1986)	CL	2-step	Op. zymosan	↔		—	—
Suematsu *et al.* (1987a)	CL	2-step	PMA	↔		↔	↑
Verspaget *et al.* (1984)	Cyt. c	1-step	PMA	?↓	↓	—	—
			Con A	↔		—	—
			CON A–cyt E	↔		—	—
			LPS	↔		—	—
	HVA	1-step	PMA	↓	↔	—	—
			Con A	↓	↔	—	—
			Con A–cyt E	↑	↔	—	—
			LPS	↓	↑	—	—
Verspaget *et al.* (1986)	Cyt. c	1-step	—	↔		↓	
			PMA	↔		↓	
			PMA–cyt E	↓		↓	
			Con A–cyt E	↔		↓	
			Zymosan	↔		↓	
			Op. zymosan	↓		↓	
			FMLP–cyt E	↔		↔	
			C5a–cyt E	↔		↔	
Verspaget *et al.* (1986)	HVA	1-step	—	↔		↔	
			PMA	↔		↔	
			Con A	↔		↔	
			Zymosan	↔		↔	
			Op. zymosan	↔		↔	
			FMLP–cyt E	↓		↔	
Verspaget *et al.* (1988)	Cyt. c	1-step	PMA	↓		↓	
	HVA	1-step	PMA	↔		↔	
Wandall and Binder (1982a, 1982b)	NBT	2-step	—	↑		↑	
		2-step	Op. LPS	↓		↔	
Ward and Eastwood (1976)	NBT	—	—	↑		↑	
Williams *et al.* (1990)	CL	2-step	FMLP	↓		↓	
			PMA	↔		↔	
			Latex	↔		↔	
Worsaae *et al.* (1982)	NBT	2-step	PMA	↔		—	—
	CL	2-step	Op. *S. aureus*	↓		—	—

Cyt. c, ferricytochrome c; CL, chemiluminescence; HVA, homovanillic acid oxidation; NBT, nitroblue tetrazolium reduction; SDC, serial density centrifugation; FMLP, formyl-methionyl-leucyl-phenylalanine; FMLP–cyt E, formyl-methionyl-leucyl-phenylalanine in combination with cytochalasin E; PMA, phorbol-12-myristate-13-acetate; PMA2, phorbol 20-oxo-20-deoxy 12-myristate-13-acetate; PMA–cyt E, phorbol-12-myristate-13-acetate in combination with cytochalasin E; *C. albicans*, *Candida albicans* in serum; Op. zymosan, opsonized zymosan; mIgG2a-SRBC, murine monoclonal anti-trinitrobenzene IgG2a antibody conjugated with trinitrobenzene-sulphonated sheep red blood cells; Con A, concanavalin A; Con A–cyt E, concanavalin A in combination with cytochalasin E; LPS, lipopolysaccharide derived from *E. coli* 0127:B8; C5a-cyt E, complement split product 5a in combination with cytochalasin E; Op. *S. aureus*, opsonized *Staphylococcus aureus* 502 A. Results are given according to the disease and the disease activity where specified in the original papers or according to the disease studied. ↑, increased activity compared to controls; ↔, no difference in activity from controls; —, no data. Where symbols lie between the columns for active/inactive this signifies that a mixed group of patients was studied. Faden and Rossi (1985) and Kellener *et al.* (1990) studied a mixed group of patients with IBD.

intracolonic PMA causes colitis in rats and rabbits (Fretland *et al.*, 1990) and LPS infused continuously into the abdominal cavity results in a proximal colitis in rats (Schiffrin *et al.*, 1991).

2.3.1.2.3 Humans Hydrogen peroxide has been used as an enema or as a cleaning agent for endoscopes and may cause mucosal damage when applied to the surface of the gut wall. "Hydrogen peroxide enteritis" can mimic an acute ulcerative, ischaemic or pseudomembranous colitis, and ranges from a reversible, clinically inapparent process to an acute, toxic fulminant colitis associated with perforation and death (Bilotta and Waye, 1989). It is conceivable that anecdotal reports of exacerbation of IBD by iron supplementation (Kawai *et al.*, 1992) are mediated by hydroxyl radical production by the Fenton reaction.

Evidence of damage to macromolecules by ROMs in IBD remains sparse. Hypochlorite is known to be involved in the inactivation of protective anti-proteases and activation of collagenases, so transforming the oxidizing potential of hypochlorite into enzyme-catalysed degradation of proteins and collagens (Weiss, 1989; Winterbourn, 1990). Increased production of hypochlorite in IBD may explain the findings of low levels of mucosal trypsin inhibitor (Playford *et al.*, 1990) and increased levels of circulating leucocyte elastase (Adeyemi *et al.*, 1985), and colorectal mucosal collagenase (Sturzaker and Hawley, 1975) in patients with IBD.

The concentration of lipid peroxides, measured using the thiobarbituric acid test, is increased in biopsies from patients with IBD and correlates well with disease activity (Ahnfelt-Rønne *et al.*, 1990). Products of arachidonic acid metabolism such as prostaglandin E_2 and leukotriene B_4 (themselves lipid peroxides) are increased in the colorecal mucosa of patients with active IBD (Rampton and Hawkey, 1984; Stenson, 1988). Activation of the arachidonic acid cascade requires the formation of a peroxy radical at the active site of cyclooxygenase (Halliwell and Gutteridge, 1989). The measurement of lipid peroxides is complicated and results need to be interpreted with caution. The thiobarbituric acid test is relatively non-specific (Gutteridge and Halliwell, 1990) and seems unlikely to distinguish between lipid peroxides resulting from enzymatic (such as prostaglandins and leukotrienes) and non-enzymatic processes (perhaps reflecting more closely damage as a result of ROM attack).

The risk of developing colonic cancer is raised in UC, particularly in those with long-standing extensive disease (Lennard-Jones *et al.*, 1990). The mechanism of this increased susceptibility is unknown, although it is tempting to speculate that it is related to inflammation and resulting oxidative damage to DNA. As yet there is little evidence to support this contention. Markowitz *et al.* (1988) have reported a decrease in constituent and oxidant-induced adenosine diphosphate ribosyl transferase (ADPRT) activity in patients with UC. This enzyme can be activated by hydrogen peroxide in experimental models (Cochrane, 1991) and these observations would fit well with the observed decreases in pro-oxidant-sensitive ADPRT activity in the most commonly dominantly inherited cancers (breast, colon, lung) (Pero *et al.*, 1990). It has been suggested that there is a natural genetic variation in the redox mechanisms controlling glutathione levels within the cells, which in turn affects the activity of ADPRT (Pero *et al.*, 1990), and this hypothesis would fit well with the low levels of glutathione found in the colonic mucosa of patients with IBD (Inauen *et al.*, 1988). In experimental models H_2O_2 activates poly(ADP-ribose) polymerase (Cochrane, 1991).

2.3.1.3 Effects of Antioxidants in Inflammatory Bowel Disease

2.3.1.3.1 Cell culture Damage to small intestinal epithelial cells by XO can be prevented by SOD and desferrioxamine (Ma *et al.*, 1991), whilst that to rat enterocytes, CaCo cells or rabbit colonic epithelial cells by XO can be decreased by catalase (Baker and Baker, 1990; Baker and Campbell, 1991; Kawabe *et al.*, 1992).

2.3.1.3.2 Animal models In rat acetic acid colitis the severity of inflammation can be decreased by methoxypolyethyleneglycol:SOD, catalase, allopurinol and SAZ (Keshavarzian *et al.*, 1990; Haydek *et al.*, 1991), and in rat carrageenan-induced colitis intestinal damage was decreased by allopurinol, SOD-polyethylene glycol and by DMSO (Moyana and Lalonde, 1991). Oxypurinol decreased morphological damage and accumulation of thiobarbituric acid-reactive substances and prevented decreases in energy-rich nucleotides in the trinitrobenzene sulphonic acid model of chronic colonic inflammation in rats (Siems *et al.*, 1992). Similarly, inflammation in a new rat model of colitis induced by mitomycin C was decreased by allopurinol, catalase and WR 2721 (a glutathione-sparing agent) (Keshavarzian *et al.*, 1992a, 1992b). In contrast, inhibition of endogenous NO formation significantly increases epithelial permeability in cat small intestine (Kubes, 1992) and enhances acute endotoxin-induced intestinal damage in the rat (Hutcheson *et al.*, 1990). Damage over a longer period, however, appears to be associated with the induction of a calcium-independent NO synthase (Boughton-Smith *et al.*, 1992c).

2.3.1.3.3 Humans Much has been written on the role of the aminosalicylates [5-ASA and sulphasalazine (SASP)] as antioxidants (Yamada *et al.*, 1990; Gaginella and Walsh, 1992). They inhibit binding of FMLP (Stenson *et al.*, 1984), attenuate the respiratory burst of neutrophils (Molin and Stendahl, 1979; Miyachi *et al.*, 1987; Suematsu *et al.*, 1987b; Williams and Hallett, 1989; Kanerud *et al.*, 1990; Gionchetti *et al.*, 1991), scavenge the hydroxyl radical and hypochlorite, and inhibit myeloperoxidase in cell free systems and

neutrophils (Aruoma *et al.*, 1987; Grisham *et al.*, 1987; Halliwell, 1987), inhibit the activity of NO (Pallapies *et al.*, 1992), and protect against oxidant-mediated damage both in cell culture and in animal models of IBD (Craven *et al.*, 1987; Dull *et al.*, 1987; Von Ritter *et al.*, 1989; Dallegri *et al.*, 1990). Ahnfelt-Ronne *et al.* (1990) have demonstrated the presence of oxidative metabolites of 5-ASA in the faeces of patients with IBD suggesting significant ROM scavenging *in vivo*. The preliminary results on the use of α-tocopherylquinone in the treatment of UC (Bennett, 1986), SOD in Crohn's disease and UC (Niwa *et al.*, 1985; Emerit *et al.*, 1989), allopurinol in pouchitis (Levin *et al.*, 1992) and allopurinol and DMSO in UC (Salim, 1992g) suggest that ROM are important in the pathogenesis of IBD, and that antioxidant activity may be important in determining therapeutic efficacy. The efficacy of allopurinol in UC supports the hypothesis that ischaemia and activation of XO plays a role in the pathogenesis of this disease (Fairburn, 1973; Grisham and Granger, 1988). However, although antioxidant activity does occur at concentrations similar to those found in the plasma of patients on oral therapy (e.g. IC_{50} of 5-ASA as a scavenger of carbon, peroxyl or nitrogen-centred radicals is 5–10 μM; Yamada *et al.*, 1990), and very much lower than those needed for inhibition of cyclooxygenase (IC_{50} 10 mM; Hawkey and Truelove, 1983) or lipoxygenase (IC_{50} 6 mM; Allgayer and Stenson, 1988), the precise mode of action of the aminosalicylates in the treatment of IBD and the relative importance of their different antioxidant properties remains unclear (Allgayer *et al.*, 1991; Halliwell 1991). Antioxidant activity is unlikely to be the only mechanism of action of aminosalicylates, since steroids, which are extremely effective in the treatment of patients with active IBD, have virtually no antioxidant activity (Levine *et al.*, 1981).

2.3.1.4 Conclusions

There is now little doubt that ROMs are produced in excess in patients with active IBD. That, at least in experimental colitis, they are rather more than irrelevant epiphenomena is indicated by the anti-inflammatory effect of specific antioxidants. Proof that this is also the case in human disease awaits the outcome of further controlled trials of specific agents interfering with ROM production. Whilst induction of NO production has been shown to occur in association with inflammation and tissue damage in both humans and in animal models, the significance of this is as yet unclear.

2.3.2 Ischaemic Colitis

The theory underlying the pathophysiology of ischaemia–reperfusion injury, and the role of free radicals in this process has been discussed in detail above. The human colon contains relatively little XO (Parks and Granger, 1986) and so the arguments supporting a role for this enzyme in the pathogenesis of small bowel ischaemic injury do not necessarily apply. It is possible that other enzymes, such as aldehyde dehydrogenase, may produce ROM and hence mucosal damage, but this remains speculative (Parks *et al.*, 1983).

There is some support for a role for free radicals in the pathogenesis of ischaemic colitis from animal studies. Murthy and Qi (1992) used a spin trap to demonstrate increased production of free radicals up to 60 min after reperfusion, whereas Douglas *et al.* (1989) demonstrated increases in malondialdehyde and conjugated dienes (presumptive measures of lipid peroxidation) in a rat model of ischaemic colitis. There is no data relating to human ischaemic colitis.

2.3.3 Behcet's Disease

This is a chronic inflammatory disease, which can affect the gut as well as other organs. There is relatively little information on the role of free radicals in this condition. Neutrophil chemiluminescence was increased in patients with intestinal Behcet's compared to normal controls (Suematsu *et al.*, 1987a) and there is some evidence for endothelial injury by neutrophil-derived oxidants (Niwa *et al.*, 1982). Preliminary studies with liposomal-encapsulated SOD demonstrated marked improvement in 12 out of 16 patients with active Behcet's disease (Niwa *et al.*, 1985).

2.4 PANCREAS

2.4.1 Increased Production of Free Radicals

2.4.1.1 Direct Evidence of Increased Production of Free Radicals

Chemiluminescence has been used to demonstrate increased free-radical activity after induction of caerulein pancreatitis, with levels peaking at about 20 min and decreasing rapidly to control values thereafter (Gough *et al.*, 1990). Electron spin resonance has been used to demonstrate increased hydroxyl radical activity in choline-deficient diet pancreatitis in the mouse (Nonaka *et al.*, 1989a).

2.4.1.2 Indirect Evidence of Increased production of Free Radicals

Guyan *et al.* 1990) have used several markers of lipid peroxidation (9-*cis*-, 11-*trans*-isomer of linoleic acid, conjugated dienes and ultraviolet fluorescent products) to demonstrate significant increases in the duodenal aspirate after secretin stimulation in patients with acute and clinic pancreatitis. They interpreted this as indicating induction of hepatic and pancreatic drug-metabolizing enzymes in the face of a shortfall of antioxidant defences, more marked in chronic pancreatitis. Subsequent studies in patients with chronic pancreatitis have confirmed decreased serum concentrations of selenium, β-carotene and vitamin E compared with healthy controls (Uden *et al.*, 1992). Basso *et al.* (1990) have measured increases in lipid peroxides in the sera of patients with chronic

pancreatitis, particularly in those with relapse, but also in patients with benign biliary tract disease and concluded that activation of ROM production appeared to reflect the inflammatory process.

Increased production of free radicals can also be inferred from studies in animal models of pancreatitis showing increases in lipid peroxidation (Nonaka *et al.*, 1989b; Schoenberg *et al.*, 1990; Dabrowski *et al.*, 1991), or decreases in levels of antioxidant defences, such as SOD and catalase (Nonaka *et al.*, 1990), pancreatic glutathione (Neuschwander-Tetri *et al.*, 1992) and sulphydryl compounds (Dabrowski and Chwiecko, 1990). Increased activity of XO is suggested in several models of pancreatitis by the protective effect of allopurinol (Sanfey *et al.*, 1985; Cassone *et al.*, 1991; Niederau *et al.*, 1992). Recent studies have suggested that, in three models of pancreatitis thought to be mediated by ROM, the conversion from xanthine dehydrogenase to oxidase is the result of reversible sulphydryl oxidation rather than irreversible proteolysis (Nordback and Cameron, 1993).

2.4.2 Production of Cell Damage

Acute pancreatitis has been induced in dogs by retrograde intraductal injections of 5% hydrogen peroxide solution and sunflower oil (Keleman and Torok, 1990) and in rats by continuous infusion of xanthine/XO or hydrogen peroxide into the coeliac artery (Tamura *et al.*, 1991a). Xanthine/XO has also been shown to be toxic, as measured by release of LDH, to *in vitro* rat pancreatic acini (Tamura *et al.*, 1991b).

2.4.3 Effects of Antioxidants

2.4.3.1 Animal Models

SOD and catalase have been shown to be effective in preventing damage in several models of acute pancreatitis, including intra-arterial infusion of oleic acid, partial obstruction of the pancreatic duct with secretin stimulation and I/R in dogs (Sanfey *et al.*, 1984), and caerulein administration or retrograde injection of sodium taurocholate in rats (Guice *et al.*, 1986; Wisner *et al.*, 1988; Schoenberg, *et al.*, 1991b). However, in other studies using rat models of pancreatitis induced by caerulein, a choline-deficient methionine-supplemented diet or sodium taurocholate, SOD and catalase decrease the amount of pancreatic oedema and may improve mortality, but do not appear to improve cell damage (Steer *et al.*, 1991; Niederau *et al.*, 1992). Saluja *et al.* (1986) found that SOD prevented pancreatic weight gain but not hyperamylasaemia in the rat caerulein model. Niederau *et al.* (1992) showed similar effects on oedema but not on cell damage in these rat models using DMSO, desferrioxamine, Ebselen and allopurinol. They suggested that any effects seen on mortality were due to the effects of these antioxidants on the extrapancreatic complications of acute pancreatitis. MacGowan *et al.* (1987) found that pretreatment with allopurinol had no effect on mortality in the rat taurocholate model. Allopurinol has been shown to prevent tissue damage in the canine I/R model, and in canine pancreatitis induced by oleic acid and partial obstruction of the pancreatic duct with secretin stimulation (Sanfey *et al.*, 1985; Cassone *et al.*, 1991). Ebselen given prophylactically improved survival in rat pancreatitis induced by caerulein or a choline-deficient methionine-supplemented diet (Niederau *et al.*, 1991). Caerulein-induced pancreatitis in the mouse can be improved by administration of glutathione monomethyl ester 1 h before the caerulein (Neuschwander-Tetri *et al.*, 1992) and pancreatitis induced by a choline-deficient methionine-supplemented diet is less severe when mice are treated with a new synthetic ascorbic-acid derivative (CV 3611) (Nonaka *et al.*, 1991).

2.4.3.2 Humans

Only one study to date has been conducted on the treatment of acute pancreatitis with antioxidants. Clemens *et al.* (1991) were unable to show any difference in the incidence or severity of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis in a prospective, randomized, double-blind, placebo-controlled trial of allopurinol. However, Salim (1991) performed a similar trial of the effect of allopurinol and DMSO in patients with pain from recurrent pancreatitis, and found significant benefit. On the basis that depletion of antioxidants is important in the pathogenesis of chronic pancreatitis, the administration of a cocktail of antioxidants was assessed for its effect on pain in this disease. Treatment with a combination of organic selenium, β-carotene, vitamins C and E, and methionine was of benefit in the initial pilot study, and in a placebo-controlled trial (Sandilands *et al.*, 1990; Uden *et al.*, 1990).

2.4.4 Conclusions

Studies of both acute and chronic pancreatitis in humans and in animals support the hypothesis that free radicals are involved in the pathogenesis of pancreatitis. There is some conflicting data from the animal work, which may in part be due to differences in the models used. It does also indicate that free radicals are not the only factors involved and suggests that activation of pancreatic enzymes are also important, particularly in the development of haemorrhagic pancreatitis (Sanfey, 1991). The findings of decreased antioxidant defences and the success of treatment reported in chronic pancreatitis with a cocktail of antioxidants and with allopurinol suggest further studies are required to establish the role of antioxidants in pancreatic disease and its prevention.

2.5 LIVER

The liver was the first organ in the gastrointestinal tract in which the role of ROMs in liver injury was established. Mitchell *et al.* (1973a, 1973b) demonstrated the roles of several drug-metabolizing enzymes in the formation of

toxic products of paracetamol and also the protective effect of glutathione. Since then, *N*-acetyl cysteine has become the accepted treatment in the management of patients with paracetamol overdose (Prescott, 1983).

The formation of ROMs within the liver occurs by one of three main mechanisms: the formation and release of ROMs by membrane-associated NADPH oxidase in stimulated polymorphs and macrophages; formation of ROMs by the action of the enzyme XO (which is formed from xanthine dehydrogenase) in reperfused ischaemic tissue; and the "leakage" of electrons on to oxygen from electron-transport systems or other proteins and cofactors involved in oxidation–reduction reactions (e.g. the microsomal oxidizing system) (Arthur, 1988). The evidence relating to the role of ROMs in the pathogenesis of liver injury will be discussed in the context of several clinical conditions: liver damage due to ethanol, paracetamol, immune-mediated mechanisms, cholestasis, iron overload, ischaemia–reperfusion, and liver transplantation and damage due to other toxins. The evidence of hepatocyte damage by ROMs will be discussed in the section on ethanol-induced liver disease, but obviously applies to the consideration of other causes of liver disease.

2.5.1 Alcoholic Liver Disease

2.5.1.1 Increased Production of Free Radicals

There are no studies in humans directly demonstrating increased production of ROM in alcoholic liver disease. However, indirect evidence does support the hypothesis that ROM are involved in the pathogenesis of ethanolic liver injury (Arthur, 1988; Tsukamoto *et al.*, 1990; Nordmann *et al.*, 1992). Basal whole blood chemiluminescence production was significantly higher in patients with alcoholic liver disease than in those with non-alcoholic liver disease and controls, and appeared to correlate well with prognosis in alcoholic liver disease (Lunel *et al.*, 1990). Chemiluminescent responses to stimulation with latex, zymosan and PMA were significantly reduced in patients with alcoholic hepatitis compared to normal controls, suggesting perhaps that cells might already be activated in the circulation. There is some evidence of increased degradation of adenine nucleotides after ethanol in humans, which may reflect increased activity of XO (Faller and Fox, 1982). Levels of phospholipid-esterified 9,11-linoleic acid isomer in blood have been used as a free-radical marker. Fink *et al.* (1985) showed a significant increase in their levels in chronic alcoholics in response to an ethanol load but not in controls. Increased levels of malondialdehyde (MDA) or diene conjugates have been found in liver from patients with alcoholic hepatitis (Suematsu *et al.*, 1981; Shaw *et al.*, 1983). Situnayake *et al.* (1990) used the measurement of total conjugated dienes by derivative spectroscopy and the molar ratio between a diene-conjugated linoleic acid isomer and the parent linoleic acid isomer as an indication of lipid peroxidation in liver biopsies from patients with alcoholic hepatitis. However, although derivative spectroscopy minima could be identified, there was no correlation with severity of disease assessed histologically, nor with other parameters taken to indicate oxidative stress (hepatic iron grade, glutathione and vitamin E lipid ratio). The findings of a significant correlation of the proportion of linoleic acid in hepatic lipids with inflammation and an inverse correlation of this ratio and the degree of iron staining with hepatic glutathione was taken to suggest that increased alcohol consumption results in hepatic lipid peroxidation.

Studies of antioxidants in blood and liver of patients with alcoholic cirrhosis also support the idea that alcoholic liver disease is associated with oxidant stress. Levels of Cu/Zn-SOD and catalase are reduced in liver from patients with alcoholic cirrhosis (Togashi *et al.*, 1992). Levels of catalase, glutathione peroxidase and glutathione in erythrocytes were significantly decreased in patients with moderate to severe alcoholic cirrhosis compared to normal controls, as were serum zinc and selenium (Gerli *et al.*, 1992). Other studies have also shown decreased levels of selenium in the serum (Aaseth *et al.*, 1983; Valimaki *et al.*, 1983; Dworkin *et al.*, 1985; Korpela *et al.*, 1985; Johansson *et al.*, 1986; Tanner *et al.*, 1986), whole blood (Dworkin *et al.*, 1985; Johansson *et al.*, 1986), platelets (Johansson *et al.*, 1986) and liver (Valimaki *et al.*, 1987; Dworkin *et al.*, 1988) of patients with alcoholic cirrhosis. Whether this reflects dietary factors, an effect of alcohol on levels of antioxidants and antioxidant enzymes directly, or is an indirect measure of oxidant stress induced by alcohol is not clear. One study has suggested that the low serum and hepatic selenium levels found in patients with liver disease is unrelated to the aetiology of that disease, and is probably related to overall nutritional status rather than dietary intake (Thuluvath and Triger, 1992).

Studies in animals also support a role for ROMs in the development of ethanol-induced liver injury. Boveris *et al.* (1983), using an *in situ* low-level chemiluminescence method for measuring ROM production in the liver, have shown that this production was significantly increased in the liver of ethanol-treated rats compared to controls. Superoxide production, as measured by the reduction of ferricytochrome c, by the perfused rat liver after ethanol intoxication is increased. This effect does not appear to be dependent on the metabolism of ethanol and can be inhibited by ibuprofen, suggesting possible involvement of prostaglandin metabolism (Bautista and Spitzer, 1992). Mobilization of iron from ferritin as a result of superoxide produced in the metabolism of acetaldehyde by aldehyde oxidase has been demonstrated *in vitro* (Shaw and Jayatilleke, 1990a). Desferrioxamine has been shown to abolish free-radical production, measured by alkane production, in a rodent model of ethanol-induced liver damage (Shaw and Jayatilleke, 1992). Studies in baboons have shown an increase in lipid peroxidation associated with ethanol, which can be

prevented by pretreatment with methionine (Shaw *et al.*, 1981). Similar changes can also be produced in rats, although very high doses of ethanol are required (Farbiszewski *et al.*, 1991; Castillo *et al.*, 1992). However, not all investigators have been able to confirm increases in lipid peroxidation and decreases in hepatic glutathione in ethanol-fed rats (Speisky *et al.*, 1985; Inomata *et al.*, 1987). Kawase *et al.* (1989) found decreases in hepatic vitamin E levels after chronic ethanol feeding, whereas Tsukamoto and Bacon (1987) found increases in hepatic levels of methionine and α-tocopherol in such animals. Possible explanations for these differences include the fasting and withdrawal states of animals and patients at the time liver biopsy specimens were collected, and also differences in the stage of liver disease studied. It has been suggested that lipid peroxidation in the liver is not increased at the initiation of hepatocyte necrosis, but only after substantial necrosis and subsequent scar formation has occurred (Tsukamoto *et al.*, 1990). This view is consistent with evidence that disrupted tissues undergo lipid peroxidation more quickly than healthy ones and that in many diseases lipid peroxidation can be prevented without preventing the tissue damage. In many cases, lipid peroxidation is a late event, accompanying, rather than causing, cell death (Halliwell and Gutteridge, 1989). More recently, it has been shown that there is an association of enhanced lipid peroxidation with liver fibrogenesis and depressed antioxidant defences (Kamimura *et al.*, 1992). Mn-SOD activity is increased in the livers of ethanol-fed monkeys, an effect which may reflect induction of the enzyme by superoxide (Keen *et al.*, 1985). Acute-phase induction of Mn-SOD has been demonstrated in intestinal epithelial cell lines (Valentine and Nick, 1992).

Administration of ethanol to rats has been shown to increase the production of ROMs by isolated liver mitochondria. This effect persists for up to 16 h after the administration of ethanol, suggesting that ethanol induces the production of ROM by liver mitochondria, but that this is not directly related to the metabolism of ethanol (Sinaceur *et al.*, 1985). Isolated rat liver microsomes oxidize ethanol by two separate pathways; cytochrome P450 and via a NADPH-dependent cytochrome P450 reductase resulting in the formation of hydroxyl radicals during electron transfer (Cederbaum, 1987). Klein *et al.* (1983) showed that chronic administration of ethanol to rats caused induction of both pathways and also increased production of hydroxyl radicals by microsomes. *In vitro*, acetaldehyde is oxidized to acetate by XO, which transfers electrons to oxygen forming superoxide. This reaction has been proposed as a mechanism of ethanol-induced liver damage (Lewis and Paton, 1982). However, it is not clear whether XO is available in sufficient amounts in the non-ischaemic liver for this to occur. More recent studies on isolated rat hepatocytes, using alkane production as a measure of free-radical generation, showed that inhibition of aldehyde oxidase and XO with a tungstate diet produced almost complete inhibition of ROM production, whereas allopurinol (which inhibits XO) only decreased ROM production by 35–50% (Shaw and Jayatilleke, 1990b). Centrilobular hypoxia, as a result of increased hepatic oxygen consumption due to the induction of microsomal enzymes by ethanol, has been proposed as one of the mechanisms of centrilobular damage in alcoholic liver disease (Bernstein *et al.*, 1973; Videla *et al.*, 1973). Oxygen venous tension in hepatic blood has been found to be lower in ethanol-fed animals (Tsukamoto and Xi, 1989) and humans (Kessler *et al.*, 1954; Iturriage *et al.*, 1980; Hadengue *et al.*, 1988). It has been shown that ethanol feeding increases vulnerability to hypoxia-induced liver necrosis (Israel and Orrego, 1981; French *et al.*, 1984) and that hypoxia results in decreased levels of ATP in ethanol-fed rats (Miyamoto and French, 1988). However, levels of XO were not measured and its role in ethanol-induced liver disease remains unclear.

2.5.1.2 *Production of Cell Damage*

Experiments on cultured hepatocytes have shown that ROMs generated using XO or glucose oxidase, or the addition of hydrogen peroxide cause cell damage as quantified by release of lactate dehydrogenase (LDH) or ^{51}Cr (Rubin and Farber, 1984; Ito *et al.*, 1992). Such damage can be prevented with a variety of antioxidants such as SOD, catalase, mannitol, thiourea, desferrioxamine, ascorbate and trolox (Starke and Farber, 1985; Zeng *et al.*, 1991; Ito *et al.*, 1992). Inactivation of cellular SOD by diethyldithiocarbamate sensitized the cells to ROM-induced cytotoxicity (Ito *et al.*, 1992). Treatment of cultured hepatocytes with *t*-butylhydroperoxide results in glutathione loss, lipid peroxidation and cell lysis, effects which can be prevented by pretreatment with α-tocopherol (Neuschwander-Tetri, 1992). The effects of hypochlorous acid and chloramines have been studied in the perfused rat liver (Bilzer and Lauterburg, 1991). Hypochlorous acid caused an increase in perfusion pressure and release of LDH associated with a decrease in bile flow. These effects could be abolished by increasing the concentration of extracellular glutathione. The chloramines had relatively little effect on the perfusion pressure, but resulted in significant increases in the biliary excretion of glutathione disulphide, indicating intracellular reduction of chloramines by glutathione.

2.5.1.3 *Effects of Antioxidants*

Allopurinol has been shown to attenuate lipid peroxidation in ethanol-fed rats (Kato *et al.*, 1990). However, this was not correlated with any possible effect on histological damage and, as discussed previously, the significance of lipid peroxidation is unclear. Despite the evidence suggesting that oxidative stress and increased oxidative metabolism may play a role in the pathogenesis of human alcoholic liver disease, it remains to be shown that treatment with specific antioxidants will modify this process.

2.5.1.4 *Conclusions*

Whilst there is some evidence that ethanol can induce increased production of ROM and that ischaemia may be involved in the pathogenesis of alcoholic liver disease, there is very little data to show that specific antioxidants can modify the disease process.

2.5.2 Paracetamol-induced Liver Damage

The mechanisms of hepatotoxicity caused by paracetamol have been reviewed in detail recently (Nelson, 1990) and will therefore be discussed relatively briefly. There is now considerable evidence that paracetamol is oxidized by hepatic cytochrome P450s to the major toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). This is detoxified by conjugation with glutathione leading to a reduction in the mitochondrial glutathione pool. NAPQI also results in depletion of cellular thiols and causes disruption of calcium homeostasis with effects on a number of enzymes including glutathione peroxidase and xanthine dehydrogenase. The established treatment of paracetamol overdose is with *N*-acetyl-L-cysteine, which is thought to act primarily by elevating intracellular glutathione concentrations.

2.5.2.1 *Increased Production of Free Radicals*

NAPQI is a powerful oxidant and has been shown to oxidize cellular thiols and pyridine nucleotides in biological systems (Blair *et al.*, 1980; Dahlin *et al.*, 1984; Albano *et al.*, 1985; Moore *et al.*, 1985; Coles *et al.*, 1988; Tirmenstein and Nelson, 1989, 1990). It has been suggested that during metabolism of paracetamol by the microsomal enzyme oxidizing system, cytochromes and/or reactive metabolites transfer electrons to oxygen, forming ROMs, which are responsible for cell injury (Farber, 1987). Superoxide formation has been demonstrated in cultured hepatocytes, and SOD, catalase and allopurinol have all been shown to protect against paracetamol-induced hepatotoxicity (Kyle *et al.*, 1987; Tirmenstein and Nelson, 1990). Increased lipid peroxidation has been found after paracetamol administration in mice and rats *in vivo* and *in vitro* (Wendel *et al.*, 1979; Fairhurst *et al.*, 1982; Albano *et al.*, 1983; Kyle *et al.*, 1987). However, the apparent dissociation between the appearance of the products of lipid peroxidation and cytotoxicity argue against lipid peroxidation as a causal mechanism in paracetamol-induced hepatotoxicity (Nelson, 1990).

2.5.2.2 *Production of Cell Damage*

Portal infusion of NAPQI into rats and mice produces necrosis in the periportal region (unpublished results quoted by Nelson, 1990). Studies of cultured hepatocytes sensitized to paracetamol by the induction of cytochrome P450 by 3-methylcholanthrene have shown that paracetamol-induced cytotoxicity was dependent on ROM generation (Gerson *et al.*, 1985).

2.5.2.3 *Effects of Antioxidants*

Paracetamol-induced hepatotoxicity can be prevented in animals with SOD, catalase and allopurinol (Kyle *et al.*, 1987; Jaeschke, 1990; Tirmenstein and Nelson, 1990), and by *N*-acetyl-L-cysteine or methionine in humans (Meredith *et al.*, 1986; Nelson, 1990). The protective effect of allopurinol in mice only occurred at high concentrations, suggesting that its effect was related to scavenging of ROMs rather than inhibition of their production by XO (Jaeschke, 1990).

2.5.3 Immune-mediated Liver Damage

Immune-mediated liver damage may occur in a number of disorders including the acute and chronic hepatitis resulting from infection, and the autoimmune disorders chronic active hepatitis, primary biliary cirrhosis and sclerosing cholangitis. The evidence that ROMs are involved in the pathogenesis of tissue damage in these diseases is sparse. Activation of phagocytes involves the production of ROMs, which have been shown to produce damage in a number of systems. However, in relation to liver damage, the only evidence is derived from studies of a rat model of liver injury induced by *C. parvum*-activated macrophages and endotoxin (Arthur *et al.*, 1985). In this model, increased production of ROMs was inferred from the presence of increased amounts of lipid peroxides in serum, which correlated with the occurrence of hepatic necrosis and their role in the development of hepatic necrosis by the protective effect of SOD. Studies *in vitro* showed that *C. parvum*-activated macrophages release significantly more ROMs than controls. There was even greater release of ROMs from isolated hepatic macrophages from rats that had received both *C. parvum* and endotoxin (Arthur *et al.*, 1986a, 1986b). There are no studies of the effect of specific antioxidants on these diseases in humans.

2.5.4 Cholestatic Liver Disease

There is very little evidence relating to the role of ROMs in cholestatic liver disease. Serum selenium and glutathione peroxidase activity are decreased in humans with intrahepatic cholestasis of pregnancy (Kauppila *et al.*, 1987). Low levels of vitamin E have been reported in patients with primary biliary cirrhosis, and in children with Alagille's syndrome or biliary atresia (Knight *et al.*, 1986; Jeffrey *et al.*, 1987; Lemonnier *et al.*, 1987; Babin *et al.*, 1988; Kaplan *et al.*, 1988; Sokol *et al.*, 1989). Serum levels of Mn-SOD are increased in patients with all stages of primary biliary cirrhosis compared with patients with other forms of chronic liver disease, although whether this causes or results from the disease process is unclear (Ono *et al.*, 1991).

In the bile-duct-ligated rat, hepatic mitochondrial lipid peroxides are increased and correlate with serum levels of alkaline phosphatase, bilirubin and alanine aminotransferase (Sokol *et al.*, 1991). Dietary vitamin E deficiency resulted in relatively higher lipid peroxide and bilirubin

levels compared to normal lipid diets. Similarly, antioxidant defences have been shown to be decreased in conjunction with increases in lipid peroxidation in the bile-duct-ligated rat (Singh *et al.*, 1992). Plasma vitamin E and serum selenium were reduced, as were liver vitamin E, glutathione, glutathione peroxidase and glutathione transferase and catalase.

2.5.5 Iron Overload (Haemochromatosis)

Iron overload is known to be toxic and potentially fatal. The major pathological effects of hepatic iron overload are fibrosis and cirrhosis, and hepatocellular carcinoma (Bonkovsky, 1991). The role of free radicals in the pathology of hepatic iron overload has been the subject of a detailed review recently (Bacon and Britton, 1990).

2.5.5.1 Increased Production of Free Radicals

There is very little data on ROM production in haemochromatosis in humans. Increases in thiobarbituric acid reactants in plasma were associated with increases in non-transferrin-bound "free" iron. However, other indices of lipid peroxidation were no different from controls (Peters *et al.*, 1985). There are no studies of *in vivo* lipid peroxidation in humans. It is also of interest that levels of antioxidant defences in liver biopsies from patients with haemochromatosis are normal (Selden *et al.*, 1980).

Animal studies have demonstrated increases in lipid peroxidation, measured as breath ethane and pentane exhalation, or hepatic thiobarbituric acid reactants, in animals treated with iron (Golberg *et al.*, 1962; Dillard and Tappel, 1979; Hultcrantz *et al.*, 1984; Britton *et al.*, 1990a; Linpisarn *et al.*, 1991). The increase in alkane exhalation was much greater in rats fed an α-tocopherol-deficient diet (Dillard *et al.*, 1984). Measurement of conjugated dienes has provided evidence of increased mitochondrial and microsomal lipid peroxidation occurring *in vivo* (Bacon *et al.*, 1983a, 1983b).

Isolated hepatocytes incubated with ionic iron rapidly undergo lipid peroxidation. Some studies have not shown a consequent decrease in viability (as indicated by uptake of trypan blue or release of enzymes). This is probably a result of short incubation times, as changes in viability lag behind increases in lipid peroxidation, and may not occur for more than 2 h after lipid peroxidation begins (Bacon and Britton, 1990). Recent studies have shown strong correlations between increased lipid peroxidation [production of thiobarbituric acid (TBA) reactants] and loss of cell viability (trypan blue staining) (Bacon and Britton, 1989). The significance of the lag between lipid peroxidation and decreases in cell viability is as yet uncertain.

2.5.5.2 Production of Cell Damage

The effects of ROMs on liver have already been discussed. That iron is involved in such effects is indicated by the prevention of cell damage by t-butyl hydroperoxide by pretreatment of rats with desferrioxamine and its potentiation by pretreatment with ferrous sulphate (Younes and Wess, 1990). The hepatotoxic effects of t-butyl hydroperoxide were also greater in livers from iron-overloaded rats (Younes and Strubelt, 1990).

2.5.5.3 Effects of Antioxidants

Treatment with iron chelators and α-tocopherol protect against lipid peroxidation and hepatocellular injury in iron-overloaded rats (Sharma *et al.*, 1990). When hepatocytes are isolated from rats, which have been pretreated with α-tocopherol, there is a significant reduction in iron-induced lipid peroxidation and improvement in cell viability *in vitro* (Poli *et al.*, 1985). Similar effects were seen when hepatocytes were incubated with iron chelators (Bacon and Britton, 1990). Treatment of moderately, but not heavily, iron-loaded rats with desferrioxamine *in vivo* inhibits the pro-oxidant activity of hepatic ultrafiltrates (Britton *et al.*, 1990b).

2.5.6 Ischaemia–Reperfusion Injury and Liver Transplantation

Oxygen radicals are thought to mediate some of the structural and functional changes associated with I/R of liver, particularly in the context of transplantation (Arthur, 1988; Parks, 1989; Clavien *et al.*, 1992). During ischaemia, oxygen supply is insufficient to maintain ATP production, resulting in a rise in intracellular calcium and accumulation of ATP metabolites. Xanthine oxidase is formed from xanthine dehydrogenase both by proteolysis and by reversible sulphydryl oxidation (McKelvey *et al.*, 1988) and produces large quantities of superoxide on reperfusion (McCord, 1985).

2.5.6.1 Increased Production of Free Radicals

Human liver is known to be rich in XO, with quoted levels being similar to those found in rats (Parks and Granger, 1986). However, there is relatively little evidence to suggest increased production of free radicals in I/R in humans. Parks *et al.* (1992) have used the appearance of allantoin and parabanic acid (uric acid oxidation products) as indicators of the occurrence of oxidant stress following human liver transplantation. Significant increases were found in the levels of these oxidation products after hepatic reperfusion and it was suggested that ROMs were produced on reperfusion, and might be responsible for the widespread tissue damage associated with reperfusion. Tan *et al.* (1992) were able to demonstrate significant increases in peripheral blood XO activity on reperfusion, suggesting that circulating XO may be an important source of ROMs following liver transplantation.

Chemiluminescence has been used to show increased free-radical production in I/R injury in isolated hepatocytes and in isolated rat livers (Caraceni *et al.*, 1992; Nunes *et al.*, 1992). Studies in isolated rat liver have shown that ischaemia results in increased conversion of

xanthine dehydrogenase to XO (Engerson *et al.*, 1987; Brass *et al.*, 1991). Brass *et al.* (1991) also found that conversion to the oxidase was accelerated in ischaemic liver by imposition of an overnight fast, or by imposing a short period of *in vivo* cardiac arrest. However, Marubayashi *et al.* (1991), whilst confirming the results of others in isolated rat liver, were unable to show any increase in the activity of XO in ischaemic rat liver *in vivo*. On reperfusion, *in vivo* levels of MDA increased significantly, even in the absence of any increase in XO. Previous studies in isolated hepatocytes have also questioned the role of XO in the mediation of tissue damage. De Groot and Littauer (1988) found that conversion of xanthine dehydrogenase to the oxidase only occurred when cells lost viability and became necrotic. Allopurinol did not protect hepatocytes against reoxygenation injury (De Groot and Brecht, 1991) and it was suggested that damage might be a result of ROMs originating from the mitochondrial respiratory chain. The relevance of XO-mediated hepatic injury has also been questioned on the basis of the timing of the conversion of xanthine dehydrogenase to the oxidase and the fact that the concentration of allopurinol needed to prevent ROM-induced injury is significantly higher than that required to inhibit XO (Engerson *et al.*, 1987; Yokoyama *et al.*, 1990; Metzger *et al.*, 1988; Parks and Granger, 1988). Perhaps one explanation for the disparity between the results in isolated rat liver and the *in vivo* preparation may by the massive release of xanthine dehydrogenase/oxidase into the circulation after 2 h of ischaemia followed by reperfusion (Yokoyama *et al.*, 1990). This correlated with a 64% decrease in endogenous hepatic xanthine dehydrogenase/oxidase activity.

A number of studies have demonstrated the depletion of antioxidants during ischaemia. SOD and hepatic GSH are decreased after periods of ischaemia or haemorrhagic shock (Shi *et al.*, 1986; Younes and Strubelt, 1988; Marubayashi *et al.*, 1989; Stein *et al.*, 1991). Furthermore, depletion of hepatic GSH induced chemically or by fasting augmented hepatic I/R-induced enzyme release and promoted lipid peroxidation (Jennische, 1984; Stein *et al.*, 1991) Benoit *et al.* (1992) have used portacaval-shunted rats as a model of chronic hepatic ischaemia, and were able to show decreases in total levels of SOD and xanthine dehydrogenase, but no significant change in catalase or glutathione peroxidase.

2.5.6.2 *Production of Tissue Damage*

The effects of ROM on liver have already been discussed. The potential involvement of ROM in I/R injury is suggested by the findings that glycogen-depleted livers are more susceptible to ischaemic injury. This is thought to be related to accumulation of hypoxanthine during anaerobic metabolism, which serves as a substrate for XO and hence enhanced production of ROM (Younes and Strubelt, 1988).

2.5.6.3 *Effects of Antioxidants*

Much of the evidence for a role of ROM in the pathogenesis of hepatic I/R injury depends on the effects of antioxidants. SOD, catalase, ascorbate, mannitol, α-tocopherol and Trolox (a hydrophilic analogue of vitamin E) all protect rat hepatocytes against ROM-induced damage, and protect the isolated rat liver from I/R injury (Attala *et al.*, 1985; Adkinson *et al.*, 1986; Marubayashi *et al.*, 1986; DeGroot and Brecht, 1991; Koo *et al.*, 1991; Wu *et al.*, 1991; Kurokawa *et al.*, 1992). Allopurinol has been found to be protective in some models, but not in others (Attala *et al.*, 1985; Nordstrom *et al.*, 1985; Adkinson *et al.*, 1986; De Groot and Brecht, 1991). A recent study on Ebselen, a seleno-organic compound, shows that it inhibits release of superoxide and NO from isolated rat Kupffer cells, and protects them from reperfusion injury (Wang *et al.*, 1992). A potential role for neutrophils in the production of tissue damage following I/R is suggested by the protection against such injury afforded by pretreatment with a monoclonal antibody against neutrophils (Jaeschke *et al.*, 1990).

2.5.6.4 *Conclusions*

Whilst there is substantial evidence that production of ROMs is increased in association with I/R injury, that hepatic antioxidants are depleted and that hepatocellular damage can be prevented by a number of antioxidants, much of the evidence is from *in vitro* studies in animals, and some of it is conflicting. The role of XO in the production of hepatocellular damage remains unclear and there are, as yet, relatively few studies in humans. Whilst caution should be used in interpreting the evidence to date, it does seem likely that ROMs do play a role in the pathogenesis of I/R injury. This hypothesis has been put to clinical use in the development of the University of Wisconsin solution. This contains allopurinol and glutathione to protect against oxidative stress, and is used for the preservation of organs prior to transplantation (Southard *et al.*, 1990).

2.5.7 Xenobiotic Liver Injury

The production of free radicals has been implicated in the mechanism of liver injury due to a number of drugs and toxins. These include adriamycin (Pritsos *et al.*, 1992), halothane (Neuberger and Williams, 1984), phenobarbital and thiopental (Kanazawa and Ashida, 1991), carbon tetrachloride (Williams and Burk, 1990), 1,1,2,2-tetrachloroethane (Paolini *et al.*, 1992), and paraquat and related bipyridylium compounds (Togashi *et al.*, 1990; De Gray *et al.*, 1991; Kanazawa and Ashida, 1991; Petry *et al.*, 1992).

2.5.8 Nitric Oxide in Liver Disease

There has been considerable interest recently in the role of NO in many disorders. The role of NO in liver disease has been the subject of a detailed review recently to which interested readers are referred (Stark and

Szurszewski, 1992). It has been hypothesized that NO mediates the hyperdynamic circulation of cirrhosis, that NO-producing drugs might be effective in the treatment of portal hypertension, that NO might be involved in the pathogenesis of tissue injury in cirrhosis and may mediate endotoxin or drug-induced hepatotoxicity (Vallance and Moncada, 1991; Whittle and Moncada, 1992; Stark and Szurszewski, 1992). It is recognized that NO is an important modulator of vascular tone in animals and humans. Induction of NO release produces similar haemodynamic responses to those found in cirrhosis, whilst the changes associated with portal hypertension and cirrhosis can be attenuated by administration of L-NMMA. Furthermore, urinary cGMP, which has been used as a marker of NO synthesis, is increased in cirrhotic humans and the hypotension associated with severe cirrhosis was ameliorated by the administration of methylene blue (a blocker of the action of NO).

3. *Free Radicals and Carcinogenesis in the Gastrointestinal Tract*

The association of chronic inflammation with the development of malignancy is well recognized. There is now much evidence from cell and animal studies that suggests that this association may be a result of phagocyte-generated oxidants (Weitzman and Gordon, 1990; Cochrane, 1991; Trush and Kensler, 1991). In summary, phagocyte-generated oxidants have been shown to cause DNA strand breaks and base modifications, be mutagenic to bacteria and mammalian cells, to cause the malignant transformation of mammalian cells, to potentiate the carcinogenicity of xenobiotics in animals and to activate procarcinogens to genotoxic intermediates. Tumour cells are relatively low in levels of antioxidant defences, including SOD and catalase (Sun, 1990). In addition, there is now epidemiological evidence that dietary antioxidants, such as vitamins A, C, D and E, phytic acid, and protease inhibitors can prevent carcinogenesis, including that of the gastrointestinal (GI) tract (Graf and Eaton, 1990; Block, 1991; Malone, 1991; Troll, 1991; Weisburger, 1991).

3.1 GASTRIC CANCER

Cancer of the stomach is most commonly seen in populations that have a preference for salted, pickled or smoked foods, which have a high content of nitrosamines, and relatively poor access to fresh fruit and vegetables (Singh and Gaby, 1991; Weisburger, 1991; Hietanen and Bartsch, 1992). Nitrosamines are known to be potent animal carcinogens and increasing evidence suggests they may be involved in human carcinogenesis (Hietanen and Bartsch, 1992). Recently, it has been suggested that infection with *Helicobacter pylori* is also a risk factor for the development of gastric cancer, possibly as a result of the induction of a chronic gastritis (O'Connor, 1992). However, hypochlohydria, leading to increased levels of *N*-nitroso compounds, and decreased vitamin C levels in gastric juice, leading to impaired antioxidant defences, may also be factors (Sobala *et al.*, 1989, 1991b; Calmels *et al.*, 1991). Nitrosamines require activation to become mutagenic and carcinogenic species. It is possible that, in chronic gastritis, phagocyte derived oxidants such as the hydroxyl radical might mediate this (Grisham and Yamada, 1992). Several epidemiological studies have shown that vitamin C and β-carotene protect against the development of stomach cancer (Block, 1991).

Studies on patients with established gastric cancer have shown decreased levels of SOD in whole blood and also gastric mucosa (Oka *et al.*, 1990b; Lan-Kai-Wei *et al.*, 1991). The histological studies suggested that the level of tissue SOD was related to the degree of differentiation of the tumour. There is only one study on the effects of antioxidants on the progression of human gastric cancer. Salim (1992h) found that treatment with allopurinol and DMSO conferred significant survival in patients who had undergone potentially curative partial gastrectomy.

3.2 COLON CANCER

Colorectal cancer is a well-established complication of ulcerative colitis (Lennard-Jones *et al.*, 1990; Ekbom *et al.*, 1990). It has been shown that inflammation enhances the formation of colonic tumours in experimental animals given known carcinogens (Chester *et al.*, 1986) and it is tempting to speculate that the long-term inflammatory response is responsible for the increased risk of malignancy in ulcerative colitis. However, there is very little direct evidence to support this. It has also been postulated that free radicals may play a part in the development of sporadic cancers (Babbs, 1990).

Increased production of ROMs has been demonstrated by an animal model of colorectal cancer (McKeever *et al.*, 1992), and in human colorectal cancer using chemiluminescence (Keshavarzian *et al.*, 1992d) and MDA as a measure of lipid peroxidation (Otamiri and Sjödahl, 1989). Levels of MDA were increased in conjunction with increases in myeloperoxidase activity, suggesting that the changes observed might be related to inflammation in and around the tumour. Mulder *et al.* (1990) have shown an increase in the level of CuZn-SOD and a decrease in the levels of metallothionein in human colorectal cancers. The significance of these findings in terms of the relationship between ROM production and the aetiology of cancer is unclear. Blood levels of α-tocopherol and selenium were found to be significantly lower in a group of patients with colorectal cancer and adenomatous polyps compared to controls, These differences were not accounted for by diet (O'Sullivan *et al.*, 1991). Similarly, higher body iron stores appear to

increase the risk of developing colorectal cancer (Stevens *et al.*, 1988). Such findings are confirmed in animal studies (Nelson, 1992; Siegers *et al.*, 1992) and also suggest that this might be due to increased production of free radicals (Younes *et al.*, 1990). Faecal unconjugated bile acids have been shown to be increased in patients with colorectal cancer or polyps (Imray *et al.*, 1992), and are known to stimulate rat colonic epithelial cell proliferation and induce the production of ROM by the rat intestinal mucosa (Craven *et al.*, 1986). Bile-salt-induced colonic epithelial cell proliferation can be abolished by the SOD-mimetic Cu(II) 3,5-diisopropylsalicylic acid (Craven *et al.*, 1986). Bile acids can also produce modification of DNA (Cheah and Bernstein, 1990; Kandell and Bernstein, 1991). Whether this is via the production of ROM is not known. Recently, it has been suggested that NO may be important in the pathogenesis of colorectal cancer complicating IBD, by the formation of carcinogenic nitrosamines (Grisham and Yamada, 1992). Antioxidants, such as 5-ASA, ascorbate, Trolox, cysteine and GSH, can inhibit formation of nitrosamines and so might prove useful in the prevention of cancer (Grisham *et al.*, 1992). Further studies of nitrosamine formation in the pathogenesis of both sporadic and IBD-associated colon cancer are needed.

Epidemiological studies have suggested that a number of naturally occurring antioxidants (e.g. selenium, vitamins A, C and E, indoles and protease inhibitors) might confer protection against colorectal cancer (Burnstein, 1992). Roncucci *et al.* (1993) have shown a decrease in the recurrence of colorectal adenomas when patients are treated with vitamins A, C and E. Antioxidants have been shown to decrease colorectal cell proliferation in patients with adenomatous polyps. Cahill *et al.* (1992) used the bromodeoxyuridine immunohistochemical technique and found significant decreases in the labelling index with supplementation with vitamin C, β-carotene and selenium. There was no difference with vitamin E. Paganelli *et al.* (1992) used the thymidine labelling index to show decreases with vitamins A, C and E. Perhaps of clinical relevance to patients with UC, recent studies in rats have shown that olsalazine, which also has antioxidant activity, can protect against azoxymethane-induced cancer (Hixson *et al.*, 1992). A recent study of 1,2-dimethylhydrazine-induced cancer in rats demonstrated significant delay in the development of hepatic metastases and prolongation of survival as a result of treatment with allopurinol and DMSO (Salim, 1992i). Phytic acid has been shown to act as an antioxidant, probably as a result of its iron-binding properties (Graf and Eaton, 1990). It has been suggested that the decreased incidence of colorectal cancer in populations taking a high-fibre diet is in fact related to the high phytate content (Graf and Eaton, 1985). Phytate has been shown to decrease the carcinogenic effects of azoxymethane and magnesium oxide (Jariwalla *et al.*, 1988; Shamsuddin *et al.*, 1988).

3.3 HEPATOCELLULAR CANCER

The increased incidence of hepatoma in haemochromatosis, cirrhosis and chronic hepatitis B infection is well known (Fargion *et al.*, 1991; Wright *et al.*, 1992). It has been suggested that the generation of ROMs and the mobilization of catalytic free iron may account for the increased risk of cancer in alcohol-induced liver disease. Rajasinghe *et al.* (1990) have demonstrated that DNA cleavage due to alcohol metabolism and the addition of acetaldehyde in isolated rat hepatocytes can be inhibited by desferrioxamine. Yoshiji *et al.* (1992) found that lipid peroxidation and the formation of 8-hydroxydeoxyguanosine were increased in rats fed on a choline-deficient L-amino-acid diet and that these changes were decreased by iron deficiency. As with other tumours, antioxidant systems are impaired in human hepatocellular carcinoma, with decreases in catalase, GSH and glutathione peroxidase (Corrocher *et al.*, 1986).

4. *Conclusions*

There is now considerable evidence suggesting a role for free radicals in the pathogenesis of inflammatory diseases and in the development of cancer. However, the effect of benefit from specific antioxidants in human disease remains to be established. Until this is done, the question of whether free radicals are yet just another inflammatory mediator, or whether their role is more central to the understanding of disease, remains open.

5. *References*

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11. Free Radicals, Muscle Fatigue and Damage

M.J. Jackson, A. McArdle *and* S. O'Farrell

1. Introduction

In recent years free-radical species have been implicated in the pathogenesis of a wide variety of disorders (see Halliwell and Gutteridge, 1989), and attention has been drawn to the possibility that these agents might mediate some of the effects of excessive or unaccustomed exercise on muscle function and structure. In addition, free radicals may play a role in a number of muscle diseases. The aim of this short review is to examine these areas, drawing particular attention to those situations where the role of free radicals is controversial or where further studies are required.

It is undoubtedly true that free radicals can cause damage to muscle and other cells, but whether they are the key mediators of tissue degeneration in many circumstances is not clear. Radical species can damage almost all constituents of the cell and there is a great deal of literature indicating that, in experimental situations, treatment of tissues with radical-generating agents can cause morphological and functional damage to tissue. For instance, although large intravenous injections of linoleic acid hydroperoxide can damage the aortic intima of rabbits (Yagi *et al.*, 1981), and both hydrogen peroxide and organic peroxides can cause functional changes in erythrocyte membranes (Van der Zee *et al.*, 1985), it does not necessarily follow that these processes are related to any aspects of human pathology. It is therefore important in studies attempting to implicate free radicals in pathological processes to demonstrate increased indices of free-radical activity in the situation (disorder or model system) under study and whether these and the ensuing damage can be prevented by inhibitors/scavengers of radical reactions. This combination of two key observations has not been reported in many of the pathological conditions of muscle in which free radicals have been implicated. In particular, elevated levels of indirect indicators of free-radical activity have been described in a large number of human pathological conditions with no evidence that the disease processes are modified by antioxidants and, conversely, protective effects of non-specific radical scavengers have been described in many experimental situations where no evidence of elevated free-radical activity has been provided. The combination of these two findings is important because either one alone is subject to possible misinterpretation. Thus indirect indicators of radical activity are notoriously non-specific and are elevated secondarily to tissue damage (Halliwell and Gutteridge, 1984), and inhibitors of radical reactions such as vitamin E may have substantial protective effects on cells unrelated to their antioxidant role (Phoenix *et al.*, 1991).

2. Exercise

The phenomenon of muscle fatigue and pain after unaccustomed or excessive exercise is well known, and

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experienced by most people at some time. In many cases the pain can be shown to reflect structural damage to the tissue induced by the exercise and, in recent years, several workers have suggested that free-radical species are involved in this damaging process (e.g. see for reviews, Quintanilha and Packer, 1983; Packer, 1984; Jackson and Edwards, 1988).

Numerous studies of the mechanisms by which muscle damage occurs during exercise have utilized a wide variety of experimental models, ranging from examination of athletes during competitive events, to isolated muscles or muscle fibres *in vitro*. Of particular importance is the form of the contractile activity that the muscle undertakes, since this appears to influence greatly the amount of damage that is produced. In concentric activity, muscle is allowed to shorten or lengthen during activation, in isometric activity, muscle is activated but not allowed to shorten, and in eccentric activity, the muscle is lengthened during activation. In both rodents and humans the muscles are injured to a greater extent when exercise involves predominantly lengthening (eccentric) contractions rather than when isometric or shortening (concentric) contractions are involved (Armstrong *et al.*, 1983; Newham *et al.*, 1983), but eccentric exercise has been relatively infrequently studied when the involvement of free-radical species in muscle damaging processes has been examined.

Initial suggestions that free-radical processes, such as lipid peroxidation, were elevated during exercise came from studies of whole-body exercise in man (Dillard et al., 1978) and rats (Brady *et al.*, 1979; Gee and Tappel, 1981). These were rapidly followed by studies of the products of free-radical reactions within the tissues of exercising animals (Davies *et al.*, 1982). These data indicated that exercise to exhaustion in rats resulted in decreased mitochondrial respiratory control, loss of sarcoplasmic reticulum integrity, increased lipid peroxidation and increased free-radical generation as shown by electron spin resonance (e.s.r.) studies. This is perhaps the most widely quoted data in support of a role for free-radical species in exercise-induced damage to skeletal muscle (and other tissues). It is notable that the exercise regime used was an endurance protocol in which the muscles were primarily contracting in a concentric manner.

Exercise can thus have deleterious effects on muscle function. These effects are generally classified as either fatigue or damage. The distinctions between these two processes are not clear. Muscle fatigue is defined as "a failure to maintain the required force" and by this definition damaged muscle is therefore also fatigued. However, the distinction between the two processes is made by the reversibility of fatigue. Fatigue is reversible over a short time period, and *in vivo* it can be of either central or peripheral origin (Gibson and Edwards, 1985). The distinction between the two processes is further clouded by the suggestion that some aspects of muscle fatigue may represent a form of low-grade damage to muscle cells (Aldrich, 1988).

Free-radical species have been implicated in the pathogenesis of both muscle fatigue and muscle damage, and the evidence for these will be discussed separately.

3. *Free Radicals and Muscle Fatigue*

Fatigue of muscles is found post-exercise and in some patients with disorders of limb or respiratory muscles. Peripheral muscle fatigue is generally characterized by the changes in force frequency relationships that occur. The process is traditionally divided into a failure of force production at either low or high frequencies of electrical stimulation.

Initial studies in the area reported that "spin-trapping" agents and vitamin E prolonged the exercise endurance of swimming mice (Novelli *et al.*, 1990), although these authors did not attempt to differentiate whether their results were due to an effect on muscle damage or fatigue. Careful studies of the diaphragm have now been undertaken to look for evidence of free-radical involvement in fatigue of this muscle. Shindoh *et al.* (1990) demonstrated a protective effect of *N*-acetylcysteine against diaphragm fatigue in rabbits *in vivo* when fatigue was induced by repetitive electrical stimulation of strips of the diaphragm *in situ*. *N*-Acetylcysteine was reported to reduce the onset of both low- and high-frequency fatigue, but did not improve the rate of recovery from either. These results are somewhat surprising in that previous data have indicated that different mechanisms underly low- and high-frequency fatigue. Following cessation of contractile activity, most studies have indicated that high-frequency fatigue reverses rapidly, whereas the force deficit apparent at low frequencies persists for considerably longer (Gibson and Edwards, 1985). Some data indicate that high-frequency fatigue is likely to be due to changes in intracellular and extracellular ion concentrations, while low-frequency fatigue is secondary to an abnormality in excitation–contraction coupling (Shindoh *et al.*, 1990). The protective effects of *N*-acetylcysteine against the onset of both forms of fatigue are therefore difficult to explain. It is possible that a common free-radical-mediated mechanism mediates both types of fatigue and that this is inhibited by *N*-acetylcysteine, or that oxidation of protein sulphydryls is common to both processes and inhibited by *N*-acetylcysteine. However, it is also entirely possible that *N*-acetylcysteine acted indirectly by improving the delivery of substrates (such as oxygen or glucose) to the exercising muscle.

More direct support for a role of free-radical species in the mechanisms underlying diaphragm fatigue was provided by Reid *et al.* (1992a, 1992b). They studied fibre bundles from rat diaphragm *in vitro* and demonstrated an apparent increase in intracellular (Reid *et al.*,

1992a) and extracellular (Reid *et al.*, 1992b) oxidant formation during contractile activity. Various antioxidant substances [catalase, superoxide dismutase (SOD) and dimethyl sulphoxide] were also found to inhibit the onset of low-frequency fatigue, although no effects on high-frequency fatigue were observed.

These data are therefore in general support of the hypothesis that free-radical species contribute to the genesis of muscle fatigue. The interpretation of these latter data is, however, complicated by the use of isolated bundles of fibres from the diaphragm as a model system. Such preparations are inherently unstable and lack longer term viability because of the trauma to muscle fibres occurring during dissection of the strips or bundles of fibres. Effects of antioxidants might therefore reflect improved preservation of tissue viability rather than effects on the fatigue process. In an attempt to clarify the possible role of free radicals and the protective effect of

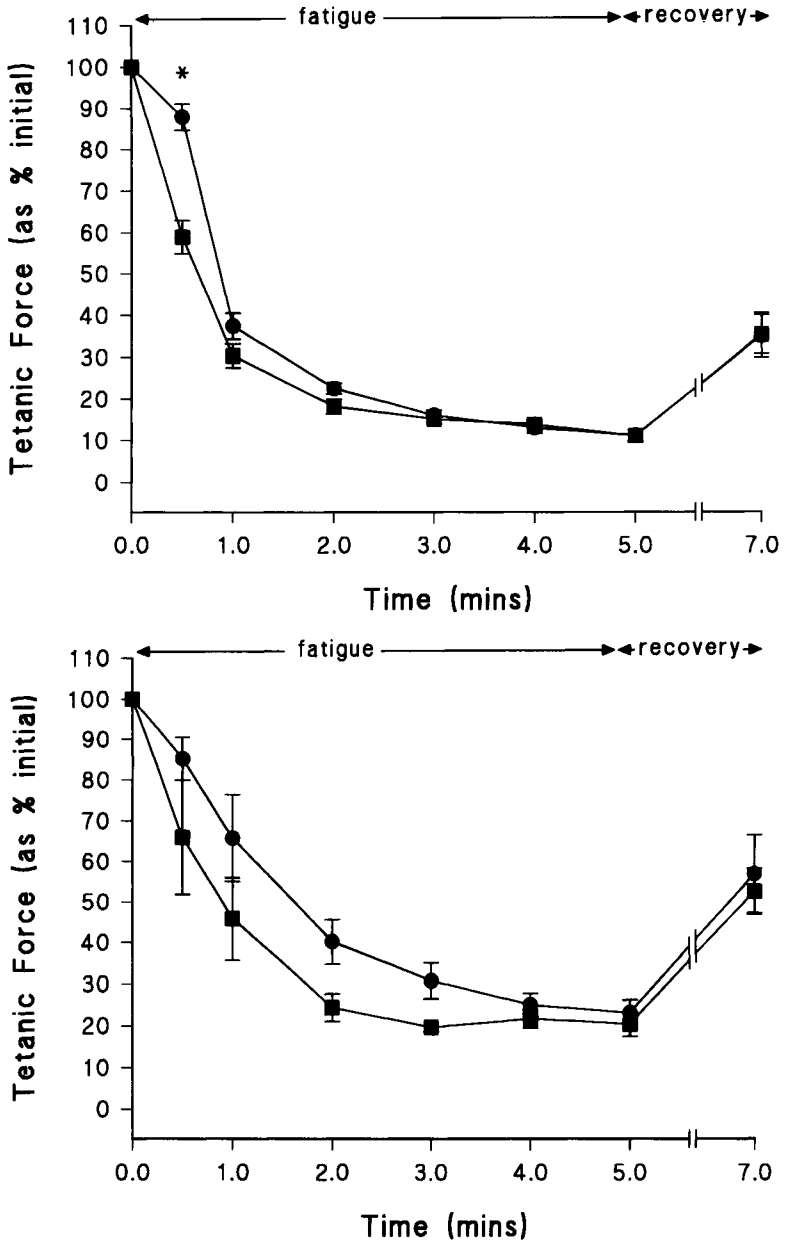

Figure 11.1 Force production by isolated mouse extensor digitorum longus (a) or soleus (b) muscles with (■) or without (●) treatment with superoxide dismutase (1000 U/ml). Muscles were stimulated at 40 Hz for 250 ms every second for 5 min. Values significantly different to control muscles, $^{*}P < 0.01$. Data derived from McArdle *et al.* (1994).

Table 11.1 Effect of superoxide dismustase on twitch characteristics of mouse muscles

	EDL muscles				*Soleus muscles*			
	Control		*SOD treated*		*Control*		*SOD treated*	
Time to peak twitch (ms)	15.6	(0.6)	15.9	(3.4)	18.4	(1.8)	20.8	(1.6)
Peak twitch (mN)	11.9	(0.5)	12.3	(5.4)	6.6	(0.6)	5.5	(1.0
Half relaxation time (ms)	17.3	(2.4)	16.8	(4.7)	23.0	(4.7)	17.6	(1.8)
Rate of increase in force (mN/ms)	0.76	(0.03)	0.77	(0.21)	0.36	(0.01)	0.26	(0.04)
Normalized maximum twitch force (mN/mg)	1.2	(0.1)	1.3	(0.5)	0.80	(0.09)	0.65	(0.11)
Normalized maximum twitch force (mN/mm^2)	15.2	(1.2)	15.9	(6.2)	10.2	(1.5)	8.6	(1.6)

Values are expressed as mean (SEM) of 4–6 muscles. EDL, extensor digitorum longus; SOD, superoxide dismutase.

antioxidants in this process, we have studied the effect of antioxidants on fatigue in an isolated rodent skeletal muscle system. Intact mouse extensor digitorum longus (EDL) or soleus muscles were used for these studies because of their improved viability *in vitro* compared to diaphragm preparations. The effect of antioxidants on the contractile characteristics of these muscles was initially studied to ensure that these compounds had no effect on contraction (unrelated to fatigue), which might complicate interpretation of the results. Fatigue was then induced using the protocol of Sacco *et al.* (1992). Examples of the results obtained for SOD are shown in Table 11.1 and Fig. 11.1. No significant variations from control untreated muscles were seen, neither were there any significant effects with desferrioxamine or vitamin E (McArdle *et al.*, 1994).

The situation concerning a possible role for free radicals in fatigue of skeletal muscle therefore remains unclear. Antioxidants may have some inhibitory role in fatigue of a diaphragm preparation but our experiments using a similar intact skeletal muscle system have not supported these conclusions. It is therefore possible that antioxidants are only beneficial where tissue viability is compromised. Further work is required to clarify this area.

4. *Free Radicals and Exercise-induced Muscle Damage*

Initial studies indicated that various indicators of free-radical-mediated lipid peroxidation were elevated in muscle from exercising animals (Brady *et al.*, 1979; Gee and Tappel, 1981; Davies *et al.*, 1982) and man (Dillard *et al.*, 1978). Contractile activity of skeletal muscle appears to be associated with an increase in an e.s.r. signal derived from free-radical species in muscle (Davies *et al.*, 1982; Jackson *et al.*, 1985), and with a loss of glutathione from the tissue (Gohil *et al.*, 1988; Jackson *et al.*, 1991). The extent of this loss of glutathione from isolated muscles undergoing contractile activity is illustrated in Fig. 11.2. Gohil and co-workers (1988) have suggested that glutathione is oxidized in muscle and rapidly transported from muscle to blood for reduction at non-muscle sites, thus providing a shuttle of reduced glutathione for the exercising muscle. Data from exercising humans are in general agreement with this proposal (Duthie *et al.*, 1990; Sastre *et al.*, 1992) as are the data shown in Fig. 11.2. However, in these *in vitro* studies shown in Fig. 11.2, we were unable to demonstrate whether the glutathione released by the muscle was in the oxidized form. In addition, the time-course of release of the glutathione does not suggest that this loss is a key step in the initiation of damage. In studies of the heart, Ferrari and co-workers (Ferrari *et al.*, 1985, 1986; Curello *et al.*, 1987) have demonstrated that there is an oxidation of intracellular glutathione during ischaemia and concluded that the extent of this oxidation is an important determinant of the vulnerability of the heart to damage during reperfusion. Our studies in skeletal

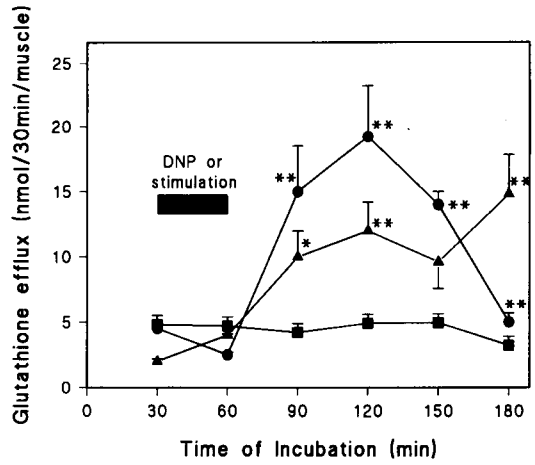

Figure 11.2 Loss of glutathione from isolated rat soleus muscles subjected to either repetitive, electrically stimulated, contractile activity (▲), treated with the mitochondrial inhibitor 2,4-dinitrophenol (●) or untreated (■). Values significantly different to resting control muscles, $^{*}P < 0.05$; $^{**}P < 0.01$. Redrawn from Jackson *et al.* (1991).

muscle do not support such a crucial role for glutathione in contraction-induced damage (Jackson *et al.*, 1991).

Repeated periods of exercise reduce the likelihood of damage to skeletal muscle during subsequent bouts of the same form of exercise and this appears to be associated with an increase in the activity of muscle SOD (Higuchi *et al.*, 1985), a reduced level of lipid peroxidation products during exercise in trained rats (Alessio and Goldfarb, 1988), and a modification of the concentration of antioxidants and activity of antioxidant enzymes in trained humans (Robertson *et al.*, 1991). Packer and colleagues (Quintanilha *et al.*, 1983; Packer, 1984) have also examined the exercise endurance of animals of modified antioxidant capacity and found that vitamin E-deficient rats have a reduced endurance capacity, while Amelink (1990) has reported that vitamin E-deficient rats have an increased amount of injury following treadmill exercise.

The concept has therefore arisen that, during high-intensity oxidative exercise, the increased formation of oxidizing free radicals (probably of mitochondrial origin) can damage muscle tissue, and that training regimes or antioxidant supplementation may reduce this by elevating muscle antioxidant capacity.

Vitamin E appears to be particularly important for preservation of muscle cell viability, since it has been shown to have significant protective effects against muscle damage induced by a number of different stresses. In addition to the effects in intact rats described by Amelink (1990), our group have also shown that vitamin E has protective effects against contractile activity-induced (Jackson *et al.*, 1983; McArdle *et al.*, 1993) and calcium ionophore-induced (Phoenix *et al.*, 1989, 1990, 1991) damage to skeletal muscle *in vitro*. The mechanisms by which this protective effect occurs are not as clear cut as some workers have proposed. The protective effects are apparent in animals fed a diet rich in polyunsaturated fatty acids (see Table 11.2) but not in animals fed a diet rich in saturated fatty acids (O'Farrell, 1994). These data are in general agreement with the concept that the excess vitamin E is preventing free-radical-mediated peroxidation of membrane polyunsaturated fatty acids. However, the protective effects also appear to be mimicked by phytol, isophytol and a number of other lipophilic, non-antioxidant substances having long hydrocarbon side chains (Phoenix *et al.*, 1989, 1991). Again therefore, it is clear that further work is required in this area to clarify the nature of the protection offered by vitamin E.

Table 11.2 Release of creatine kinase activity from isolated rat soleus muscles of different vitamin E status

	Vitamin E supplemented	*Vitamin E deficient*
Untreated	0.26 (0.04)	0.72 (0.32)
Electrically stimulated	0.88 (0.23)	2.90 (0.70)
Calcium ionophore treated	2.84 (0.32)	5.22 (0.46)

Values are presented as mU/min/mg and mean (SEM) of 4–6 muscles. Muscles were either incubated without any further treatment, stimulated for 30 min with repetitive tetanii or treated with the calcium ionophore (A23187 – 20 μm) for 30 min. Data presented represent efflux over 90–120 min post-treatment. Animals were fed either a vitamin E-deficient or vitamin E-supplemented diet (Phoenix *et al.*, 1990) for 6 weeks prior to study. Data derived from O'Farrell (1994).

An area of substantial current interest is the relationship between free-radical-induced damage to muscle and the role of calcium in contraction-induced damage. We have undertaken studies of isolated skeletal muscles to specifically look at this area.

These data have indicated that loss of muscle-cell viability is associated with a loss of calcium homeostasis (Jones *et al.*, 1984) leading to an influx of calcium from the extracellular fluid (McArdle *et al.*, 1992) and activation of various calcium-dependent degradative pathways (Jackson *et al.*, 1984). We have attempted to define the relationships between these degradatory pathways and oxygen radicals. Vitamin E-deficient mouse muscles are more susceptible than supplemented muscles to contraction-induced damage in this system (Jackson *et al.*, 1983) and this appears to be explained by a greater sensitivity to calcium-induced degeneration (Phoenix *et al.*, 1990; McArdle *et al.*, 1993). One possible explanation for these data is that the vitamin E-deficient and control muscle suffer a similar failure of calcium homeostasis during excessive contractile activity, and that this leads to increased free-radical activity. In addition, e.s.r. studies have indicated that the increase in the "stable" free-radical signal in muscles subjected to this form of contractile activity (Jackson *et al.*, 1985) also occurs following a failure of muscle calcium homeostasis (Johnson *et al.*, 1988).

The pattern emerging is therefore complicated in that there is evidence of activation of both calcium-dependent degradative pathways and oxygen radicals in muscle during excessive isometric or concentric activity. The relative role and importance of these two processes is currently unclear.

Eccentric exercise has been infrequently studied from the point of view of free-radical processes, but where damage to skeletal muscle specifically induced by eccentric contractions has been studied, conflicting data have been reported. In a detailed study of damage to mouse EDL muscle induced by eccentric contractions, Zerba *et al.* (1990) found that treatment of animals with polyethylene glycol–SOD significantly reduced the amount of injury which was present 3 days post-exercise in mice of various ages. However, in a study of animals undertaking lengthening contractions during downhill walking, Warren *et al.* (1992) could show no protective effect of vitamin E supplementation. Nevertheless, studies of human subjects undertaking eccentric exercise have reported changes in blood parameters indicative of increased free-radical activity (Packer and Viguie, 1989).

In summary, therefore, the evidence seems convincing that exercise modifies circulating and tissue concentrations of antioxidants and enzyme activities. It is much less certain that the fatigue or damage to skeletal muscle associated with various forms of excessive or unaccustomed exercise is initiated by free radical-mediated degradation. Considerably more work is required in this area to clarify the underlying pathogenic mechanisms.

5. *Ischaemia/Reperfusion Damage to Muscle*

Skeletal muscle is relatively insensitive to ischaemia and reperfusion-induced damage, but damage is now known to occur in a number of situations, such as following revascularization of the acutely ischaemic limb, or after prolonged use of a tourniquet during complicated orthopaedic surgery. This reperfusion damage can result in a considerable deficit in muscle function following restoration of the blood supply (Patterson and Klenerman, 1979; Klenerman *et al.*, 1980; Patterson *et al.*, 1981). Recent work has indicated that such situations are associated with an increased production of free radical species (Patterson *et al.*, 1981; Lindsay *et al.*, 1990; Pang, 1990; Walker, 1991) and that antioxidants provide some protection against the damage (Perler *et al.*, 1990; Oredsson *et al.*, 1991). However, much further work remains to be undertaken on the nature and source of the relevant radical species, and on the application of these studies to the clinical situation.

6. *Muscle Diseases*

Free radicals have been investigated as a possible pathogenic mechanism in various muscle diseases. The role of vitamin E and selenium deficiency in the aetiology of the animal disorders, incorrectly termed "nutritional muscular dystrophy" (Bradley and Fell, 1980), initially prompted studies of the role of these antioxidants and of the possible involvement of free radicals in the human muscular dystrophies. Despite a large number of studies in this area, the results of supplementation studies with antioxidants have been unrewarding (Griffiths *et al.*, 1985; Gamsdorp *et al.*, 1986; Jackson *et al.*, 1989). However, patients with Duchenne muscular dystrophy have elevated levels of indirect indicators of lipid peroxidation, such as diene conjugates (Hunter and Mohamed, 1986) or thiobarbituric acid reactive products (TBARs) (Kar and Pearson, 1979; Hunter and Mohamed, 1986). This may reflect the very substantial amount of ongoing muscle damage that characterizes this severe form of muscular dystrophy. Halliwell and Gutteridge (1984) have indicated that degenerating tissue can form non-specific indicators of lipid peroxidation, which might provide an explanation for the changes in this disorder. It is perhaps pertinent that in an exact biochemical animal model of this disease (the *mdx* mouse), which lacks the same muscle protein (dystrophin), there is much less active muscle degeneration and indicators of lipid peroxidation are normal (Foxley *et al.*, 1991).

Free radicals have also been postulated to play a role in the pathogenesis of malignant hyperthermia. Duthie and co-workers (Duthie and Arthur, 1987; Duthie *et al.*, 1989) have studied the possible role of free radicals in the porcine form of this disorder and have found evidence for abnormal free-radical production by erythrocytes. This was a somewhat unexpected finding since considerable data suggested that the fundamental genetic defect is associated with the muscle sarcoplasmic reticulum, possibly within the gene for the ryanodine receptor (MacLennan *et al.*, 1990). Duthie and co-workers have also undertaken a supplementation study with vitamin E in stress-susceptible pigs (Duthie and Arthur, 1989). Vitamin E supplementation produced a significant fall in the circulating activity of the muscle-derived enzyme creatine kinase, indicating a reduction in the elevated basal level of muscle damage in these animals, although no protection of the animals against the mortality associated with stress was observed. These interesting observations are difficult to explain in the light of the likely fundamental defect in this disorder, but might be an *in vivo* correlate of the *in vitro* protective effect of vitamin E against calcium-mediated muscle damage reported by Phoenix *et al.* (1989, 1991).

The myopathy associated with chronic alcohol abuse has also been associated with increased free-radical activity (Martin and Peters, 1985) as have various other toxicity syndromes affecting muscle, such as cocaine toxity (Kloss *et al.*, 1983). Little work appears to have been undertaken on the possible role of free radicals in the inflammatory myopathies, although, by analogy with other inflammatory disorders, this is likely to be an area worthy of further study.

In conclusion, it is apparent that the lack of definitive methods for assessment of free radicals in muscle and other tissues inhibits clear conclusions to be drawn concerning the relevance (or lack of relevance) of these substances in muscle pathology. In particular, the relevance of free radicals to the deficits in muscle function (fatigue and damage), which occur with exercise, is still unclear despite extensive study. This and other areas require much further examination.

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12. Free Radicals, Oxidative Stress and Diabetes Mellitus

Alan J. Sinclair *and* Joseph Lunec

1. Introduction

Free-radical reactions are implicated in the pathogenesis of a growing number of disorders including Parkinson's Disease (Dexter *et al.*, 1989), rheumatoid arthritis (Blake *et al.*, 1985), cancer (Cerutti, 1991) as well as the ageing process (Harman, 1993). Although the exact nature and identification of the cellular and biochemical mechanisms that contribute to the long-term complications of diabetes mellitus remain elusive, evidence is now accumulating that increased oxidative stress, secondary to free-radical mechanisms, may be involved (Wolff, 1987; Jennings and Barnett, 1988; Baynes, 1991; Sinclair, 1993).

It is generally believed that the pathogenesis of Type 1 (insulin-dependent) diabetes involves both humoral and cell-mediated autoimmune destruction of pancreatic beta cells leading to absolute insulin deficiency (Eisenbarth, 1986) whereas Type 2 (non-insulin-dependent) diabetes is a degenerative metabolic disorder associated with insulin resistance, and defects in insulin secretion whose pathogenesis involves strong hereditary and environmental components (De Fronzo, 1988). However, the spectrum of tissue and organ damage is similar in both types suggesting a common mechanism in the disease process. Although the majority of diabetic patients develop vascular lesions, these vary in time of presentation, site of involvement and severity. This suggests that other factors (e.g. free radicals) may mediate the effect of an intrinsic mechanism. The nature of this mechanism is not known but, in this review, we consider the evidence linking free radicals to vascular tissue damage in diabetes mellitus.

1.1 The Vascular Lesion in Diabetes

Diabetes continues to be a major cause of excessive morbidity, severe disability and premature death in Western populations. In developed countries, the cost of diabetes to society may be estimated to be as high as 5% of the total health costs, much of which relates to the chronic vascular complications of this disorder (Williams, 1991). The vascular lesion in diabetes consists of (1) microangiopathy, distinguished by thickening of capillary basement membranes resulting in increased vascular permeability, which is clinically manifested as diabetic retinopathy (Fig. 12.1a) and/or nephropathy (Fig. 12.1b), and (2) macroangiopathy (Fig. 12.2),

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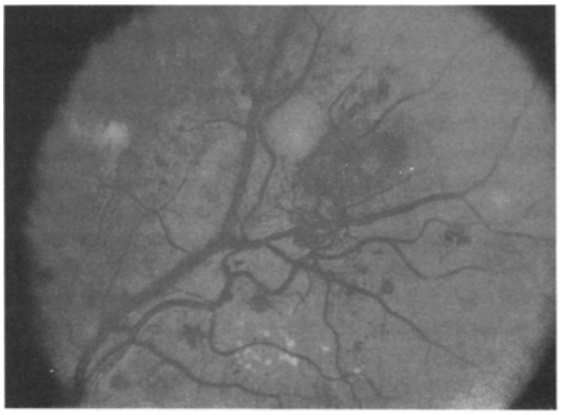
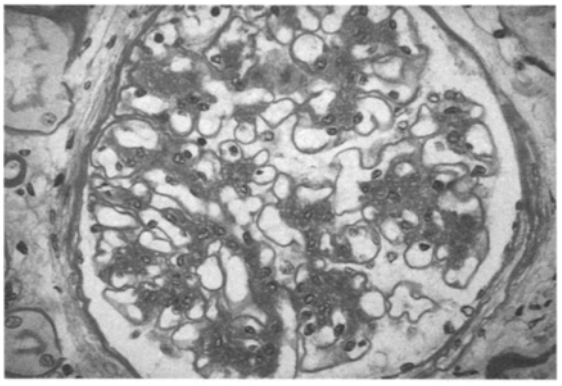


(a)



(b)

Figure 12.1 (a) Diabetic microangiopathy: pre-proliferative retinopathy. (b) Diabetic microangiopathy: glomerular disruption and degeneration in diabetic nephropathy.

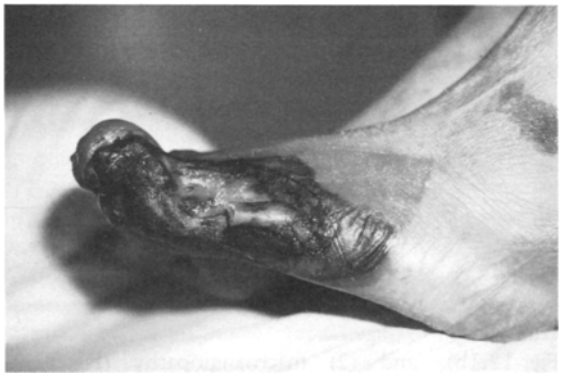


Figure 12.2 Diabetic macroangiopathy: severe peripheral vascular disease and gangrene of the foot.

which consists of atheromatous involvement of large blood vessels, morphologically similar to non-diabetic atheroma, but tending to occur earlier and be more extensive.

2. *Free Radicals and Diabetes Mellitus*

Diabetes represents a state of increased oxidative stress (a high pro-oxidant/antioxidant ratio), which is mainly based on evidence of increased lipid peroxidation or by indirect evidence of reduced antioxidant reserve in both animal and human models of diabetes. This area has recently been reviewed by Lyons (1991). Table 12.1 provides a summary of the studies of lipid peroxidation in patients with diabetes. In the initial Japanese studies in which the thiobarbituric acid reaction was used to measure peroxides, the absence of specific clinical details relating to the type of diabetes, or presence or absence of diabetic complications, makes interpretation of the relationship between peroxidation and diabetic vascular disease imprecise. In the study by Jennings *et al.* (1987), circulating diene conjugates were higher in a group of diabetic patients with microangiopathy ($n = 26$) compared with patients without complications ($n = 36$) and controls ($n = 36$) who had similar levels. The type of diabetes or level of glycaemia bore no relationship to the degree of peroxidation. Diene conjugation was also increased in a group of elderly diabetic patients with complications (microangiopathy and/or macroangiopathy, $n = 22$) compared with an age-matched group of non-diabetic controls ($n = 24$) (Mooradian, 1991). A group of patients without complications ($n = 23$) had a level of diene conjugates intermediate between the level found in control subjects and the level in patients with diabetic complications. The levels of glycaemia, triglycerides and phospholipids were similar in both diabetic groups, suggesting that increased availability of lipid substrate is not the primary cause of increased diene conjugate formation in patients with complications. However, this study did not match patients for disease duration and did not specify the number of patients with microangiopathy.

In contrast, Collier *et al.* (1988) reported decreased diene conjugates in a young group (mean age 31 years) of 34 patients with Type 1 diabetes (most of whom had evidence of diabetic retinopathy) compared with an age-matched group of non-diabetic controls (mean age 30 years). Although the authors concluded that the results of their study did not support the concept that free radicals play a significant role in the development of diabetic vascular disease, this view may be incorrect for two reasons. Firstly, the diene conjugate, PL-9, 11-LAR′, was measured in this latter study; the validity of this compound as a marker of free-radical activity has been questioned by others, since it has a non-peroxide origin and because of its isomeric specificity, 18:2 (9-*cis*, 11-*trans*) (Thompson and Smith, 1985; Sinclair *et al.*, 1989). This

Table 12.1 Studies of serum/plasma lipid peroxides in human diabetes modified from Lyons (1991)

Author (Year)	*Subjects*	*Method of measurement*	*Results in patients*
Sato *et al.* (1979)	110 DM (?Type) 331 C	TBA/fluorescence	↑ LP in "angiopathy" group
Nishigaki *et al.* (1981)	31 DM (?Type) 32 C	TBA/fluorescence	↑ LP in HDL fraction
Kaji *et al.* (1985)	60 DM (Type 2) 71 C	TBA/fluorescence	↑ LP ? complications
Jennings *et al.* (1987)	62 DM (Type 1/2) 36 C	Diene conjugates	↑ DCs in "MA" group Normal in "no MA" group
Collier *et al.* (1988)	34 DM (Type 1) 35 C	Non-peroxide DCs	↓ DCs in patients (+/− complications)
Mooradian (1991)	45 DM (Type 2) 24 C	DCs	↑ DCs in "MA"and "no MA" group (MA > no MA)
Sinclair *et al.* (1992)	50 DM (Type 2) MA:25;No MA:25; 40 C	TBA/DCs	No difference between patients and controls

TBA, thiobarbituric acid reactivity; DCs, diene conjugates; MA, microangiopathy; LP, lipid peroxides; DM, diabetic patients; C, controls; HDL, high-density lipoprotein.

is because peroxidation of lipid systems would be expected to generate a similar quantity of the stereoisomer, 18:2 (10-*trans*, 12-*cis*) but this has not been detected in human fluids and tissues. Secondly, low levels of the PL-9, 11-LA′ isomer may reflect competition for lipid substrate during increased activity of the peroxidation pathway in diabetes. This interpretation has been previously considered by others (Lyons, 1991).

Overall, and contrary to others (Lyons, 1991), we feel that the weight of scientific evidence indicates that lipid peroxidation is increased in diabetes irrespective of whether complications are present or not. The presence of microangiopathy or severe atheroma is likely to increase the degree of peroxidation even further.

Reduced scavenger capacity is deduced from studies demonstrating low plasma and cellular levels of antioxidants such as glutathione, vitamin E, thiols, magnesium and ascorbic acid, as well as reduced levels of scavenger enzymes such as neutrophil glutathione peroxidase and red cell superoxide dismutase (Lyons, 1991; Sinclair *et al.*, 1992).

Collier *et al.* (1990) extended their studies relating to oxidative stress and diabetes by demonstrating that the levels of several free-radical scavengers (red cell superoxide dismutase, plasma thiols) were significantly reduced in 22 type 2 diabetic patients (mean age 53 years) in comparison with 15 control subjects (mean age 51 years). No significant differences in red cell lysate thiols or plasma caeruloplasmin were observed between the groups. These findings were consistent with an increase in free-radical activity in type 2 diabetes, which is in contrast to their findings in a previous study of type 1 diabetes discussed above (Collier *et al.*, 1988).

Several other studies have measured caeruloplasmin levels in diabetic subjects, either as serum copper or by immunological techniques. This copper-containing protein is regarded as a physiological inhibitor of lipid peroxidation and acts by virtue of its ferroxidase activity, converting iron (II) to iron (III) by electron transfer. Most have demonstrated increased serum levels of copper or immunoreactive caeruloplasmin (Solerte *et al.*, 1984), although the study by Collier *et al.* (1990) found no difference between patients and controls, and a previous study found low caeruloplasmin concentrations in diabetic serum (Mateo *et al.*, 1978). Concentrations of iron-binding proteins (transferrin, ferritin) have also been measured in patients with diabetes. These act as secondary antioxidants by making iron unavailable for participation in iron-catalysed radical reactions. A previous study reported levels of transferrin to be normal in pooled diabetic sera (McMillan, 1974) but more recently Jones *et al.* (1988) found significantly elevated levels of transferrin, ferritin and caeruloplasmin in 67 middle-aged diabetic subjects compared with 37 non-diabetic controls. These observations suggest that increased secondary antioxidant activity of diabetic sera

may represent a response to increased oxidative stress in diabetes.

Ascorbic acid (vitamin C) depletion is the most consistent evidence of compromised antioxidant status in diabetes with reports of reduced levels and altered metabolic turnover in several tissues in experimentally induced diabetes in animals (Rikans, 1981; Yew, 1983; McLennan *et al.*, 1988) and in patients with diabetes (Som *et al.*, 1981; Jennings *et al.*, 1987; Sinclair *et al.*, 1991).

In a recent study, serum ascorbate concentrations were significantly reduced in a group of elderly diabetic patients ($n = 40$, mean age 69 years) in comparison with an age-matched group of non-diabetic controls ($n = 22$, mean age 71 years), and this reduction was more pronounced in those patients with microangiopathy (Sinclair *et al.*, 1991). Diabetic patients were shown to have a high serum dehydroascorbate/ascorbate ratio indicative of increased oxidative stress. Ascorbate deficiency was partially corrected by vitamin C supplementation, 1 g daily by mouth, but the obvious disturbance in ascorbate metabolism in the diabetic patients was accentuated, since serum ascorbate concentrations fell (after the initial rise) despite continued vitamin C supplementation (Fig. 12.3).

A more recent study, which measured three established "markers" of free-radical activity in addition to serum ascorbic acid in two groups of elderly diabetic patients (with and without retinopathy), found no significant differences in any of the markers between patients and age-matched controls despite significant depletion of ascorbic acid in patients with diabetes, especially those with retinopathy (Sinclair *et al.*, 1992). These rather paradoxical findings suggest the existence of a complex interrelationship between the levels of individual antioxidant molecules in cells and tissues.

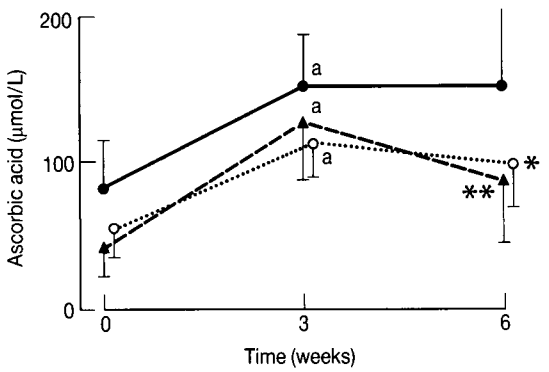
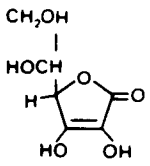


Figure 12.3 Effects of 6 weeks' vitamin C (ascorbic acid) supplementation in both patients and controls (from Sinclair *et al.*, 1991). -•-, Controls; ··○··, no complications group; -- ▲ --, complications group. [a]$P < 0.001$ compared with 0 weeks in all groups. *$P < 0.05$ compared with 3 weeks. **$P < 0.01$ compared with 3 weeks.

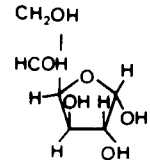
A cooperative interaction between vitamin E and ascorbic acid has already been clarified *in vitro* (Packer *et al.*, 1979) experiments. Although knowledge of this interaction taking place *in vivo* is limited, a recent study in streptozocin-diabetic rats has provided evidence that, at least in experimental diabetes, a synergistic relationship is likely. Young and co-workers (1992) demonstrated a marked increase in oxidative stress with reductions in plasma ascorbate and retinol, and increases in markers of lipid peroxidation, malondialdehyde (MDA) and diene conjugates, in the plasma and red blood cells of untreated streptozocin (STZ)-induced diabetic rats (Young *et al.*, 1992). Vitamin E levels were unchanged. Insulin treatment normalized MDA and ascorbate levels, but ascorbate supplementation (in the absence of insulin) had no effect on MDA and diene conjugates, but increased both plasma retinol and vitamin E levels.

Complex alterations in antioxidant status within different tissues in diabetic animals has been demonstrated in several studies. In one study, 12 weeks of STZ-induced diabetes in female Wistar rats led to marked changes in the levels of tissue-scavenging enzymes (in comparison with controls), with increases in catalase, glutathione reductase and superoxide dismutase (SOD) in the pancreas, whereas the liver's antioxidant capacity was lowered with decreases in both catalase and SOD reported (Wohaieb and Godin, 1987). Reversal of these changes occurred after insulin treatment. In contrast, a recent study of alloxan-diabetes in male Sprague–Dawley rats of 6 weeks revealed that several tissues actually displayed marked resistance to lipid peroxidation (Parinandi *et al.*, 1990). In this latter study, tissue homogenates of both heart and kidney were incubated in the presence of Fe^{2+}/ascorbate for 2 h and assayed for evidence of lipid peroxidation. Both organs yielded significantly lower levels of thiobarbituric acid-reactive substances (TBARs) and lipid hydroperoxides. Diabetic hearts and kidneys also contained higher levels of glutathione. These findings may represent an adaptive response to increased oxidative stress in diabetes but further studies are required to define these intricate relationships.

Many of the discrepancies reported in the literature relating to lipid peroxidation and in diabetes are partly due to differences in the study populations, but limitations of the methods used to quantify lipid peroxidation is probably a major factor. The TBA test is still the most frequently used measurement of lipid peroxidation *in vivo*, and remains a sensitive and simple method. It has, however, been criticized for a number of reasons including its susceptibility to interference from prostaglandins, carbohydrates, and bile pigments, and because of uncertainties about the exact identity of substances taking part in the thiobarbituric acid (TBA) reaction (Gutteridge, 1986). This remains a highly controversial area, and even more sophisticated and direct methods of measuring free radicals such as electron spin resonance (e.s.r.) might not necessarily equate with



L - Ascorbic Acid



D - Glucose

Figure 12.4 Molecular structures of L-ascorbic acid and D-glucose.

radical production *in vivo* because the compounds that capture the radicals (spin traps) may actually perturb the system under measure. In addition, peroxidation in the plasma may not be a reflection of tissue damage. For instance, although lipid peroxidation is thought to be important in the development of atherosclerosis, it seems to be peroxidation in the arterial wall that matters, not peroxidation in the plasma (Steinberg *et al.*, 1989). These results also emphasize the present-day difficulties of directly measuring free radicals and extrapolating *in vitro* peroxidation to an *in vivo* situation (Gutteridge and Halliwell, 1990).

It should be remembered that some of the established antioxidants have other metabolic roles apart from free-radical scavenging. The finding of reduced antioxidant defences in diabetes, for example, may not be prima fascie evidence of increased oxidative stress, since alternative explanations may operate. For example, this may reflect a response to reduced free-radical activity as suggested by the results of a previous study (Collier *et al.*, 1988). In the case of ascorbate, an alternative explanation has been proposed by Davis *et al.* (1983), who demonstrated competitive inhibition of ascorbate uptake by glucose into human lymphocytes. This view is supported by the similar molecular structure of glucose and ascorbic acid (see Fig. 12.4) and by a report of an inverse relationship between glycaemic control and ascorbate concentrations in experimental diabetes in rats. Other investigators, however, have not demonstrated this relationship (Som *et al.*, 1981; Sinclair *et al.*, 1991).

3. *Free Radicals and Islet Cells in Diabetes*

More recently, free radicals have been postulated to mediate part of the beta cell cytotoxicity of interleukin-1 (IL-1), a peptide hormone produced by macrophages, monocytes and other cell types, which may have a pathogenetic role in Type 1 diabetes (Nerup *et al.*, 1988). Direct evidence of free-radical involvement in this pathogenetic model of IDDM appears to be lacking but several lines of evidence support the idea: firstly, IL-1 induces free-radical formation in cells such as eosinophils and endothelial cells (Matsubara and Ziff, 1986; Pincus *et al.*, 1986); secondly, beta cells are exquisitively sensitive to free radicals and may have the lowest free-radical scavenger potential of any cell type in the body (Malaisse, 1982; Asayama *et al.*, 1986).

Yamada and colleagues demonstrated in studies of non-obese diabetic (NOD) mice that large doses of nicotinamide given prior to the onset of diabetes completely prevents the occurrence of glycosuria, and has a therapeutic role when given immediately after the onset of diabetes (Yamada *et al.*, 1982). The histological lesion in NOD mice is similar to that produced by STZ whose beta-cell toxicity is thought to be mediated by alterations in NAD and free-radical metabolism (Oberley, 1988). Pretreatment with nicotinamide can prevent the diabetogenic action of STZ in experimental diabetes (Schein *et al.*, 1967).

The iron chelator, desferrioxamine, has been shown to prevent chronic islet cell allograft inflammatory tissue damage in mice, presumably by chelating iron and preventing hydroxyl radical formation via the Fenton reaction (Bradley *et al.*, 1986). Desferrioxamine and nicotinamide also appear to act synergistically and in a further study by the same group, combined treatment was shown to protect islet allografts in NOD mice to a greater degree than using either agent alone (Nomikos *et al.*, 1986).

Asayama and co-workers investigated the effect of isolated and combined deficiencies of vitamin E and selenium on islet function and free radical scavenging systems in male Sprague–Dawley rats (Asayama *et al.*, 1986). Insulin secretory reserve was impaired in each group of animals deficient in either antioxidant or in combined deficiency. Manganosuperoxide dismutase levels in pancreatic islets were lower in each deficient group compared with controls with combined deficiency producing an additive effect. Glutathione peroxidase activity was markedly decreased in selenium-deficient animals but catalase activities did not change in any of the deficiency groups. A previous study by Slonim *et al.* (1983) demonstrated that administration of vitamin E prior to STZ or alloxan in male Wistar rats protects them against the diabetogenic effects of both agents. Depletion of vitamin E resulted in an enhanced susceptibility to the diabetogenic action of STZ using doses that are normally non-diabetogenic.

Taken together, these observations emphasize the critical importance of maintaining the antioxidant potential of the pancreatic beta cell in order to ensure both its survival and insulin secretory capacity during times of increased oxidative stress.

4. *Criteria for a Role of Free Radicals in Diabetic Vascular Disease*

Several general considerations apply before a causative role of free radicals in diabetes can be established (Table 12.2). There must be a chronological relationship between the onset of increased oxidative stress and the later manifestations of diabetes. Data relating to free-radical reactions prior to the development of diabetes in humans are not available but a number of recent studies in experimental models of diabetes in animals suggest a disturbance of redox chemistry takes place before diabetes is present. In a small study of diabetic-prone BB Wistar rats, expired pentane (as a marker of free-radical-induced lipid peroxidation) increased more than 150% at the time of manifestation of diabetes in comparison to a control group, where pentane expiration remained constant (Pitkanen *et al.*, 1991). In several animals, this increase was observable before the onset of glycosuria. In the same study, expired pentane did not increase in a group of rats made diabetic by STZ. Both treatment with insulin (and withdrawal of insulin resulting in ketosis) did not affect pentane levels in the diabetic group. In another study, both vitamin C (ascorbic acid) and vitamin E status were measured in a group of spontaneously diabetic BioBreeding (BBdp) rats during development, and before the onset of diabetes (Behrens and Madere, 1991). At 30 and 64 days, levels of ascorbic acid and its metabolite, dehydroascorbic acid, were significantly higher in the plasma and spleen of the diabetes-prone rats compared with the control group, but this difference disappeared by 113 days. No differences were seen in the liver, adrenals, thymus and pancreas at any time. In contrast, lower levels of vitamin E were observed in the adrenal gland, thymus and pancreas of the BBdp rats. These changes may be secondary to specific genetic and physiological factors operating in this strain of rat, although an alternative possibility is that an imbalance in oxidative stress is present prior to the onset of diabetes.

Table 12.2 Criteria for free radicals as a causative role in disease

General considerations	
1	Chronological relationship
2	Dose dependency
3	Beneficial effects of antioxidants
Related to diabetes	
4	Free radicals universally present in tissues affected by diabetic process
5	Free radicals have a direct "toxic" effect on tissues
6	Under certain conditions, glucose can induce free-radical production
7	Cellular and extracellular mechanisms that exist to limit the toxicity of free radicals (antioxidants) are known to be deficient in diabetes

Other considerations such as demonstrating a direct correlation between the level of oxidative stress and tissue damage in diabetes and showing that antioxidant therapy leads to prevention, arrest or regression of diabetic complications are also important and must be the basis of future well-designed studies.

Free radicals, however, satisfy other criteria required for an important role in the pathogenesis of diabetic vascular disease:

(1) They are universally present in tissues affected by the diabetic process: in endothelial cells, retina, lens, peripheral nerve cells and kidney.

(2) They have a direct "toxic" effect on tissues. Both free radicals and lipid hydroperoxides have been reported to be directly cytotoxic for vascular endothelial cells. Using a model of acute lung injury (involving systemic activation of complement and leucocyte aggregation by cobra venom factor) in rats, endothelial cell destruction in interstitial pulmonary capillaries was prevented by pretreatment with the vigorous scavenger of hydroxyl radicals, dimethyl sulphoxide (Ward *et al.*, 1983). In this study, infusion of ionic iron potentiated the tissue damage but desferamine mesylate (an iron chelator) afforded protection from the lung injury. These data provide some of the early evidence which suggests that conversion of leucocyte-produced hydrogen peroxide into hydroxyl radicals (by a Fenton reaction) may be an important step in both complement and neutrophil-dependent tissue injury.

Free-radical-induced oxidation of low-density lipoprotein (LDL) may be another mechanism that leads to tissue injury. Following incubation with endothelial or smooth muscle cells, LDL oxidizes and becomes toxic to proliferating fibroblasts (Morel *et al.*, 1983a).

The cytotoxicity of LDL can also be inferred from the study by Blake *et al.* (1985). In this study of human cultured endothelial cells, stored sera from patients with necrotizing arteritis demonstrated an enhanced tendency to develop oxidized LDL, which correlated closely with endothelial cell cytotoxicity. This process appears to require the presence of both oxygen and transition metal ions such as iron in the presence of a reducing agent (Gebicki *et al.*, 1991). There is considerable evidence that transition metals are involved in cell-induced modifications of LDL including the inhibitory effects of EDTA and desferrioxamine (Hiramatsu *et al.*, 1987). A role for O_2^- in LDL modification by endothelial cells and fibroblasts comes from studies showing inhibition of LDL oxidation by SOD (Steinbrecher, 1988).

(3) Under certain conditions glucose molecules can induce free-radical production (see section on non-enzymatic glycosylation of protein).

(4) Cellular and extracellular mechanisms, which exist

to limit the toxicity of free radicals (antioxidants), are known to be deficient in diabetes (Sinclair *et al.*, 1992).

5. *Mechanisms of Free-radical Damage in Diabetes*

Several distinct biochemical mechanisms have been proposed to account for free-radical-induced vascular damage in diabetes.

5.1 NON-ENZYMATIC GLYCOSYLATION OF PROTEIN

Circulating [e.g. immunoglobulin G (IgG)] or structural (e.g. arterial wall collagen or glomerular basement-membrane proteins) proteins exposed to sustained hyperglycaemia undergo non-enzymatic glycosylation to form fluorescent Amadori adducts (Fig. 12.5). Via a series of slow chemical rearrangements (a process known as Maillard or browning reactions), these adducts are converted to advanced glycosylation end-products (AGEs) that continue to accumulate on long-lived proteins and are thought to contribute to the chronic complications of diabetes by causing irreversible damage by protein–protein cross-linking (Brownlee *et al.*, 1984; Baynes, 1991). Three of these products have been identified and are known to be increased in diabetic collagen: pentosidine, carboxymethylhydroxylysine and carboxymethyllysine and provide indirect evidence for a diabetes-related increase in oxidative damage to protein (Baynes, 1991). Concentrations of these products in tissues do not appear to be sufficiently high to cause significant physical or chemical alterations in collagen, but it has been suggested that other as yet unidentified non-fluorescent cross-links may be present as a larger fraction, which may accumulate and have more of an impact on collagen's structural integrity (Baynes, 1991).

Fluorescence changes in glycated proteins may be, at least in part, associated with oxidation of the constituent amino acid by oxygen radical attack. This hypothesis is supported recently by Le Guen *et al.* (1992). La Bella and Paul (1965) associated tyrosine oxidation with age-related changes. Le Guen *et al.* however, found an association with tryptophan oxidation leading to kynurenine formation, previously reported as markers of free-radical attack on tryptophan (Griffiths *et al.*, 1992). It has been reported that when AGE proteins or AGE peptides formed *in vitro* by exposure to glucose are injected into normal rats, they become covalently attached to matrix proteins in vessel walls. This appears to promote endothelial cell permeability and margination of monocytes (Esposito *et al.*, 1989; Kirstein *et al.*, 1990). Specific receptors for proteins modified by AGEs have recently

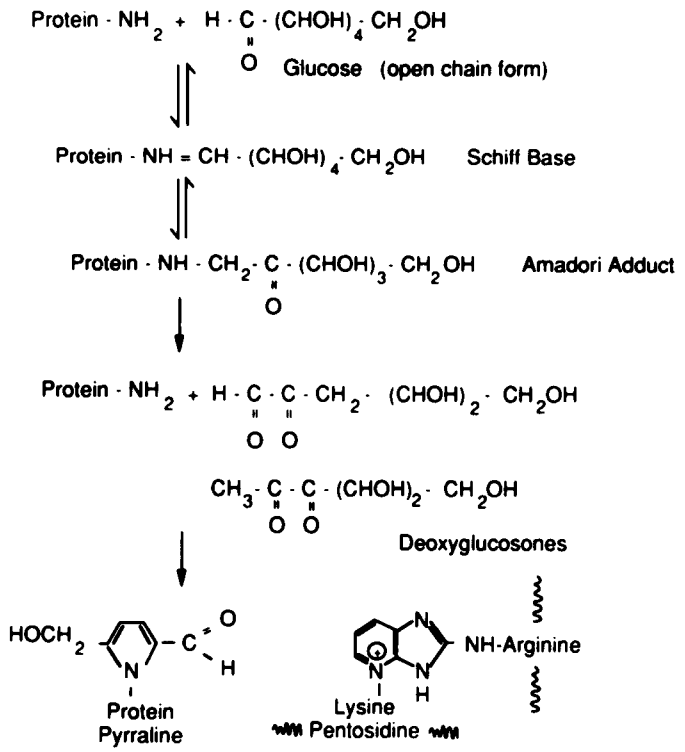


Figure 12.5 Chemistry of advanced glycosylation end-products.

been identified on both murine and human macrophage cells, and are thought to participate in the clearance of AGE-modified proteins (Radoff *et al.*, 1988; Vlassara *et al.*, 1988).

Makita *et al.* (1991) recently measured serum and tissue (arterial wall) AGEs in type 1 and type 2 diabetic patients, and controls at different levels of renal function using a new competitive, whole-cell radioreceptor assay for AGE-modified proteins. The mean AGE content of arterial-wall samples from diabetic patients was significantly higher than that of samples from non-diabetic patients. Diabetic patients with end-stage renal disease had higher levels of AGEs in arterial-wall collagen than diabetic patients without renal failure. Circulating serum AGE levels were also significantly elevated in diabetic patients compared with non-diabetic controls. However, additional studies suggested that increased AGE concentrations in tissues of diabetic patients with end-stage renal disease reflect decreased removal of AGE-modified proteins rather than increased production.

Several lines of evidence suggest a close link between protein fluorescence and free radicals in diabetes:

(1) Exposure of protein amino groups to MDA (formed by the degradation of lipid peroxides) or to oxygen radicals directly, generated by transition metals and hydrogen peroxide, induce fluorescence indistinguishable from that attributed to Amadori-adduct formation (Chio and Tappel, 1969), and leads to the formation of cross-links (Lunec *et al.*, 1985).

Amadori products appear to be a ready source of superoxide radicals, for example, in the fructosamine assay (Jones *et al.*, 1987), which suggests that glycation of protein increases its susceptibility to oxidative damage. Jones *et al.* (1987) suggested a possible mechanism of superoxide production from glycated protein. *In vitro* superoxide production occurs rapidly at alkaline pH and forms the basis of the fructosamine assay for glycated proteins. At physiological pH, however, superoxide production is much slower, but nevertheless measurable. These results were confirmed by Sakurai and Tsuchiya (1988) who also demonstrated oxidation of NADH in the presence of lactate dehydrogenase by glycated proteins, which could be inhibited by SOD. The chemical species responsible was found also to be weakly chemiluminescent (Gillery *et al.*, 1989).

(2) Fluorescence of both collagen (Monnier *et al.*, 1986) and IgG (Jones *et al.*, 1988) are associated with diabetic microangiopathy. Monnier *et al.* (1986) found that measured collagen-linked fluorescence in skin biopsies from both patients with type 1 diabetes ($n = 41$, age range 29–52 years) and controls ($n = 25$, age range 28–41 years) was significantly correlated with the severity of retinopathy as well as arterial and joint stiffness. Jones *et al.* (1988) found increased fluorescence of serum IgG in diabetic patients with retinopathy ($n = 38$, mean age 56 years) in comparison with diabetic subjects without complications ($n = 31$, mean age 53 years) and controls ($n = 26$, mean age 47 years) whose level of fluorescence was similar. Fluorescence of serum albumin was similar in both diabetic groups but significantly elevated in comparison with the control subjects. Fluorescence was not correlated with type or duration of diabetes.

(3) In a recent placebo-controlled study, 2 month's vitamin E treatment in patients with type 1 diabetes resulted in a significant dose-dependent fall in glycosylated proteins independent of changes in plasma glucose (Ceriello *et al.*, 1991). Dose-related falls in both labile and stable fractions of haemoglobin A_1 also occurred.

(4) Glucose may auto-oxidize (like other alphahydroxyaldehydes) and generate hydroxyl radicals in a transition-metal-catalysed reaction, and induce both fragmentation and conformational changes in glycated proteins (Hunt *et al.*, 1990).

This process, referred to as "auto-oxidative glycosylation" can be inhibited by chelating agents [ethylenediamine tetracetic acid (EDTA), diethylenetriamine pentaacetic acid (DETAPAC)] or by a strong reducing environment indicating the critical importance of metal-catalysed reactions and oxidative chemistry in these changes. In addition, the auto-oxidation of monosaccharides, such as glucose, generates hydrogen peroxide and is likely to be increased because of the higher glucose levels. The auto-oxidation of monosaccharides is catalysed by trace metal ions up to a maximum rate but metal ions are not essential for the reaction to proceed. E.s.r., with spin trapping, has been used to demonstrate the generation of hydroxyl and hydroxyalkyl radicals in this reaction (Thornally, 1985). The relevance of these *in vitro* changes to glucose-dependent modifications of proteins *in vivo* is uncertain, and further studies are required to identify specific carbohydrate-derived products of auto-oxidative glycosylation (e.g. the ketoimine adduct to protein) (Baynes, 1991).

5.2 SORBITOL (POLYOL) PATHWAY

This pathway, in which the rate-limiting enzyme aldose reductase (AR) uses NADPH as a cofactor to catalyse the reduction of glucose to sorbitol (Fig. 12.6), has been implicated in the pathogenesis of diabetic complications (Dvornik, 1987). AR is widely distributed in mammalian tissues that are susceptible to diabetic vascular damage, such as the peripheral nerve, retina, glomerulus or lens. During hyperglycaemia there is increased flux through the polyol pathway and intracellular sorbitol accumulates, causing functional and structural changes in tissues through several mechanisms, including exerting a

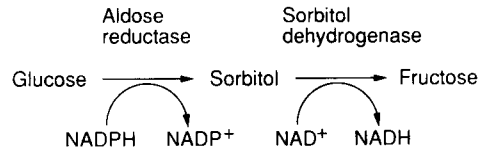


Figure 12.6 The polyol pathway. NADPH, reduced nicotinamide adenine dinucleotide (NAD) phosphate; NADH, reduced NAD.

powerful osmotic effect and depleting myoinositol, which is an important component of membrane phospholipids and determinant of neuronal Na^+/K^+-adenosine triphosphatase (ATPase). Increased polyol pathway activity results in a depletion of both NADPH and NAD^+ with consequent major alterations in cellular redox potentials. This leads to an intracellular deficiency of glutathione (GSH), which is usually regenerated by NADPH-dependent GSH reductase and renders cells less likely to cope with increased oxidative stress. This may underly ascorbate deficiency in diabetes, since GSH is required to regenerate ascorbate from dehydroascorbate. The close relationship between ascorbic acid and the polyol pathway has been observed in rats, where the usual dramatic fall in ascorbate following the induction of diabetes is prevented by both aldose reductase inhibitors and myo-inositol (Yue *et al.*, 1989).

Inhibitors of AR have been demonstrated to prevent a wide variety of biochemical, functional and structural alterations in animal models of diabetes. Early studies demonstrated arrest of both early cataract development and nerve conduction velocity. At least 30 clinical trials of AR inhibitors have been published involving nearly 1000 patients in total. However, there is little impressive data of their efficacy up to now but, rather than undermine the hypothesis linking excess polyol pathway activity to diabetic complications, it may reflect methodological difficulties and trial design errors.

A relationship between polyol pathway activity and reduction in endothelium-dependent relaxation in aorta from chronic STZ-diabetic rats has recently been reported (Cameron and Cotter, 1992). In agreement with several previous studies (Oyama *et al.*, 1986; Kamata *et al.*, 1989), endothelial-dependent relaxation was defective in the diabetic rats but the deficit was prevented by prior treatment with an AR inhibitor. The mechanism underlying the defect has been speculated to be due to decreased production of endothelium-derived relaxing factor (EDRF) or nitric oxide, NO (Hattori *et al.*, 1991). It has been speculated that these vascular abnormalities may lead to diminished blood flow in susceptible tissues and contribute to the development of some diabetic complications. NO is synthesized from the amino-acid L-arginine by a calcium-dependent NO synthase, which requires NADPH as a cofactor. Competition for NADPH from the polyol pathway would take place during times of sustained hyperglycaemia and consequently lead to vascular relaxation deficits. In contrast, using thoracic aortic ring and bioassay procedures, Langenstroer and Pieper (1992) recently reported that diabetic rat aorta releases more spontaneous EDRF than control aorta, but this is offset by an increased production of superoxide radicals, which are known to be inhibitory or to inactivate EDRF (Gryglewski *et al.*, 1986). Addition of SOD increased endothelial relaxation, providing further evidence of an interaction between free radicals and vascular function in diabetes.

5.3 FREE RADICALS AND ATHEROSCLEROSIS

Various lines of evidence indicate a relationship between lipid peroxidation (due to free radicals) and atherogenesis (Table 12.3): (1) lipid peroxides are directly cytotoxic to vascular endothelium (Ward *et al.*, 1983; Blake *et al.*, 1985); (2) circulating lipid peroxides are markedly raised in patients with extensive atherosclerosis, and in diabetic patients with vascular complications (Stringer *et al.*, 1989); and (3) atheromatous plaques are loaded with lipid peroxides and lipid peroxidation products (Glavind, 1952). These do not identify the mechanism of accelerated atherogenesis in diabetes. One hypothesis is that, at sites close to the vessel wall, free-radical-induced oxidation (peroxidation) of LDL takes place leading to rapid uptake into both monocytes and macrophages (via specific scavenger and high-affinity receptors) (Lyons, 1991). This results in foam-cell formation, which is characteristic of the early fatty-streak lesion of atheroma (Fig. 12.7). Some of the lipid peroxidation products generated within the LDL particle covalently bind to the amino-acid residues of apolipoprotein B (apoB) and produce modifications in apoB, which have a high affinity to the macrophage scavenger receptors (Hoff *et al.*, 1989).

Glycosylation of LDL may involve free-radical reactions and itself lead to oxidative damage (Steinbrecher *et al.*, 1984; Esterbauer *et al.*, 1992). However, it appears that glycated LDL is poorly recognized by the classical LDL receptor, but is preferentially recognized by a high-

Table 12.3 Lipid peroxidation and atherosclerosis

Evidence for a link
1 Lipid peroxides are directly cytotoxic to vascular endothelium
2 Circulating lipid peroxides are markedly raised in patients with extensive atheroma
3 Atheromatous plaques are loaded with lipid peroxide products
4 Probucol treatment slows progression of atheroma in the Watanabe rabbit
5 Oxidation and glycation of LDL: may lead to enhanced uptake of LDL by monocytes and macrophages

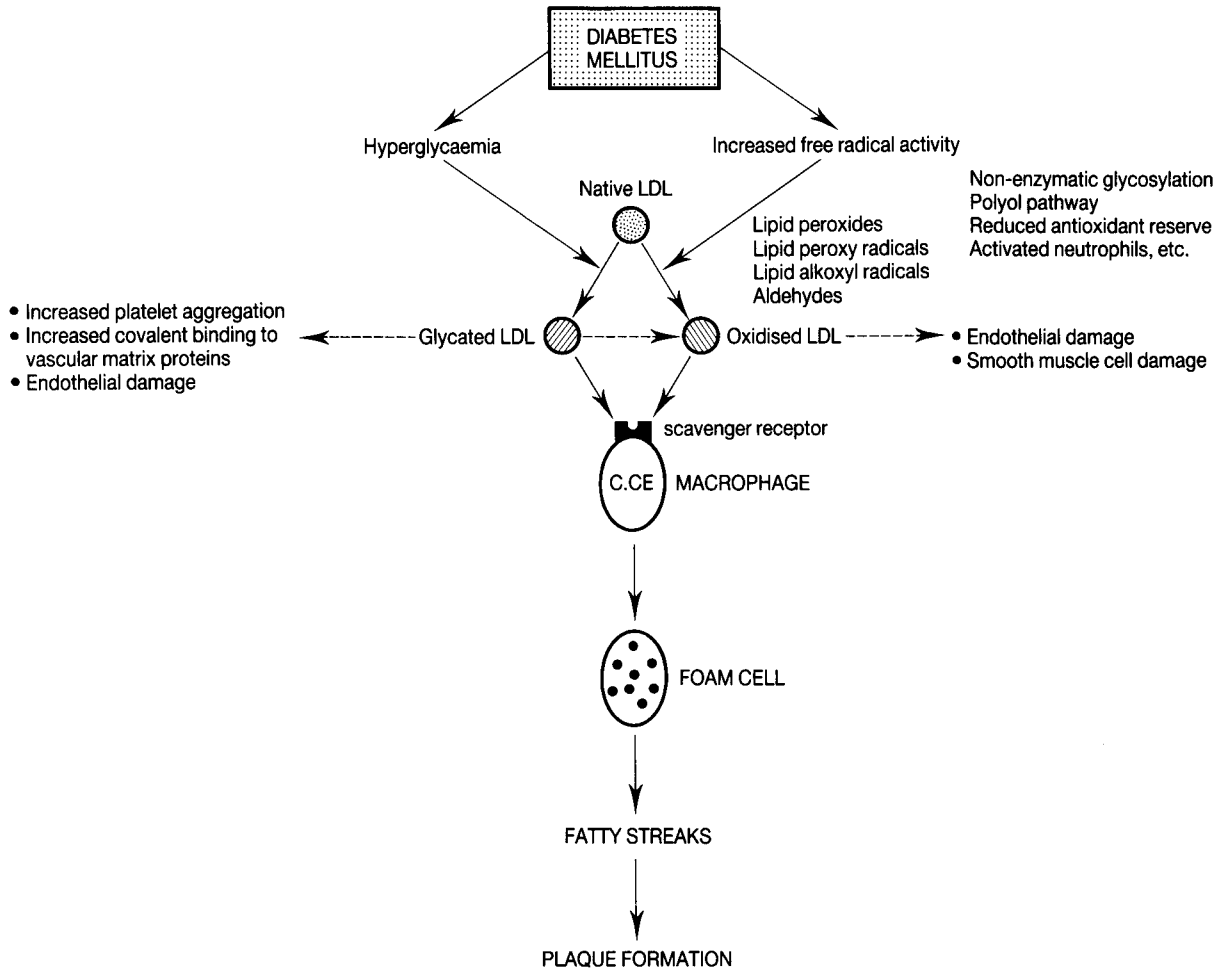


Figure 12.7 Schematic diagram showing the role of LDL glycation and oxidation in foam-cell formation [adapted from Lyons (1991) and Esterbauer *et al.* (1992)]. C, cholesterol; CE, cholesterol ester.

capacity low-affinity pathway on monocytes–macrophages, increasing uptake by these cells and therefore elevating foam-cell formation (Lyons, 1991). Oxidized LDL is present in plaques and is cytotoxic to various vascular wall cells: monocyte–macrophages, smooth muscle cells and fibroblasts (Morel *et al.*, 1983b; Yla-Herttuala *et al.*, 1989). Oxidized LDL is also known to be taken up avidly by cultured murine monocyte–macrophages and to be chemotactic for human monocytes. In addition, a spectrum of procoagulant effects are induced by oxidized LDL, including the inhibition of anti-thrombin III and platelet activation.

There have been more than 20 studies relating to the prevention of atherosclerosis by antioxidants. *In vitro*, several studies have shown that antioxidant treatment (e.g. vitamin E) inhibits both oxidation and the formation of cytotoxic LDL (Steinbrecher *et al.*, 1984; Parthasarathy *et al.*, 1986; Esterbauer *et al.*, 1987). *In vivo*, vitamin E supplementation prevents LDL oxidation in diabetic rats and its conversion to a cytotoxic form (Morel and Chisholm, 1989). Chronic marginal deficiency of vitamin E and C is associated with the development of atherosclerotic-like lesions in rodents, piglets and primates (Liu *et al.*, 1984). The antioxidant, probucol, has been shown to reduce LDL modification and lipid storage in macrophages, and slow the progression of atheroma in the Watanabe Heritable Hyperlipidaemic rabbit, a fact which provides further support for the link between free radicals and atherogenesis (Carew *et al.*, 1987).

Epidemiological studies in Europe reveal an inverse relationship between plasma vitamin E levels and the incidence of ischaemic heart disease (Gey and Puska 1989), and the risk of angina pectoris appears to increase with low plasma levels of vitamins E, A and C (Riemersma *et al.*, 1991). These interesting observations require further population-based controlled intervention trials with specific supplements of antioxidant vitamins (Gey *et al.*, 1991).

5.4 OTHER MECHANISMS OF TISSUE DAMAGE IN DIABETES INVOLVING FREE RADICALS

Further research is required to establish whether free-radical-induced damage is a primary event in diabetes. Tissue damage, which is associated with inactivation of antioxidants and release of metal ions that are potent catalysts of free radical reactions, can lead to lipid peroxidation. This raises the possibility that the diabetic process itself or other factors may increase free-radical activity following direct tissue damage.

Essential hypertension, whose prevalence is increased nearly two-fold in the diabetic population, may be another source of free-radical activity. The vascular lesions of hypertension can be produced by free-radical reactions (Selwign, 1983). In the recent Kuopio Ischaemic Heart Risk Factor Study in Finnish men, a marked elevation of blood pressure was associated with low levels of both plasma ascorbate and serum selenium (Salonen *et al.*, 1988). A few studies report a hypotensive effect of supplementary ascorbate in patients with hypertension, but the actual changes in both systolic and diastolic pressure after ascorbate were not statistically significant in comparison with placebo (Trout, 1991).

However, it remains a valid argument (as in the case of atheroma) that increased oxidative stress may be secondary to (rather than a cause of) endothelial damage in hypertension due to other mechanisms.

Free radicals are by-products of *prostaglandin metabolism* and may even regulate the activity of the arachidonate pathway. Arachidonic acid, released from lipids as a result of activation of phospholipases by tissue injury or by hormones, may be metabolized by the prostaglandin or leukotriene pathways. The peroxidase-catalysed conversion of prostaglandin G_2 to prostaglandin H_2 (unstable prostanoids) and the mechanism of hydroperoxy fatty acid to the hydroxy fatty acid conversion both yield oxygen radicals, which can be detected by e.s.r. (Rice-Evans *et al.*, 1991).

A "thrombotic" tendency is present in diabetes due to an imbalance between prostacyclin and thromboxane. Lipid peroxides and newly generated free radicals are thought to inhibit the vasodilator and anti-platelet effects of endothelial-derived prostacyclin, but stimulate platelet cyclooxygenase activity, thereby promoting the production of thromboxane A_2. This leads to vasoconstriction and platelet aggregation – the concept of "peroxide vascular tone" (Halliwell and Gutteridge, 1989).

Polymorphonuclear leucocytes (PMNs) employ a system comprising myeloperoxidase, hydrogen peroxide, and a halide factor to kill microorganisms and tumour cells. This process is sometimes loosely called the "respiratory burst", which refers to the sudden rise in oxygen consumption by the phagocytosing neutrophils that is independent of the mitochondrial electron transport chain.

Superoxide is produced by the NADPH oxidoreductase (oxidase), which is a membrane-bound enzyme complex containing a flavoprotein that catalyses the transfer of single electrons from NADPH in the cytosol to extracellular oxygen. NADPH is mainly supplied by the hexose monophosphate shunt. In resting cells, the oxidase complex is inactive and disassembled, but is rapidly reconstituted and activated by chemotactic mechanisms or phagocytosis (Baggiolini and Thelen, 1991).

Following a chemotactic or phagocytic stimulus (e.g. complement C5a), vast amounts of superoxide radicals are released into the vacuole, engulfing the microorganism and dismutate either spontaneously or enzymatically to hydrogen peroxide, which has bactericidal properties. *In vitro*, phagocytic responsiveness can be enhanced by a process called priming, involving pretreatment with agents such as cytokines, bacterial endotoxins, activators of protein kinase C, etc. The equation for the reaction is:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\cdot -} \quad (12.1)$$

Chronic granulomatous disease is a rare inherited disorder characterized by the failure of neutrophils, eosinophils, monocytes and macrophages to produce the respiratory burst (Curnutte and Babior, 1987); this leads to recurrent bacterial and fungal infections often starting within the first year of life.

Several reports indicate evidence of immune dysfunction in diabetes, although it is still not absolutely clear if all diabetics (e.g. those with good glycaemic control) have more frequent infections (Bern and Busick, 1985). Large numbers of highly reactive PMNs are present during infection and inflammation, and may promote a state of increased oxidative stress. Hiramatsu and Arimori (1988) have demonstrated that mononuclear cells in diabetics with hypertriglyceridaemia have a greater than normal ability to produce superoxide when stimulated by phorbol esters. In the light of the high incidence of hypertriglyceridaemia in patients with diabetes (particularly type 2), this may also be relevant to the increased oxidative stress observed in diabetes.

6. *Strategies to Modulate Free-radical Activity in Diabetic Vascular Disease (Table 12.4)*

Prevention of vascular disease is one of the goals of a study in progress in Sweden, in which newly diagnosed diabetic children have been randomized in a double-blind study where one group receives placebo and the other a preparation containing ascorbic acid, β-carotene, nicotinamide, selenium and vitamin E (Ludvigsson, 1992). Future research with antioxidants may attempt to prevent the onset of pancreatic beta-cell destruction in the prediabetic phase of susceptible individuals.

Table 12.4 Strategies to modulate free radical activity in diabetic vascular disease

1	Prevention
	Antioxidant therapy
2	Glycaemic control
	Reduction in:
	Non-enzymatic glycosylation
	Monosaccharide autooxidation
	Polyol pathway
3	Routine antioxidant vitamin supplementation
	Replete:
	Vitamin C/E
	Improve:
	Blood pressure
	Immune function
	Reduce:
	Inflammatory reactions
	Ischaemic injury
	Vascular thrombosis
4	Calcium antagonists and ACE inhibitors
	Reduction in:
	Lipid peroxidation
	Hypertension
5	Gliclazide
	Reduction in:
	Level of glycaemia
	Lipid peroxidation

Improving glycaemic control may not only reduce the rate of non-enzymatic glycosylation and monosaccharide autooxidation, but lower polyol pathway activity. In addition, it should have a beneficial effect on other haemodynamic and hormonal factors involved in the development of diabetic vascular disease. However, in studies of diabetic retinopathy, rapid control of glucose levels by intensive insulin therapy has been shown to worsen vascular disease initially and it could be postulated that a sudden improvement in retinal blood flow promotes further free-radical damage as part of a reperfusion–ischaemic injury.

Routine antioxidant vitamin supplementation, e.g. with vitamins C and/or E, of the diabetic diet should be considered. Vitamin C depletion is present in all diabetics irrespective of the presence of vascular disease. A recent study demonstrated no significant difference between the dietary intake of vitamin C (the main determinant of plasma ascorbate) in patients with diabetes and age-matched controls, confirming the view that ascorbate depletion is secondary to the diabetic process and suggesting that diabetic patients require additional intakes of the vitamin to maintain optimal levels (Sinclair *et al.*, 1994). Antioxidant supplementation may have additive beneficial effects on a wide variety of processes involved in diabetic vascular damage including blood pressure, immune function, inflammatory reactions, non-enzymatic glycosylation, ischaemic injury and vascular thrombosis.

At the present time, however, the most appropriate dose, formulation, method and site of administration of antioxidants is not known. A theoretical disadvantage of giving certain antioxidants in high doses (e.g. vitamin C or selenium) is that free-radical reactions may be promoted (a "pro-oxidant" action) rather than inhibited. These areas of doubt need clarifying. Another therapeutic option involves the use of calcium antagonists and angiotensin-converting enzyme (ACE) inhibitors. These drugs have anti-hypertensive, coronary vasodilator and renal haemodynamic effects in addition to free-radical-scavenging properties. Nifedipine and verapamil have been shown to depress lipid peroxidation (Ondrias *et al.*, 1989). More recent studies have shown that verapamil, nicardipine and diltiazem prevent the occurrence of alloxan diabetes (partly mediated by free radicals) in rats, presumably by inhibiting Ca^{2+} inflow into pancreatic beta-cells, although the exact interaction is not known (Katsumata *et al.*, 1992).

The ACE inhibitor, captopril, has been shown to scavenge free radicals *in vitro* and reduce superoxide release in stimulated neutrophils (Chopra *et al.*, 1989). This property was thought to be due to the presence of the sulphydryl group (as in glutathione) but treatment with enalapril (which does not contain a sulphydryl group) also seems to lead to a fall in lipid peroxidation in diabetic patients (Bain *et al.*, 1991). Further research needs to establish the physiological significance of these changes.

Several studies have demonstrated that treatment of diabetic patients with the sulphonylurea, gliclazide, is associated with a fall in lipid peroxidation, protein fluorescence and beneficial effects on platelet function (Florkowski *et al.*, 1988; Jennings *et al.*, 1992). These changes were seen to be independent of changes in glycaemic control.

Aminoguanidine has been shown to prevent the formation of AGEs (Brownlee *et al.*, 1986) and treatment with this drug prevents retinal pericyte loss in STZ-induced diabetic rats (Hammes *et al.*, 1990). More recently, functional and structural impairments in peripheral nerves are ameliorated by aminoguanidine in male Wistar rats (Yagihashi *et al.*, 1992). These data suggest that inhibitors of non-enzymatic glycosylation may have a future role in the treatment of diabetic complications.

7. *Future Studies*

It is important that future studies of antioxidant treatment in patients with specific disorders should be well designed (randomized, double-blind, placebo-controlled) prospective studies that utilize the most up-to-date methodology to assess free-radical activity.

Future research should also focus its attention on the factors/mechanisms that regulate free-radical activity *in vivo*. The complex interrelationship between cellular and extracellular levels of antioxidants needs to be clarified, and factors that govern the synthetic rate of the scavenging enzymes, for example, SOD or catalase will provide further insight into cellular redox control.

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13. DNA Damage by Free Radicals: Carcinogenic Implications

Okezie I. Aruoma *and* Barry Halliwell

1. Introduction

From the early days of free-radical research (Fenton, 1894; Gomberg, 1900; Haber and Weiss, 1934) to the present interest in the use of antioxidants to circumvent cancer (Ames, 1983; Willet and MacMahon, 1984; Bal and Foerster, 1991; Block *et al.*, 1992; Dorant *et al.*, 1993), the words "free radical" have continued to capture the imagination of scientists.

Cancer is one of the diseases in which a role has been implicated (see Table 13.1) for free radicals. Comprehensive accounts of the involvement of reactive oxygen species in human diseases may be found in Halliwell and Gutteridge (1989), Aruoma (1993) and in Cheeseman and Slater (1993).

It is well established that aerobes constantly produce small amounts of oxygen-derived species, such as the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), the latter being generated by the enzyme myeloperoxidase in neutrophils (for reviews, see Di Guiseppi and Fridovich, 1984; Halliwell and Gutteridge, 1989; Weiss, 1989). Exposure of living organisms to background levels of ionizing radiation leads to homolytic fission of oxygen–hydrogen bonds in water to produce highly reactive hydroxyl radicals, $^{\cdot}OH$ (for review, see von Sonntag, 1987). Hydroxyl radicals can also be generated when H_2O_2 comes into contact with certain transition-metal ion chelates, especially those of iron and copper. In general, the reduced forms of these metal ions (Fe^{2+}, Cu^{+}) produce $^{\cdot}OH$ at a faster rate upon reaction with H_2O_2 than the oxidized forms (Fe^{3+}, Cu^{2+}), and so reducing agents such as $O_2^{\cdot -}$ and ascorbic acid can often accelerate $^{\cdot}OH$ generation by metal ion/H_2O_2 mixtures (Halliwell and Gutteridge, 1990a). Another potentially physiologically important source of $^{\cdot}OH$ is the interaction of nitric oxide radical ($NO^{\cdot}$) with $O_2^{\cdot -}$ (Beckman *et al.*, 1990).

Aerobes have evolved antioxidant defences to protect

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Table 13.1 Some clinical conditions in which oxygen free radicals are thought to be involved

Brain
Parkinson's disease
Neurotoxins
Vitamin E deficiency
Hyperbaric oxygen
Hypertensive cerebrovascular injury
Aluminium overload
Allergic encephalomyelitis (demyelinating diseases)
Potentiation of traumatic injury

Eye
Photic retinopathy
Occular haemorrhage
Cataractogenesis
Degenerative retinal damage
Retinopathy of prematurity

Heart and cardiovascular system
Atherosclerosis
Adriamycin cardiotoxicity
Keshan disease (selenium deficiency)
Alcohol cardiomyopathy

Kidney
Metal ion-mediated nephrotoxicity
Aminoglycoside nephrotoxicity
Autoimmune nephrotic syndromes

Gastrointestinal tract
NSAID-induced GI tract lesions†
Oral iron poisoning
Endotoxin liver injury
Diabetogenic actions of alloxan
Halogenated hydrocarbon liver injury
FFA-induced pancreatitis†

Cancer

Inflammatory–immune injury
Rheumatoid arthritis

Inflammatory–immune injury (*continued*)
Glomerulonephritis
Autoimmune diseases
Vasculitis (hepatitis B virus)

Alcoholism

Ageing

Radiation injury

Iron overload
Nutritional deficiencies (Kwashiorkor)
Thalassaemia and other chronic anaemias treated with multiple blood transfusions
Dietary iron overload (red wine, beer brewed in iron pots)
Idiopathic haemochromatosis

Red blood cells
Fanconi's anaemia
Sickle cell anaemia
Favism
Malaria
Protoporphyrin photo-oxidation

Lung
Bronchopulmonary dysplasia
Mineral dust pneumoconiosis
Bleomycin toxicity
Hypoxia
Cigarette-smoke effects
Emphysema
ARDS (some forms)†
Air pollutants (ozone, SO_2, NO_2)

Ischaemia–reperfusion
Stroke/myocardial infarction
Organ transplantation

† ARDS, Adult respiratory distress syndrome; NSAID, non-steroidal anti-inflammatory drug; FFA, free fatty acid; GI, gastrointestinal. Skin injury due to solar radiation, porphyria, contact dermatitis and photosensitizers may also involve free-radical mechanisms.

themselves against the oxygen-derived species generated *in vivo*. These defences include enzymes (such as superoxide dismutases, catalase, and glutathione peroxidases), low molecular mass agents (such as α-tocopherol and ascorbic acid), and proteins that bind metal ions in forms unable to accelerate free-radical reactions (Di Guiseppi and Fridovich, 1984; Halliwell and Gutteridge, 1990a, 1990b; Sies, 1991). Oxidative stress results when oxygen-derived species are not adequately removed. This can happen if antioxidants are depleted and/or if the formation of oxygen-derived species is increased beyond the ability of the defences to cope with them (Sies, 1991).

2. *DNA as a Target of Free-radical Damage*

Subjecting cells to oxidative stress can result in severe metabolic dysfunctions, including peroxidation of membrane lipids, depletion of nicotinamide nucleotides, rises in intracellular free Ca^{2+} ions, cytoskeletal disruption and DNA damage. The latter is often measured as formation of single-strand breaks, double-strand breaks or chromosomal aberrations. Indeed, DNA damage has been almost invariably observed in a wide range of mammalian cell types exposed to oxidative stress in a number

Table 13.2 Methods used to subject cells to oxidative stress that has produced increased intracellular DNA damage (see Halliwell and Aruoma, 1991)

Elevated O_2 concentrations
Exposure to activated phagocytic cells
Exposure to "redox cycling" drugs (e.g. alloxan, paraquat, menadione)
Exposure to cigarette smoke
Exposure to ozone
Exposure to ionizing radiation
Direct addition of hydrogen peroxide or organic peroxides
Exposure to "autoxidizing" agents (e.g. dihydroxyfumarate, pyrogallol, adrenalin)
Exposure to xanthine oxidase[a] plus its substrates (xanthine, hypoxanthine)
Addition of TNF

[a] Care must be taken in the use of commercial xanthine oxidase, which is often heavily contaminated with proteases and other material directly injurious to cells.

of different ways (Table 13.2). Oxidative stress (Larrick and Wright, 1990) and DNA damage (Zimmerman *et al.*, 1989) also occur when some mammalian cells are exposed to tumour necrosis factors. Oxidative stress may additionally play some role in the carcinogenicity of several compounds (Floyd, 1990; Frenkel, 1992) including asbestos, cigarette smoke (Leanderson and Tagesson, 1989, 1992; Loft *et al.*, 1992; Kiyosawa *et al.*, 1990), nitroso compounds (Chung and Xu, 1992; Kelly *et al.*, 1992) and certain metals, such as nickel (Kasprzak, 1991; Kasprzak *et al.*, 1992). There is also considerable interest in the relationship between oxidative stress and non-genotoxic carcinogenesis.

3. *Possible Mechanisms of DNA Damage Induced by Oxidative Stress*

Why does oxidative stress cause DNA damage? In the case of externally generated oxygen-derived species (e.g. when cells are incubated with H_2O_2, activated phagocytes or xanthine oxidase plus its substrates), damage is usually inhibited by adding catalase, showing that H_2O_2 is needed. Superoxide dismutase (SOD) does not usually cause much inhibition, which could mean either that $O_2^{\cdot-}$ is not involved in the DNA damage, or that SOD does not enter cells easily. That the latter interpretion is correct in at least some cell systems is shown by several observations that SOD can protect cells from the toxicity of H_2O_2 or organic hydroperoxides under conditions where SOD can enter the cells (e.g. Kyle *et al.*, 1988; Nakae *et al.*, 1990).

It has been known for many years that neither $O_2^{\cdot-}$ nor H_2O_2 causes any strand breakage in DNA, if the reaction mixture is carefully freed of transition metal ions (see e.g. Rowley and Halliwell, 1983). Our later work (Aruoma *et al.*, 1989a, 1989b, 1991) confirmed this inability of $O_2^{\cdot-}$ or H_2O_2 at physiologically relevant concentrations to damage DNA by looking for chemical changes in the purine and pyrimidine bases (see below). Hence, DNA damage by oxidative stress *in vivo* is unlikely to involve direct attack of $O_2^{\cdot-}$ or H_2O_2 upon the DNA.

Two explanations of the DNA damage have been advanced (Fig. 13.1). First, it is possible that the damage is due to $^{\cdot}OH$ radical formation, a proposal first clearly stated by Mello-Filho *et al.* (1984). Thus, it is envisaged that H_2O_2, which crosses biological membranes easily, can penetrate to the nucleus and react with iron and/or copper ions to form $^{\cdot}OH$. Because of the high reactivity of $^{\cdot}OH$ and its resultant inability to diffuse significant distances within the cell (Halliwell and Gutteridge, 1990a), this mechanism is only feasible if the $^{\cdot}OH$ is generated from H_2O_2 by reaction with metal ions bound upon or very close to the DNA. One possibility is that these metal ions are always present bound to the DNA *in vivo*. For example, copper ions are thought to be present in chromosomes (Lewis and Laemmli, 1982; Dijkwel and Wenink, 1986; Prutz *et al.*, 1990). Copper ions bind to guanine-rich sequences in DNA (Geierstanger *et al.*, 1991) and are very effective in promoting H_2O_2-dependent damage to isolated DNA and to DNA within chromatin *in vitro* (see below). A second possibility (suggested by Halliwell, 1987) is that the metal ions are released within the cell as a result of oxidative stress and then bind to the DNA. Thus, just as oxidative stress causes rises in intracellular free Ca^{2+} (Orrenius *et al.*, 1989), it may cause rises in intracellular free iron and/or copper ions by interfering with normal intracellular sequestration mechanisms. Some of these released ions may then bind to DNA and make it a target for oxidative damage.

A second explanation of the ability of oxidative stress to cause DNA damage is that the stress triggers a series of metabolic events within the cell that lead to activation of nuclease enzymes, which cleave the DNA backbone. Oxidative stress causes rises in intracellular free Ca^{2+}, which can fragment DNA by activating Ca^{2+}-dependent endonucleases (Orrenius *et al.*, 1989; Farber, 1990; Ueda and Shah, 1992) in a mechanism with some of the features of apoptosis (see Wyllie, 1980). An example of apoptosis is the killing of immature thymocytes by glucocorticoid hormones, which activate a cell-destructive process that apparently involves DNA fragmentation by a Ca^{2+}-dependent nuclease.

These two mechanisms (DNA damage by $^{\cdot}OH$ or by activation of nucleases) are not mutually exclusive, i.e. they could both take place (Fig. 13.1). Indeed, there is evidence consistent with the existence of both mechanisms. Their relative importance may depend on the cell type used and on how the oxidative stress is imposed (Halliwell and Aruoma, 1991). For example, chelating agents that bind iron ions into chelates unable to generate $^{\cdot}OH$ (such as desferrioxamine,

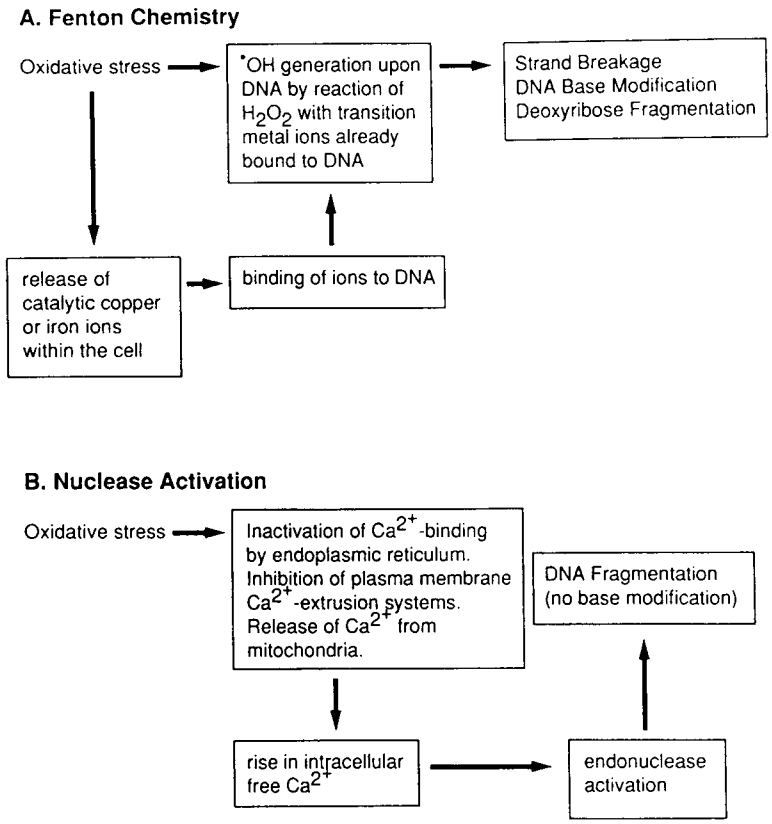

Figure 13.1 Hypotheses to explain DNA damage resulting from exposing cells to oxidative stress.

desferrithiocin and phenanthroline) can often protect cells against DNA damage and other toxic effects of oxidative stress (Mello-Filho *et al.*, 1984; Imlay and Linn, 1988; Halliwell and Aruoma, 1991). The effects of desferrioxamine are variable, since in general it does not cross cell membranes readily, although it appears to enter some cell types (such as hepatocytes) more readily than it enters others.

The evidence for a role played by metabolic changes in the DNA damage produced in cells as a result of oxidative stress is also strong (Birnboim, 1988; Larsson and Cerutti, 1989; Orrenius *et al.*, 1989). Menadione and other quinones (which "redox cycle" within cells to give $O_2^{\cdot-}$ and H_2O_2) appear to produce DNA strand breaks in hepatocytes by Ca^{2+}-dependent activation of an endonuclease. DNA damage could be inhibited by preventing the rise in Ca^{2+} using Ca^{2+} chelators. Oxidative stress can also sometimes activate and/or cause changes in the subcellular location of protein kinase C (Kass *et al.*, 1989; Larsson and Cerutti, 1989). Cantoni *et al.* (1989) found that the Ca^{2+}-chelator quin 2 inhibited H_2O_2-induced DNA strand breakage, although it did not inhibit iron ion-dependent $^{\cdot}OH$ generation from H_2O_2 *in vitro* under their reaction conditions (its effect on copper ion-dependent $^{\cdot}OH$ formation was not examined). Of course, even if transition-metal ion–quin 2 complexes are capable of catalysing $^{\cdot}OH$ formation, the chelator could still protect by removing metal ions from the vicinity of the DNA, so that any $^{\cdot}OH$ generated no longer attacks this molecule (Halliwell and Gutteridge, 1990a). It is also possible that chelators such as desferrioxamine and phenanthroline interfere with changes in cell Ca^{2+} metabolism in response to oxidative stress. It is clear that attempting to elucidate the mechanism of DNA damage in the nucleus of cells subjected to oxidative stress by adding free-radical scavengers or metal-ion chelators to the surrounding media is unlikely to give unambiguous answers.

4. *The Physiological Importance of DNA Damage Induced by Oxidative Stress: Carcinogenic Implications*

Why is it important to understand the mechanism of DNA damage by oxidative stress? Oxidative stress, imposed by a variety of mechanisms (including increased O_2 concentrations) has been convincingly shown to be mutagenic to bacterial and mammalian cells (reviewed by Halliwell and Aruoma, 1991; see also, Essigmann and

Wood, 1993; Feig and Loeb, 1993; Marnett and Burcham, 1993). For example, *Escherichia coli* mutants lacking SOD activity show greatly enhanced rates of spontaneous mutation (Touati, 1989; Prieto-Alamo *et al.*, 1993). Moraes *et al.* (1990) studied the pattern of mutations obtained in a gene of a shuttle plasmid when simian cells transfected with this plasmid were exposed to H_2O_2. Both single base changes and deletions were observed. The majority of base changes were at GC base pairs, the GC → AT base transition being predominant. Treatment of the plasmid with H_2O_2 *in vitro* before transfection did not produce an increased number of mutations (unless iron ions were added), consistent with the inability of H_2O_2 to react directly with DNA. McBride *et al.* (1991) found similar results when single-stranded M13mp2 DNA was incubated with Fe^{2+} ions under aerobic conditions and then transfected into *E. coli*. Cheng *et al.* (1992) showed that 8-hydroxydeoxyguanosine residues in DNA can lead to G → T and A → C substitutions.

Mutations induced by oxidative stress may lead to cancer. Ionizing radiation is well known to be both mutagenic and carcinogenic. Since much of the cell damage caused by such radiation involves $^{\cdot}OH$ production by homolytic fission of the oxygen–hydrogen bonds in water, $^{\cdot}OH$ can probably be classified as a complete carcinogen. Base-pair changes and some frameshifts are the commonest mutations observed in cells exposed to ionizing radiation (for review, see Breimer, 1988, 1990). Chemical changes in the DNA bases, single- and double-strand breaks, and enhanced expression of certain proto-oncogenes have also been described (von Sonntag, 1987). However, the precise relationship between these different events and the development of cancer is uncertain. Thus, the chemical changes in DNA may themselves somehow lead to cancer (for discussion, see Floyd, 1990). An unrepaired lesion in DNA may be bypassed in an error-prone fashion. Resynthesis of DNA after excision repair may conceivably introduce errors.

There are many steps between a healthy cell and a malignant tumour (Trush and Kensler, 1991; Frebourg and Friend, 1992). Cancer biologists have often referred to at least three stages: initiation (an irreversible change in DNA), promotion (probably involving changes in gene expression), and progression (further changes in DNA leading to the eventual production of a malignant tumour), although these distinctions have become less clear cut in recent years. Both Zimmerman and Cerutti (1984) and Weitzman *et al.* (1985) showed that a clone of C3H mouse fibroblasts exposed to activated human neutrophils or to hypoxanthine plus xanthine oxidase underwent malignant transformation. Nassi-Calo *et al.* (1989) showed that H_2O_2 also transformed these cells, an action prevented by the chelating agent *o*-phenanthroline. The ability of oxidative stress to induce transformation has also been shown in human lung fibroblasts (Weitberg and Corvese, 1990). Weitzman *et al.* (1988) reported that DNA isolated from C3H mouse fibroblasts that had been transformed by exposure to activated neutrophils, could sometimes transform another cell line, NIH-3T3, when the DNA was transfected into the latter cells.

Although most attention has been paid in the literature to the action of oxygen-derived species as promoters of carcinogenesis, their ability to damage DNA and produce alterations in gene expression implies that they could be involved in all stages of carcinogenesis (Trush and Kensler, 1991). Indeed, it has been argued (see e.g. Totter, 1980; Ames, 1989) that continuous oxidative damage to DNA by free-radical mechanisms is a significant cause of cancer in humans and explains why cancer incidence increases sharply with age. Of course, DNA damage resulting from oxidative stress (or from any other mechanism; Oller and Thilly, 1992) need not necessarily lead to cancer. Low levels of damage may be efficiently repaired with a minimal risk of error (Breimer, 1991; Lindahl, 1993). High levels of oxidative stress may lead to cell death, so that initiated cells do not remain in the organism. Thus, an intermediate level of oxidative stress is most likely to predispose to malignancy. Another relevant observation may be the ability of low-level oxidative stress to stimulate cell proliferation (Burdon and Rice-Evans, 1989; Goligorsky *et al.*, 1992).

It is interesting to note the association of chronic inflammation (involving phagocytic production of $O_2^{\cdot-}$ and H_2O_2) with malignancy in such human diseases as ulcerative colitis, Crohn's disease and reflux esophagitis (Weitzman and Gordon, 1990). Cerutti *et al.* (1989) showed that one difference between a clone of mouse epidermal cells that was promotable by xanthine/xanthine oxidase and a non-promotable clone was that the latter had lower levels of SOD and catalase, and was more sensitive to killing by oxygen-derived species. Thus, increased antioxidant defences, by protecting against cell death resulting from oxidative stress, may sometimes conceivably, and ironically, lead to increased cancer.

Another interesting observation is that exposure of DNA to $^{\cdot}OH$ can render the DNA antigenic, an observation perhaps relevant to the formation of anti-DNA antibodies in some human chronic inflammatory diseases (Blount *et al.*, 1992; Alam *et al.*, 1993).

5. *Probing the Mechanism of DNA Damage in Cells Exposed to Oxidative Stress: Current Status*

We have already commented that it is difficult to gain information about the mechanism of oxidative stress-induced DNA damage by using antioxidants and scavengers. Another means of implicating free radicals as damaging agents is to use what we have called a "fingerprint" approach: if a free radical produces a unique

pattern of chemical change in a biological molecule, then observation of the same pattern *in vivo* is evidence that the radical attacked that molecule. The damage pattern must be unique to that radical. Therefore, we and others (e.g. Carmichael *et al.*, 1992; Epe *et al.*, 1988, 1993) set out to characterize the chemical changes produced in DNA by different oxygen-derived species. We used the technique of gas chromatography/mass spectrometry with selected ion monitoring, largely developed for work with DNA by Dizdaroglu (reviewed in Dizdaroglu, 1991). We concentrated on damage to the purine (adenine, guanine) and pyrimidine (cystosine, thymine) bases and found, as expected, that $O_2^{\cdot-}$ and H_2O_2 at physiologically relevant concentrations do not themselves cause any base damage in DNA (Aruoma *et al.*, 1989a, 1989b, 1991).

Several studies (for reviews, see Steenken, 1989; Dizdaroglu, 1991) had already shown that $^{\cdot}OH$ reacts in a multiplicity of ways with all four DNA bases. Thus, $^{\cdot}OH$ can add to guanine residues at C4, C5 and C8 positions to give hydroxyguanine radicals that can have various fates. For example, addition of $^{\cdot}OH$ to C8 of guanine produces a radical that can be reduced to 8-hydroxy-7,8-dihydroguanine, oxidized to 8-hydroxyguanine (8-OH-Gua) (Aida and Nishimura, 1987; Oda *et al.*, 1991) or can undergo ring opening followed by one-electron reduction and protonation to give 2,6-diamino-4-hydroxy-5-formamidopyrimidine, usually abbreviated as FapyGua. Figure 13.2 shows the structures of some of these products. Similarly, $^{\cdot}OH$ can add on to C4, C5, or C8 of adenine. Among other fates, the C8 $^{\cdot}OH$ adenine radical can be converted into 8-hydroxyadenine (8-OH-Ade) by oxidation or can undergo ring opening followed by one-electron reduction to give 5-formamido-4,6-diaminopyrimidine (FapyAde). Pyrimidines are also attacked by $^{\cdot}OH$ to give multiple products. Thus, thymine can form *cis*- and *trans*-thymine glycols (5,6-dihydroxy-6-hydrothymines), 5-hydroxy-5-methylhydantoin, 5,6-dihydrothymine and 5-hydroxymethyluracil. Cytosine can form several products, including cytosine glycol and 5,6-dihydroxycytosine (Fig. 13.2). [for reviews, see Dizdaroglu (1991) and Steenken (1989)]. In addition, $^{\cdot}OH$ generation within whole cells or isolated chromatin can result in the formation of cross-links between DNA bases and amino-acid residues in nuclear proteins.

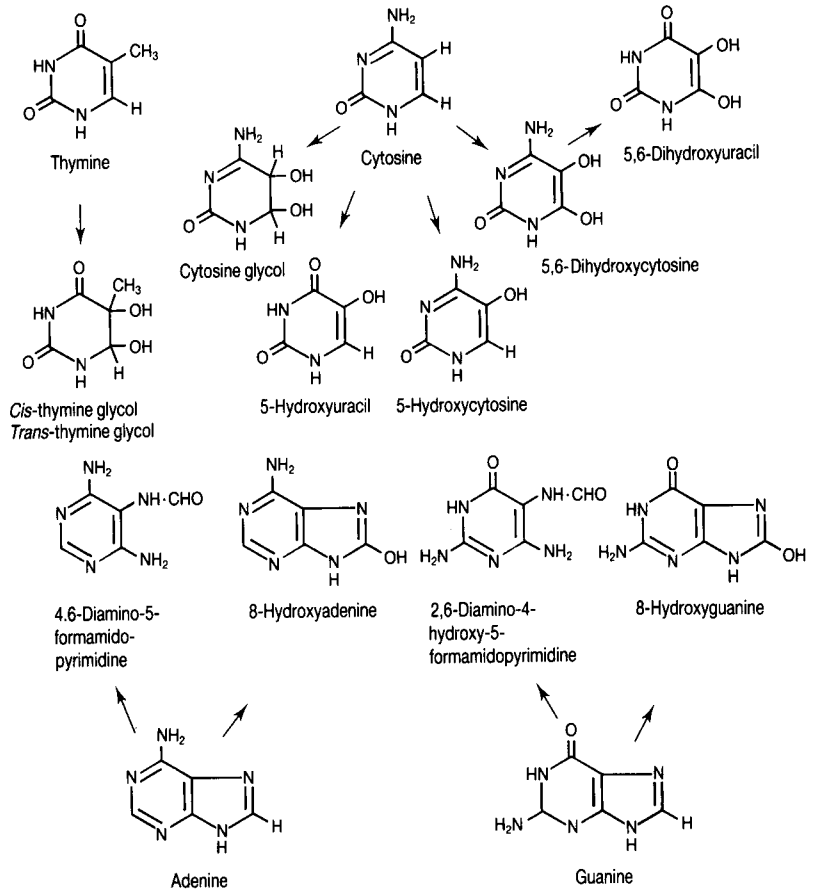

Figure 13.2 Some of the end-products that result from attack of hydroxyl radicals upon the bases of DNA.

Thus, thymine–tyrosine, thymine–aliphatic amino acid and cytosine–tyrosine links have been identified in isolated calf thymus chromatin subjected to γ-irradiation, or treated with metal ions and H_2O_2 (Dizdaroglu, 1991; Nackerdien *et al.*, 1991).

Molecular biologists have examined the likely physiological effects of these various lesions in DNA. 8-OH-Gua (and perhaps 8-OH-Ade) may lead to mutations by inducing mis-reading of the base itself and possibly of the adjacent bases (Breimer, 1991; Shibutani *et al.*, 1991; Tchou and Grollman, 1993; Retel *et al.*, 1993). Thymine glycol may have some mutagenic action and it can be lethal if not removed from the DNA before replication (Evans *et al.*, 1993). Ring-fragmented bases, as well as thymine glycol, can block DNA replication (Evans *et al.*, 1993). Abasic sites, which can also result from attack of $^{\cdot}OH$, can be mutagenic as well (Breimer, 1991).

It is clear that $^{\cdot}OH$ produces multiple changes in DNA, whereas $O_2^{\cdot-}$ and H_2O_2 have no effect. What about other oxygen-derived species? Singlet oxygen is able to produce limited strand breakage in isolated DNA and its ability to modify the DNA bases is also limited. For example, Sies (1993) found small amounts of 8-OH-Gua and FapyGua, but no other significant base changes in DNA exposed to singlet O_2 generated by the thermal decomposition of an endoperoxide. Thus, singlet O_2 seems specific for guanine and does not induce the extensive pattern of DNA base modification produced by $^{\cdot}OH$. Peroxyl and alkoxyl radicals might be expected to react preferentially with guanine, whereas HOCl would be expected to chlorinate $-NH_2$ groups. Oxides of nitrogen may deaminate DNA bases, whereas $NO^{\cdot}$ might react with $O_2^{\cdot-}$ to give $^{\cdot}OH$ and other toxic agents (Beckman *et al.*, 1990; Wink *et al.*, 1991; Van der Vliet *et al.*, 1994). More work is required to characterize these reactions in detail, but we doubt that any species other than $^{\cdot}OH$ produces the extensive pattern of base modification shown in Fig. 13.2. Several authors have reported that peroxidizing lipids damage DNA but, in interpreting the data, one must bear in mind that peroxidizing lipids produce a range of reactive oxygen species including $^{\cdot}OH$, H_2O_2, singlet oxygen, peroxyl radicals and alkoxyl radicals (Gutteridge and Halliwell, 1990), and the exact contributions of these species to the DNA damage observed need to be determined. Lipid peroxides also decompose at body temperature in the presence of transition-metal ions to give a huge range of products, including carbonyl compounds, such as malondialdehyde (MDA) and the unsaturated aldehyde 4-hydroxy-2-*trans*-nonenal, which has been shown to be mutagenic to mammalian cells (Canonero *et al.*, 1990). If these aldehydes are generated in the vicinity of DNA, they may be able to combine with it to form distinctive products. Thus, MDA reacts with adenine, cytosine, and guanine (Stone *et al.*, 1990a, 1990b), and a guanine-MDA adduct has been identified in human urine (Hadley and Draper, 1990). The product of reaction of hydroxynonenal with deoxyguanosine has also been characterized (Sodum and Chung, 1988).

Humans are constantly exposed to background levels of ionizing radiation, which will generate some $^{\cdot}OH$ *in vivo*. This radical may also arise by reaction of metal ions with H_2O_2 *in vivo*. Thus, it is not surprising to find that repair systems have evolved to remove at least some of the lesions in DNA that can result from attack of $^{\cdot}OH$ (for review, see Breimer, 1991).

Some of the modified DNA bases shown in Fig. 13.2 and their nucleosides (base-deoxyribose), have been detected in the urine of humans and other mammals. Thus, 8-OH-Ade, 7-methyl-8-hydroxyguanine, thymine glycol, thymidine glycol, hydroxymethyluracil, 8-OH-Gua and 8-hydroxydeoxyguanosine have been detected in mammalian urine (Ames, 1989; Stillwell *et al.*, 1989; Fraga *et al.*, 1990). The presence of these products in urine suggests that oxidative damage to the DNA bases does occur *in vivo* and that repair systems are active to cleave modified bases from DNA. However, it is possible that some of the modified bases excreted originate from the diet or from the metabolism of gut flora (This is unlikely for the nucleosides which will probably not cross the gut). In addition, it is possible that DNA released from dead and dying cells within an organism undergoes rapid oxidative damage, since cell disruption can increase free-radical reactions (see Halliwell and Gutteridge, 1984). 8-Hydroxydeoxyguanosine may arise from deoxyguanosine triphosphate (dGTP) that has become oxidized *in vivo*: the damaged dGTP may be removed before incorporation into DNA, in a process that has been called "sanitization of the nucleotide pool" (Mo *et al.*, 1992). Hence, one must be cautious in using the amounts of modified DNA bases excreted from the body as an index of the extent of repair of oxidative DNA damage in healthy cells.

6. *Measurement of 8-Hydroxyguanine and 8-Hydroxydeoxyguanosine*

6.1 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The development of a high-performance liquid chromatography (HPLC)-based technique, coupled with highly sensitive electrochemical detection, for the measurement of 8-hydroxydeoxyguanosine (8-OH-dGua) released from DNA by enzymic digestion was the main impetus that has led to the popularity of this product for measurement as a putative index of oxidative DNA damage (Kasai and Nishimura, 1984; Kasai *et al.*, 1984; Floyd *et al.*, 1986), although HPLC techniques for other bases have been described (e.g. Berger *et al.*, 1990; Kojima *et al.*, 1989). The studies of Aruoma *et al.* (1989a, 1989b, 1991) and others helped to validate the

choice of 8-OH-dGua by showing that 8-OH-Gua is a major product of damage to DNA by biologically relevant oxygen-derived species such as $^{\cdot}OH$ and $^1O_2$, i.e. if one had to pick a single product to measure, 8-OH-Gua would be the one to choose.

HPLC-based measurement of 8-OH-dGua (Floyd *et al.*, 1986) has rapidly become popular as a means of gaining some information about damage to DNA in intact cells and whole organisms. For example, the amount of 8-OH-dGua in the DNA of certain subpopulations of rat liver mitochondria was found to be considerably higher than in rat liver nuclear DNA, leading to proposals about the role of mitochondrial DNA damage in ageing and carcinogenesis (Richter *et al.*, 1988). The steady-state level of mitochondrial 8-OH-dGua in human heart has been reported to rise with age (Hayakawa *et al.*, 1992). Exposure of numerous cell types to oxidative stress has been reported to increase the 8-OH-dGua content of their DNA (reviewed by Ames, 1989; Floyd, 1990). Oxidative DNA damage (as 8-OH-dGua) has been measured in human sperm and the amount detected shown to increase in subjects with low intakes of ascorbic acid (Fraga *et al.*, 1991).

These pioneering measurements of 8-OH-dGua have produced evidence that oxidative damage to DNA does occur *in vivo*, although care must be used in interpreting the data. One must be especially cautious in attempting to use levels of 8-OH-dGua (or of any other single product) as a quantitative measure of DNA base damage by oxygen-derived species such as $^{\cdot}OH$ (Halliwell and Aruoma, 1991). When $^{\cdot}OH$ attacks DNA bases, radicals are formed that can have different fates, depending on experimental conditions. For example, attack of $^{\cdot}OH$ on guanine can lead to formation of 8-OH-Gua by oxidation of the C8 OH adduct radical, but this radical can lead to other products as well (such as FapyGua). Hence, different amounts of 8-OH-Gua can result from attack of the same amount of $^{\cdot}OH$ on guanine in DNA. This is one reason why changes in 8-OH-dGua levels do not necessarily reflect changes in the amount of free-radical attack on DNA (Halliwell and Aruoma, 1991, 1993). To take some examples, iron ion-dependent systems generating $^{\cdot}OH$ cause substantial formation of FapyGua as well as of 8-OH-Gua in DNA (Aruoma *et al.*, 1989a, 1989b), whereas systems containing copper ions and H_2O_2 appear to favour 8-OH-Gua over FapyGua (Aruoma *et al.*, 1991; Dizdaroglu *et al.*, 1990). When mammalian chromatin is isolated, suspended in aqueous buffer solution and exposed to radiation-generated $^{\cdot}OH$, the relative amounts of 8-hydroxypurines and formamidopyrimidines generated depend on the environment provided by gases used to saturate the aqueous solution. For example, anoxic conditions favour formamido-pyrimidines over 8-hydroxypurines (Gajewski *et al.*, 1990).

Thus, HPLC-based analysis of 8-OH-dGua as a method of measuring oxidative DNA damage, despite its undoubted value, has intrinsic limitations. Another problem is the frequency with which co-eluting peaks occur when HPLC is applied to complex biological fluids: a peak must never be assumed to represent 8-OH-dGua merely on the basis of its retention time. Electrochemical behaviour, and (if possible) absorbance or fluorescence spectra should be checked for identity with those of authentic 8-OH-dGua. Mass spectrometry is the technique of choice for unequivocal identification, if sufficient material is available.

One must also be careful in interpreting the results of such measurements. An increase in the 8-OH-dGua content of DNA in a cell might mean increased oxidative damage, but decreased repair could also explain it. Thus, do mitochondria repair oxidized DNA more slowly than the nucleus does and so show higher levels of 8-OH-Gua (Richter *et al.*, 1988)? Another problem to be considered is that of dead cells. Cells die constantly in the human body. Dead, disrupted cells are known to undergo lipid peroxidation faster than healthy cells (Halliwell and Gutteridge, 1984): perhaps they also undergo oxidative DNA damage faster as well, so that excretion of DNA-base damage products is not necessarily a reflection of the extent of oxidative DNA damage in healthy cells. Food often contains oxidized lipids (Addis and Warner, 1991; St Angelo, 1992) generated during storage and cooking and perhaps DNA is also damaged. The damaged DNA could be hydrolysed in the intestine, and the damaged base products absorbed and eventually excreted. An alternative explanation of the results of Fraga *et al.* (1991) would be that ascorbate-depleted sperm die faster and their DNA then oxidizes.

In addition, interpretation of measurements of base-damage products excreted in urine as an index of "whole-body oxidative DNA damage" presupposes that these products are not significantly metabolized after cleavage from the DNA. Knowing the tremendous capacity of mammals to metabolize "foreign compounds", this seems unlikely. Thus enzymes exist that can deaminate DNA bases: perhaps they can also metabolize modified bases (Agarwal and Parks, 1978; Glantz and Lewis, 1978; Geborek *et al.*, 1992). These areas remain to be explored.

6.2 GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Characterization of various types of damage to DNA by oxygen-derived species can be achieved by the technique of gas chromatography–mass spectrometry (GC-MS), which may be applied to DNA itself or to DNA–protein complexes such as chromatin (Dizdaroglu, 1991). For GC-MS, the DNA or chromatin is hydrolysed (usually by heating with formic acid) and the products are converted to volatile derivatives, which are separated by gas chromatography and conclusively identified by the structural evidence provided by a mass spectrometer. Stable isotope-labelled bases may be used as internal standards

(Dizdaroglu, 1993). High sensitivity and selectivity of detection can be achieved by operating the mass spectrometer in the selected ion monitoring (SIM) mode. In SIM, the mass spectrometer is set to monitor several ions derived by fragmentation of a particular product during the time at which this product is expected to emerge from the GC column. The GC-MS-SIM technique can be used to study the precise mechanism by which DNA is damaged in cells subjected to oxidative stress. Thus, if damage is due to 'OH generation, then the whole spectrum of base products characteristic of 'OH attack should be detected, as has been observed in chromatin isolated from γ-irradiated human cells in culture (Nackerdien *et al.*, 1992), from murine hybridoma cells after treatment with H_2O_2 (Dizdaroglu *et al.*, 1991b) and in DNA isolated from primate tracheal epithelial cells exposed to ozone or to cigarette smoke (Halliwell and Aruoma, 1991). If, for example, singlet O_2 were responsible for the DNA damage, then a much more limited range of products should be measurable, such as 8-OH-Gua and FapyGua (Floyd *et al.*, 1989; Devasagayam *et al.*, 1991). The results of GC-MS analysis of modified bases in DNA have usually been expressed as nanomoles of modified bases per milligram of DNA (equivalent to pmol/μg DNA). However, it is easy to convert these data into the actual number of bases modified. Dividing the amount of nmol bases/mg DNA by 3.14 (or multiplying by 0.318) gives the number of modified bases per 10^3 bases in DNA, i.e. 1 nmol/mg DNA corresponds to about 318 modified bases per 10^6 DNA bases.

6.3 COMPARISON OF THE HPLC AND GC-MS METHODS

The HPLC-based measurement of 8-OH-dGua is a highly sensitive method, largely because of the use of electrochemical detection, introduced by Floyd *et al.* (1986). Floyd *et al.* (1986) quote a detection limit of 20 fmol, or one 8-OH-dGua per 10^6 nucleosides. Shigenaga *et al.* (1990) quote 5–50 fmol on 40–100 μg samples of DNA.

How much 8-OH-dGua does the technique measure in cellular DNA? Floyd (1990) quotes background levels in cells or tissues (not deliberately subjected to oxidative stress) as 0.5–2.0 8-OH-dGua residues per 10^5 guanosines, or between one and five 8-OH-dGua residues per 10^6 DNA bases (assuming that DNA has, on average, 25% guanine bases). Ames (1989) gives similar figures (one 8-OH-dGua per 130,000 bases in rat liver nuclear DNA, or eight per 10^6 bases, but 1/8000 in mitochondrial DNA, or 125 per 10^6 bases). Most other scientists obtain broadly comparable results (some published data are summarized in Table 13.3). The range is between three and 50 8-OH-dGua per 10^6 bases. Part of the variation might be accounted for by an effect of age (Fraga *et al.*, 1990) in some rat tissues. By contrast, commercial calf thymus DNA (frequently used in studies *in vitro*) contains much more 8-OH-dGua than freshly isolated DNA (Table 13.2). Floyd *et al.* (1989) quote a range of 8–320 8-OH-dGua per 10^6 bases, depending on the batch of commercial DNA used.

The GC-MS-SIM technique also provides high sensitivity and selectivity, and can be applied directly to chromatin (Dizdaroglu, 1991; Dizdaroglu *et al.*, 1991a). The highest sensitivity for a compound under analysis (generally about 5 fmol per compound for products of DNA base modification) is achieved by monitoring the most abundant characteristic ion in its mass spectrum. For initial processing of DNA samples, 50–100 μg of DNA has usually been used but smaller amounts are feasible, since usually only 0.1–0.4 μg of hydrolysed and derivatized DNA is injected on to the GC column for actual analysis.

The lowest background level of a base modification in DNA or in chromatin measurable by currently used GC-MS-SIM techniques corresponds to one modified base in approximately 10^6 bases (Dizdaroglu, 1991). This makes the technique broadly comparable in sensitivity to measurement of 8-OH-dGua by HPLC with electrochemical detection. The exact sensitivity achieved is affected by the GC-MS instrument used and the type of column. In most cases, it is not the absolute sensitivity of the technique that matters but the "background" levels of base modification in untreated DNA, or DNA from "unstressed" cells or tissues (e.g. Lutgerink *et al.*, 1992). Comparison of Tables 13.3 and 13.4 shows that GC-MS-SIM is sensitive enough to detect the levels of 8-OH-dGua that have been recorded by HPLC analysis. GC-MS has been used to show that human cancers appear to contain elevated levels of DNA base damage products (Malins and Haimanot, 1991; Olinski *et al.*, 1992), suggestive of increased oxidative damage and/or decreased repair of such damage *in vivo*.

Commercial calf thymus DNA usually contains 0.5–1.0 nmol of 8-OH-Gua per mg, as measured by GC-MS after formic acid hydrolysis and trimethylsilylation. This corresponds to 159–318 bases/10^6 bases, within the range of HPLC-determined values stated by Floyd *et al.* (1989) (see Table 13.3). Values up to 570 8-OH-dGua per 10^5 guanines were also obtained in commercial calf thymus DNA using a ^{32}P-post-labelling technique (Lutgerink *et al.*, 1992). To date, fewer studies on DNA freshly isolated from cells and tissues have been done with GC-MS-SIM than with HPLC, but the figures available show around 40 8-OH-Gua per 10^6 DNA bases (Table 13.4), generally higher than the figures recorded by HPLC (Table 13.3).

6.3.1 Some Comments on the Discrepancy

Modifications of DNA bases affect cell metabolism and may be related to carcinogenesis (Ames, 1989; Cerutti *et al.*, 1989; Breimer, 1990, 1991; Olinski *et al.*, 1992; Lindahl, 1993), so it is important to understand this apparent discrepancy. What are the possible explanations? First, HPLC may underestimate the real amount

Table 13.3 "Baseline" levels of 8-hydroxydeoxyguanosine in DNA as determined by HPLC (calculation assumes that guanine is, on average, 25% of the DNA bases)

Source of DNA	Content of 8-OH-dGua		Reference
	Per 10^5 guanines	*Per 10^6 DNA bases*	
Rat liver	1.21±0.45	≈3	Conway *et al.* (1991)
(total DNA or nuclear DNA)	4.0±1.3		
	3.1±0.4	≈8	Denda *et al.* (1991)
	7.4±0.9	≈19	Hinrichsen *et al.* (1990)
	1.3–3.33	3–8	Fraga *et al.* (1990)
		≈20	Heig *et al.* (1990)
Mouse liver (total DNA)	0.96±0.37	2.4	Adachi *et al.* (1993)
	0.6–1.4	1.5–3.5	Kasai *et al.* (1986)
	≈20	≈50	Faux *et al.* (1992)
Hamster liver	≈7	≈17	Roy *et al.* (1991)
Rat liver (mitochondrial DNA)	≈13	≈33	Richter *et al.* (1988)
Human phagocytes	0.3±0.008[a]	≈1	Kiyosawa *et al.* (1990)
	—	<10	Floyd *et al.* (1986)
Commercial calf thymus DNA	3–128	8–320	Floyd *et al.* (1989)
	—	26.8±12.6	Aiyar *et al.* (1990)
	22±2	≈55	Kiyosawa *et al.* (1990)
	—	25	Mouret *et al.* (1991)
	—	70	Lu *et al.* (1991)
	6	15	Park and Floyd (1992)
Rat kidney DNA	2.4–4.7	≈6–12	Fraga *et al.* (1990)
	≈1.7	≈4	Sai *et al.* (1991)
	≈1	≈3	Umemura *et al.* (1991)
Hamster kidney DNA	≈3	≈7	Roy *et al.* (1991)
HeLa cell DNA	0.6–1.4	1.5–3.5	Kasai *et al.* (1986)
Salmonella typhimurium DNA	0.6–1.4	1.5–3.5	Kasai *et al.* (1986)

[a] From non-smokers.

Table 13.4 GC-MS-based measurement of 8-hydroxyguanine in DNA (after acidic hydrolysis of DNA, unless otherwise stated)

Source of DNA	8-OH-Gua		Totally modified bases[a] (per 10^6 DNA bases)	Reference
	DNA (nmol/mg)	*Bases/10^6 DNA bases*		
Commercial calf thymus DNA	0.5–1.0	159–318	≈640	Aruoma *et al.* (1989a, 1989b, 1991)
Mouse liver[b] mitochondrial DNA	—	≈3500	—	Hayakawa *et al.* (1991)
Fish liver	0.13±0.06	≈41	—	Malins and Haimanot (1990)
Chromatin isolated from murine hybridoma cells[c]	—	35–40	≈138	Dizdaroglu *et al.* (1991b)
Human breast tissue	0.13±0.03	≈41	—	Malins and Haimanot (1991)
Human neutrophils	0.20–0.23	64–73	—	Malins and Haimanot (1991)
Human brain	—	14		Olinski *et al.* (1992)
Human lung	—	73–97		Olinski *et al.* (1992)

[a] Total of all the modified bases measured in the DNA.
[b] GC-MS analysis after enzymic hydrolysis of DNA.
[c] Freshly isolated chromatin. Chromatin after extensive dialysis gives higher figures.

of 8-OH-dGua in DNA. Second, GC-MS may overestimate it, e.g. if the derivatization and hydrolysis procedures somehow artefactually cause formation of 8-OH-Gua. Third, both errors could occur. Before discussing these potential explanations in detail, it is worth pointing out that isolation of DNA from cells may introduce some oxidative modification, particularly if phenol-based methods are used (Claycamp, 1992), since oxidizing phenols produce a wide range of reactive radicals (Halliwell and Gutteridge, 1989). This is one of the reasons why extraction of chromatin for analysis may be preferable (Dizdaroglu, 1991; Dizdaroglu *et al.*, 1991a). This technique is milder than those used for DNA extraction and may minimize the loss of extensively fragmented DNA and of DNA that has become cross-linked to protein as a result of oxidative damage.

6.3.2 Can HPLC Underestimate 8-OH-dGua?

Once "up and running", the HPLC technique is fairly reliable and does not treat DNA as "roughly" as do the preparation procedures that are used for GC-MS. However, HPLC analysis requires complete extraction of DNA from the cell and complete enzymic hydrolysis before quantitative measurement of 8-OH-dGua can be achieved. First, extraction of DNA that has undergone extensive oxidative modification and fragmentation (Table 13.4) may be impaired, because of the easy loss of small DNA fragments and of cross-linking of the DNA bases to amino-acid residues in nuclear proteins (Dizdaroglu, 1991). Hence this HPLC method may underestimate DNA damage in cells that have been subjected to intense oxidative stress.

Second, the efficiency of exonucleases and endonucleases in hydrolysing DNA is greatly affected by modification of the bases (Dizdaroglu *et al.*, 1978; Breimer, 1991). For example, Maccubbin *et al.* (1991) reported that the presence of 8-OH-Gua severely inhibits digestion of dinucleotides by phosphodiesterase. Thus it is not always certain that modified bases are completely hydrolysed from DNA, especially when published hydrolysis techniques are transplanted from one laboratory to another and not revalidated.

Third, the HPLC technique usually measures 8-OH-dGua, and not 8-OH-Gua, which is separated on the columns used. Frenkel *et al.* (1991) showed that acid pH (frequently used for nuclease P_1 digestion) can promote hydrolysis of 8-OH-dGua to 8-OH-Gua, causing a loss of HPLC-detectable material. Extensive free-radical damage might also lead to release of modified guanine from the DNA backbone to leave an abasic site. Frenkel *et al.* (1991) considered these artefacts and, using a more complex HPLC technique than is commonly employed, they found that DNA extracted from murine epidermal cells contains baseline 8-OH-dGua levels of at least 30 per 10^6 DNA bases, closer to the values measured by GC-MS (Table 13.4) than to those measured by HPLC (Table 13.3).

6.3.3 Artefacts in GC-MS Techniques?

The GC-MS techniques give much more chemical information than does measurement of only 8-OH-dGua. However, the hydrolysis and derivatization procedures are lengthy and tedious, and may destroy some modified bases, e.g. hydroxymethyluracil (Djuric *et al.*, 1991). It has also been speculated that they might create artefacts, e.g. if the amounts of modified bases increase during the preparation procedures. Data suggest that formic acid hydrolysis does not create additional 8-OH-Gua in DNA (Halliwell and Dizdaroglu, 1992) but the question is currently open as to whether the derivatization procedures might do so.

We do not know the "baseline" level of products of DNA damage by oxygen-derived species *in vivo*, since different measurement techniques give different results. Greater attention must be given to resolving these methodological questions and validating the assays being used before the widespread adoption of such methods as a true index of oxidative DNA damage.

7. *Antioxidant Therapy: Points for Consideration*

Free-radical generation occurs normally in the human body, and rates of free-radical generation are probably increased in most diseases (see Table 13.1). Their importance as a mechanism of tissue injury is still uncertain, largely because the assays used to measure them have, until recently, been primitive. The development of new assays applicable to humans (such as the assays of oxidative DNA damage described above) should allow rapid evaluation of the role of free radicals in disease pathology and provide a logical basis for the therapeutic use of antioxidants. A rationale is presented in Fig. 13.3. Attempts to use antioxidants in the treatment of human disease can be divided into three main areas:

(1) Administration of antioxidants that occur naturally in the human body, such a α-tocopherol, glutathione or SOD.
(2) Administration of synthetic antioxidants and/or chelating agents that suppress iron ion-dependent free-radical reactions. Some enzyme inhibitors may be appropriate here, for example, xanthine oxidase inhibitors.
(3) The possibility that drugs developed to protect against other mechanisms of tissue injury might have an additional physiological action because they have antioxidant properties.

However, it must not be forgotten that mechanisms of tissue injury in human disease are very complex and it may not be possible to clearly identify the role played by

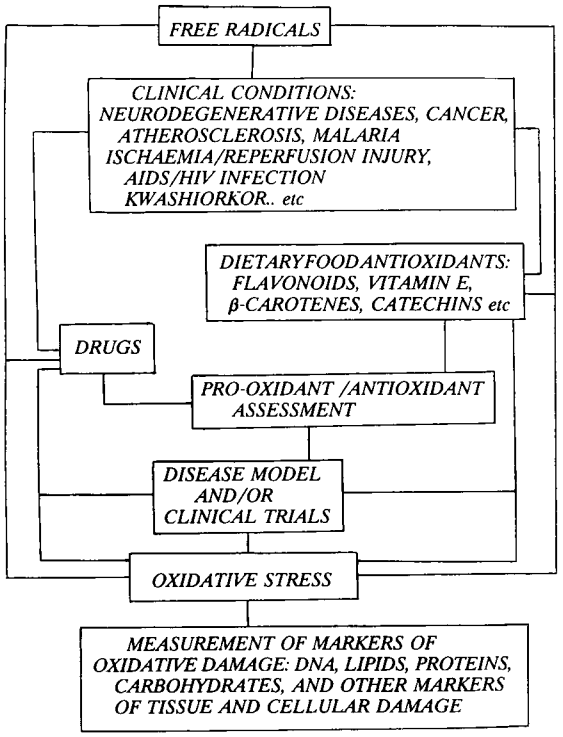

Figure 13.3 A rationale for considering the effect of drugs and proposed antioxidants on oxidative stress in biological systems (from Aruoma, 1994).

free radicals in the process. Nevertheless, these difficulties should not inhibit further research. The rationale presented in Fig. 13.3 may become useful (Aruoma, 1994). It is envisaged that drugs with multiple mechanisms of protective action including antioxidant properties may become available in attempts to minimize tissue injury in human disease.

At least some of the antioxidant nutrients are essential to human health, and others (such as carotenoids) may be highly beneficial, particularly in preventing cancer (e.g. Block *et al.*, 1992). However, we do not yet know what dietary intakes are optimal. In principle, this could be investigated by varying the dietary intake of antioxidants and measuring free-radical damage in the human body. This is one of our current research directions.

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9. References

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14. Free Radicals in Lung Inflammation and Environmental Exposure to Pollutants

M. Yeadon

1. Introduction

The lung is unique in operating in an environment of high partial pressure of oxygen and continual exposure to airborne materials. In addition, the principal physiological role of the lungs in gas exchange dictates the complex and somewhat vulnerable structure that characterizes pulmonary tissue. As a result of these factors, the potential for injury to one or more components of the ventilation or perfusion subassemblies in the lung is great, and the consequences of failure to respond appropriately to biological and chemical stressors severe.

The lung is the only organ in the body that is perfused with the entire cardiac output and must be in optimal condition for efficient gas exchange. Yet the lung can be damaged in many ways: by inhaled gaseous and particulate materials, by systemic xenobiotics and by endogenous processes. A common motif that links such injurious stimuli is that the lung tissue responds to the insult with an inflammatory response. Inflammation is classically characterized by redness, heat, pain, swelling and loss of function. The lung is capable of producing analogues of these classical indices: redness is expressed as alterations in the tone of the pulmonary and more particularly the tracheobronchial circulation, resulting in airway erythema and plasma exudation. Swelling can occur locally in the airway wall itself to narrow the calibre of the airway lumen, or in the alveolar regions during the gross oedema, which is observed in acute lung injury such as adult respiratory distress syndrome (ARDS). It has been argued that the pulmonary analogue of pain, or more particularly the hyperalgesia, which is a common feature of inflammatory pain, is bronchial hyperreactivity (Adcock and Garland, 1993). In both analogues, the threshold stimulus required to evoke a response (nociception or bronchoconstriction) is reduced.

The ultimate mode of reduction in function, which, if sufficiently severe, results in death, is blood oxygen desaturation and accumulation of carbon dioxide. However, the inflammatory changes that the insults listed above can evoke and contribute to interference with the perfusion/ventilation relationship are varied and have different expressions in diverse lung disorders: bronchial hyperresponsiveness after ozone or nitrous oxide

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inhalation, excessive airflow limitation and eosinophil recruitment in asthma, mucus hypersecretion in bronchitis, neutrophil accumulation and alveolar oedema in ARDS, fibrotic changes in emphysema and others. It is now accepted that there may be an additional common factor that underlies the inflammatory response, (particularly in the lung because of the persistent high partial pressure of oxygen), which is that reactive oxidizing species (ROS) could be important factors in inflammatory lung injury.

Some of the evidence that ROS contribute to varying degrees to initiation and/or progression of some of the modes of inflammatory lung dysfunction and injury will be outlined. No attempt has been made to review the entire literature in the area of oxidants and lung injury: the body of information is extensive and the reader interested in particular facets is referred to some excellent articles. The particular focus on epithelial and endothelial injury in animal models and their disparate consequences reflects a personal interest and bias. This evidence is largely confined to whole animal studies, and experiments with human and animal tissue and cells *in vitro*, principally because of the limitations of current therapeutic agents whose main activity is to act as protective molecules against the potentially damaging ROS. However, some of the experimental therapeutic approaches in humans are discussed.

The terms free radical and reactive oxidizing species are often used synonymously and incorrectly. Free radicals are species that possess an unpaired electron and, as such, are capable of initiating oxidative chain reactions. Thus free radicals can be considered a subset of a larger group of ROS, some of which may oxidize cellular components directly and thus alter their biochemical behaviour. The therapeutic strategy and physicochemical requirements of molecules designed to ameliorate the effects of these oxidizing stressors may differ depending on the particular ROS or process involved. In the biophysical and medicinal chemical arenas, the distinction could be very important. However, for the purposes of this review, the author will not discuss the distinction further, since it has been covered comprehensively in other parts of this volume.

2. *Endogenous Sources of ROS in the Lung*

Almost all living creatures require oxygen to act as the ultimate electron acceptor in a series of chemical reactions. In these, oxygen is reduced to the level of water and the bond energy of the substrates thus concommitantly oxidized is liberated. Oxygen is able to perform these functions because it can be progressively oxidized by successive one-electron additions, but it is this property that provides the basis for the toxicity associated with imbalance in pro-oxidant/antioxidant systems in the lung. This imbalance in favour of pro-oxidant factors constitutes an oxidant "burden" or "stress", which, if unopposed, may lead to tissue damage.

Indeed, when present in concentrations sufficient to overwhelm normal antioxidant defences, ROS may be the principal mediators of lung injury (Said and Foda, 1989). These species, arising from the sequential one-electron reductions of oxygen, include the superoxide anion radical, hydrogen peroxide, hypochlorous ions and the hydroxyl radical. The latter species is thought to be formed either from superoxide in the presence of iron ions (Haber–Weiss reaction; Junod, 1986) or from hydrogen peroxide, also catalysed by ferric ions (Fenton catalysis; Kennedy *et al.*, 1989).

The major sources of endogenous ROS are activated polymorphonuclear leucocytes (neutrophils and eosinophils) and phagocytic cells, such as alveolar and interstitial macrophages. On activation, the polymorphonuclear leucocytes undergo a respiratory burst during which oxygen consumption is abruptly elevated and superoxide is produced as a result by means of the NADPH-oxidase and peroxidase pathways (Sagone *et al.*, 1989). Stimuli to the respiratory burst are diverse and include bacteria, immune complexes, complement fragments, phorbol esters, opsonized zymosan, calcium ionophores, platelet activating factor, *N*-formyl peptides and leukotriene B_4 (LTB_4). ROS release can also be enhanced by pre-exposure of the cells to "priming" agents, such as tumour necrosis factor (TNF) and cytochalasin B. The "primers" do not greatly stimulate ROS release but potentiate the subsequent response to other agents (Follin *et al.*, 1991). During acute inflammatory responses within the lung, all these cell types can release ROS in significant quantities. The cells are known to be present and activated, and mediators known to be stimuli to ROS release can also be demonstrated in bronchoalveolar lavage fluid. A growing body of literature on the effects of ROS on lung cellular and tissue function *in vitro* and *in vivo* supports the concept that ROS may play a role in the pathophysiology of a number of inflammatory diseases of the lung. Epithelial and endothelial cells (Phan *et al.*, 1989; Friedl *et al.*, 1989; Keenan and Franke, 1981) can also release ROS including nitric oxide (NO) (Moncada *et al.*, 1991). In addition, ROS may also be produced by other NADPH-dependent oxidases and monooxygenases, such as the haemoprotein cytochromes P450 (Schenkman and Gibson, 1983) during the metabolism of endogenous steroids and xenobiotics.

Other enzyme systems may also be directly or indirectly involved in the generation of ROS in the lung, including those of the eicosanoid pathway, the mitochondrial electron transport system, and aldehyde, glucose and xanthine oxidases (Parks and Granger, 1986). These systems may also be relevant to lung damage. For example, the oedematous pulmonary injury that results from cessation of blood flow for a period followed by reinstatement of

blood supply (reperfusion injury) arises because of an increased permeability of the vascular endothelium, which has been ascribed to deleterious effects of ROS generated from xanthine oxidase.

In many human lung diseases there is evidence for an important role for the inflammatory response in the pathology of the disorders, although it is far from clear which aspects of inflammatory cell function are most important. While the inflammatory nature of the disorder has been well recognized in bronchitis, ARDS and idiopathic fibrosis, it is only in the last few years that there has emerged a realization that asthma is also a chronic inflammatory lung disease (Barnes *et al.*, 1988). The eosinophil in particular seems to play a key role, through release of ROS and cytoplasmic granule products, both in the delayed ("late") allergic response in asthma and the development of bronchial hyperreactivity (Frigas and Gleich, 1986). Whether the eosinophilic lung inflammation is important for hyperreactivity is in dispute (Morley, 1993). However, both alveolar macrophages (Damon *et al.*, 1989) and peripheral blood neutrophils (Meltzer *et al.*, 1989) from asthmatic patients appear to be primed for ROS release, and there is a significant correlation between the extent of superoxide release and the degree of airway hyperreactivity. Thus, although the identification of the "key inflammatory cell" in asthma is still awaited, the influence of inflammatory processes in asthma is now widely agreed.

3. *Exogenous Sources of ROS in the Lung*

At rest, humans typically ventilate at the rate of 5 l/min, a value that is matched by the cardiac output. Air contains numerous particles and gases, which have potential for pulmonary injury. These include organisms, organic and inorganic dusts, and pollutants, such as ozone, and oxides of nitrogen and sulphur. All of these can produce oxidative damage to pulmonary tissue, either directly or indirectly through induction of inflammatory responses. Silica-containing dusts such as asbestos can act as Fenton catalysts in the lung after inhalation and entrapment (Kennedy *et al.*, 1989) and are toxic to the macrophages normally able to clear inhaled particulates. Alveolar macrophages having phagocytosed these minerals release LTB_4 and TNF, which recruit and activate neutrophils (Tsujimoto *et al.*, 1986). Thus, independent of the initial event, a cycle of inflammatory cell-derived focal ROS production may be established. It is unclear how this leads to a fibrotic lung but it is possible that ROS either directly, or indirectly through production of lipid membrane peroxidation products, are stimulators of fibroblast proliferation.

It is well established that ROS may be produced in the body in response to foreign compounds and there are examples where the lung is the target tissue, including the herbicide paraquat and the anti-neoplastic bleomycin. Although the mechanism of toxicity is not yet unequivocally established, it has been shown that compounds such as paraquat can accept electrons from haemoproteins during metabolism by cytochrome P450 and subsequently undergo redox cycling by forming free radicals in anaerobic conditions. Reaction with oxygen then produces superoxide anion radicals. However, this mechanism of ROS-dependent lung toxicity is not unequivocally established, as it has been reported that production of the paraquat radical by lung tissue is not blocked by the cytochrome P450 inhibitor SKF 525-A (Baldwin *et al.*, 1975). However, the survival of rats after paraquat has been enhanced by pretreatments (endotoxin and hyperbaric oxygen) that induce lung antioxidant enzymes (Frank *et al.*, 1989) or by desferrioxamine (Van Asbeck *et al.*, 1989), and thus this data may be taken to mean merely that other entities than cytochrome P450 are responsible for the reduction of paraquat, rather than implying that ROS are not involved in paraquat lung injury. Indeed, Waintrub and colleagues (1990) showed that xanthine oxidase, whose activity is elevated in lung following paraquat administration in rats, may be a good candidate for such an entity. Metabolic activation of substances to form toxic entities is a recognized motif in toxicology and, while the liver is the major organ in the body responsible for the metabolism of xenobiotics, the lung is the second largest source of P450 cytochromes. Therefore the possibility of production of proximate toxins by metabolizing enzymes in the lung must always be considered a potential mechanism of toxicity.

Bleomycin is a glycopeptide antibiotic that induces lung fibrosis in experimental animals (Jordana *et al.*, 1988). While bleomycin is used as an anti-cancer agent, there is dose-limiting pulmonary fibrotic damage, which may be related to injury produced by ROS, either via lipid peroxidation or DNA strand breakage. In mice, the injury occurs only in the lung and *in vitro* can be accelerated by hyperoxia and inhibited by superoxide dismutase.

Exposure to ozone and other common gas-phase pollutants adversely affects lung function in animals and humans. Even after exposures of low magnitude for short periods of time, the responsiveness of the lungs to agents that contract airway smooth muscle is greatly enhanced. This phenomenon is termed bronchial hyperresponsiveness (BHR) and is a characteristic feature of asthma. Ozone and nitrogen dioxide are potent oxidants, and both are able to to initiate a radical process (Mustafa, 1990). There is evidence that the oxidant stress, which is imparted by exposure to ozone and other urban pollutant gases, has an important role in some instances of acute worsenings of asthma, and may additionally increase the incidence of emphysema and fibrotic lung disorders. Ozone and nitrogen dioxide exposure recruits

neutrophils to the lung and, while these cells may be pivotally involved in the longer term consequences, they are not thought to have a significant role in the acute effects of these gases. This may also be the case for acute lung injury induced by exposure to hyperoxia, where depletion of neutrophils prior to exposure has no protective effect against the oedema that develops (Lurie *et al.*, 1988). In addition fasting or administration of buthionine sulphoximine (which both reduce glutathione levels) or induction of isoforms of P450 increase the susceptibility of mice to hyperoxic lung injury (reviewed by Doelman and Bast, 1990), suggesting that oxygen can injure the lung through generation of ROS.

4. *Antioxidant Defence Systems in the Lung*

The lungs are adapted for their function and are not passive victims of oxidative attack. Biochemical systems provide a battery of enzymic and non-enzymic antioxidant defences to prevent, limit or reverse oxidant damage in the lung. This subject has been excellently reviewed (Heffner and Repine, 1989; Sies, 1991). Briefly, such systems include the enzymes superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide. This enzyme exists in at least two forms: a copper–zinc-centred protein located in lung cytosol and a manganese-containing form, which is principally located in mitochondria. Catalase is able to reduce hydrogen peroxide to water at very high rates, a reaction that can also be catalysed by glutathione peroxidase, using reduced glutathione as co-substrate. Several forms of the selenium-containing and selenium-independent glutathione peroxidase enzymes have been described, which can in addition reduce lipid hydroperoxides to the corresponding alcohols using glutathione as the source of reducing electrons. Thus glutathione, a sulphur-containing tripeptide, plays an important part in maintaining the integrity of ROS-sensitive cellular components, both by acting as a direct antioxidant or as a cofactor. Glutathione becomes oxidized in the process and its cellular concentration is kept in dynamic equilibrium by the enzyme glutathione reductase. This reaction consumes NADPH as the source of reducing equivalents, supplied by the pentose phosphate pathway, which in turn requires ATP. Under severe oxidative stress, glutathione may become depleted and the energy charge of cells falls. Indeed, biochemical determinations of these parameters have been used in animal studies to evaluate oxidative stress in lung tissue after ozone exposure (Yeadon *et al.*, 1992). Methionine sulphoxide reductase (Weissbach and Brot, 1981) should also be considered as an antioxidant enzyme system, since it is able to catalyse reduction of this oxidizable residue thus potentially restoring protein function. α-Antitrypsin, an endogenous inhibitor of neutrophil elastase, possesses such a vulnerable methionine residue. Its oxidative inhibition by components of cigarette smoke are thought to contribute to the development of emphysema in vulnerable individuals.

Non-enzymatic endogenous antioxidants also serve to counteract the pro-oxidant environment in the lungs. Ascorbic acid acts as an antioxidant under certain conditions, although *in vitro* experiments have shown that pro-oxidant activity is also possible. Ascorbate, along with urate, are considered to be important molecules in the extracellular defence of the lung, being present at high concentrations in airway surface liquid (Hatch *et al.*, 1989). Ascorbate can behave as a free-radical scavenger and may interact with oxidized glutathione to return this important molecule to its active reduced form, although this has been disputed. In the lipid membrane of the cell, the hydrophobic vitamin E inhibits lipid peroxidation through scavenging of lipid peroxides and radicals. Other endogenous compounds, which together may play an important role in antioxidant defence of the lung, include albumin and other plasma components such as free cysteine, which are exuded on to the airway luminal surface during acute inflammation. Increased resistance to ROS by elevated expression of antioxidant enzymes after non-lethal oxidant stress is a motif of adaptive responses to ROS. When rats were exposed to 100% oxygen for 7 days, they all died. However, when the protocol was changed so that exposure was intermittent (7 out of 10 days, with normoxic days between), lung antioxidant enzymes were induced and all the animals survived (Frank *et al.*, 1989). Cytokines, such as interleukin-1 (IL-1) and TNF, which are released from lung macrophages during inflammation, are also able to induce lung antioxidant enzymes and thus afford greater resistance of rats to hyperoxia (White *et al.*, 1989). This is an interesting observation in view of the well-known priming effect of certain cytokines including TNF on neutrophil ROS release (She *et al.*, 1989). Perhaps co-induction of antioxidant enzymes in lung tissue coupled with priming of neutrophil superoxide release provides a means of limitation of oxidant damage in tissues to which inflammatory cells have migrated, while enhancing the physiological activity of neutrophils in host defence? Another possible mechanism for detoxification of ROS endogenous has recently been reported. Varani *et al.* (1991) showed that manganese ions and glycine can protect lungs of glucose/glucose oxidase-challenged rats, probably by promoting hydrogen peroxide disproportionation and oxidation of sacrificial amino residues. There may be other pathways to be described.

5. *Pulmonary Targets of ROS*

The responses of any tissue to a strong oxidant stimulus are progressive and range in severity from pertubation of

the cellular redox status to overt toxicity and cell death. Of the cellular targets for oxidant-induced damage to tissue, few are unique to the lungs, but the consequences of impairment of function of the epithelial and endothelial barriers are diverse. Polyunsaturated fatty acids comprise a substantial proportion of the lipid in cellular membranes and are susceptible to oxidation by an initiation reaction involving hydrogen atom abstraction. Subsequent propagation reactions can occur that produce lipid peroxides, alkanes, alkenes, ketones and hydroxyacids. Some of the initial reactions can be performed by lipoxygenase and cyclooxygenase enzymes, which produce unstable hydroperoxy intermediates, and which can be activated by oxidant stress on the cell. Cellular function may be altered directly through alterations of membrane fluidity and integrity, which can affect intracellular ion concentrations and through mitochondrial damage, alter the energy charge of the cell. Some oxidized fatty acids act as calcium ionophores. Uncontrolled elevation of intracellular calcium concentrations is implicated in destruction of cells by ROS. Proteins may also be modified by ROS, especially at labile thiol-group-containing residues. These may be oxidized directly, or react with lipid peroxidation products such as 4-hydroxy-2,3-transnonenal. This may adversely affect the function of cell-surface receptors and ion channels. Elastase is an enzyme that can degrade extracellular matrix in the lung and is derived from activated polymorphonuclear leucocytes. Elastase is normally inhibited by α_1-antitrypsin but oxidation at a crucial methionine residue prevents its inhibitory action on elastase. Thus it has been proposed that chronic oxidant stress, through unmasking of excess elastase enzyme activity, may play a crucial role in the development of lung matrix breakdown and fibrosis, which characterizes emphysema. In addition, modifications of proteins are sufficient to inhibit the catalytic activity of some enzymes, such as adenylate cyclase, glucose-6-phosphatase and DNA polymerase, key proteins in regulation of cellular function. Deoxyribose sugars on the outside of DNA strands are most likely the target for hydroxyl radicals and, in addition, lipid peroxide products have been implicated in DNA damage.

It is misleading to consider that ROS are always deleterious, and that to prevent release or action of all ROS will be of therapeutic value. One could reason that some ROS are released without control or purpose, as "by-products" of the normal metabolism of eicosanoids, or during oxidative phosphorylation in the mitochondria. However, during normal function, inflammatory cells appropriately release ROS both intracellularly into vacuoles and extracellularly in order to kill foreign organisms in host defence. Also, nitric oxide is a radical species whose principal role in the lung appears to be the control of pulmonary vascular tone and platelet function. Nevertheless, there are clear examples where frustrated phagocytosis could explain an excessive release of ROS in the lungs, such as during the chronic inflammatory response to inhaled silica-containing dusts. The alveolar macrophages, unable to degrade the engulfed particles, are a source both of ROS and of chemotactic factors, which recruit other ROS-releasing cells, such as neutrophils. It seems more reasonable to consider that, under some circumstances, the cells in the lung may be exposed to or generate ROS in an inappropriate way, and that this oxidant stress may be responsible for some aspects of pulmonary dysfunction in animal models of lung disease and also in human lung diseases.

It is not necessary to invoke cell death in diseases in which ROS may play a role: temporary alterations in cell function such as cell–cell tight junctions in endothelial and epithelial layers can have profound consequences for lung function. This could be expressed as bronchial hyperreactivity or increased ease with which active sensitization to foreign proteins might occur through the airway (Matsumara, 1970). Evidence is presented that ROS in the lung, either endogenously generated in the course of inflammation, or of exogenous origin, are credible mediators of some of the pathology of lung diseases. Examples are taken principally from preclinical work, but with references to human disease where information permits.

6. *Lung Epithelium and Airway Reactivity*

Increased responsiveness to inhaled constrictors of airway smooth muscle or BHR is a characteristic feature of asthma (Boushey *et al.*, 1980). It is now accepted that epithelial injury is also widely observed in asthmatic airways (Laitenen *et al.*, 1985) and is probably the main, though not the sole, cause of BHR. One of the reasons for this assumption is that BHR is not reproducibly observed *in vitro* in linear strip preparations of human or animal airways taken from subjects in which BHR was confirmed during life. However, loss of normal geometry of the airways when preparing strips from tubes may account for this discrepancy (Sparrow *et al.*, 1990). While it is generally assumed that epithelial denudation is required for BHR, it is probably more correct to say that there is evidence of epithelial dysfunction rather than its total loss. Various explanations have been offered for the change in airway function that accompanies epithelial damage, including increased permeability to a variety of molecules, exposure of intraepithelial sensory nerve terminals (Barnes, 1986), loss of a relaxant factor (Hay *et al.*, 1987) or deficits in epithelial metabolism (Yeadon *et al.*, 1990, 1992). Is it conceivable that all the mechanisms have a part to play in the clinical manifestation of BHR in asthmatics. It is noteworthy that many chemical or biological factors that are able to induce BHR in animals and humans are also known trigger factors for

the emergence or exacerbation of asthma, such as inhaled toluene diisocyanate (Gordon *et al.*, 1985), 15-hydroxyperoxyeicosatetraenoic acid (HPETE) (Folkerts *et al.*, 1983), ozone (Yeadon *et al.*, 1992), respiratory virus infection (Empey *et al.*, 1976) and specific allergic challenge (Daffonchio *et al.*, 1989). All of these stimuli impose oxidative stress on pulmonary tissue either directly or indirectly through release of mediators, such as eicosanoids, or recruitment of inflammatory cells capable of producing ROS locally in lung tissue. Thus it seems possible that a proportion of BHR results from epithelial injury secondary to an inability to withstand oxidative stress.

In guinea pigs, brief inhalational exposure to low concentrations of ozone are sufficient to induce a substantial degree (10–20-fold reduction in threshold concentration) of BHR to a variety of inhaled substances, such as histamine and substance P (Yeadon *et al.*, 1990). This occurs despite there being no detectable injury to the epithelial layer at times when BHR is clearly established, although ozone is known to damage the epithelium profoundly after greater exposures (Plopper *et al.*, 1973). In addition, the BHR is dependent on the route, being great for bronchoconstrictors delivered via the airways and small for those given intravenously. It is unclear how ozone damages lung epithelium and induces BHR in humans, but in guinea pigs there is no requirement for recruitment of inflammatory cells or local generation of arachidonic acid metabolites for expression of BHR. Indeed, the phenomenon of BHR is not one of non-specific enhancement of bronchial responses to all inhaled bronchoconstrictors, since it has been shown that ozone induces BHR to histamine, substance P, citric acid, salbutamol and others, but not to leukotriene D_4 (Yeadon *et al.*, 1993b). Instead, there appears to be a direct oxidant effect of ozone inhalation on lung tissue, which selectively affects responses to certain compounds, although the reasons for this are unclear. After 2 h exposure to 3 p.p.m. ozone, the lung concentrations of oxidized glutathione and of gluthathione–protein mixed disulphides were significantly increased, whilst that of reduced gluathione was reduced (Yeadon *et al.*, 1992). There were no such changes in the heart. As further evidence of localized oxidative stress in the lung, the concentration of ATP was reduced and that of AMP increased, with a resultant fall in the energy charge of pulmonary cells, although this was not statistically significant. Ascorbic acid was used as an antioxidant in an attempt to prevent ozone-induced BHR and, although large doses (0.3–1 g/kg) were required, complete protection against the effects of ozone was provided. Since the epithelial layer was histologically normal after ozone exposure in the absence of ascorbic acid, what were the effects of ozone that resulted in such a clear-cut change in pulmonary response to inhaled bronchoconstrictors, such as histamine and substance P? In cats, Katsumata and colleagues have demonstrated very clearly that ozone inhalation produces BHR to inhaled acetylcholine, which is prevented by intravenous PEG-conjugated SOD (Katsumata *et al.*, 1989). Thus ozone may produce oxidative stress directly or via conversion to, or stimulation of the release of, superoxide anions.

It is well established that a number of peptidase enzymes in the lung control the rate of degradation, and thus biological activity of endogenous and exogenous peptides (Shore and Drazen, 1989). The key enzyme in degradation of substance P in airways is neutral endopeptidase (NEP; EC 3.4.24.11: enkephalinase) (Johnson *et al.*, 1985) and characterized inhibitors of NEP were used to investigate the activity of NEP in guinea-pig airways before and after ozone exposure. Bronchoconstrictor responses to inhaled substance P in guinea pigs were greatly enhanced after administration of inhibitors of NEP, showing that, under healthy conditions, the presence of airway NEP was of paramount importance in regulating the biological activity of the peptide. The extent to which inhibition of NEP increased the bronchoconstrictor potency of substance P in guinea pigs was identical to the extent of BHR induced by ozone exposure. Furthermore, inhibitors of NEP were not able to increase responsiveness to inhaled substance P in guinea pigs, which had first been rendered hyperreactive to substance P by prior exposure to ozone (Yeadon *et al.*, 1990, 1992). Moreover, if animals were pretreated with ascorbic acid to prevent induction of BHR by ozone exposure, inhibitors of NEP were then able to enhance reactivity to substance P in the same way as in naive animals (Yeadon, unpublished data). Taken together, these data strongly suggest that, after brief exposures to ozone, induction of BHR results from a selective functional inhibition of airway epithelial NEP activity with little, if any, overt injury to the epithelial layer. This functional inhibition of NEP appears to arise through oxidative stress resulting from ozone exposure, since an antioxidant prevented both induction of BHR and loss of the enzyme. Recovery of normal reactivity to substance P occurs by 24 h after induction with ozone and, once recovered, inhibitors of NEP again can modulate the responsiveness to inhaled peptides (Yeadon, unpublished data).

Much of the pulmonary NEP activity is believed to reside in the epithelium, as has been demonstrated in the ferret (Borson *et al.*, 1986), and thus it is likely that inhaled ozone would preferentially destroy luminal NEP before affecting any enzymes in the vasculature, which may degrade peptides delivered by the intravenous route. This may explain the route-dependency of BHR after ozone in guinea pigs. Further evidence that the oxidant effects of inhaled ozone are selective is provided by the findings that pressor responses to angiotensin I (which requires conversion by ACE to angiotensin II) were not altered by ozone exposure (Yeadon *et al.*, 1992).

Ozone is known to be a powerful oxidant capable of both initiating peroxidation in lipids and reacting with

proteins directly (Heffner and Repine, 1989). For example, cysteine, methionine and histidine residues of proteins can be oxidized and may result in cross-linking of the polypeptide chain, thus altering protein function. Ozone has been shown to move the position of the glutathione redox couple to a more oxidized state, and it is known that the functions of a number of cellular enzymes are extremely sensitive to the status of this redox couple and the formation of protein–glutathione conjugates. NEP may share this susceptibility. Alternatively, direct inactivation of NEP could occur by oxidative modification of essential amino acids, as is the case for α-antitrypsin. Interestingly, with regard to oxidant processes, NEP contains 12 cysteine residues, probably involved in the formation of internal disulphide bonds, which may be oxidizable.

This oxidative inactivation of a pulmonary enzyme is apparently not an isolated phenomenon but may underlie, in several species of animals at least, BHR induced by several agents. This suggests the possibility that oxidative stresses resulting from inflammatory responses in the lung are also capable of modulating epithelial function. BHR induced in guinea pigs by inhalation of the agent most commonly associated with occupational asthma in man, toluene diisocyanate, also appears to be due to loss of airway NEP activity (Sheppard *et al.*, 1988). Virus infection is known to be a predisposing factor for asthma and in rats (Piedimonte *et al.*, 1990) infection with Sendai virus (Parainfluenza type I) induced a marked increase in sensitivity to neurogenic peptide-mediated inflammatory stimuli in the airways, consistent with a loss of NEP. In guinea pigs, infection with the same agent has been shown to increase airway reactivity, reduce airway NEP activity and produce insensitivity to the enhancing effects of exogenous NEP inhibitors (Dusser *et al.*, 1989). In none of these model systems has the effect of antioxidants yet been studied. However, in mice infected with influenza A virus, a massive infiltration with polymorphonuclear leucocytes into the alveolar spaces was accompanied by activation of the cells. Rates of release of superoxide *ex vivo* were increased by 15–70-fold over control cells, hydrogen peroxide formation in the lungs was enhanced and lung fluid ascorbate concentrations were reduced (Buffington *et al.*, 1992). Although no direct evidence that antioxidant defences were overwhelmed was presented, it is conceivable that oxidative stress arising out of lung inflammation could produce changes in lung function. Indeed, in a separate study, Akaike and colleagues (1990) demonstrated that influenza-infected mice, given a lethal dose of virus, were protected by an injection of a long-lived SOD conjugate or by allopurinol, a xanthine oxidase inhibitor. It is intriguing that removal of ascorbic acid from the diets of guinea pigs produces a slowly developing BHR (Mohsenin *et al.*, 1988). Perhaps ongoing oxidative stress in the lungs in healthy animals is kept in check by secretion into the airway surface liquid of several antioxidant species, including ascorbate? Certainly ozone exposure in guinea pigs and humans produces a similar decline in lung function, and in the concentration of ascorbate and vitamin E in lung fluid (Hatch *et al.*, 1989, 1990). Concentrations of extracellular oxidants may thus provide a useful guide to the degree of oxidative stress encountered by lung tissue in infection, exposure to inhaled pollutants and in inflammation.

7. *Pulmonary Vascular Endothelium*

Endothelial cells line the entire vascular tree and serve many functions, including those of a regulated physical barrier, and a secretor of vasodilator and anti-inflammatory factors (Bassenge, 1992). The integrity of the endothelial layer may be more important in the lung than in any other organ, because of the rapid and gross alterations in pulmonary function, which result from oedema in this compressible tissue. ARDS is a form of acute lung injury now almost universally agreed to result from severe alterations in endothelial cell function (MacNaughton and Evans, 1992). The effect of elevation of endothelial layer permeability ranges from mild respiratory impairment to a fluid imbalance, presenting as overwhelming pulmonary oedema. It is still unclear what are the pathophysiological causes of the damage to the endothelium, but a number of hypotheses have been proposed and addressed experimentally.

Oxidative stress in the lung imparted by ventilation of anaesthetized dogs with 100% oxygen is able to induce lung oedema and other physiological alterations consistent with injury to the vascular endothelium (Wagner *et al.*, 1989), changes which were ameliorated by pretreatment with *N*-acetylcysteine. If ROS can injure and increase the permeability of the pulmonary endothelial barrier, what might be the sources of ROS in ARDS? There is strong evidence that neutrophils contribute to endothelial damage in ARDS (for review, see Repine, 1992), although the case for a sole or central role for neutrophils is at odds with the observation that patients with severe neutropaenia can develop ARDS (Ognibene *et al.*, 1986). However, there may be several origins of the clinical condition termed ARDS, and numerous preclinical studies have been published showing the importance of neutrophil-derived ROS and other mediators in acute oedematous lung injury in animals induced by neutrophil activators. In the majority of ARDS cases, the lung tissue is populated by large numbers of neutrophils, many of which are adherent to the damaged vascular endothelium (Weiland *et al.*, 1986). It is not enough to locate neutrophils in the lung to implicate them in endothelial cell injury, since neutrophils can increase transiently in the lung without producing pathology (Martin *et al.*, 1989). However, in ARDS these cells are present because they have been recruited by chemotactic factors, presumably released from lung tissue, which are found in elevated

amounts in the bronchoalveolar lavage (BAL) fluid of ARDS patients and, in addition, neutrophil degranulation products such as elastase are elevated (Gadek, 1992) providing evidence of activation. On activation, with or without priming, neutrophils are capable of releasing ROS, which have a variety of effects in the lung. Oxidants can inactivate anti-proteases *in vitro* and, indeed, ARDS patients have elevated amounts of oxidized inactivated anti-proteases in BAL fluid, and increased quantities of hydrogen peroxide in their breath (Sznadjer *et al.*, 1989). In *ex vivo* studies of neutrophils derived from ARDS patients, there have been reports of enhanced releases of ROS (Zimmerman *et al.*, 1983). There is no question that in contrived experiments in isolated lungs *in vitro*, neutrophils and neutrophil-derived ROS are capable of producing acute lung injury in accord with their proposed role in human ARDS. Shasby and colleagues (1982) showed that the lungs of rabbits were made oedematous by the action of stimulated human neutrophils and also showed convincingly that the ability of neutrophils to injure the lungs was closely related to their production of superoxide. When neutrophils were used from humans suffering from chronic granulomatous disease, in which the inflammatory cells do not produce ROS, no injury to the rabbit lungs occurred. Eosinophils in physiologically relevant numbers are also able to injure the isolated lung: phorbol ester-stimulated eosinophils added to the perfusate of isolated rat lungs produced oedema and endothelial cell damage (Rowen *et al.*, 1989). These effects were not produced by addition of the corresponding amount of phorbol ester alone, nor by non-activated eosinophils and were blocked by addition of catalase to the preparation. Taken together, data of this kind strongly suggests that, in the course of an inflammatory response, tissue injury could occur independently of proteases and principally due to oxidant mechanisms.

In healthy individuals, lipid peroxidation and tissue injury in response to ROS release is mitigated by the removal of free iron by binding proteins such as transferrin. In ARDS patients, however, the serum concentrations of this molecule was lower than those in normal individuals (Krsek-Staples *et al.*, 1992). Although transferrin was raised in BAL fluid, it is arguable that it is the vascular endothelial side that is most vulnerable to injury resulting from interactions between iron and neutrophil-derived ROS. It is generally agreed that there is an increased pulmonary burden of ROS in ARDS, but there is considerable debate as to the place of ROS in acute lung injury in this disease. Definitive statements must await the testing of putative therapeutic agents, which inhibit the production or consequences of ROS generation in ARDS.

It is not obvious what pharmacological or biochemical profile such agents need have to be effective. It has been proposed that ROS are responsible for the oedematous tissue injury, which can be produced by a period of ischaemia followed by reperfusion. In models of this process in lung (Grosso *et al.*, 1989), induction of the enzyme xanthine oxidase has been demonstrated and protection afforded by enzymic antioxidants such as catalase. In ischaemia–reperfusion injury models and in others in which ROS are believed to be pivotal factors in lung injury, the timing of interventions with antioxidants is very important. Gannon and colleagues (1990) demonstrated this elegantly using two models of lung injury: glucose/glucose oxidase/lactoperoxidase and cobra venom factor in rats. When SOD and catalase were administered simultaneously with the inflammatory challenges, clear protection was observed, but if a delay of 4–20 min after challenge was allowed, little or no protection was obtained. This study could be interpreted as meaning that ROS have an initiating role and are not important thereafter. However, it is also as likely that extracellular ROS, which are available to extracellular enzymes, are crucial in initiating a process of further ROS-dependent injury inside cells. It is assumed that ROS are generated principally by inflammatory cells, which are then encountered by the target cells from the extracellular direction. In this way, lung injury in response to ROS might be prevented by antioxidants of a qualitatively different nature at different times after challenge, and this could have important implications for the treatment of disorders such as ARDS.

In pursuit of this concept, a series of water-soluble and lipid-soluble antioxidants were investigated in an isolated, perfused, ventilated guinea-pig lung preparation in which intravascular hydrogen peroxide induces concentration-related lung injury manifest as increases in lung weight and pulmonary perfusion pressure, as well as increases in vascular and airways reactivity (Yeadon *et al.*, 1991a). A single, submaximally effective concentration of hydrogen peroxide was chosen and the ability of a number of antioxidants to prevent lung oedema was tested. The arachidonic acid 5-lipoxygenase (5-LO) inhibitor BW B70C (Payne *et al.*, 1991) was protective but this was not due to 5-LO inhibition, since the 5-lipoxygenase activating protein (FLAP) inhibitor MK886 was ineffective. BW B70C also binds iron and may scavenge peroxyl radicals. Catalase was completely protective, presumably by rapid removal of the challenging agent. SOD was also active, though less protective than catalase. Interestingly, while the lipophilic iron chelator 1,10-phenathroline was very active, the hydrophilic chelator, maltol, clearly provided no protection. The lipophilic peroxyl radical scavenger, Ionox 220, a BHT dimer, was also able to prevent oedematous lung injury induced by hydrogen peroxide. The most likely interpretation of these findings is that hydrogen peroxide acutely damages the lung by oxidative injury to the vascular endothelium, resulting in high permeability oedema. The oxidative mechanism probably involves iron-dependent generation of other ROS, resulting in peroxidation of lipids in cell membranes: this possibility is

reviewed by Farber *et al.* (1990). The principle of the importance of free iron ions in oxidant lung injury has been addressed in a number of studies. Bhalla and colleagues (1987) using rats showed that exposure to gases such as ozone, nitrogen dioxide and sulphur dioxide produces increases in pulmonary and tracheobronchial permeability, and that these effects were markedly exacerbated by pretreatment with aerosolized iron sulphate. These findings were confirmed in guinea pigs by Louie *et al.* (1989), and are suggestive of a Fenton or Haber–Weiss catalytic production of hydroxyl radicals. Since Louie and colleagues were able to demonstrate protection with SOD and catalase, it is likely that the primary injury is by superoxide anions and hydrogen peroxide, as inferred for ozone-induced BHR in cats from the findings of Katsumata *et al.* (1989). Thus, extracellular ROS may initiate oxidant processes in cell membranes, such as in the vascular endothelium, which continue in a separate microenvironment (the cell membrane or subcellular organelles), which may not be accessible to water-soluble agents. There is some evidence that this process might occur in the lungs of ARDS patients. ROS can lead to oxidization of lipids and the production of dienes. In order to provide indirect proof of radical production, Cavaliere and colleagues (1989) repeatedly sampled plasma and BAL fluid from ARDS patients for dienes during the development of the syndrome. Plasma levels of dienes in the at-risk patients were initially higher than healthy controls but declined steadily in patients who survived. In those patients who died from ARDS, the plasma and also BAL fluid diene concentrations rose markedly with time. This information suggests that an appropriate strategy for intervention in this disorder might be to use lipid peroxyl radical scavengers once ARDS is diagnosed: it may already be too late to use water-soluble antioxidants, including enzymes.

In addition to altering airway reactivity via oxidative changes in epithelial cells, ozone inhalation also stimulates microvascular leakage of plasma proteins (Yeadon *et al.*, 1991b), which is thought to be a marker for the presence of an inflammatory response in the tissue. The vessels affected are thought to be those of the tracheobronchial circulation rather than the pulmonary bed itself (Erjefelt and Persson, 1989). This microvascular leakage differs from the effect of intravascular oxidants on the classical pulmonary endothelium, which results in high permeability oedema, and may be involved in the pathogenesis of ARDS. In the case of the tracheobronchial circulation, leakage of plasma proteins appears to be a co-ordinated response to inflammatory stimuli, and the effect of ozone shows that inhaled ROS can themselves directly act as an inflammatory signal in this bed, as can ROS generated by inflammatory cells. Plasma extravasation into the upper airways, which are principally perfused by the tracheobronchial circulation, can be estimated by administering Evans blue dye intravenously and determining the amount of dye per unit weight of tissue after a procedure. In guinea pigs exposed to ozone, pretreatment with ascorbic acid prevented the increase in Evans blue found in tracheal tissue. After brief exposures to ozone, little enhancement of plasma leakage was observed, although BHR was already established. However, if neutral endopeptidase was first inhibited by intravenous injection of phosphoramidon and thiorphan, brief exposures to ozone were then sufficient to induce a marked extravasation of plasma proteins (Yeadon *et al.*, 1991b). This suggests that inhaled ROS are able to stimulate airway nerves, which, in rodents at least, leads to the local release of neuropeptides, which are potent stimulators of microvascular leak (Barnes, 1987). If guinea pigs were first pretreated with the antioxidant ascorbic acid, ozone was not able to induce plasma protein leakage into the upper airways. Interestingly, whilst ascorbate could protect the tracheobronchial circulation from ozone, ascorbate was only able to prevent induction of BHR induced by short exposures to ozone. When ozone exposure duration was increased, ozone was again able to induce BHR even after ascorbate pretreatment but plasma leakage was still suppressed. This might suggest that the two pulmonary "subassemblies", the airway epithelium and the vascular endothelium of the tracheobronchial circulation, are differentially susceptible to ROS injury, either because of inherent differences or because of the degree of protection afforded by endogenous antioxidants.

8. *Oxidant Stress and Lipid Mediators*

It is widely believed that a mechanism whereby ROS may damage cells is via the generation of lipid peroxides, which may result in alteration of membrane function. Current evidence suggests that superoxide anions and hydrogen peroxide injure cells as a result of the generation of a more potent oxidizing species. In this regard, a cellular source of ferric iron is considered a necessary catalyst (Farber *et al.*, 1990). However, on a less drastic scale, exposure to hydrogen peroxide, lipid peroxides or other strong oxidants can stimulate the release and metabolism of arachidonic acid, a precursor which is extensively metabolized to biologically active lipids such as prostanoids and leukotrienes. Perhaps a lesser exposure to ROS can prime the metabolism of arachidonic acid? The generation of lipid mediators from substrates derived from membranes such as arachidonic acid occurs through peroxidation of substrate. For the enzyme cyclooxygenase, it has been proposed that the level of peroxide tone and hydroperoxide content in the membrane is a critical step in activation of the enzyme (Hemler and Lands, 1980; Lands, 1984), and this may be true also for other enzymes utilizing arachidonic acid, such as the lipoxygenases. Thus, since ROS can stimulate the release of substrate and provide elevated membrane peroxide tone, in addition to potential modulation of

intracellular calcium concentrations (Orrenius *et al.*, 1989), it is perhaps unsurprising that ROS have been reported to elevate eicosanoid generation in lung tissue (Dennery *et al.*, 1989; Farrukh *et al.*, 1989).

Asthma is now recognized as a chronic, T cell-dependent, inflammatory disease, characterized by mastocytosis and eosinophilia in blood and bronchoalveolar lavage fluid (Holgate *et al.*, 1992). There is evidence for activation and priming of inflammatory cells in asthmatic lung (Damon *et al.*, 1989), for elevated release of arachidonic acid metabolites in blood and BAL (Wenzel *et al.*, 1990) and for ongoing oxidant stress in the lungs of asthmatics (Amantuni and Safarian, 1984, 1986a). A considerable proportion of asthmatics are sensitized to one or more of a variety of common allergens, such as house dust mite or grass pollen. When provoked by specific allergen by inhalation, an acute bronchoconstrictor response and, in some individuals, a late (inflammatory) response are evoked (Beasley *et al.*, 1989). Animal and clinical studies with agents that prevent the synthesis or action of leukotrienes have confirmed that products of the 5-LO pathway are important mediators of the acute bronchoconstrictor response to airway provocation in asthma, not only by allergen, but also by exercise and other stimuli (Israel *et al.*, 1990; Findlay *et al.*, 1992; Finnerty *et al.*, 1992). Animal experiments support the contention that leukotrienes also contribute to the prolonged eosinophilic lung inflammation that occurs 4–48 h after challenge (Yeadon *et al.*, 1993a).

Actively sensitized guinea pigs have been used as a model of leukotriene-dependent bronchoconstriction resulting from specific allergen inhalation (Payne *et al.*, 1988). However, this experimental system is not considered a "model of asthma" for a variety of reasons, partly because the reactivity of the animals to inhaled mediators is normal. By a combination of active sensitization and ozone exposure, we have produced an animal system that is more like asthma in that it combines specific allergen sensitivity and markedly elevated airway reactivity (Yeadon and Payne, 1989). Induction of BHR by ozone involves oxidative stress in the lung and may elevate lipid peroxide tone in airway tissue. The pharmacology of elevation of responsiveness to allergen by ozone is different from induction of BHR to mediators, such as acetylcholine and substance P, in that there is no dependency on route of challenge for expression of the heightened response: increased responses to both intravenous and inhaled allergen are obtained after ozone exposure, and for inhaled but not intravenous mediators like acetylcholine (Yeadon and Payne, 1989). This finding suggests that, for allergic responses, ozone does not operate simply by increasing epithelial permeability, and could be interpreted as a priming event for mast cell activation or 5-LO activation by allergen. Indeed, 5-LO is sensitive to cellular redox status through complex mechanisms, probably related to the redox status of the iron-containing moiety and glutathione (Hatzelman and Ullrich, 1987). In addition, the increased responsiveness to allergen does not arise because of a generalized hyperreactivity to the mediators released by allergen, since ozone does not induce BHR to inhaled or intravenous leukotriene D_4 (LTD_4) (Yeadon *et al.*, 1993b). The LTD_4 receptor antagonist ONO-1078 (Ishii *et al.*, 1990) does block responses to allergen after ozone exposure, confirming that leukotrienes remain important mediators of the acute bronchoconstrictor response to allergen. It therefore appears likely that the effect of ozone and consequent pulmonary oxidative stress is to increase the "releasability" of mediators stimulated by allergen provocation and may occur because of changes such as elevated peroxide tone in the lipid membranes of target cells. Under these conditions of ozone-induced hyperreactivity, the 5-LO inhibitor BW B70C (Payne *et al.*, 1991) is a markedly more potent inhibitor of acute pulmonary bronchoconstrictor responses to inhaled allergen than in normoreactive animals (Yeadon *et al.*, 1993c). Whether this effect depends on the 5-LO inhibitory activity of BW B70C or some other property such as iron chelation is unknown. However, this finding may have implications in the therapy of allergic asthma with 5-LO inhibitors of this chemical class, such as zileuton (Hui *et al.*, 1991). It is possible that in asthmatic patients with marked airway hyperreactivity, 5-LO inhibitors of certain chemical classes such as redox compounds but not others, will be more potent against allergen provocation than in atopic normoreactive volunteers in whom some initial trials with compounds such as these are performed. Secondly, if BHR to allergen results from an increased propensity to metabolize arachidonic acid, compounds which are not 5-LO inhibitors but which can scavenge lipid peroxyl radicals may reduce the extent to which leukotrienes are released in response to allergen.

Perhaps in chronic asthma, the continued presence of activated inflammatory cells such as eosinophils which may release ROS contributes to the heightened releasability of lipid mediators such as leukotrienes? When leukotrienes are elaborated, can further inflammatory cell recruitment, activation and ROS release compound this process? Pulmonary oxidative stress resulting from environmental pollutants not only exacerbates asthmatic responses to allergen provocation (Molfino *et al.*, 1991) but also to exercise-induced asthma (Bauer *et al.*, 1986). For these variants of asthma, common features are chronic, eosinophilic lung inflammation, airway hyperreactivity and leukotriene-dependent bronchoconstriction. The hypothesis that inflammatory cell-derived ROS "primes" leukotriene generation could link variants of asthma and suggests that chronic administration of a scavenger of lipid peroxyl radicals may gradually reduce the severity of asthma. Such studies await the development of suitable agents.

9. *ROS and Airway Mucus*

A characteristic feature of many chronic, inflammatory lung diseases such as asthma and bronchitis is an increased mucus burden in the airways. To what extent this results from mucus hypersecretion, altered physical characteristics of mucus or inadequacies in mucociliary clearance is still unclear, but in both asthma and bronchitis, mucus contributes to the morbidity of the disorders and perhaps, through mucus plugging of small airways, to mortality. While airway mucus serves a vital role in defence of the mucosal surfaces against microorganisms and inhaled particulates, secretion can be modulated in several ways that are potentially disadvantageous. Mucus release can be increased acutely by inflammatory lipid mediators such as 15-hydroxyeicosatetraenoic acid (15-HETE) (Yeadon *et al.*, 1994) and by inhalation of ozone (Bergofsky, 1991). The rheology of mucus may be altered by changes in composition, the production of mucus can be chronically increased by up-regulation of the number of mucus secreting cells in the airway mucosa and mucus clearance is affected by inhibition of ciliary function. There is evidence that exogenous and, to a limited extent, endogenous ROS can influence many of these aspects of regulation of airway mucus behaviour. For example, it is recognized that inhalation of oxidizing stimuli such as ozone (Allegra *et al.*, 1991), cigarette smoke or sulphur dioxide (Koenig, 1988) reduces mucus flow rate, probably through a direct effect on ciliary beat frequency. *In vitro*, guinea-pig lung epithelial cells were stimulated to release mucin by challenge with xanthine and xanthine oxidase and, although this response was correlated with enhanced production of arachidonic acid metabolites, principally $PGF_{2\alpha}$, the stimulatory effect was abolished by coincubation with catalase (Adler *et al.*, 1989). More direct evidence comes from the work of Kobayashi and colleagues (1992) who showed that hydrogen peroxide at modest concentrations also inhibited ciliary beating in a reversible manner in sheep tracheal explants *in vitro*. The inhibition was delayed in onset at these concentrations and probably indirect. At threshold cilioinhibitory concentrations, hydrogen peroxide did not acutely injure the cells, as evidenced by the lack of release of lactate dehydrogenase, and appeared to involve activation of protein kinase C-dependent mechanisms. In principle, it is possible for inflammatory cell-derived ROS to have a cilioinhibitory effect *in vivo* and in chronic inflammatory lung diseases could contribute to abnormal airway mucus. Chronic exposure to inhaled oxidizing environmental pollutants such as sulphur dioxide (Turner *et al.*, 1985; Berry *et al.*, 1992) and ozone (Hotchkiss *et al.*, 1991), at concentrations unlikely to induce airway hyperreactivity, up-regulate airway mucus content by inducing hyperplastic and metaplastic changes in epithelial and secretory cells. Hotchkiss and colleagues showed that a brief exposure to ozone was sufficient to initiate subsequent metaplastic changes to mucus-secreting cells. Both stimuli recruit inflammatory cells, particularly neutrophils, to lung tissue within a few hours of initiating exposure (Seltzer *et al.*, 1986). It is possible therefore that the effects of these gases on airway epithelial mucosubstances is indirect, via inflammatory cell-derived mediators, or direct, via oxidant injury to epithelial cells. Most irritant and inflammatory stimuli that result in increased mucus secretion in the airways are able to impart oxidative stress in lung tissue, such as virus infection and pollutant gas inhalation. Epithelial tissue responds rapidly to these stimuli and, in rats, up-regulation of mucin gene mRNA occurs promptly after Sendai virus inoculation or sulphur dioxide challenge (Jany *et al.*, 1991). This induction by oxidizing irritants of expression of high levels of mucin gene mRNA is one of the early events in mucus cell differentiation and hypersecretion. It is intruiging to speculate that the so-called "mucolytic" drug', *N*-acetylcysteine, which enhances the rate of reversal of mucus hypersecretion in cigarette smoke exposed rats (Rogers *et al.*, 1988), might act by diminishing the signal that leads to elevation of mucus glycoprotein synthesis at the level of gene expression. Interestingly, in ozone-exposed conscious sheep, *N*-acetylcysteine prevents both acute and chronic elevation of mucus secretion rate, and reduction in mucus flow rate (Allegra *et al.*, 1991). Perhaps in chronic lung diseases such as asthma and bronchitis, endogenously generated ROS stimulate mucus synthesis and release, and the mucolytic properties of *N*-acetylcysteine reside in its ability to ablate the oxidative stress.

10. *ROS in Human Lung Diseases and Therapeutics*

In preclinical studies, the potential for exogenous and endogenously generated ROS to produce physiological changes consistent with those seen in lung diseases is established. Oxidizing gases can alter epithelial function both biochemically and physically, and increase the airways reactivity to subsequent challenge, either with direct-acting stimuli or immunological triggers, and can additionally enhance the development of immune responses in the airways. ROS can increase endothelial cell permeability in the upper airways, which might contribute to lowered pulmonary function at rest, and exacerbate airflow changes in asthma induced by precipitating events. In the alveolar-capillary bed of the pulmonary circulation, exogenous and endogenous, inflammatory cell-derived ROS can fatally alter fluid barrier function in animals. Oxidant stressors, from any source, enhance the release and metabolism of arachidonic and other membrane-derived lipids, producing

highly biologically active products, some of which are implicated in lung diseases such as asthma. Leukotrienes recruit inflammatory cells, which when activated release ROS and other mediators. ROS can, in addition, either directly or indirectly through release of lipid mediators stimulate airway mucus production. This may be an acute effect, or a chronic one through up-regulation of mucin synthesis and induction of differentiation of mucin-producing cells. In diseases such as asthma and ARDS, there is limited evidence, which suggests that oxidant stress is encountered in the lung during the course of the disorders (Barnes, 1990; Doelman and Bast, 1990).

Clinically, the unanswered question is whether ROS are principal mediators of any aspect of altered physiology or whether they are casually linked to the disease progress. Data of this kind appears to be a prerequisite for rational development and testing of "antioxidants", which could have as targets one or more of a variety of oxidizing or oxidized molecular species. Even if one were convinced of the case for a causal role for ROS in the initiation of lung diseases, current clinical options are limited to dietary antioxidant substances such as ascorbate, selenium and vitamin E, or a few experimental molecules such as *N*-acetylcysteine, the aminosteroid lazaroids or long-acting enzymic antioxidants. Trials in asthma with dietary antioxidants have been reported but the outcomes have not been clear. Ascorbate at 100 mg/kg orally over 3 days has been shown to block induction of bronchial hyperreactivity to inhaled methacholine by nitrogen dioxide (Mohesenin, 1987), in accord with similar data in animals. A number of studies have indicated that oxidative stress occurs in the lungs of asthmatics. The ratio of lipid peroxides to vitamin E and SOD in lung lavage fluid was elevated over non-asthmatics and increased further with disease exacerbation (Amantuni and Safarian, 1984, 1986a). These findings were extended (Krylov *et al.*, 1984) in that concentrations of hydroperoxides, malondialdehyde and conjugated dienes were increased in lung lavage fluid during asthmatic episodes. Evidence of increased lung ROS has also been reported in defined variants of asthma, such as exercise-induced bronchoconstriction (EIB). During exercise, the levels of lavage lipid peroxides rose and of catalase fell in patients with EIB, changes which were not observed in controls (Amantuni and Safarian, 1986b). Indirect evidence of ROS generation in asthmatic lungs is provided by the findings of Aderele *et al.* (1985) who showed that plasma concentrations of ascorbate were significantly lower in asthmatic, compared with control children. In addition, both Bibi *et al.* (1988) and Malmgren *et al.* (1986) found that erythrocyte and plasma glutathione peroxidase activity was lower in asthmatic children than in healthy controls, and fell further during asthmatic attacks. Furthermore, glutathione (GSH) peroxidase activity was particularly low in aspirin-sensitive asthmatics. These findings were confirmed and extended by Novak *et al.* (1991), who concluded that plasma antioxidant capacity in asthmatic children was lower than in non-asthmatics. These findings, however, are in conflict with those of Ward *et al.* (1984) who reported that plasma selenium concentrations were normal in asthmatics and that GSH peroxidase activity in plasma was actually higher than in non-asthmatics. Higher glutathione levels have also been reported in asthmatic lavage fluid compared with normals (Smith *et al.*, 1993). On balance, this evidence is in accord with the contention that ROS can modify airway reactivity, that there is an elevated ROS burden in the lungs of asthmatics (probably derived from inflammatory cells), which is further increased during disease exacerbations, but that pulmonary antioxidant adaptation varies between individuals. This broad hypothesis underlies a number of clinical studies with ascorbate, as one of the best-tolerated antioxidants that can be used in man at this time, although it should be recognized that many antioxidants can act as pro-oxidants under some conditions. This possibility can confound the interpretation of results of clinical studies. Mohsenin and co-workers (1983) reported that 1 g orally of ascorbate significantly reduced airway reactivity in asthmatics. In contrast, others failed to find such an effect (Cockcroft *et al.*, 1977; Kreisman *et al.*, 1977; Malo *et al.*, 1986). However, Schachter and Schlesinger (1982) found that oral ascorbate reduced the falls in lung function that followed exercise in susceptible asthmatics. In a less acute situation, a daily dose of 1 g of ascorbate for 14 weeks reduced the frequency and severity of asthmatic attacks compared with placebo (Anah *et al.*, 1980), a benefit which disappeared on withdrawal of the supplement. Again in contrast, Anderson and co-workers (1983) failed to demonstrate therapeutic effects of ascorbate in asthmatic children over a period of 6 months. There is considerable doubt whether any effects seen with ascorbate are attributable to its antioxidant properties. Mohsenin *et al.* (1983) suggest that the broncholytic effects are due to bias in the metabolism of arachidonic acid towards relaxant prostanoids. While it has been shown that *in vitro* ascorbate releases prostanoids from human lung slices, examination of such data does not support the hypothesis that ascorbate acts by preferentially enhancing the production of relaxant, rather than contractile prostanoids.

In bronchitics, there have been reports of elevated serum-conjugated dienes, hydroperoxides and aldehydes, and a claim of clinical efficacy as well as normalization of these parameters after vitamin E therapy (Kleiner *et al.*, 1990). However, these patients were given combined therapy including steroids and thus the effect of vitamin E alone cannot be assessed. *N*-Acetylcysteine administered to chronic bronchitics increased plasma cysteine from a below-normal baseline but it has not been shown that this intervention had any effect on the disease process, the dosing being of short duration, nor were there short-term effects of the release of ROS from blood neutrophils (reviewed by MacNee *et al.*, 1991). A

combination of human SOD and catalase has been administered to chronic bronchitics for a period of 48 days (Chirila *et al.*, 1987). Although marked benefits were claimed, the clinical data are all provided on an analogue scale and so it is difficult to reassess the improvements reported.

Plasma ascorbate and ubiquinol-10 levels were much reduced in ARDS patients compared with normals, and hydroperoxides were found in lavage of individuals with ARDS but not healthy controls (Cross *et al.*, 1990). This evidence of oxidative stress may be accompanied by rapid antioxidant adaptation, since Leff and colleagues (1993) have reported that, in patients at risk from ARDS, elevation of serum manganese SOD and catalase activity were useful predicters of the probability of development of ARDS. Those who went on to develop ARDS had higher levels of the antioxidant enzymes. Preliminary trials with the antioxidant *N*-acetylcysteine have been performed in ARDS patients, for which there is a good rationale, as *N*-acetylcysteine is now approved therapy in paracetamol overdosage in which liver damage is considered to be secondary to oxidant production. *N*-Acetylcysteine administration resulted in elevated plasma cysteine and glutathione, as well as increased erythrocyte glutathione: these changes were associated with improvements in chest radiographic oedema scores and oxygenation variables (Bernard, 1991). However, these changes were not large and further studies are required to clarify the value of *N*-acetylcysteine in ARDS.

In other inflammatory lung diseases there is evidence of ongoing oxidative stress. Idiopathic pulmonary fibrosis is a chronic and usually fatal inflammatory disorder characterized by accumulation of alveolar macrophages and neutrophils in the lower respiratory tract, with parenchymal injury and alveolar fibrosis. The inflammatory cells in the airway spontaneously release elevated amounts of superoxide and hydrogen peroxide (Cantin *et al.*, 1987), and, in addition, alveolar macrophages possess increased levels of components of the NADPH-oxidase system (Jaffe *et al.*, 1989). The epithelial lining fluid concentration of reduced glutathione is significantly lower than in healthy individuals (Cantin *et al.*, 1989) and, although levels can be raised by inhalation of nebulized glutathione, over the subsequent 3 days there was an increase in the concentration of the oxidized form (Borok *et al.*, 1991), suggesting that the glutathione was utilized as an antioxidant. However, there are no data available on any therapeutic benefit that might result from this strategy.

11. *Concluding Remarks*

In animals it is clear that both endogenous and exogenous ROS can produce acute injury to pulmonary cells *in vitro* and *in vivo*, and that a variety of antioxidant substances can reduce such damage. However, although there is evidence of an increased oxidant burden in the lungs of humans with many lung disorders, a confident extrapolation that antioxidants will be of clinical benefit cannot be made, principally because there have been few trials in man with antioxidants. Those which have been reported so far, although encouraging, are not conclusive in this regard. However, is this failure to demonstrate efficacy more to do with the dose and mechanism of action of compounds that have been tested, rather than the hypothesis of a primary causative role for ROS in lung injury being in doubt? In terms of preclinical work, better antioxidants with improved therapeutic indices are needed. It is reassuring that, in terms of possible predictive value, several experimental models show pathological similarities with responses in man. For example, the mechanisms of lung injury induced by oxidizing gases (Mauderly, 1984), silica-containing minerals (Vallyathan *et al.*, 1988) and some xenobiotics such as paraquat (Smith, 1986) appear to be very similar in animals and man.

There are several fundamental questions that remain unanswered. What makes a case for the use of antioxidants in lung disease? Evidence that there is an elevated burden of oxidants and products of oxidant processes in the lungs, and changes in antioxidant systems do not prove that ROS are causal agents in pathology. Why use exogenous antioxidants when up-regulation of endogenous systems occur? What profile should such antioxidants have? Do they need to act in the aqueous or lipid biophases? What could be expected from a good antioxidant in acute or chronic lung diseases in which there is an increased oxidant burden? In terms of design, discovery and development of novel antioxidants, it is likely from the variety of chemical structures being patented at a high rate that new avenues exist, such as the lazaroid aminosteroids (Thomas *et al.*, 1993). In addition, experimental systems of oxidant injury ranging from lung cells, through isolated tissues and organ preparations to intact animals will allow rational evaluation of such new entities.

12. *References*

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15. *Free Radicals and Liver Injury*

Kevin H. Cheeseman

1. *Introduction*

In the study of classical biochemistry the liver has always been the animal tissue that has been studied most intensively. This fact is obvious from any textbook of biochemistry, as is the reason: the liver contains in abundance all of the enzymes and substrates of the intermediate biochemistry pathways and is a tissue that readily lends itself to practical investigation. For similar reasons, the liver has been at the centre of research into free-radical biochemistry. It is a rich source of all the antioxidant enzymes and most of the non-enzymic biological antioxidant compounds that are so important in counteracting free-radical-induced tissue injury; it is a ready source of cell-membrane preparations (notably microsomes) that are such convenient substrates for lipid peroxidation studies; it is the principal organ of drug metabolism and detoxification, which are closely associated with free-radical studies; and it was the liver that was the target organ of the first chemical compound to be discovered to exert its toxic activity through a free-radical mechanism, carbon tetrachloride (CCl_4). The discovery of the free-radical mechanisms involved in CCl_4 hepatotoxicity was a watershed in free-radical toxicology and in the biochemistry of free radicals in general, revealing their importance in pathological processes, and underlying the development of techniques that would prove invaluable in the advancement of this field of research. Since free radicals have become accepted into the orthodoxy of biochemistry and medicine, the spotlight has been less on the liver, and more on other organs such as the heart and the brain. But the liver remains an important tissue to be considered in this field, not just as a source of experimental material but as an important target organ in the clinical situation, as will be discussed below.

2. *Liver Structure and Function*

The liver is a wedge-shaped organ of some 1.5 kg in adult humans, which, in terms of blood circulation, is interposed between the gastrointestinal tract and the rest of the body. The blood supply to the liver is from the hepatic portal vein (80%) and the hepatic artery (20%), the former bringing a rich supply of nutrients direct from the intestinal tract and the latter supplying the liver with oxygen. Blood drains from the liver by the hepatic vein. The position of the liver enables it to act as a processor of the absorbed nutrients, and to control their storage

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and supply to the other tissues; one of the principal functions of the liver is to regulate the concentration of nutrients in the blood. Its position also lays the liver open to exposure to any toxic substances absorbed via the intestinal tract and, while the liver is the organ best equipped to perform detoxification reactions, it is also the most likely to be affected by toxins absorbed via this route. The liver is also an endocrine organ, synthesizing bile and secreting it into the duodenum to facilitate the absorption of lipids. The bile is also an important route of secretion of detoxified xenobiotics.

One of the reasons that the liver has always been a popular source of material for experimental biochemists is that it contains relatively few different cell types and very little connective tissue. The parenchymal cells of the liver (usually referred to simply as "hepatocytes" comprise the bulk of the tissue, representing some 80% of the liver volume. Thus, when determinations are made using liver homogenates, the determinations represent an average of the whole organ but principally reflect the biochemistry of the hepatocytes. These cells are responsible for the major functions of the liver, nutrient metabolism, drug metabolism and bile synthesis, but the other cell types should not be ignored. The other cell types are: (1) the biliary epithelial cells, which line the intrahepatic bile ducts; (2) the sinusoidal cells, endothelial cells that line the small blood vessels of the liver; (3) the Kupffer cells, macrophage cells that reside in the liver sinusoids; and (4) fat-storing cells, otherwise known as Ito cells or lipocytes, which store vitamin A and are intimately involved in fibrosis. The hepatocytes are arranged in a muralium structure, wall-like sheets one-cell thick lined by very thin, flat sinusoidal cells with no basement membrane between them, allowing the maximum surface area for absorption of nutrients from the blood. The portal vein, hepatic artery and bile duct, and their respective venules, arterioles and ductules, form a bundle of parallel vessels that infiltrate the whole tissue. Any section through the liver reveals clusters of three vessels, the portal triads, arranged in hexagonal arrays around a central vein, with irregular lines of hepatocytes running from the portal triads to the central vein. This array is known as the hepatic lobule and is a useful reference for referring to the various areas of the liver, although the liver acinus is a better model of the functional unit of the liver. Using the hepatic lobule concept, the hepatocytes around the area around the portal triad is known as the periportal area (approximately equivalent to zone I on the acinus model) and the area around the central vein is known as the centrilobular area (approximately equivalent to zone III in the acinus model). The remaining area is known as the intermediate zone or mid-zone (zone II). This nomenclature is useful in describing the pathology of the liver, for example, toxic liver injury often results in death of hepatocytes around the central veins of the lobule and this is referred to as centrilobular necrosis. It also has some real physiological basis in that the hepatocytes of the various zones do appear to have different characteristics. The periportal cells are exposed to higher concentrations of any substances absorbed from the intestinal tract, nutrients and toxins, and there is also a gradient of oxygen across the lobule from periportal zone to centrilobular zone. There is also increasing evidence that the different zones possess different enzyme activities; for example, different cytochrome P450 isozymes are expressed in different zones of the liver. Clearly, this could be of great significance to the localization of cell injury induced by chemicals that are detoxified or activated by cytochrome P450.

3. *Free Radicals in Experimental and Clinical Liver Injury*

3.1 CARBON TETRACHLORIDE-INDUCED LIVER INJURY

Acute exposure to CCl_4 causes fatty degeneration (steatosis) and necrosis of the liver, chronic dosing with lower levels causes fibrosis and eventually cirrhosis. The hepatotoxic properties of CCl_4 were discovered when it was briefly used as a general anaesthetic at the end of the 19th century and extensively used as an anti-helminthic agent in the 1920s. It was also extensively utilized as a degreasing and dry-cleaning agent, and was readily available to the general public. Few people these days are exposed to CCl_4 and it can no longer be considered a clinically important hepatotoxin. Nevertheless, it remains an experimental hepatotoxin of great importance and most significantly in the field of free-radical toxicology. It was the first toxic chemical to be shown to exert its toxicity through a free-radical mechanism and is is still by far the best understood experimental model of its type.

The seminal papers on this topic were published almost simultaneously in 1966 by Trevor Slater in England and Richard Recknagel in the United States (Recknagel and Ghoshal, 1966; Slater, 1966). These two had pulled together the fragments of information gleaned from a decade or more of intensive research into the mechanisms of hepatotoxicity of CCl_4, added original findings of their own, and both come to the conclusion that CCl_4 toxicity probably involved the generation of a free-radical metabolite of CCl_4 and the stimulation of lipid peroxidation. They both felt that lipid peroxidation was likely to be the key cytotoxic event in this model. This suggestion was not universally accepted by biochemists reluctant to believe that free radicals even existed in biological systems, let alone exerted significant pathological effects.

In the subsequent years, the mechanism of CCl_4 hepatotoxicity has been more fully characterized, although the picture is still not complete. CCl_4 is metabolized by cytochrome P450 in the endoplasmic

reticulum of the liver to a reactive free radical, the trichloromethyl free radical ($CCl_3^{\cdot}$), in what is essentially an electron capture reaction:

$$CCl_4 + e^- \rightarrow CCl_3^{\cdot} + Cl^- \qquad (15.1)$$

Cytochrome P450 has evolved as a detoxifying enzyme in animal cells but the metabolism of CCl_4 is a classical example of bioactivation of a chemical substance to a more toxic intermediate. The involvement of cytochrome P450 in the activation of CCl_4 explains why the toxicity of this substance is principally targeted to the liver, the organ that contains the highest concentration of this enzyme. It probably also underlies the centrilobular focus of the hepatotoxicity, the centrilobular zone containing a higher activity of cytochrome P450 in general and particularly of an isozyme of cytochrome P450 (cytochrome P450 IIE1) that is especially active towards CCl_4. The biliary epithelial cells of the liver are devoid of detectable cytochrome P450 activity and consequently are completely resistant to CCl_4, even when isolated and incubated with the toxin *in vitro* (Parola *et al.*, 1988). The involvement of cytochrome P450 also explains why certain inducers of the drug metabolizing system (e.g. phenobarbitone, ethanol) exacerbate the toxicity of CCl_4: prior administration of such compounds leads to enhanced activity of the enzyme responsible for activating the hepatotoxin. The involvement of cytochrome P450 IIE1 in activating halogenated hydrocarbons, including CCl_4, has recently been reviewed by Raucy *et al.* (1993). An interesting point mentioned in that review is that this cytochrome is found not only in the endoplasmic reticulum (ER) of the hepatocytes but also in the plasma membrane, where its activity was reported to be some 33% of the activity in the ER. This would suggest an alternative locus of activation for CCl_4 and alternative mechanisms of cytolysis by this compound. However, in studies with purified plasma membranes, we were unable to demonstrate activation of CCl_4 or stimulation of lipid peroxidation (Le Page *et al.*, 1988).

Between 1966 and 1980, it was possible to postulate with a degree of credibility that the primary product of CCl_4 metabolism was the $CCl_3^{\cdot}$ radical, from indirect evidence such as the covalent binding of radiolabelled CCl_4 to proteins and lipids, and from studies with other halogenated methanes. However, the production of $CCl_3^{\cdot}$ had not been demonstrated definitively. This was eventually achieved using the technique of electron spin resonance (e.s.r.) spectroscopy with spin trapping, when two groups reported trapping of $CCl_3^{\cdot}$ in rat liver microsomes and isolated rat hepatocytes *in vitro*, and even in rat liver *in vivo* (Poyer *et al.*, 1980; Albano *et al.*, 1982). Similarly, for some time $CCl_3^{\cdot}$ was universally described as a very reactive free radical that was single handedly responsible for the toxicity of the halocarbon, but its reactivity had never actually been determined. This objective was eventually achieved using the technique of pulse radiolysis, whereby the $CCl_3^{\cdot}$ radical was generated in solution by radiolysis and its reactivity with a variety of biological compounds was determined accurately (Packer *et al.*, 1980; Forni *et al.*, 1983). These studies confirmed that $CCl_3^{\cdot}$ is indeed a reactive free radical but also demonstrated something that had hitherto not been suspected: that in aerobic systems $CCl_3^{\cdot}$ reacts very rapidly with oxygen to form the trichloromethyl free radical ($CCl_3OO^{\cdot}$), a radical that is even more reactive than $CCl_3^{\cdot}$ and more likely to oxidize biological targets. In retrospect, the rapid reaction of a carbon-centred radical such as $CCl_3^{\cdot}$ with oxygen should have been predictable. It is now felt that both radical species are likely to be involved in the hepatotoxicity of CCl_4, as discussed below.

Early in these investigations, CCl_4 had been demonstrated to stimulate lipid peroxidation in rat liver homogenates (Comporti *et al.*, 1965), in rat liver microsome preparations supplemented with NADPH (Slater, 1967) and in rat liver *in vivo* (Recknagel, 1967). Increasingly, isolated rat hepatocytes were used as an experimental system and CCl_4 was shown to cause lipid peroxidation in these cells *in vitro* (Gravela *et al.*, 1979). Another early observation was that radiolabelled CCl_4 could be detected covalently bound to protein and to lipid in rat liver microsomes, and in rat liver *in vivo* (Reynolds, 1967). This covalent binding was also consistent with reactions of the $CCl_3^{\cdot}$ radical. There was a tendency, which to some extent remains, for those in the field to be divided into three camps: those who believed lipid peroxidation to be the main mechanism of hepatotoxicity, those who favoured covalent binding as the principal mechanism, and those who believed that cell death probably involved both processes. In fact, the CCl_4 model is one of the few putative models of lipid peroxidation-induced cell injury to fulfil the essential criteria needed to prove the involvement of lipid peroxidation in causing the tissue injury: (1) lipid peroxidation should precede cell death, rather than be a consequence of it; and (2) prevention of lipid peroxidation should also prevent cell death.

With regard to criterion (1), Recknagel (1967) demonstrated early in the proceedings that lipid peroxidation, measured as the appearance of conjugated dienes in the microsomes prepared from the livers of CCl_4-treated rats, was detectable within 15 min of dosing, preceding the appearance of necrosis by nearly 6 hours. With regard to criterion (2), the administration of antioxidant compounds such as α-tocopherol (Biasi *et al.*, 1991) and promethazine (Poli *et al.*, 1989a) inhibit CCl_4-induced lipid peroxidation and necrosis of rat liver *in vivo*, and inhibit lipid peroxidation and cell death in rat hepatocytes *in vitro*.

The present view of the mechanism of acute CCl_4 hepatotoxicity is that it is metabolized by cytochrome P450 to $CCl_3^{\cdot}$, a large proportion of which reacts with oxygen to form $CCl_3OO^{\cdot}$. Neither radical can leave

the membrane for reasons of solubility and reactivity. $CCl_3^{\cdot}$ covalently binds to lipids and proteins of the membrane, which in itself may be the cause of inhibition of enzymes associated with the membrane and particularly the "suicidal" destruction of cytochrome P450. $CCl_3OO^{\cdot}$ oxidizes polyunsaturated fatty acids and initiates lipid peroxidation of the ER membrane. The membrane is badly damaged and associated enzyme activities (glucose-6-phosphatase, cytochrome P450) are inhibited. Moreover, the process of lipid peroxidation releases a variety of carbonyl products (Poli *et al.*, 1985), many of which are biologically active. The significance of reactive products of lipid peroxidation such as 4-hydroxynonenal (4-HNE) is that they combine reactivity with diffusibility and are capable of spreading the damage away from the original locus of activation of the hepatotoxin, something of which the free-radical metabolites are not capable. 4-HNE and other hydroxyalkenals react with thiol and amino groups of proteins, and inhibit a wide range of enzyme processes (Esterbauer *et al.*, 1990). Notwithstanding that the liver is possessed of a variety of enzymes that can metabolize hydroxyalkenals, they are still likely to be of great significance to the overall cytolytic process.

The ultimate cause of cytolysis remains obscure, but calcium homeostasis is certainly deranged and this may be the final insult. The calcium pump of the ER and the mitochondria is inactivated very soon after CCl_4 activation, preventing them from carrying out their task of sequestering calcium and maintaining a submicromolar concentration of calcium in the cytosol (Recknagel *et al.*, 1989). Elevation of cytosolic calcium concentrations will cause an activation of a number of enzymes (lipases, proteases and endonucleases) and may contribute to cell death. For example, phospholipase A_2 (PLA_2) is activated in isolated hepatocytes exposed to CCl_4 (Glende and Pushpendran, 1986). PLA_2 hydrolyses phospholipids into lysophosphatides and releases arachidonic acid, so a sustained elevation of calcium could seriously disrupt the structure of the plasma membrane with lethal consequences. However, PLA_2 activation does not seem to be an obligatory step for cytolysis, since inhibition of this enzymes is not protective and exposure of cells to low CCl_4 concentrations can cause cytolysis without activating PLA_2 (reviewed by Recknagel and Glende, 1992). Given that uncontrolled PLA_2 activation would be extremely destructive it seems likely, however, to contribute in some part to the overall hepatotoxicity of CCl_4. It also seems likely that, in the welter of disturbed cell metabolism caused by CCl_4 (protein synthesis inhibition, disturbed calcium homeostasis, phospholipase activation, depletion of cell energy supplies, lipid peroxidation, covalent binding, etc.), it will be extremely difficult to ever identify a single step that is, ultimately, uniquely responsible for cell death.

It has been emphasized here that the CCl_4 model of liver damage was the first example of free-radical-mediated cell damage and remains the best characterized. Other experimental models of free-radical-mediated cell damage have since been extensively studied, using in particular redox-cycling compounds such as quinones and paraquat, and also paracetamol (discussed below). Because these are all free radical mediated and can all be considered to involve "oxidative stress", there is the danger that generalizations begin to obscure the essential differences between the individual examples. One important difference that separates the CCl_4 model from the others mentioned here concerns glutathione (GSH), depletion of which forms an essential step in the toxic mechanism of the redox-cycling compounds but which is not depleted in CCl_4 poisoning. Similarly, lipid peroxidation is an important aspect of CCl_4 hepatotoxicity but is not normally observed in hepatocytes treated with redox-cycling agents.

The above discussion emphasizes the central position of the parenchymal hepatocyte in CCl_4 hepatotoxicity. This is justifiable in that other liver cell types are resistant to the toxicity of this compound and, since CCl_4 toxicity can be demonstrated in isolated hepatocytes incubated *in vitro* in the absence of other cell types, it demonstrates that non-parenchymal cells are not a necessary requirement for manifestation of toxicity. Nevertheless, there is evidence that non-parenchymal cells may be involved in the full manifestation of CCl_4 hepatotoxicity *in vivo*. A recent interesting example of this line of thinking concerns the proposed role of Kupffer cells. Edwards *et al.* (1993) have demonstrated that treatment of rats with gadolinium chloride, which selectively kills Kupffer cells, strongly protects the rats against the hepatotoxic effects of CCl_4 administered subsequently. They hypothesize that "modestly damaged" hepatocytes release substances (as yet unidentified) that activate Kupffer cells, causing them to release cytotoxic products including cytokines, reactive oxygen intermediates, eicosanoids and proteolytic enzymes. The activation of Kupffer cells is further proposed to attract activated neutrophils to the site of damage, releasing more inflammatory mediators and amplifying the inflammatory response and the liver damage. Other work in this area concerns the effect of high doses of vitamin A, which are hepatotoxic *per se* and which also potentiate the hepatotoxicity of a variety of xenobiotics, including CCl_4. Sipes and coworkers (ElSisi *et al.*, 1993a, 1993b) have studied this effect and shown that vitamin A pretreatment greatly increases the lipid peroxidation observed when CCl_4 is administered without apparently increasing the bioactivation of CCl_4, or decreasing hepatic GSH or vitamin E. Administration of antioxidant enzymes only active in the extracellular space [polyethylene gycol (PEG) superoxide dismutase and PEG-catalase] blocked both the enhanced lipid peroxidation and the potentiated hepatotoxicity of CCl_4 implicating a role for reactive oxygen species released by non-parenchymal liver cells. Vitamin A treatment activates Kupffer cells and they are believed to be

responsible for this effect: blocking Kupffer cell activity with methyl palmitate also prevented the potentiation of CCl_4 hepatotoxicity with vitamin A. Thus, while the many isolated hepatocyte studies demonstrate clearly that hepatocytes are certainly the initial site of CCl_4 activation and that the presence of non-parenchymal cells is not a requirement for CCl_4-dependent cytolysis, non-parenchymal cells such as the Kupffer cells may well be involved in amplifying the damage *in vivo*. Activated macrophages have also been implicated in the hepatotoxicity of galactosamine (Al-Tuwaijri *et al.*, 1981) and paracetamol (Laskin and Pilaro, 1986).

There are other very good reasons to consider the interactions of the various different cell types present in the liver. The above discussion of CCl_4 hepatotoxicity has focused on the acute toxicity of this compound, as most experimental studies have done. However, it has long been recognized that CCl_4 is a very versatile experimental tool for the biochemical pathologist and can also be used for studying chronic liver injury. When administered repeatedly in small doses over a prolonged period of time (several weeks), CCl_4 does not cause sufficient liver necrosis to be acutely lethal but causes a fibrosis of the liver: it can therefore be used as an experimental model of liver cirrhosis. Poli's group in Turin have used this model very successfully to investigate the mechanism of stimulation of fibrosis and to demonstrate the protective properties of the antioxidant vitamin, α-tocopherol (Parola *et al.*, 1992). In these studies, chronic CCl_4 treatment stimulated a fibrotic response in the liver, measurable as increased mRNA for procollagen, increased collagen levels and an increase in the fibrogenic transforming growth factor β_1 (TGFβ_1) cytokine. Of great interest is the observation that increasing the hepatic concentration of α-tocopherol, by elevating the dietary concentration of the vitamin, blocked the CCl_4^- stimulated overexpression of TGFβ_1 and of procollagen mRNA. An overview of this experimental model is as follows. Hepatocytes are the initial site of activation and damage, releasing substances that activate Kupffer cells; activated Kupffer cells release the fibrogenic cytokine TGFβ_1 and this stimulates fat-storing cells (Ito cells) to synthesize collagen and initiate fibrosis, ultimately producing cirrhosis. The ability of vitamin E to block this chain of events suggests a role for lipid peroxidation early on in the proceedings, either simply in preventing hepatocyte cytolysis or, more specifically, preventing the release of lipid peroxidation products possessed of the ability to activate Kupffer cells. The significance of these studies is that they remind us that even a tissue as relatively simple as the liver is composed of multiple cell types and that these cells interact in a complicated fashion to produce the final manifestation of tissue damage. Researchers investigating the involvement of free radicals in the toxicity of compounds such as CCl_4 have, for very good reasons, hitherto concentrated on relatively simple experimental systems but are now learning to consider more complex models that better replicate the physiological situation.

3.2 ETHANOL-INDUCED LIVER INJURY

Alcohol abuse is a major clinical problem in many countries and has been the subject of investigation for many years by those interested in determining the molecular basis of ethanol-induced liver damage (see Lieber, 1990). These intensive and extended efforts have revealed much about the metabolism of ethanol in the liver and about the toxicity of its primary oxidative product, acetaldehyde. They have not, however, fully elucidated the molecular mechanisms that lead to the typical features of alcoholic liver injury: steatosis, necrosis and eventually cirrhosis.

It is implicitly accepted that the hepatotoxicity of ethanol is associated with its metabolism and this seems to be well characterized. The major route of ethanol metabolism is through alcohol dehydrogenase (ADH) to produce acetaldehyde, whose reactivity has led to many efforts to prove its culpability in the tissue damage associated with alcohol abuse. The acetaldehyde is rapidly removed, however, by aldehyde dehydrogenase. These two consecutive enzymatic oxidations both produce NADH that is not readily reoxidized and the perturbation of the redox status of the hepatocyte forced by ethanol metabolism has also been incriminated, particularly with regard to the development of fatty liver. Alternative routes of metabolism of ethanol involve catalase in the liver peroxisomes and cytochrome P450 in the endoplasmic reticulum. Acetaldehyde is always the product. The relative importance of these various routes is a matter of some continuing debate: all are agreed that the major quantitative route is through ADH but the significance of the cytochrome P450-dependent system is likely to be increased under conditions of ethanol abuse as it is an inducible enzyme.

Free radicals have long been implicated in the pathogenesis of alcoholic liver injury, this belief being stimulated by and dating back to the revelations concerning CCl_4 in the 1960s. Most work has centred on the putative role of lipid peroxidation in this tissue injury, beginning with investigations by Di Luzio and colleagues (Comporti *et al.*, 1967) proceeding through other reports of lipid peroxidation products such as conjugated dienes (Corongiu *et al.*, 1983; Shaw *et al.*, 1983) being increased by the administration of ethanol and supported to some extent by reports of disturbed antioxidant status in alcoholic patients (Ward and Peters, 1992) and experimental animals (Shaw *et al.*, 1983). Detecting free radicals and their products is always fraught with problems and hard evidence has been difficult to come by, but research on this topic has continued and may now be bearing fruit.

In common with CCl_4, a free-radical metabolite of

ethanol has been detected and proposed as an important toxic intermediate. The hydroxyethyl free radical ($CH_3C^{\cdot}HOH$) is probably a minor product of ethanol metabolism under normal circumstances but may be of significance. Albano and colleagues (1988, 1991) have led the field in studying this radical and demonstrated that it is produced by metabolism of ethanol by cytochrome P450. Unlike CCl_4, ethanol induces cytochrome P450, the specific isozyme induced being P450IIE1. This isozyme is not only particularly active towards ethanol, it also appears to be less well coupled than other P450 forms and prone to producing larger amounts of reactive oxygen species (ROS), including superoxide and hydrogen peroxide. Thus, the induction of this type of cytochrome P450 by alcohol abuse is likely to lead to increased production not only of the free-radical metabolite of the inducer but also of oxygen free radicals and related products. The latter may place some stress on the cell in terms of requiring GSH to remove hydrogen peroxide and the carbon-centred hydroxyethyl radical could conceivably be an initiator of lipid peroxidation. The real significance of this route of metabolism remains to be established.

The product of ethanol's better-characterized oxidative metabolism, acetaldehyde, may also be involved in generation of damaging free radicals. Acetaldehyde may be metabolized by xanthine oxidase and so generate superoxide and hydrogen peroxide, further increasing oxidative stress in the cell (Shaw and Jayatilleke, 1987). The elucidation of the mechanism of ethanol-induced liver injury has been hampered by the multiple routes of metabolism available and by the complex sequelae of production of acetaldehyde, large amounts of NADH and induction of cytochrome P450IIEl, etc. The evidence for free-radical involvement is not overwhelmingly convincing but nor is there a convincing alternative. It is likely that, in such a complex situation, more than one mechanism of damage is involved and that the relative importance of different mechanisms may alter with the duration of the toxic insult and with other influencing factors, particularly the diet. Alcoholics rarely consume a vitamin-rich diet along with their source of alcohol, and antioxidant vitamin deficiency may contribute to the overall problem. In this context, the observations of Kawase *et al.* (1989) may be pertinent. This study, coming from the group of Lieber that has led this field for many years, concerns the influence of vitamin E on ethanol-mediated liver injury and vice versa. The effects of chronic ethanol treatment were not dramatic or clear cut, but it may be of some significance to the human situation that the combination of ethanol treatment together with the existence of low levels of vitamin E in the experimental animals was associated with the highest levels of tocopherol quinone (the oxidized product of tocopherol) in the liver microsomes.

A relatively recent study of significance to the putative involvement of lipid peroxidation in ethanol-induced liver injury is that by Kamimura *et al.* (1992). In this study the authors have measured an increased level of the reactive carbonyl product of lipid peroxidation, 4-HNE, in rat liver after chronic ethanol treatment. It is the conclusion of these researchers that lipid peroxidation is not involved in the necrosis induced by ethanol but may be of significance in the fibrogenesis. These data were discussed by the authors with regard to the mechanisitic link between lipid peroxidation and fibrogenesis reported by Chojkier *et al.* (1989) and may be compared to the CCl_4 study of Parola *et al.* (1992) discussed above.

3.3 IRON OVERLOAD AND LIVER INJURY

Iron overload can be either primary (genetic) or secondary (acquired) in nature. Genetic haemochromatosis is reported to be one of the most common inherited disorders, and is characterized by the excessive absorption of iron from the intestine and its deposition in several internal organs, particularly the liver (Gordeuk *et al.*, 1987). The presence of high iron concentrations in the parenchyma over several years inevitably leads to cirrhosis and is also associated with hepatocellular carcinoma. Secondary iron overload is associated with disorders treated with frequent blood transfusions (e.g. thalassaemia), with alcoholism and with porphyria cutanea tarda (PCT) amongst others. There is a strong correlation between the hepatic iron concentrations, and the degree of liver injury and the lowering of iron concentrations by phlebotomy (in genetic haemochromatosis) or chelation therapy (in secondary haemochromatosis) is known to be protective.

The well-established role of transition metal ions in catalysing the generation of free radicals from non-radical precursors and in amplifying free-radical-mediated damage to biomolecules underlies the long-held conviction that free radicals are involved in the pathogenesis of tissue injuries caused by iron overload. Nevertheless, the specific mechanisms of damage to the liver induced by iron overload have not been convincingly described. A role for increased lipid peroxidation has often been mooted and seems likely given the ready and very well-documented ability of iron to stimulate lipid peroxidation in almost any biological membrane system from isolated liver microsomes to whole cells *in vitro*. Animal models of iron overload, mostly using rats, have been widely used. Increased levels of lipid peroxidation products have been reported in rats treated with iron either as an intraperitoneal injection or in the diet. These have included exhaled ethane and pentane (Dillard and Tappel, 1979) and thiobarbituric acid-reactive substances (TBARs) (Golberg *et al.* 1962) in the liver. Using conjugated dienes as their index of lipid peroxidation, Bacon *et al.* (1983, 1985) deduced that the liver mitochondria were most sensitive to iron-overload-induced damage and that peroxidative damage to the endoplasmic reticulum required higher iron concentrations.

Mitochondrial respiratory control is affected and also the ability of mitochondria to sequester calcium. The former seriously affects the hepatic energy state and thereby many aspects of cell metabolism. Calcium pump activity is also diminished in the ER and the possible consequences of disturbed calcium homeostasis in the hepatocyte could be far reaching. Endoplasmic reticulum enzymes, including enzymes of drug metabolism, are inactivated by iron overload. Hepatic lysosomes are also a target of iron overload with apparently increased fragility. Lipid peroxidation is implicated in all these examples of damage to hepatic organelles and the combined effect of such phenomena could well be cytolysis. Iron overload is characterized by fibrosis and eventually cirrhosis. Iron-induced lipid peroxidation causes an increase in the transcription of procollagen in cultured fibroblasts (Chojkier *et al.*, 1989) and it is reasonable to consider similar mechanisms in the liver *in vivo*. Thus, hepatic fibrosis may be the result of both iron-induced hepatocyte cell death and direct effects of iron in stimulating the synthesis of collagen. The reader is reminded of the discussion (above) concerning the involvement of lipid peroxidation and vitamin E in the control of fibrosis in the chronic CCl_4 model. It will be of interest to see if the two experimental situations involve similar mechanisms, centred on lipid peroxidation as a crucial process in the overall pathology. If this proves to be the case, one would expect to see a protective effect of α-tocopherol. α-Tocopherol deficiency is known to exacerbate iron-overload-induced liver damage (Dillard *et al.*, 1984) and Omara and Blakley (1993) have recently shown a protective effect with this antioxidant vitamin against acute lethal effects of iron overload in mice, but a convincing demonstration of protection by α-tocopherol administration against chronic iron-induced injury has not yet been made.

With regard to the increased incidence of hepatocellular carcinoma in genetic haemochromatosis, it may be of significance that iron can induce strand breaks in DNA in hepatic nuclei and mitochondria *in vitro* (Shires, 1982; Hruszkewycz, 1984). As with peroxidative damage to cell membranes, iron-dependent oxidative damage to DNA is easy to predict from experiments *in vitro* and difficult to prove *in vivo*. There is an increasing body of evidence linking oxidative stress with DNA damage and there is also a great deal of current research concerning redox control of gene regulation. Oxidative stress is proposed to be an important mechanism of carcinogenesis by both routes (reviewed by Guyton and Kensler, 1993). These lines of evidence can only lend support to the proposal that hepatocarcinoma of iron overload is induced by oxidative free-radical damage.

A particular instance of iron overload being associated with liver injury, with free radicals again being implicated, is the hepatic porphyria and hepatocarcinoma induced by polyhalogenated aromatic chemicals. This is described separately below.

3.4 POLYHALOGENATED AROMATIC CHEMICALS, IRON AND HEPATIC PORPHYRIA

Polyhalogenated aromatic chemicals are of great interest to toxicologists because they evince an array of toxic effects in animals and man, and because many of them are environmental pollutants. This diverse group of compounds includes fungicides, such as hexachlorobenzene, polychlorinated biphenyls and the notorious "dioxin" more properly known as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Hepatic porphyria is one of the most striking toxic effects of these compounds and is characterized by the loss of activity of a key enzyme in haem biosynthesis, uroporphyrinogen decarboxylase, leading to the accumulation of uroporphyrin. The similarity of this toxic syndrome to PCT has raised the possibility that it may be a useful model of the latter condition in man. Both PCT and the hepatic porphyria induced by polyhalogenated aromatics are exacerbated by iron overload but the biochemical basis of this effect is as yet unknown.

TCDD is possibly the most potent inducer of cytochrome P450 in existence and its toxicity is strongly associated with this property. The receptor for TCDD that is involved in the induction of cytochrome P450 (the Ah receptor) has been implicated in the hepatotoxicity of TCDD; inbred strains with a low-affinity receptor are resistant to both the P450 induction and the porphyria. In sensitive strains of mice, the hepatic porphyria is greatly exacerbated by concomitant treatment with iron and is alleviated by lowering iron stores (see Smith *et al.*, 1989). A current interpretation of these observations is that the combination of induction of cytochrome P450 and iron overload are involved in inhibition of uroporphyrinogen decarboxylase and onset of porphyria (Smith *et al.*, 1989). According to this hypothesis, the induction of a poorly coupled cytochrome P450 leads to the chronic release of ROS such as hydrogen peroxide and the presence of "free" iron inevitably leads to the generation of reactive oxidizing free radicals such as the hydroxyl radical. The specificity of the damage for the uroporphyrinogen decarboxylase enzyme requires the invocation of a specific inhibitor being generated as a consequence of the free-radical generation and an oxidized form of uroporphyrinogen has been suggested.

It is now clear that iron overload *per se* can cause hepatic porphyria in sensitive strains of mice given sufficient time (several months) so the role of the polyhalogenated aromatic chemicals can be seen as actually accelerating and amplifying the effect of the iron rather than the other way around. This rules out the necessary generation of a reactive metabolite of, for example, TCDD. Further, induction of cytochrome P450 is therefore, not a prerequisite for the onset of porphyria. In fact, the association between sensitivity to P450 and porphyria, respectively, is not perfect. Ruling out these other possible mechanisms has, rather by default, lent support

to the role of iron-induced free-radical generation in the onset of porphyria. Lipid peroxidation has occasionally been linked with TCDD-induced porphyria (Stohs *et al.*, 1984) and it has been reported that a strain of mouse resistant to porphyria is also resistant to iron-induced lipid peroxidation (Cheeseman *et al.*, 1985). The mechanistic link between lipid peroxidation and porphyria is obscure, however, not least because the affected enzyme is located in the cytosol rather than a membrane.

As well as inducing porphyria in the livers of treated animals, polyhalogenated aromatic chemicals can induce hepatomas in rats. Human PCT sufferers also have an increased risk of developing hepatocellular carcinoma in middle age. It is presently unclear whether there is a causal connection between the porphyria and the cancer in the liver, or if they are separate phenomena. Iron overload is of considerable interest in the carcinogenic effects of the polyhalogenated aromatics. Mice treated with iron alone or with hexachlorobenzene alone do not develop hepatomas, but mice treated with both develop hyperplastic nodules and most progress to carcinomas (Smith *et al.*, 1989). Again, iron-catalysed free-radical generation is implicated in the carcinogenic process and it is of possible significance that the nucleus of hepatocytes is found to contain iron inclusions (Smith *et al.*, 1989). Iron-overloaded mice are also found to excrete larger amounts of oxidized DNA products than controls (Faux *et al.*, 1992). This is an experimental model that may benefit from the application of modern molecular biology techniques that have been applied to the investigation of the molecular basis of action of other carcinogens and may provide some interesting insights in return.

3.5 PEROXISOME PROLIFERATORS

The classic example of a peroxisome proliferator is clofibrate. This compound was developed as a drug for the treatment of hyperlipidaemia disorders. Given to rodents, it induces the proliferation in the liver of peroxisomes and, at high concentrations over a prolonged period of time, induces the development of hepatomas. A number of other compounds are now known to possess this property, many of them structural analogues of clofibrate (Reddy and Rao, 1986).

The role of peroxisomes in hydrogen peroxide metabolism has led to the proposal that the carcinogenic properties of peroxisome proliferators is associated with generation of ROS. Peroxisomes are equipped with a variety of flavin oxidases that generate hydrogen peroxide and also contain catalase that, in normal circumstances, removes the peroxide. The effect of peroxisome proliferators is to induce the activity of the flavin oxidases rather more than catalase activity. Hence, an overproduction of hydrogen peroxide may not be matched by the protective power of catalase, and it is suggested that peroxide leaks out, resulting in the generation of oxidizing free radicals and in genetic damage (Rao and Reddy, 1987). Clofibrate also acts as an inducer of a specific form of cytochrome P450, another potential source of oxidizing species (Gibson *et al.*, 1982). Clofibrate treatment has been linked with increased lipid peroxidation in the liver (Lake *et al.*, 1987).

In their review some years ago, Reddy and Rao (1986) cited several lines of evidence for peroxisome-proliferation-mediated oxidative stress being associated with hepatocarcinogenesis. They mentioned the sustained increase in hydrogen peroxide production, the detectable increased levels of hydrogen peroxide in the livers of treated animals, increased lipid peroxidation associated with treatment and marked inhibition of hepatocarcinogenesis by antioxidant compounds. However, definitive studies remain to be carried out.

3.6 PARACETAMOL-INDUCED LIVER INJURY

Paracetamol, otherwise known as acetaminophen, is a valuable and widely used analgesic and anti-pyretic. It is safe when used at the recommended dose level but causes hepatic necrosis when administered in high doses. Severe liver injury due to accidental or deliberate paracetamol overdose is an unfortunately rather common occurrence, and is often fatal. This clinical situation justified research into its hepatotoxic effects originally but it has developed into an experimental model in its own right. The mechanism of paracetamol toxicity is not completely clear but many aspects of this model have been well described. At non-toxic doses, paracetamol is metabolized in the liver, mostly by conjugation to glutathione and sulphate. In overdose conditions, these pathways are saturated and paracetamol is available for metabolism by cytochrome P450, which activates it to the very reactive intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI is also metabolized by conjugation to GSH or by GSH-mediated reduction. High concentrations of paracetamol thus leads to depletion of hepatocyte GSH by a combination of conjugation and oxidation. GSH depletion allows NAPQI to react with other cellular molecules, and a high level of covalent binding to proteins has been demonstrated and suggested to be involved in cell death (Andersson *et al.*, 1989). The principal question about paracetamol hepatotoxicity centres around the relative importance of the covalent binding of NAPQI and the oxidative stress induced by GSH depletion. Studies with NAPQI suggest that covalent binding to protein may be more important in the toxicity of paracetamol than oxidative reactions. On the other hand, lipid peroxidation has been reported to be a consequence of treating hepatocytes with paracetamol and protection has been observed with antioxidant compounds (McLean and Nuttal, 1978). Thus, it is possible that both arylation and oxidative reactions are involved in paracetamol-induced necrosis.

3.7 QUINONES, HYDROPEROXIDES AND RELATED EXPERIMENTAL AGENTS

In the field of free radicals and liver injury there is a vast body of work concerning a group of compounds that have proven to be of great value as experimental models but are of little clinical significance. The most frequently used compounds are quinones (particularly menadione), paraquat and diquat, bromobenzene, and organic hydroperoxides, particularly cumene hydroperoxide and *t*-butyl hydroperoxide (see Poli *et al.*, 1989b).

Menadione and paraquat are redox-cycling compounds that are readily one-electron reduced by flavoproteins such as NADPH:cytochrome P450 reductase to free radicals that themselves readily reduce oxygen to produce superoxide radicals and regenerate the parent compound. Redox-cycling of this kind can generate large amounts of superoxide and deplete NADPH, with relatively little of the toxic chemical (Thor *et al.*, 1982). The superoxide dismutates spontaneously and enzymically to produce hydrogen peroxide that is metabolized by GSH peroxidase at the expense of cellular GSH concentrations. GSH depletion is a common feature of the toxic effects of menadione, paraquat, bromobenzene and organic hydroperoxides. However, whereas lipid peroxidation is observed with the latter compounds, it is not seen with menadione or paraquat treatment. In these cases, the cytotoxicity appears to be caused by oxidation of critical protein thiols and subsequent loss of calcium homeostasis. Influx of calcium results in the stimulation of a variety of enzymic processes including proteases, phospholipases and endonucleases that are destructive and can ultimately kill the cell (Orrenius *et al.*, 1989). The loss of calcium homeostasis has been thought of as the final common pathway of cell death for the many diverse hepatotoxic agents that have been studied. Bromobenzene, metabolized by cytochrome P450 to an epoxide substrate for GSH transferase, and acrolein (a reactive aldehyde metabolite of allyl alcohol) stimulate lipid peroxidation in liver cells and antioxidant compounds, such as Trolox, are hepatoprotective (Comporti, 1989). GSH depletion is important and GSH must be depleted below a certain critical threshold before toxicity is manifest.

Organic peroxides such as cumene hydroperoxide and *t*-butyl hydroperoxide have extensively been used as experimental agents. They provoke lipid peroxidation in hepatocytes, probably by the generation of alkoxyl and peroxyl radical intermediates after reaction with cytochrome P450. Other cytotoxic mechanisms are probably involved including protein thiol and non-protein thiol oxidation and deranged calcium homeostasis (Jewell *et al.*, 1986). In fact, the addition of cumene hydroperoxide to isolated bile duct cells, devoid of cytochrome P450 activity, still results in cell death but lipid peroxidation is not detectable (Parola *et al.*, 1990).

The above studies have generated a huge body of invaluable data concerning the effects of oxidative stress on cells. In particular the importance of GSH in cell protection and the role of disturbed calcium homeostasis in cell killing have been greatly illuminated.

3.8 COMMON THREADS AND DISCREPANCIES

In the above descriptions of liver injury induced by a variety of chemical agents, several common threads should be apparent along with some unresolved discrepancies. The CCl_4 model remains the best characterized of the experimental models described here. Lipid peroxidation can be readily demonstrated and seems clearly associated with cell death in that both can be prevented by antioxidant treatment. The involvement of lipid peroxidation in ethanol hepatotoxicity is less obvious. In the hepatotoxicity caused by iron overload, polyhalogenated aromatics and peroxisome proliferators, lipid peroxidation has been demonstrated but not always convincingly or consistently. The presence of "free" iron is proposed to cause lipid peroxidation and fibrosis in iron overload, and porphyria in some animals, particularly in the presence of polyhalogenated aromatic chemicals. The generation of ROS is proposed as a causal mechanism in the hepatotoxicity of ethanol, peroxisome proliferators and polyhalogenated aromatics. Clearly, the same general mechanisms are being invoked to explain liver injuries with different characteristics. According to these proposals, CCl_4, peroxisome proliferators and ethanol should all also cause porphyria; iron overload should exacerbate the carcinogenicity of peroxisome proliferators; fibrosis and cirrhosis should be a common feature of all these models; vitamin E might be expected to be protective against all of these toxic liver injuries. This is apparently not the case, or such common features and mechanisms of action would have been reported. The message here is that we must look more carefully for sophisticated and specific mechanisms of toxicity and resist the tendency to overgeneralize and invoke reactive free radicals *faute de mieux*. This is not in any way to disclaim a role for free radicals in these models of hepatotoxicity: given the circumstances, it seems highly probable that free radicals must be generated in most of the situations cited above. We must also recognize and investigate more intensively the possibility that free radicals may be capable of acting in a specific manner against a specific target, and by mechanisms more sophisticated than hitherto suspected. In this way the discrepancies mentioned above may be explained along with the common threads.

3.9 REPERFUSION INJURY IN LIVER ALLOGRAFTS

Liver transplantation is increasingly used for treatment of end-stage liver disease and thousands of transplants are

now performed each year. There are still many problems of primary non-function or dysfunction of the graft, however, and mortality rates are still high, ranging from 15% to 25% within 1–2 years of operation. This is very often a problem of preservation injury. One of the most significant advances in extending the viable storage time and post-operative function of the liver has been the introduction and widespread use of University of Wisconsin (UW) preservation solution. This solution was designed to prevent the well-documented deleterious effects of hypothermia: it contains substrates for the regeneration of ATP and ADP, agents to prevent acidosis, agents to prevent cell swelling and antioxidant compounds to prevent the action of reactive oxygen free radicals (Belzer and Southard, 1988). It is well documented for many tissues that ischaemia results in the degradation of ATP and accumulation of hypoxanthine with concomitant conversion of xanthine dehydrogenase to xanthine oxidase (XO) (Omar *et al.*, 1991). Reperfusion with oxygen-containing medium results in XO-catalysed metabolism of hypoxanthine and generation of superoxide and hydrogen peroxide. This sequence of events has been heavily implicated in ischaemia–reperfusion injury of many tissues. For this reason, UW solution is equipped with antioxidant compounds and these are glutathione, the naturally abundant intracellular antioxidant thiol compound, and allopurinol, included to inhibit XO and thus prevent XO-mediated generation of superoxide and hydrogen peroxide. Nevertheless, the actual role of free radicals in reperfusion injury to the liver eludes exact definition.

Unlike the liver injury that occurs with toxic chemicals (see above), reperfusion injury affects not only the hepatocytes but also the endothelial cells that line the sinusoids as an important primary target. It is thought that there may be some aspects of preservation–reperfusion injury that are unique to the liver and UW solution is apparently more effective in this tissue than in others (Clavien *et al.*, 1992). The principle characteristic of this injury is that it involves damage to the sinusoidal cells such that leucocytes and platelets are stimulated to adhere on reperfusion.

It is known that, while the injury is not manifest until reperfusion with oxygenated solution commences, the duration of the period of preservation is the major determinant of the extent of the preservation–reperfusion injury. Hence, changes in the liver during the ischaemic period prepare the liver for injury when oxygen is reintroduced. The target of preventive measures should therefore be the ischaemic changes or the events that occur immediately after reperfusion. In the latter case, reactive oxygen free radicals may be involved.

Under normal conditions the sinusoidal endothelial cells are flat thin cells lying close against the hepatocyte surface, separated only by the periplasmic space. Cold preservation of the liver causes the endothelial cells to round up and detach, although they are not actually killed. This is the most obvious symptom of damage to the endothelial cells during the ischaemic period. On reperfusion, further evidence that the cell surface has been altered is the adherence of leucocytes. The generation of free radicals by XO appears to be involved in the promotion of leucocyte adherence and can be prevented by allopurinol or SOD treatment just before reperfusion. The actual mechanisms of free-radical involvement in leucocyte adherence are presently unclear but are postulated to involve activation of PLA_2 and release of leukotriene B_4, and possibly also the induction of overexpression of adhesion molecules on the endothelial cell surface and also on the surface of hepatocytes and bile duct cells. Adherent leucocytes can produce numerous inflammatory mediators including more oxygen free radicals, cytokines, platelet activating factor (PAF) and proteases. This amplification of the inflammatory response further damages the liver. Apart from the aforementioned XO system, there are several potential sources of reactive oxygen intermediates in liver reperfusion injury. The Kupffer cells that reside in the sinusoids release superoxide when activated. Similarly, neutrophils stimulated to invade and adhere generate large amounts of superoxide using their plasma-membrane NADPH oxidase. At present it seems certain that production of reactive free radicals is stimulated during preservation-reperfusion injury and e.s.r. studies have been used to detect some of them (Connor *et al.*, 1992), but the precise source of these intermediates and their role in effecting the liver damage is not yet well defined. They appear to be just a part of the overall picture, which is the typically confused scene of an acute inflammatory response involving a whole host of mediators: cytokines, proteases, PAF, eicosanoids, etc. produced by both the resident Kupffer cells, the invading neutrophils, and also the endothelial and epithelial cells that are ultimately damaged.

The role of UW solution in protecting the transplanted liver is also not clear, despite the rational basis of its design. The actual role of some of its components may not be what was originally intended. Discussion of the other components is not appropriate here but, as stated above, UW solution contains allopurinol and GSH as antioxidant constituents. A derivative of UW solution, Carolina rinse solution (Gao *et al.*, 1991a), also includes a chelating agent, desferrioxamine, to mop up any stray iron ions that may catalyse free-radical generation. Carolina rinse certainly appears to be even more effective than UW solution when used as the reperfusion solution: sinusoidal endothelial cells were protected, Kupffer cells were inhibited from activation and overall survival rates of animal receiving grafts were greatly increased (Gao *et al.*, 1991b). Nevertheless, the precise mechanism of action of this solution is presently unclear, and its developers have on the one hand suggested that its efficacy is not due to the presence of antioxidant compounds but to other components (Gao *et al.*, 1991b),

whilst at the same time demonstrating that Carolina rinse is effective in preventing free-radical formation during liver reperfusion (Connor *et al.*, 1992). Clearly, as in the whole area of tissue reperfusion injury, there is scope for a great deal more work but we should be encouraged by the advances already made.

3.10 ISCHAEMIC HEPATITIS

Hepatic reperfusion injury is not a phenomenon connected solely to liver transplantation but also to situations of prolonged hypoperfusion of the host's own liver. Examples of this occurrence are hypovolemic shock and acute cardiovascular injury (heart attack). As a result of such cessation and then reintroduction of blood flow, the liver is damaged such that centrilobular necrosis occurs and elevated levels of liver enzymes in the serum can be detected. Particularly because of the involvement of other organs, the interpretation of the role of free radicals in ischaemic hepatitis from this clinical data is very difficult. The involvement of free radicals in the overall phenomenon of hypovolemic shock has been discussed recently by Redl *et al.* (1993). More specifically, Poli (1993) has reported preliminary data on markers of free-radical production during ischaemic hepatitis. These markers mostly concerned indices of lipid peroxidation in the serum and also in the erythrocytes of affected subjects, and a correlation was seen with the extent of liver injury. The mechanisms of free-radical damage in this model will be difficult to determine in the clinical setting, but the similarity to the situation with transplanted liver suggest that the above discussion of the role of XO activation, Kupffer cell activation and induction of an acute inflammatory response would be also relevant here. It will be important to establish whether oxidative stress is important in the pathogenesis of ischaemic hepatitis and in the problems of liver transplantation discussed above, since it would suggest that antioxidant therapy could be of real benefit.

4. *Concluding Remarks*

As stated at the beginning of this article, the liver is the most intensively studied animal tissue in biochemistry. In the context of the role of free radicals in human diseases, the liver is not obviously at centre stage, since heart disease and cancer are more important in the industrialized world than, for example, cirrhosis. Free-radical biochemistry of the liver will remain a fertile area of work, however, not least because so many original ideas and techniques are developed there and then applied to the study of other tissues. The increasing use of liver transplantation, following the acceptance of kidney and heart transplants as almost routine, will surely increase the interest in the study of ischaemia–reperfusion injury in this organ, just as the study of that phenomenon in the heart has been a massive area of endeavour.

This paper has concentrated on free-radical-mediated damage to the liver, but an allied topic that has not been dealt with here concerns antioxidant biochemistry. There is at present a huge interest in the role of antioxidant nutrients in the prevention of human disease, particularly atherosclerosis, as detailed elsewhere in this volume. The principal antioxidant nutrients of interest include vitamin E (RRR-α-tocopherol) and the carotenoids. These are carried around the body in the lipoproteins and the liver is at the centre of lipoprotein metabolism. At present, the role of the liver in processing lipoproteins and controlling the distribution of fat-soluble antioxidant micronutrients is not fully understood. Given the interest in using natural (and also synthetic) antioxidants as protective agents against heart disease and other disorders, this is a topic that is likely to attract considerable attention in the near future.

5. *Dedication*

I would like to dedicate this paper to all of my colleagues at the Institute of General Pathology in Turin, who have made such important contributions to this subject, and have been such consistent and generous friends for so long. Forza Toro.

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16. The Conioses: Organotypic Inflammatory Oxidative Responses to Environmental Particulate Pollutants

Peter H. Evans

I will show you fear in a handful of dust.
The Waste Land (T.S. Eliot)

1. Introduction

Exposure of living organisms to exogenous environmental agents is a potentially major source of oxidative stress. Such agents may themselves be free radicals, for example, free-radical contaminants formed by thermocombustion in cigarette smoke, or alternatively they may be non-radicals *per se* but be capable of inducing the generation of free radicals by subsequent redox interactions with the biological host organism. Humans are exposed to a large number and variety of compounds, materials and influences that exert a deleterious oxidative effect. These include: chemicals, e.g. drugs, pesticides and metals; particulate dusts, e.g. silica and asbestos; gases, e.g. ozone and nitrogen oxides; and irradiation, e.g. ultraviolet (UV) light and ionizing radiation. The combination of the exogenous sources, be they communal, occupational or personal pollutants, together with the physiological endogenous production of free-radical oxidants, ensures that there is a continuous but varying flux of potentially injurious reactive oxygen metabolites (ROMs) with which the body must be able to deal efficiently and effectively. It is fortunate that the customary physiological antioxidant mechanisms with their dependence on the dietary supply of nutritional antioxidants are generally effective in fulfilling this critical protective role. The advisability and necessity of supplementary antioxidants in the prevention and treatment of the so-called oxidative diseases, be they in the form of specific nutrient supplements or pharmacological

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compounds, is a topic of growing interest. The generally low toxicity associated with the antioxidant micronutrient vitamins and trace elements, together with their synergistic mechanism of action and their wide spectrum of involvement in various organs and pathologies in which oxidative stress has been implicated, means that they hold a central role in combating the adverse effect of tissue-damaging ROMs. The identification and development of efficacious antioxidant agents and drugs with the promise of enormous therapeutic benefits in combating the major degenerative diseases constitutes a major challenge for nutritional medicine, medicinal chemists, pharmacologists and the pharmaceutical industry.

2. *The Conioses: Organotypic Responses to Particulate-induced Injury*

While dust in the geochemical sense is associated with major adverse ecological effects on the biosphere and stratosphere, as evident in the catastrophic volcanic eruptions of Mount St Helens and Mount Pinatubo, its more insidious and chronic influence on human health may not be so readily apparent. The Greek word *konis* provides the etymological basis of the nosological term relating to the dust-induced group of diseases, the conioses. The entry points, modes of access and organ targets, of the variety of environmental particulates and dusts into the body, are several and various. Inhalation of airborne particles in the form of cigarette smoke, Diesel fumes, or general environmental and occupational exposure to mineral dusts, can result in pneumoconiosis, be it silicosis, asbestosis or coalworkers' pneumoconiosis. Such diseases may be characterized by fibrotic, emphysematous, granulomatous and cancerous pulmonary pathology. Particles may also gain access to the gastrointestinal tract. Clearance of previously inhaled dust from the lung following transfer by the mucociliary transport mechanism via the trachea and pharynx, results in subsequent particle ingestion. Particulates may also be ingested as additives or contaminants in food, drinks and drugs. Alternatively, particles may penetrate directly through the skin or enter via the various body orifices. Less obviously, particulate material can possibly gain access indirectly into other organs of the body via the intravascular system or even, as has been proposed, via the olfactory nerve directly into the brain. The pathology, pathogenic mechanisms and treatments of the dust and particle-related diseases, provides a multifarious aspect on the role of oxidative interactions.

2.1 LUNG

By its intrinsic structure and function, the primary organ exposed to environmental dust is of course the lung.

2.1.1 Direct Free-radical Particulate Reactions

While by common perception, mineral dusts may appear as particularly inert materials, inorganic particles can participate in a variety of chemical and cellular reactions, some of which are mediated by free radicals.

2.1.1.1 Surface Reactions

X-Ray irradiation of quartz or silica particles induces an electron-trap lattice defect accompanied by a parallel increase in cytotoxicity (Davies, 1968). Aluminosilicate zeolites and clays (Laszlo, 1987) have been shown by electron spin resonance (e.s.r.) studies to involve free-radical intermediates in their catalytic activity. Generation of free radicals in solids may also occur by physical scission of chemical bonds and the consequent formation of "dangling bonds", as exemplified by the "freshly fractured" theory of silicosis (Wright, 1950; Fubini *et al.*, 1991). The entrapment of long-lived metastable free radicals has been shown to occur in the tar of cigarette smoke (Pryor, 1987).

2.1.1.2 Redox Element Reactions

The involvement of transition-metal ions, especially iron and copper, in catalysing free-radical interactions (Halliwell and Gutteridge, 1985), is of direct significance to the pathogenesis of the dust-related lung diseases. The surface properties of quartz and its associated redox reaction, has been proposed to be a factor in the genesis of silicosis (Marasas and Harington, 1960). Likewise with asbestos, and in particular the iron-rich amphiboles namely crocidolite and amosite, the *in vitro* catalytic generation of superoxide and hydroxyl radicals from hydrogen peroxide (Weitzman and Graceffa, 1984) causes the hydroxylation of guanine residues and strand scission of purified DNA (Kasai and Nishimura, 1984). Asbestos-catalysed lipid peroxidation of lung microsomes, both by NADPH-dependent (Fontecave *et al.*, 1987) and NADPH-independent (Gulumian *et al.*, 1983) mechanisms, is mediated by iron. The hypothesis that decontrolled iron, partially sequestered in indigestible porous bodies, may act as an ultimate toxin and carcinogen has been proposed (Kon, 1978), and it is interesting to note that, in this context, zeolite minerals possess these particular physical and biological characteristics. Zeolites are characterized by their open microporous structure and chemically by their active hydrophilic, ion-exchange and catalytic functions (Newsam, 1986). These properties are crucial to the widespread industrial use of the synthetic non-fibrous zeolites in the chemical and petroleum cracking industry. Mössbauer spectroscopy of the fibrous zeolitic mineral erionite has revealed iron to be present (Evans *et al.*, 1987). Human environmental (Baris *et al.*, 1981) and experimental (Wagner *et al.*, 1985) exposure to fibrous

erionite is associated with an extremely high prevalence of lung fibrosis and mesothelioma.

Ferruginous bodies are formed in the lungs following exposure to asbestos, and are composed of mineral fibres coated with the iron-rich protein ferritin (Pooley, 1972). The ability of superoxide derived from stimulated polymorphonuclear leucocytic neutrophils (PMNs) to mobilize iron from ferritin stores (Biemond *et al.*, 1984) and the catalytic effect of iron in producing lipid-peroxidative changes by stimulated PMNs (Thomas *et al.*, 1986), suggests that ferruginous asbestos bodies may well possess a pathogenic effect and are not merely markers of fibre exposure. Mössbauer spectroscopy of autopsied lung tissue of asbestos workers has indicated the presence of considerable quantities of iron (Stroink *et al.*, 1987).

In addition to the well-characterized role of iron in catalysing redox interactions, other metallic contaminants, for example, nickel, may also contribute. *In vivo* toxicity studies have demonstrated the capacity of nickel particulate compounds to induce tumours following intraperitoneal injection (Pott *et al.*, 1987). Such activity is proportional to their phagocytic uptake, and to the associated respiratory burst and generation of PMN-derived reactive oxygen metabolites (ROMs), a proposed pathogenic mechanism (Evans *et al.*, 1992a).

2.1.2 Phagocyte-derived Free Radicals

As a consequence of the inhalation of mineral dusts, infiltration into the lung of inflammatory phagocytic cells, namely PMN and macrophages, occurs (Rola-Pleszczynski *et al.*, 1984). Analysis of the cell populations of the rat pleural cavities after injection with asbestos and silica dust also showed both degranulation and reduction of the mast cell population (Edwards *et al.*, 1984), and it is of interest to note that histamine augments the particle-stimulated generation of macrophage superoxide production (Diaz *et al.*, 1979).

The capacity of activated phagocytic cells to generate various ROMs, namely superoxide and hydroxyl radicals, hydrogen peroxide and hypochlorite, contributes to the anti-microbial defence mechanism that is initiated when an organism encounters a foreign organism. However, the toxicity of ROMs when directed against the host organism's own molecules and cells, renders the activated phagocyte a potentially auto-injurious agent (Babior, 1984). The availability of a range of endogenous enzymic and chemical antioxidants fortunately limits the actual toxicity thus produced.

Phagocyte-derived ROMs have been implicated in the pathogenesis of a number of pulmonary diseases, including emphysema, acute respiratory distress syndrome, and various environmental diseases such as asbestos-related fibrosis and cancer (Mossman and Marsh, 1985). The relatively high oxygen tension in pulmonary tissue renders the lung prone to oxidative stress (Edwards and Lloyd, 1988).

The initial demonstration using an *in vitro* chemiluminescent technique, of the capacity of various fibrous, non-fibrous and metallic air-pollutant particles to stimulate ROM production by alveolar macrophages (Hatch *et al.*, 1980), and the similar action of asbestos dusts to stimulate the respiratory burst of human blood PMN (Doll *et al.*, 1982), inaugurated many subsequent investigations into the role of phagocyte-derived ROM in the pathogenesis of the pneumoconioses and allied cancerous dust-induced diseases. Confirmation of the capacity of alveolar macrophages to generate ROM when stimulated by asbestos particles has also been demonstrated utilizing the SOD-inhibitable cytochrome c reduction assay (Case *et al.*, 1986). Inhibition studies using superoxide dismutase (SOD), catalase and the iron chelator desferrioxamine, with asbestos-treated mouse peritoneal macrophages using the nitroblue tetrazolium (NBT) reduction technique, have suggested a significant role for iron in mediating the cell toxic activities (Goodlick and Kane, 1986). In addition to the increased lipid peroxidation, which occurs with asbestiform dusts, similar results following *in vitro* exposure of alveolar macrophages to isometric particles like quartz is also found (Gabor *et al.*, 1975). While the pathogenic activity of the various mineral dusts is evidently not simply related to their capacity to stimulate phagocyte-derived ROMs (Gormley *et al.*, 1985), chemiluminescent studies using human PMN have indicated that erionite, a fibrous zeolite mineral and potent carcinogen, is extremely active in stimulating the generation of ROMs (Evans *et al.*, 1989a). Erionite has also been shown to be active in stimulating macrophage superoxide production as measured by cytochrome c reduction (Hansen and Mossman, 1987). Likewise, the related zeolite mineral clinoptolite is an active stimulant of macrophage chemiluminescence (Velichkovsky *et al.*, 1983). Evidence of the genotoxic and clastogenic activity of erionite to cultured cells has also been presented (Poole *et al.*, 1983; Kelsey *et al.*, 1986).

The PMN luminol-dependent chemiluminescent technique may have significant potential application in identifying and monitoring particulate carcinogenic agents, however, a detailed evaluation of the validity of the system as an *in vitro* experimental model system is required. Furthermore, consideration of the extrapolation of any *in vitro* assay for particle toxicity to the *in vivo* situation, would need to be carefully evaluated by additional anatomical and aerodynamic considerations. These concern the degree of particle penetration into the alveoli following inhalation, together with a range of factors governing the modification and elimination of particles from the lung, by mucociliary transport, phagocytosis, biodegradation, and adsorption of serum proteins and surfactants (Desai and Richards, 1978; Jaurand *et al.*, 1984).

Mesotheliomas are the characteristic asbestos-related tumours of the pleura. Exposure of explants of human pleura to crocidolite blue asbestos in culture produces a

proliferative cellular response (Rajan *et al.*, 1972), and rat pleural mesothelial cells are capable of phagocytosing chrysotile white asbestos fibres (Jaurand *et al.*, 1979). However, when human mesothelial cells were exposed to amosite brown asbestos, no production of free radicals was detectable by e.s.r. (Gabrielson *et al.*, 1986).

2.1.3 Oxidative Effects

Mineral dust-induced ROMs contributes to pulmonary fibrosis, malignancy, hypersensitivity and emphysema (Doelman *et al.*, 1990; Kamp *et al.*, 1992). The involvement of ROMs in pulmonary fibrotic reactions is indicated by the participation of PMN oxidants in the autoactivation of latent collagenase (Weiss *et al.*, 1985). Prolyl hydroxylase, a key enzyme in collagen fibril formation, has been shown to be dependent on the reaction of superoxide with prolyl residues (Myllylä *et al.*, 1979).

In addition to the inflammatory fibrotic effects, prooxidant states have also been proposed to play a significant pathogenic role in carcinogenesis and in tumour promotion (Trush and Kensler, 1991). The classic tumour promoter phorbol myristate acetate (PMA) is an active stimulant of PMN ROMs, indicating the importance of chronic inflammation in the process (Cerutti, 1985). Activated PMNs have been shown to produce mutational effects (Weitzman and Stossel, 1981), cytogenetic changes (Weitberg *et al.*, 1983), and the malignant transformation of co-cultured cells (Weitzman *et al.*, 1985). The association of PMN ROMs with DNA-base modification (Frenkel and Chrzam, 1987), illustrates the putative promotional and mutational mechanism.

Oxidation of enzyme sulphydryl groups by free radicals (Armstrong and Buchanan, 1978) and by the longer lived chloroamines (Weiss *et al.*, 1983), contributes to enzymic dysfunction and to the decrease in serum sulphydryl levels, primarily mercaptoalbumin, found in coalworkers with rheumatoid inflammatory disease (Thomas and Evans, 1975). Formation of the *S*-nitroso adduct of plasma mercaptalbumin is thought to provide a metastable reservoir for intravascular nitric oxide release (Stamler *et al.*, 1992).

2.1.4 Modulatory Actions

In vivo studies into the pertinent parameters related to the pathogenicity of mineral dusts, have indicated that the particle fibriform morphology and dimensions are of particular significance, with fibres longer than *c.* 8 μm and thinner than *c.* 0.25 μm being the most carcinogenic (Stanton *et al.*, 1981). However, the exclusive applicability of the so-called "Stanton hypothesis" of fibre toxicity continues to be a topic of scientific debate (Dunnigan, 1984). Specific mineral dust samples of the fibrous clays palygorskite and attapulgite, can also produce mesotheliomas when injected into experimental animals (Wagner *et al.*, 1987). Milled and unmilled glass fibres of varying size distribution differ in their ease of phagocytosis, their capacity to stimulate PMN ROM production (Yano *et al.*, 1991), and in their ability to stimulate a dose-dependent release of prostaglandins and lysosomal beta-glucuronidase from perfused alveolar macrophages (Forget *et al.*, 1986), the longer fibres being the most active. Ultrastructural examination of the effects of asbestos fibres on cultured macrophages have revealed that several macrophages may be involved in attempting to phagocytose the longer fibres, resulting in partial endocytosis and release of lysosomal enzymes from the incompletely fused phagosome membrane (Johnson and Davies, 1981). The extracellular release of ROMs by particle-stimulated human PMNs (Test and Weiss, 1984), which occurs at the site of membrane attachment (Vissers *et al.*, 1985), appears to be related to the delay in completion of endocytosis (Ohno *et al.*, 1982), and a similar process implicating the proposed phenomenon of "frustrated phagocytosis" has been hypothesized as of direct pathogenic significance for the carcinogenic effects of mineral fibres and plastic films (Archer and Dixon, 1979). Reports that ROM release is dependent on the size of the stimulant particle and the corresponding area of perturbed cell membrane is consistent with the finding of increased stimulatory activity of fibrous particles (Hansen and Mossman, 1987).

As well as the specific effects of particulate size, morphology and ionic composition, the surface electrostatic charge exhibited by dusts is another particle parameter that determines its interaction with biological components (Light and Wei, 1977). Using laser-Doppler velocimetry to measure particulate surface charge, the coating of poly-2-vinyl pyridine-*N*-oxide produces a decrease in electrophoretic mobility accompanied by a corresponding lowering of cytotoxicity (Davies and Preece, 1983). The adsorption of serum proteins by silica alters the immunological reactivity of adsorbed albumin (Scheel *et al.*, 1954) and may also stimulate chemotactic factor generation and complement activation (Yano *et al.*, 1984), a process of significance to the accumulation and activation of PMNs. Covalent protein derivatization of the surface of amosite asbestos inhibits its cytolytic action, whilst retaining a significant ability to induce the release of arachidonate metabolites from a macrophage-type cell line (Evans *et al.*, 1983).

The effect of a variety of endogenous modifying factors influencing the activity of inhaled dusts is pertinent to their reactivity *in vivo*. Priming of elicited peritoneal macrophages by prior exposure to chrysotile asbestos produces an enhanced chemiluminescent response to subsequent *in vitro* stimulation by particulates (Donaldson and Cullen, 1984). Generation of PMN ROMs is modulated by cell contact and by the secretory products of activated macrophages, with the stimulation of surface-adherent PMNs being greatly exacerbated in response to exposure to tumour necrosis factor (Nathan, 1987). *In vivo* and *in vitro* exposure of alveolar macrophages to fibrous minerals stimulated an

enhanced secretion of both tumour necrosis factor and interleukin-1 (Brown *et al.*, 1991a).

2.1.5 Co-carcinogenesis

Epidemiological studies into the combined effect of smoking and occupational exposure to asbestos on lung cancer mortality, suggest a synergistic mode of action (Selikoff *et al.*, 1968). With regard to mesothelioma, the epidemiological evidence indicates that asbestos may be acting as a tumour promoter (Browne, 1983). Mechanistic investigations have shown that asbestos directly catalyses the oxidation of 6-hydroxybenzo(a)pyrene, a metabolite of benzo(a)pyrene and the major carcinogen in cigarette smoke, to form the reactive intermediate compound 6-oxobenzo(a)pyrene radical, a reaction not inhibited by desferrioxamine (Graceffa and Weitzman, 1987). The metabolic systems for the activation and deactivation of xenobiotics in the lung play an important role in the toxicity of reactive electrophiles (Minchin and Boyd, 1983), and microsomal-mediated oxidation of benzo(a)pyrene by asbestos and superoxide-induced reactions has been demonstrated (Byczkowski and Gessner, 1987). However, an extramicrosomal bioactivation pathway of procarcinogens by a phagocyte-dependent mechanism has been proposed (Roman-Franco, 1982), and the enhanced mutation toward V79 Chinese hamster cells of benzo(a)pyrene by particle-stimulated alveolar macrophages (Romert and Jenssen, 1983), provides convincing evidence in support of the postulated role of activated inflammatory cells in the carcinogenic process. The adsorption of environmental carcinogens to asbestos indicates that a carrier mechanism may also be operative (Harvey *et al.*, 1984), with ROMs exerting a co-carcinogenic effect by catalysing the covalent binding of polycyclic hydrocarbons to DNA (Monny and Michelson, 1982). The generation of a hydroxyl radical by the catalytic effect of asbestos-bound iron on cigarette-smoke oxidants further contributes to DNA damage (Jackson *et al.*, 1987).

2.2 GUT

While the lung is the major organ exposed to airborne dusts, such agents may also be swallowed following mucociliary transport and removal from the lung. Contaminating particles contained in food and drink also gain direct access into the gut.

2.2.1 Cancer

Animal experiments into the effect of ingestion of chrysotile asbestos demonstrated an accumulation of cellular debris within the lumen of the ileum and colon consistent with cytotoxic changes of the mucosal lining cells (Jacobs *et al.*, 1978). The question as to whether asbestos causes tumours in the gastrointestinal tract in humans is a topic of concern, however, the evidence remains equivocal (Levine, 1985).

2.2.2 Inflammatory Bowel Disease

Granulomatous reactions to particulate abrasives present in toothpaste (Miller, 1976) has led to their postulated involvement in Crohn's disease (Sullivan, 1990). The presence of aluminium phosphate crystals (Roge *et al.*, 1991) and aluminosilicate deposits in the Peyer's patches in the gut (Powell *et al.*, 1991), have likewise been linked with inflammatory bowel disease. The success of elemental diets in the treatment of Crohn's disease (Sanderson *et al.*, 1987), as well as the proposed effect of removing dietary antigens, is also consistent with the resultant accompanying removal of particulate material. The infiltration of PMNs into the gut, and accompanying production of free-radical oxidants, is considered to be a significant pathogenic feature of intestinal inflammation (Koningsberger *et al.*, 1988), and it has been suggested that the pathogenesis of toothpaste-mediated inflammatory bowel disease could be caused by the chronic particle-induced stimulation of infiltrating phagocytes with the associated generation of ROMs (Evans *et al.*, 1990). Indeed, isolated blood PMNs from patients with inflammatory bowel disease exhibit an increased chemiluminescent response, indicative of chronic ROM generation (Faden and Rossi, 1985).

2.2.3 Particle Migration

Migration of particulates through the intestinal mucosa has been shown to occur in patients taking bismuth treatment for dyspepsia (Nwokolo *et al.*, 1992), a process mediated by gastrointestinal phagocytes or via the process of persorption whereby particles may pass between mucosal cells (Volkheimer, 1977). An increase in intestinal permeability in Crohn's disease has been found to precede clinical relapses (Wyatt *et al.*, 1993). Additionally, the identification of ingested asbestos fibres, particularly the longer fibres, in the thoracic lymph duct of rats (Sebastien *et al.*, 1980), indicates that mineral fibres are capable of penetrating the gastrointestinal wall. Experimental studies in baboons have furthermore demonstrated that migration of ingested asbestos particles to other organs of the body, namely heart, spleen and pancreas, is possible (Kaczenski and Hallenbeck, 1984). Exposure of pregnant rats to ingested chrysotile asbestos have also shown the capacity of fibres to penetrate even the placenta (Cunningham and Pontefract, 1974), although no teratogenic effects were demonstrable. *In vitro* studies of exposed blastocytes indicate that trophoblastic cells take up fibres only when the zona pellucida is removed (Schneider and Maurer, 1977).

2.3 SKIN

Elephantiasis, oedema of the lower leg consequent to obstruction of the lymphatic drainage, is endemic in bare-footed subjects in the Cameroons and other regions of Africa. The identification of aluminosilicate clay

particles in the dermis of the foot of an African patient with elephantiasis has been related to the associated characteristic histological cellular and fibrotic tissue reactions to penetrating soil microparticles, and induction of so-called podoconiosis (Blundell *et al.*, 1989; Price and Plant, 1990). Analogous granulomata have been observed in auxiliary lymph nodes in humans exposed to talcum powder and deodorant sprays containing aluminosilicate (Williams and Freemont, 1984). Such findings indicate that microparticles are capable in particular circumstances of penetrating the skin in susceptible individuals.

Direct entry of alumina adjuvants through the skin may occur by the use of therapeutic vaccines, with a resultant transient uptake of aluminium in the brain (Redhead *et al.*, 1992). The injection of talc (magnesium silicate)-containing drugs intended for oral consumption has been shown to induce progressive pulmonary fibrosis in drug abusers (Paré *et al.*, 1989).

Exposure of women to cosmetic talcum powder, has been related to the observed presence of the characteristic phyllosilicate talc particles in normal and in malignant ovary tissue (Henderson *et al.*, 1979). The experimental demonstration in rats of migration of talc particles from the vagina to the ovary, illustrates the potential route of entry into the body (Henderson *et al.*, 1986).

2.4 JOINTS

Pathological reactions induced by various crystalline endogenous particulates, for example, uric acid crystals in gout, have been implicated in several of the arthopathies (Dieppe *et al.*, 1979). Such inflammatory insults have been proposed to arise from defects in anti-mineralization control mechanisms in connective tissue (Grant *et al.*, 1992). PMNs may also generate ROMs when exposed to urate and a variety of other particulates found in the inflamed synovia, namely calcium hydroxyapatite, calcium hydrogen orthophosphate dihydrate (brushite) in chondrocalcinosis, and calcium pyrophosphate dihydrate in "pseudo gout" patients (Higson and Jones, 1984). PMN ROM production may be further exacerbated by complement, resultant from its adsorption and activation at the urate crystal surface (Abramson *et al.*, 1982). Such crystals may also provoke a specific antibody reaction, which potentiates additional *de novo* urate crystal formation (Kam *et al.*, 1992). It is of interest to speculate as to whether the observed rare co-existence of gout and rheumatoid arthritis is due to the antioxidant function of soluble urate (Ames *et al.*, 1981).

Although commonly formed from endogenous material, the occurrence of synovial crystals formed following environmental exposure to exogenous agents is indicated by the identification of both aluminium phosphate and aluminium silicate particulates (Netter *et al.*, 1983, 1991). It is noteworthy in this context that arthritic symptoms have been reported following the acute exposure of individuals to aluminium as a consequence of the major water pollution incident in Camelford, Cornwall (Coutts, 1990).

2.5 BRAIN

The presence of particles in the brains of experimental rats and humans exposed to asbestos has been reported (Pontefract and Cunningham, 1973; Auerbach *et al.*, 1980). In experimental studies, particles of Teflon, a reflux paste, enter the brain via intravascular transport when injected into the bladder (Aaronson *et al.*, 1993). Encephalitic reactions to accumulated calcium oxalate crystals in the brain as a result of infusions of glucose surrogate polyol solutions have been described (Peiffer *et al.*, 1984). Such studies indicate the capacity of particulates to enter the brain and thus pose a potential pathological threat to the functioning of the central nervous system (CNS).

2.5.1 Cancer

A case report of primary lymphoma of the brain in an asbestotic welder has been linked to a dysfunctional immune system (Bianchi *et al.*, 1983), and an epidemiological study of a large cohort of asbestos insulation workers has revealed a small, though not statistically significant, excess prevalence of head tumours (Seidman *et al.*, 1982). The identification of microscopic endogenous magnetite biomineralized particles in human brains has been proposed to be linked to the controversial cancer-promoting effects of electromagnetic fields (Kirschvink *et al.*, 1992).

2.5.2 Alzheimer's Disease

The aetiopathogenesis of Alzheimer's disease is evidently related to an ill-defined and complex interplay of age-related, genetic and environmental neurotoxic factors, the most widely mooted of putative agents being aluminium.

2.5.2.1 Plaque Particulates

The identification, using analytical microprobe and solid-state "magic-angle" nuclear magnetic resonance (NMR) techniques, of aluminosilicate deposits in the cores of the pathognomic senile plaques in the brains of Alzheimer subjects (Candy *et al.*, 1986) has prompted widespread scientific and public concern, and controversy with regard to the possible aetiological role of environmental aluminium and aluminosilicates in senile dementia (Walton, 1991).

The development of specific and reliable analytical procedures for the detection, location, and quantification of mineral particles in biological tissues (Henderson and Barr, 1988) has provided both the experimental techniques and additional evidence for detecting aluminosilicates in Alzheimer brains (Singhrao *et al.*, 1990). The association of asbestos-related disease with severe

Alzheimer-type lesions also suggests the possible pathogenic role of particulates in causing dementia (Bianchi *et al.*, 1986), and inhalation of putative prophylactic aluminium dust by miners has been associated with subsequent cognitive dysfunction (Rifat *et al.*, 1990). The persorption of intact starch granules through the gut and uptake into the brain has been hypothesized to contribute to the pathogenesis of senile dementia (Freedman, 1991).

While the role of aluminium as a neurotoxin is well recognized, its presence and proposed aetiological involvement in the pathogenesis of Alzheimer's disease remains a matter of dispute (Landsberg *et al.*, 1992). It may be envisaged that the presence of aluminosilicate deposits within senile plaques may conceivably arise either from the co-precipitation of soluble aluminium and silicon moieties (Birchall and Chappell, 1988), or from the deposition of preformed environmental aluminosilicate particulates. Aluminium ions may be carried bound to plasma transferrin via tranferrin receptors into the brain tissue (Roskams and Connor, 1990). The experimental transfer of aluminium ions directly from the nose to the olfactory regions of the brain by retrograde axonal transport has been demonstrated (Perl and Good, 1987), and it has been proposed that microparticles may likewise gain access into the brain from the atmospheric environment along the olfactory nerve (Roberts, 1986). Indeed, pathological examination of the region of the brain particularly affected in Alzheimer's disease indicates a close olfactory connection (Mann *et al.*, 1988). However, the occurrence of similar pathological features in an individual with a malformation characterized by the absence of a peripheral olfactory connection, would seem to provide evidence inconsistent with this hypothetical mode of entry (Arriagada *et al.*, 1991). Alternatively, indirect access via the intravascular system across the blood–brain barrier may possibly occur by a proposed "Trojan horse" monocyte-mediated mechanism (Williams and Blakemore, 1990). Experimental injury to the brain, by either local cold lesions or hyperosmolar shock, increases the permeability of the blood–brain barrier by a variety of cellular and biochemical oxidative mechanisms (Wei *et al.*, 1986; Vorbrodt *et al.*, 1993). While evidence of a breakdown in the permeability of the blood–brain barrier in Alzheimer's disease has been presented (Scheibel, 1987), it continues to be a matter of contention (Rozemuller *et al.*, 1988).

2.5.2.2 *Inflammatory Oxidative Stress*

Evidence for a neuroimmunological involvement in Alzheimer's disease is accumulating. Activation of the complement cascade by beta amyloid (Rogers *et al.*, 1992), the recruitment, proliferation and activation of microglia in intimate juxtaposition to the senile plaques (Davis *et al.*, 1992), and the increased synthesis of microglia-derived pro-inflammatory cytokine interleukin-1 (Griffin *et al.*, 1989) is indicative of a chronic inflammatory response, although convincing evidence of specific immune antigen–antibody reactions remains to be presented. It is noteworthy that, within the cerebellum of Alzheimer's brains where diffuse plaques predominate and microglia are sparse, incomplete activation of the complement cascade occurs (Lue and Rogers, 1992). In this context, it may be of significance that the nucleation of protein deposition by mineral surfaces (McPherson and Shlichta, 1988) and the proposed role of aluminosilicate particulates in Alzheimer's disease may be related to the demonstrated capacity of amorphous aluminosilicate to promote aggregation and deposition of beta amyloid fibrils (Edwardson *et al.*, 1992). Aluminosilicate clay kaolin particles have been found to enhance aggregation of synthetic beta amyloid peptide 1–42 *in vitro* (Evans, Edwards and Harrington, unpublished observation). Controversial *in vitro* (Pike *et al.*, 1991) and *in vivo* studies (Frautschy *et al.*, 1991) into the neurotoxicity of beta amyloid plaque cores and synthetic beta amyloid peptides have indicated that the neurotoxic activity appears to be associated with the aggregated and aged form rather than the soluble form of amyloid. Amyloid fibril aggregation is promoted by metal-catalysed oxidation (Dyrks *et al.*, 1992), and neurotoxicity is inhibited by the addition of vitamin E *in vitro* (Behl *et al.*, 1992), indicating the involvement of free radical interactions. The possible nucleation effects of aluminosilicate deposits have likewise been implicated in the crystal seed replication of the infection amyloidoses, which characterize the spongiform encephalopathies (Brown *et al.*, 1991b).

Immunochemical studies have identified a proliferation of microglia, the brain macrophages, around senile plaques (Haga *et al.*, 1989). The *in vitro* generation of ROMs by microglia has been demonstrated using chemiluminescent and spectrophotometric techniques in a number of experimental models (Colton and Gilbert, 1987; Sonderer *et al.*, 1987), and the production of nitric oxide (NO) has been related to the expressed cytotoxicity to co-cultured neurons, an action which is augmented by SOD, which stabilizes NO by the removal of superoxide (Boje and Arora, 1992). Down's syndrome subjects with chromosome 21 trisomy and the associated increased expression of SOD activity exhibit an Alzheimer-type pathology, which may be related to a comparable redox mechanism. Experimental findings concerning the stimulated *in vitro* production of ROMs by PMNs (Evans *et al.*, 1989b) and microglia (Evans *et al.*, 1992b) when exposed to model aluminosilicate particles have provided some evidence consistent with the proposed hypothetical mechanism concerning the postulated analogous chronic stimulatory activity of the fibrillar co-deposits of aluminosilicates and amyloid within the Alzheimer senile plaque cores, hence the neonosological term "cephaloconiosis" (Evans *et al.*, 1991, 1992c). The identification by electron microscopy and microprobe analysis of aluminosilicate kaolin-type particles in the Alzheimer

brain (Henderson, personal communication), and the capacity of kaolin to generate hydroxyl radicals (Ghio *et al.*, 1990) suggests a comparable aetiopathogenic mechanism. Kaolin is directly toxic to cultured neuroblastoma cells (Banin and Meiri, 1990) and silica particles, while inducing axonal degeneration when injected directly into experimental rodent brains, failed, however, to produce evidence of amyloid deposition (Rees and Cragg, 1983).

3. *Antioxidant Defence Mechanisms and Therapy*

The natural endogenous physiological antioxidant systems for controlling the potentially injurious effects of environmentally exacerbated oxidative stress, may be modulated by a number of various nutritional and pharmacological supplementary interventions.

3.1 PHYSIOLOGICAL

There are a variety of physiological, cellular and biochemical mechanisms that exist to ensure that the organism can generally effectively counter the adverse effects of oxidative insults.

The lung, by virtue of its direct and extensive contact with the atmospheric environment, is unique in its potential vulnerability to toxic agents and, as such, requires a particularly efficient defence mechanism (White and Ripine, 1985). Whereas a major portion of the larger particles are entrapped within the nose, the smaller particles which enter the trachea and eventually the lung alveoli, are removed via the ciliary transport mechanism or phagocytosed by the alveolar macrophages prior to their migration elsewhere. Extracellular fluids contain a wide variety of free-radical scavengers (Halliwell and Gutteridge, 1990), with the glycoproteins of the extracellular tracheobronchial mucus (Cross *et al.*, 1984), and catalase in the lung epithelial lining fluid forming a secondary antioxidant defence (Cantin *et al.*, 1990).

A major contribution of the free-radical scavenging activity in blood plasma is attributable to the macromolecular proteins (Wayner *et al.*, 1985) of which albumin is a primary component and trapping agent (Holt *et al.*, 1984). Serum sulphydryl levels, primarily albumin-related, are decreased in subjects with rheumatoid complicated coalworkers' pneumoconiosis, indicative of exacerbated inflammatory ROM production (Thomas and Evans, 1975). Experimental asbestos inhalation in rats leads to an adaptive but evidently insufficient response by an increase in endogenous antioxidant enzymes (Janssen *et al.*, 1990). Protection of the vascular endothelium against iron-mediated ROM generation and injury is afforded by the iron sequestrant protein ferritin (Balla *et al.*, 1992).

Within the brain, an organ which consumes about 20% of the body's oxygen, antioxidant protection is provided by glutathione, ascorbate, vitamin E, and more specifically the histidine derivatives carnosine and homocarnosine (Kohen *et al.*, 1988).

3.2 NUTRITIONAL

A variety of micronutrient dietary components may perform a protective function against the toxicity of mineral dusts (Evans *et al.*, 1987). *In vitro* studies have indicated that asbestos-induced toxicity in microsomes is antagonized by ascorbate (Khan *et al.*, 1990). In smokers, it has been calculated that the excess daily dietary requirement for vitamin C is about 30 mg (Cross and Halliwell, 1993). Nicotinamide protects against the cytotoxicity of mineral dusts towards pulmonary macrophages (Nadeau *et al.*, 1989). Vitamin E, in addition to functioning as a primary free-radical scavenger in cell lipomembranes, inhibits the generation of phagocyte-derived ROM by its action on protein kinase C (Sakamoto *et al.*, 1990). Asbestos-induced hyperplasia of cultured tracheal epithelia is inhibited by retinol methyl ether (Mossman *et al.*, 1980), while the results of ongoing chemopreventive studies into the therapeutic use of retinol and beta carotene in a population of asbestos workers are awaited with particular interest (Malone, 1991). Trace elements like selenium and thiol amino acids may also protect against oxygen toxicity in the lung (Forman *et al.*, 1983).

Within the gut, oxidative damage may be prevented by phytic acid, obtained from cereals and vegetables (Graf *et al.*, 1987), and by soluble non-starch polysaccharides like pectin (Kohen *et al.*, 1993). The use of antioxidant vitamins in the treatment of inflammatory bowel disease has also been suggested (Evans *et al.*, 1990).

Supplementation with antioxidant micronutrients, for example, vitamin E and selenium, in the elderly and in dementia subjects (Tolonen *et al.*, 1985) has indicated that such treatments may be of some limited benefit. The value of ascorbate as a cerebroprotective antioxidant against excitotoxic neuronal injury has been proposed (Grünewald, 1993).

3.3 PHARMACOLOGICAL

The development of the concept concerning the use of a variety of therapeutic agents and measures by which oxidative-related injury may be treated is of relevance to the burgeoning evidence implicating ROM in the pathogenesis of particulate-mediated diseases.

Experimental *in vitro* investigations utilizing liposomal-encapsulated and polyethylene glycol-conjugated SOD and catalase have demonstrated the potential value of such means in countering oxidative asbestos-related diseases (Freeman *et al.*, 1985; Mossman *et al.*, 1986). In addition to using supplementary endogenous antioxidant enzymes, the use of iron chelators like desferrioxamine

has been the topic of extensive investigation. However, while desferrioxamine caused inhibition, mobilization of iron from crocidolite by citrate and EDTA chelators enhanced redox activity, and reductants like ascorbate may result in comparable paradoxical pro-oxidant effects (Lund and Aust, 1991). The non-steroidal anti-inflammatory drug ibuprofen inhibits oxidant lung injury in rabbits by an iron chelation-dependent mechanism (Kennedy *et al.*, 1990). The development of alternative effective, convenient and safe iron chelators for clinical use, for example, the hydroxypyridones, is a topic of major research effort (Hershko and Weatherall, 1988).

It is of interest to note that the reported benefit of desferrioxamine in reducing the rate of disease progression in Alzheimer's disease patients may not only be related to its ability to chelate and remove aluminium – the putative toxin – but also to its capacity to inhibit iron-mediated hydroxyl-radical production (McLachlan *et al.*, 1991). The 21-aminosteroid drugs, the hyperbolical named Lazeroids, exhibit iron-chelating attributes, and have been shown to be effective in countering traumatic and ischaemic brain injury by an antioxidant mechanism (Sato and Hall, 1992). As a consequence, they may also prove to be of potential value in the treatment of Alzheimer's disease. Reports (Jenkinson *et al.*, 1989; McGeer *et al.*, 1990), though disputed (Beard *et al.*, 1991), of a negative association between Alzheimer's disease and rheumatoid arthritis have led to the suggestion that the long-term intake of anti-inflammatory drugs by rheumatoid patients may inhibit the development of dementia. Allied pathological (Namba *et al.*, 1992) and clinical studies (McGeer *et al.*, 1992) on the prevalence of dementia in leprosy subjects has implicated the particular use of the anti-inflammatory drug dapsone. Dapsone, a lipophilic drug, inhibits intracellular and extracellular PMN-derived ROMs, whereas ascorbate inhibits extracellular ROMs only (Anderson *et al.*, 1987). However, another study on the use of non-steroidal anti-inflammatory drugs (NSAIDs) on cognitive performance in the elderly revealed no significant effect (May *et al.*, 1992). The purported prophylactic use of Japanese herbal medicines to combat neuronal ageing has been related to their free-radical scavenging activity (Hiramatsu *et al.*, 1992). Inhibition of the pro-inflammatory effects of cytokine interleukin-1 by recombinant endogenous interleukin-1 receptor antagonist in experimental rats is associated with alleviation of excitotoxic neuronal damage, an action which has also been related to the anti-inflammatory effect of lipocortin 1 (Relton and Rothwell, 1992).

It has been proposed that the capacity of anti-inflammatory drugs to scavenge and inhibit ROM production is pertinent to their therapeutic effect (Halliwell *et al.*, 1988). NSAIDs, namely indomethacin, inhibit superoxide generation by stimulated guinea-pig macrophages (Oyanagui, 1976) and the scavenging effects of PMN-derived ROMs by the sulphasalazine metabolite 5-aminosalicylic acid, and pentoxyfylline, contributes to the drug's efficacy in the treatment of inflammatory bowel disease (Tamai *et al.*, 1991) and experimental lung injury (McDonald, 1991). The SOD mimetic, copper 3,5-diisopropyl salicylic acid, has been shown to inhibit PMN ROM stimulation by the tumour promoter PMA (Kensler and Trush, 1983).

The use of thiol agents to counter lung oxidative damage illustrates a promising therapeutic approach. Experiments utilizing a glutathione aerosol (Buhl *et al.*, 1990), oral dosage with the cysteine precursor L-2-oxothiazolidine-4-carboxylate (Taylor *et al.*, 1992) and 2-mercaptopropionylglycine (Ayenne *et al.*, 1993) have demonstrated significant antioxidant capability. The thiol broncholytic drug *N*-acetylcysteine has been shown to reduce PMN-induced ROM cytotoxicity in co-cultured lung epithelial cells (Simon and Suttorp, 1983) and, interestingly, has been reported to be of value in the treatment of silicosis (Margolis and Margolis, 1974). The allied anti-rheumatic thiol drug penicillamine, used with limited success in the treatment of rheumatoid coal-workers' pneumoconiosis (Evans, 1977) has been proposed to act via a free-radical scavenging mechanism (Munthe *et al.*, 1982), although paradoxically it has been reported to produce a stimulation of PMN and monocyte ROM generation *in vitro* (Evans *et al.*, 1987) and *in vivo* (Hurst *et al.*, 1986).

While it may be desirable to limit the excess, chronic and inappropriate generation of phagocyte-derived ROMs, it is necessary to achieve this without simultaneously compromising their vital bactericidal function. The orally effective iron chelator 2,3-dihydroxybenzoate has been shown to decrease PMN ROM generation without a corresponding loss of bactericidal activity to phagocytosed *Staphylococcus aureus* (Boxer *et al.*, 1978).

4. *Conclusion*

Humans are exposed to a wide variety of environmental particulates of differing size, morphology, chemical composition, crystal structure, durability and reactivity. The penetration and migration of particles into the organs of the body by various means, entails the potential risk of a diverse range of acute and chronic foreign-body inflammatory organotypic reactions, the so-called conioses. A growing body of evidence indicates that such interactions, whether directly or indirectly mediated primarily by the various phagocytic cells of the body, involve the generation of free-radical oxidants. The accompanying resultant oxidative stress may be a potent source of cellular dysfunction, carcinogenicity and even cell death. Augmentation of the body's antioxidant defensive mechanisms by nutritional supplementation and pharmacological intervention, provides an increasingly promising avenue for future experimental and clinical research into countering the pathogenic effect of the

ubiquitous and potentially hazardous environmental particulate agents.

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17. Reactive Oxygen: Recent Therapeutic Intervention Strategies

Jill A. Panetta *and* John M. McCall

1. Introduction

This review generally describes the injury mosaic of ischaemia–reperfusion injury and then discusses the role of reactive oxygen in that process. The ischaemia–reperfusion injury process is characterized by metabolic and ionic changes, protease activation, changes in the arachidonic acid cascade, lipid peroxidation and inflammatory injury. Figure 17.1 gives a comprehensive overview of the complete injury cascade. Not surprisingly, an injury cascade as complex as that described in Fig. 17.1 has invited many therapeutic interventions. Calcium channel blockers, NMDA antagonists, lipid peroxidation inhibitors, diuretics, iron chelators, magnesium, opiate antagonists, anti-inflammatories and more have all been tried with varied success in models of central nervous system (CNS) injury. This review emphasizes the therapeutic role of compounds that modulate reactive oxygen–mediated injury. Injury processes in the CNS are also emphasized. Our review is organized into the following sections: Introduction, Enzyme-related therapeutic approaches, Enzymatic antioxidants/derivatives, Exogenous antioxidants and the Inflammatory cascade. Structures of new compounds are usually included in the text. We have concentrated on the recent literature.

The major cellular target for oxygen radical damage is membrane polyunsaturated fatty acids, although proteins may also be vulnerable to oxidation. Radical formation and lipid peroxidation are catalysed by free iron. Tissue acidosis and reactive oxygen both facilitate the release of iron and thus promote lipid peroxidation. Membrane lipid peroxidation can contribute to injury-induced fatty acid release and calcium influx. Lipid peroxidation can amplify the threat of increased intracellular calcium by increasing membrane permeability and/or impairing calcium extrusion mechanisms and mitochondrial sequestration. From this, we and others have concluded that compounds that inhibit lipid peroxidation and thus control reactive oxygen injury will show many activities that are directly and indirectly linked to their antioxidant activities.

Lipid peroxidation (see Fig. 17.2) is a chain reaction that can be attacked in many ways. The chain reaction can be inhibited by use of radical scavengers (chain termination). Initiation of the chain reaction can be blocked by either inhibiting synthesis of reactive oxygen species (ROS) or by use of antioxidant enzymes like superoxide dismutase (SOD), complexes of SOD and catalase. Finally, agents that chelate iron can remove free iron and thus reduce Haber–Weiss-mediated iron/oxygen injury.

Inflammatory injury is tied to both neutrophil release of neutral proteinases and a respiratory burst with production of superoxide anion and hydrogen peroxide

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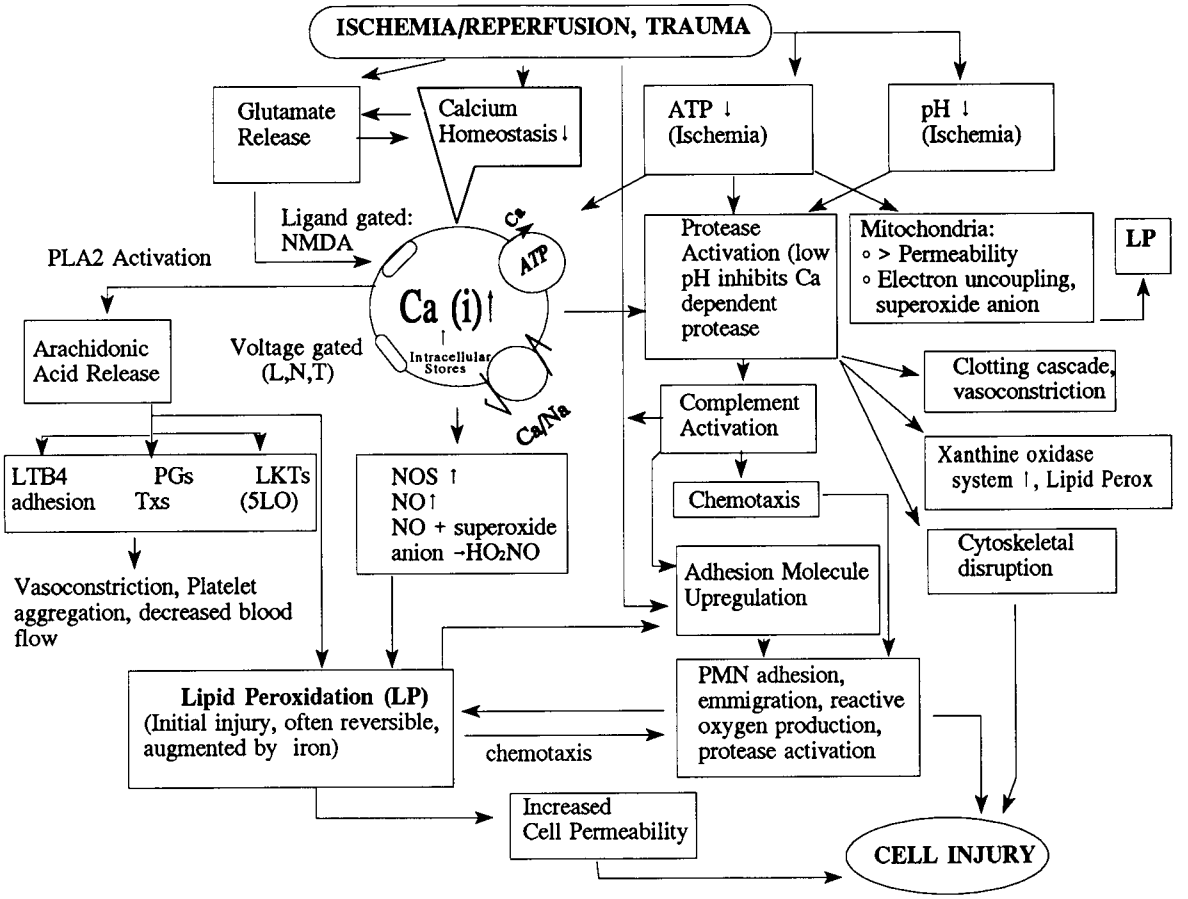


Figure 17.1 Injury mosaic. PLA_2, phospholipase A_2; LKTs, leukotrienes; LTB_4, leukotriene B_4; PGs, prostaglandins; 5LO, 5-lipoxygenase; NO, nitric oxide; NOS, nitric oxide synthase; TXs, thromboxanes.

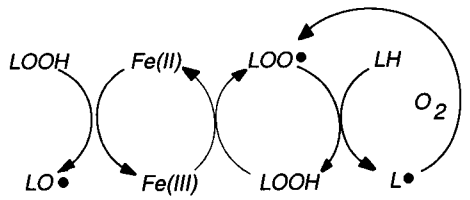


Figure 17.2 Lipid peroxidation scheme. LH, a polyunsaturated fatty acid; LOOH, lipid hydroperoxide; LOH, lipid alcohol; L˙, lipid radical; LOO˙, lipid hydroperoxyl radical; LO˙, lipid alkoxyl radical. *Initiation*: the LH hydrogen is abstracted by reactive oxygen (e.g. lipid alkyl radical, lipid alkoxy radical, lipid hydroperoxyl radical, hydroxy radical, etc.) to produce a new lipid alkyl radical, L˙. *Propagation*: the lipid alkyl, alkoxyl or hydroperoxyl radical abstracts hydrogen from the neighbouring LH to generate a new L˙ radical.

by neutrophils, monocytes and macrophages. The subsequent generation of hypochlorous acid and secondary chloramines via myeloperoxidase action on hydrogen peroxide can augment oxidation. Recruitment, adhesion and extravasation through endothelial cells as well as lymphocyte antigen recognition, proliferation and differentiation into effector cells, which target the antigen, can all be players in this process.

Inflammatory cells are sources of reactive oxygen and nitric oxide. In addition to the respiratory burst mentioned above, neutrophils and, to a greater extent, monocytes and macrophages convert L-arginine to nitric oxide. Superoxide anion, hydrogen peroxide and nitric oxide are all key players in the initiation of lipid peroxidation. In addition, reactive oxygen plays an important role in adhesion biology. When exposed to either reactive oxygen challenge (Bradley *et al.*, 1993) or ischaemia/reperfusion injury (Perry and Granger, 1992), endothelial cells up-regulate expression of adhesion molecules on their surface. Thus, ROS are central to lipid peroxidation, protein oxidation and to the inflammatory cascade.

2. *Enzyme-related Therapeutic Approaches*

The level of reactive oxygen and nitrogen species is endogenously controlled by a variety of enzymatic systems. Glutathione *S*-transferase catalyses the reaction between glutathione and reactive electrophiles to form thioethers. Oxidized glutathione (GSSG) reductase maintains levels of reduced glutathione. SOD and catalase promote the sequential one-electron reduction path from oxygen to water. These enzymes offer a point of intervention in the cascade of injury.

During periods of ischaemic metabolism, superoxide anion is produced by mitochondrial dysfunction, as a by-product of various enzyme–substrate reactions (xanthine/xanthine oxidase) and by auto-oxidation of many low molecular weight molecules. Electron transport chains in mitochondria and endoplasmic reticulum are major sources of superoxide. Normally, over 90% of the oxygen taken up by the body is reduced to water in a four-electron process by mitochondrial cytochrome oxidase. When mitochondrial function breaks down, some of the electrons "leak" from the usual electron carriers on to oxygen, forming superoxide anion. This is augmented by hyperoxia. Superoxide anion is not itself particularly reactive and it does not cross cell membranes very well. However, it can become more dangerous by either accepting a proton or by dismuting to hydrogen peroxide. During ischaemia, lactic acidosis can lead to protonation of some of the superoxide anion. The lipophilicity of lactic acid facilitates this process by making the proton donor more available. Protonated superoxide anion can better penetrate the membrane where it can initiate lipid peroxidation. Auto-oxidation of catecholamines, ascorbic acid, glutathione and thiols by oxygen with transition-metal catalysis can also produce superoxide anion.

Superoxide anion production is associated with injury to the CNS. Endogenous SOD and glutathione peroxidase levels have been measured in male Wistar rats subjected to middle cerebral artery occlusion with temporary bilateral carotid artery occlusion. Cytosolic Cu/Zn-SOD levels decreased the infarcted area of brain while glutathione peroxidase did not, suggesting a protectant role for SOD in ischaemia (Imaizumi *et al.*, 1989). Additional evidence for the pathological role of superoxide anion in infarction and oedema following focal cerebral ischaemia comes from transgenic mice that overexpress Cu/Zu-SOD. These animals show reduced brain oedema and infarct size compared with non-transgenic mice (Kinouchi *et al.*, 1991). SOD and conjugates of SOD [polyethylene glycol (PEG)-SOD, divinylether maleic acid (Pyran)-SOD], which have longer circulating half-lives than SOD itself have been reviewed (Greenwald, 1990). Human recombinant SOD has been found to protect CA_1 hippocampal neurons from a transient ischaemic insult in gerbils (Uyami *et al.*, 1992). SOD has also been shown to decrease cord paralysis and motor nerve damage when administered during the reperfusion phase in a rabbit model of spinal cord ischaemic injury (Cuevas *et al.*, 1989).

Tasdemiroglu *et al.* (1993) have shown, in a rabbit unilateral carotid occlusion model, that Evans Blue dye leaks into the brain after ischaemia–reperfusion injury. This breakdown in the blood–brain barrier was inhibited by SOD, which was administered after 55 min of ischaemia. The authors concluded that focal cerebral ischaemia and reperfusion produce a vascular endothelial injury at the blood–brain barrier and that SOD, a blood-borne protectant enzyme, can inhibit this injury (Tasdemiroglu *et al.*, 1993). This suggests that antioxidants do not need access to the brain to protect this organ. Tanno has also observed breakdown of the blood–brain barrier after fluid percussion (traumatic) brain injury in the rat. Hypoxia after injury exacerbated the regional breakdown of the barrier to circulating proteins. The influence of hypoxia was evident at 6 h after injury and delayed recovery in the afflicted animals (Tanno *et al.*, 1992). In related work, Karlsson *et al.* (1993) have shown that extracellular superoxide dismutase C (EC-SOD C), a secretory tetrameric Cu- and Zn-containing glycoprotein, has a high affinity for heparin and heparan sulphate. Human recombinant EC-SOD-C, when administered intravenously (i.v.), was found to bind to the vasculature wall. This binding was dependent on heparin affinity. The authors project that the affinity for heparan sulphate proteoglycan in the vascular wall should make recombinant human EC-SOD C a protector against vascular wall-derived oxidants (Karlsson *et al.*, 1993).

In a prospective randomized trial, human SOD given i.v. for 5 days after multiple traumatic injury, attenuated multiple organ failure evaluated over 14 days. The results, although preliminary, are encouraging (Marzi *et al.*, 1993). PEG-SOD has been evaluated in 104 patients with severe head injury at three different doses. Patients were treated at an average of 4 h after injury. Although results versus placebo were not significant, a higher dose trial is being planned. The use of Cu/Zu-SOD injections in refractory Crohn's disease has shown promise in preliminary clinical trials (Emerit *et al.*, 1989).

Another area of active research is the development of stable low molecular weight metal complexes, which could serve as SOD mimics. Fridovich has described a complex of manganese (III) with desferral, which can catalyse the dismutation of superoxide anion *in vitro* and can protect green algae against paraquat toxicity (Beyer and Fridovich, 1989). This manganese-desferral complex was evaluated in models of circulatory shock and also found to improve survival rate (de Garavilla *et al.*, 1992).

Fe(II)-tetrakis-*N*, *N*, *N'*, *N'*(2-pyridylmethyl-2-aminoethyl)amine (Fe-TPEN) and Fe(III)-tris[*N*-(2-pyridylmethyl)2-aminoethyl]amine (Fe-TPAA) are novel iron complexes containing lipophilic and neutral ligands.

They have been shown to inhibit the reduction of cytochrome c catalytically by superoxide anion and to protect *Escherichia coli* against paraquat toxicity (Nagano *et al.*, 1989). Recently a series of (salen)–manganese complexes have been developed as low molecular weight SOD mimics. Several of these (salen)–manganese complexes demonstrated the same level of SOD activity as the iron or manganese SOD mimics just described. The SOD activity was maintained over weeks in solution with these (salen)–manganese complexes, suggesting that they are far more stable than the previously described SOD mimics. These (salen)–manganese SOD mimics have shown protection against anoxia-induced damage using a hippocampal slice preparation and against neurotoxin-induced degeneration of dopaminergic neurons in mice (Baudry *et al.*, 1993).

Hydrogen peroxide or ROS derived from hydrogen peroxide can serve as important mediators of CNS tissue injury. Hydrogen peroxide can be produced directly by inflammatory cells, by monoamine oxidase deamination of monoamines, such as dopamine, or by chemical or enzymatic conversion of superoxide anion to hydrogen peroxide by superoxide dismutase (SOD). Mitochondria are rich in SOD, and much of the superoxide that they produce becomes hydrogen peroxide. Hydrogen peroxide, like superoxide anion, is not highly reactive. Unlike superoxide anion, hydrogen peroxide does cross cell membranes. Thus, it can position in areas of the cell where it can, with the help of transition metals, cause important injury to the lipid bilayer. Hydrogen peroxide can react with Fe(II) to yield hydroxyl radical, which reacts at a diffusion-controlled rate. Hydrogen peroxide can be detoxified by catalase to form water. It can also be transformed by myeloperoxidase to hypochlorous acid, which can, in turn, convert primary amines to *N*-chloroamines. These halogenated species are particularly important for phagocytic cells, which use hypochlorous acid and its derivatives to destroy their targets.

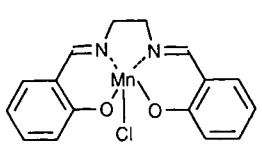
An increase in brain levels of hydrogen peroxide was reported during reperfusion in a temporary unilateral carotid artery occlusion model in the gerbil. Brain hydrogen peroxide levels during reperfusion correlated with the severity of the neurological deficit (Patt *et al.*, 1988). A source of superoxide anion and hydrogen peroxide during post-ischaemia reperfusion is the enzyme xanthine oxidase. When gerbils were fed a tungsten-rich diet to inhibit xanthine oxidase, brain oedema and levels of hydrogen peroxide were reduced during reperfusion. Xanthine oxidase has been shown to be highly concentrated in cerebral vascular endothelium. In a rat spinal cord injury model, xanthine dehydrogenase and xanthine oxidase activity were measured using a new flurometric assay and shown to be high in the injured cord. Allopurinol, an irreversible inhibitor of xanthine oxidase, was shown to be effective in this rat spinal cord injury model. Preliminary studies of the use of allopurinol in pouchitis (Levin *et al.*, 1992), and allopurinol and DMSO in ulcerative colitis (Saum, 1992) have demonstrated promise. Another potent inhibitor of xanthine oxidase is amflutizole. Propentofylline (HWA-285), a xanthine derivative, reduced infarct size by 27% in a focal cerebral ischaemia model in gerbils and significantly protected the CA_1 hippocampal neurons, even when it was given 1 h after injury.

Catalase, the antioxidant enzyme that converts hydrogen peroxide to water, has been evaluated in combination with SOD in a model of focal ischaemic stroke in the rat. Both enzymes were conjugated with polyethylene glycol to increase the circulatory half-life. They significantly reduced infarct volume (Liu *et al.*, 1989).

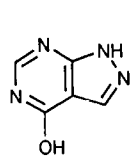
A combination of PEG-SOD and PEG-catalase was compared to nimodipine in a prospective, blinded, randomized study in which piglets were subjected to hypoxic ischaemic brain injury. Carotid arteries were ligated and blood pressure was reduced by haemorrhage. After 15 min, pO_2 was reduced. At 30 min, the ligation was released and normal oxygen was restored. The treatment protocols and vehicle were not significantly different. The authors concluded that neither PEG-SOD/PEG-catalase, nimodipine nor the two regimens in combination ameliorate hypoxic injury to the brain in the newborn pig when given 5 min after reoxygenation.

2.1 NITRIC OXIDE SYNTHASE

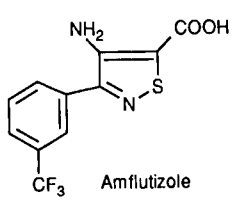
L-Arginine is converted by NO synthase (NOS) to nitrogen oxide. NO has recently been recognized as potentially pathological. Normally, NO is an important messenger molecule that regulates immune function and the dilation of blood vessels. It also serves as a neurotransmitter. However, NO is produced in excess during ischaemia. The NOS is activated by, among many stimulants, calcium influx. NO is itself a reactive free radical (Lowenstein and Snyder, 1992). It can react with the superoxide anion to form the peroxynitrite anion that is readily protonated to form HOONO, particularly if the pH is down because of lactic acidosis. This HOONO is



(Salen)-Manganese Complex



Allopurinol



Amflutizole

a particularly dangerous material because it can move across membranes and later decompose to form hydroxyl radicals and NO. Thus, peroxynitrite may deliver hydroxyl radicals to sites that they would not normally penetrate (Beckman *et al.*, 1990). Since NO is a vasodilator, its conversion to peroxynitrite can have vasoconstrictor effects (Laurindo *et al.*, 1991). Interestingly, NOS is widespread in the brain (Hope *et al.*, 1991), which is particularly susceptible to ischaemia–reperfusion injury. NOS exists as both a constitutive and inducible enzyme, and as tissue selective isotypes. Inhibition of NOS thus provides a particular challenge.

The genes of the inducible and the constitutively expressed forms of NOS have been cloned and expressed. The expression of inducible NOS in the brain tissue of animals with experimentally induced neurological disorders (borna disease virus and rabies virus in rats), herpes simplex virus (mice) and experimental allergic encephalitis (in rats) suggests that NO produced by induced NOS may be a toxic factor in the pathogenesis of neurological diseases (Koprowski *et al.*, 1993).

During ischaemia, NOS is activated by calcium influx or by cytokines like tumour necrosis factor (TNF) or by lipopolysaccharide (LPS) and NO is produced in excess. It has been proposed that the excitotoxic effect of glutamate, which contributes to ischaemia-induced neuronal damage, is mediated by increased production of NO via a chain of events that includes increases in intracellular calcium (via glutamate activation of NMDA receptors), calcium activation of NOS, production of NO and peroxynitrite, and induction of lipid peroxidation. In fact, *N*-nitro-L-arginine, a selective inhibitor of NOS, has been shown to prevent glutamate-induced neurotoxicity in cortical cell cultures (Dawson *et al.*, 1991).

N-Nitro-L-arginine methyl ester (L-NAME) is an inhibitor of NOS; L-NAME reportedly reduces the volume of cortical and striatal infarct after middle cerebral artery occlusion in the rat. This protection can be reversed by co-injection of L-arginine. L-NAME also reduced the excitotoxic damage induced by NMDA injection. Finally, the authors showed that L-NAME reduced glutamate efflux produced by ischaemic injury in rats. The authors concluded that NOS induced by NMDA receptor overstimulation is a key event in the neuronal injury cascade (Buisson *et al.*, 1993).

In contrast to the deleterious effects of arginine described by Buisson, L-arginine was shown to decrease infarct size caused by middle cerebral artery occlusion in spontaneously hypertensive rats. L-Arginine is a precursor for NO synthesis by NOS. The authors attributed the protection to dilation of cerebral blood vessels by NO (Morikawa *et al.*, 1992). These examples illustrate the difficulty that the NO villain/protector paradox presents to us.

The NO donor, C87-3754, reportedly attenuates the injury induced in cats by splanchnic artery occlusion of the coeliac, superior mesenteric and inferior mesenteric arteries (2 h) followed by 2 h of reperfusion. The NO donor, given 10 min prior to reperfusion, protected while attenuating the shock-induced decline in release of endothelium-derived relaxant factor (EDRF) from mesenteric artery rings stimulated by acetylcholine (ACh). C87-3754 also significantly decreased polymorphonuclear neutrophil (PMN) adherence to the superior mesenteric venous endothelium *in vitro* (Carey *et al.*, 1992).

Further support for the hypothesis that NO may play a mediatory role in focal ischaemic stroke was provided by a study in which N^{-G}-nitro-L-argine was given intraperitoneally (i.p.) to 6-day-old rat pups. The drug protected against hypoxic–ischaemic injury (Trifiletti, 1992).

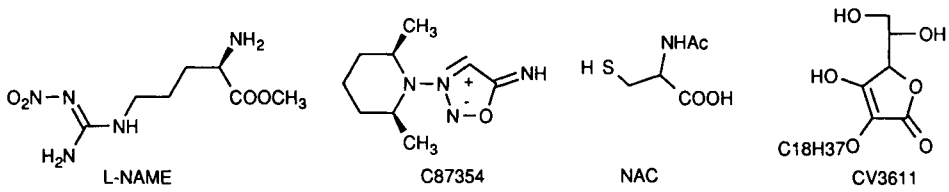
3. *Endogenous Antioxidants/Derivatives*

Normally, the cascade from oxygen to water is well controlled by SOD, catalase and endogenous antioxidants such as glutathione, ascorbate and vitamin E. Vitamin E is the most important membrane-bound antioxidant. However, during ischaemia, the local control of ROS is lost, thus reactive free radicals can attack the membranes and lipid peroxidation begins. Endogenous antioxidants can be supplemented. This section describes this supplementation strategy.

Ascorbic acid (vitamin C) is one of the body's endogenous water-soluble antioxidants. Modifications on the ascorbic acid structure have led to some very interesting compounds, such as a novel series of 3-O-alkyl ascorbic-acid derivatives. They have been found to be inhibitors of lipid peroxidation (Nihro *et al.*, 1991). This antioxidant activity is directly related to the lipophilicity of the alkyl chain, suggesting that the lipid chain may anchor the antioxidant portion of the molecule in the membrane.

Kaneko *et al.* (1993) have described a group of lipophilic ascorbic-acid analogues that have been studied in cultured human umbilical vein endothelial cells that were first incubated with test drug and then exposed to lipid hydroperoxides. Although ascorbate itself did not protect the endothelial cells, derivatives like CV3611 protected. Pretreatment was necessary. CV3611 was synergistic with vitamin E. The authors concluded that these lipophilic antioxidants incorporate into endothelial cell membranes where they are effective inhibitors of lipid peroxidation. In contrast, lipophobic antioxidants were not effective in their hands (Kaneko *et al.*, 1993).

Ohnishi (Sakamoto *et al.*, 1991) has described an oligomeric derivative of prostaglandin B_2 (PGB_2) and ascorbic acid. In a rat bilateral carotid occlusion–reperfusion injury complicated by haemorrhagic hypotension, this compound reduced α-phenyl-*t*-butyl nitrone (PBN) spin-trapped radicals and thiobarbituric acid-reactive products (TBARs) (a measure of lipid peroxidation) in isolated



brain homogenate. This (PG-COO-ascorbyl)$_n$ derivative also reduced mortality in the rodent model (Sakamoto *et al.*, 1991).

Roberts and Morrow (Morrow *et al.*, 1990) have described a non-cyclooxygenase-derived prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that is formed from membrane phospholipids by lipid oxidation. $PGF_{2\alpha}$ is released from the phospholipids by phospholipase A_2 (PLA_2). These oxidized lipids may account for some of the changes in fluidity and membrane integrity that characterize peroxidized membranes because they are remarkably distorted molecules relative to normal membrane lipids. $PGF_{2\alpha}$ levels are increased in carbon tetrachloride hepatotoxicity, an animal model of lipid peroxidation (Morrow *et al.*, 1990).

Thiols are also important protection against lipid peroxidation. Glutathione (γ-Glu-Cys-Gly) is used by several glutathione-dependent enzymes such as free-radical reductase (converts vitamin E radical to vitamin E), glutathione peroxidase (reduces hydrogen peroxide and lipid hydroperoxides to water and to the lipid alcohol, respectively), and others. In addition, the thiol group of many proteins is essential for function. Oxidation of the thiol of calcium ATPases impairs function and leads to increased intracellular calcium. Thiol derivatives such as the ovothiols (1-methyl-4-mercaptohistidines) (Shapiro, 1991) have been explored as therapeutics.

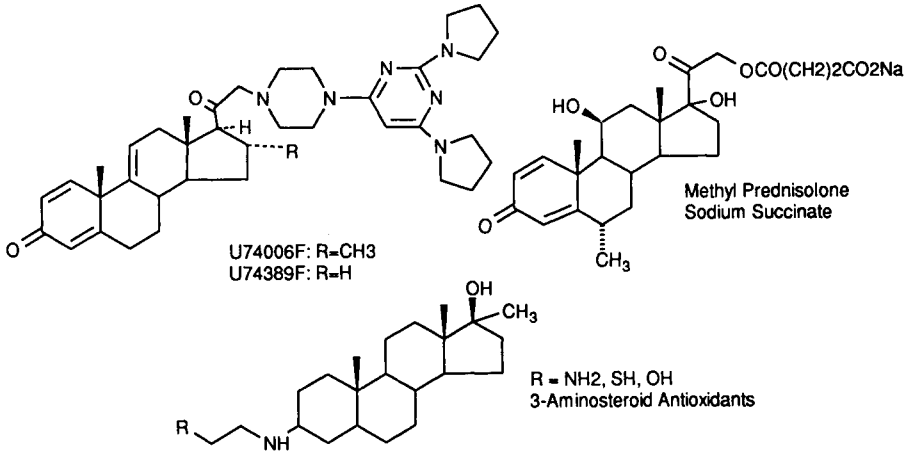
In a percussive brain injury model in cats, *N*-acetylcysteine (NAC) preserved normal hyperventilation when administered either prior to or 30 min post-insult (Ellis *et al.*, 1991). NAC was also found to be active in a model of acute immunological alveolitis in the rat in which intratracheal immunoglobulin G (IgG) anti-bovine serum albumin antibodies and i.v. antigen caused lung damage. Oral NAC prevented the injury. The authors showed that NAC also inhibited hypoxanthine/xanthine oxidase (superoxide anion)-induced lung damage *in vitro*. They attributed the *in vivo* activity to inhibition of free-radical-induced damage (Sala *et al.*, 1993).

4. *Exogenous Antioxidants*

4.1 STEROIDS

Steroids have long been used in the treatment of neurological injury. These highly lipophilic compounds have a high affinity for membrane bilayers. This property is key to their role in inhibition of lipid peroxidation.

The 21-aminosteroids (lazaroids) have been explored in a variety of models. Tirilazad mesylate (U74006F) is in an advanced clinical trial in head and spinal cord injury, subarachnoid haemorrhage and ischaemic stroke. Significant efficacy on clinical vasospasm and improved 3-month outcome as well as safety have been reported after the completion of Phase II trials (Kassell *et al.*, 1993). Pharmacological activity for U74006F and its close analogue U74389 have been reported in over 300 publications. These 21-aminosteroid compounds lack hormonal activity, inhibit lipid peroxidation and exert dramatic physicochemical effects on cell membranes. They are active in a variety of CNS injury models (Braughler *et al.*, 1989; Hall *et al.*, 1991a) and in degenerative disease models such as the guinea-pig experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis



(Karlik *et al.*, 1991). They have also shown impressive activity in models of hyperoxic lung injury (Richards *et al.*, 1992), ischaemic gut injury (Badylak *et al.*, 1990) and transplant (Quayumi *et al.*, 1992) among others.

A family of 3-aminosteroids reportedly inhibit lipid peroxidation while acting as anti-inflammatories. These activities were attributed to the combination of the steroid and the amine as neither component was, by itself, effective (Spyriounis *et al.*, 1993). In this sense, they resemble the 21-aminosteroid lazaroids. These 3-aminosteroids most probably inhibit lipid peroxidation through a physicochemical mechanism.

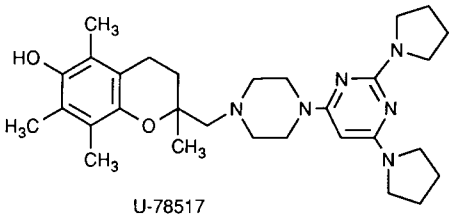
Methylprednisolone (MPSS) was shown to be clinically effective in human spinal-cord injury during NASCIS II (National Acute Spinal Cord Injury Study II) (Bracken *et al.*, 1990). MPSS is now being compared to tirilazad mesylate in the NASCIS III trials, which are underway. In addition, high doses of MPSS given to multiple sclerosis patients reduced blood–brain barrier abnormalities during treatment as shown by gadolinium-diethylenetriamine pentaacetate (DTPA)-enhanced magnetic resonance imaging (MRI). Although new lesions appeared within a month, reversal of the barrier abnormalities was said to contribute to the accelerated recovery from multiple sclerosis relapse (Miller *et al.*, 1992). MPSS is neuroprotective in many animal models at high non-hormonal doses; dose–response curves are often an inverted U-shape (Hall, 1992). MPSS is a modest inhibitor of lipid peroxidation.

Mooradian (1993) has studied the antioxidant properties of 14 steroids in a non-membranous system in which the fluorescence of the protein phycoerythrin was measured in the presence of a lipid peroxyl radical generator (ABAP). Oxidation of the protein produces a fluorescent species. Quenching of fluorescence by a test compound indicates antioxidant activity. Oestrone, testosterone, progesterone, androstenedione, dehydroepiandrosterone, cortisol, tetrahydrocortisone, deoxycorticosterone and aldosterone had no significant antioxidant properties. Oestradiol and 17β-oestradiol had antioxidant activity (Mooradian, 1993). This work highlights the importance of membrane incorporation that can unmask the lipid peroxidation inhibitory activity of many steroids that are not inherently antioxidant.

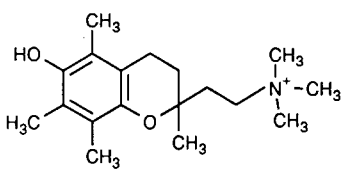
4.2 PHENOLS

Phenols are important antioxidants, with vitamin E being the most important endogenous phenolic membrane-bound antioxidant. Membrane levels of vitamin E are maintained through recycling of the vitamin E radical with ascorbate and thiol reductants. Vitamin E is a mixture of four lipid-soluble tocopherols, α-tocopherol being the most effective radical quencher. The reaction of α-tocopherol with alkyl and alkylperoxyl radicals of methyl linoleate was recently reported. These are facile reactions that result in mixed dimer adducts (Yamauchi *et al.*, 1993).

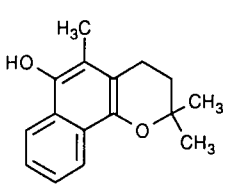
The benzopyran portion of vitamin E has been incorporated into several new lipid peroxidation inhibitors, for example, U78517 and MDL73404. U78517 contains the same amino side chain as tirilazad mesylate (U740067), while MDL73404 replaces the lipophilic side chain of vitamin E with a trimethylethanaminum chain. U78517 is effective in several different animal models of traumatic CNS injury and asthma. Hall has reported submicromolar potency against iron-mediated *in vitro* lipid peroxidation, as well as protection in a mouse model of head injury and a gerbil model of ischaemic stroke. U78517F is more efficacious acutely than either vitamin E or trolox (Hall *et al.*, 1991b). In addition, U78517F inhibits tracheal eosinophil influx that is stimulated by antigen challenge (Johnson *et al.*, 1992). MDL73404 was found to reduce myocardial infarct size in rats subjected to coronary ligation followed by reperfusion (Grisar *et al.*, 1991). MDL73404 reduced serum levels of TBARs (lipid peroxidation indicator), increased cardiac



U-78517



MDL 73404



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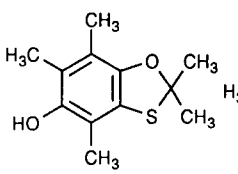

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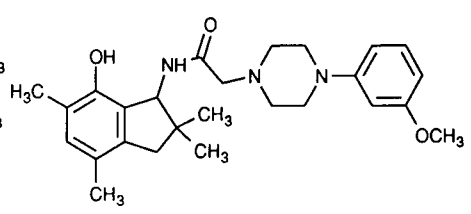


OPC14117

output and heart rate and decreased infarct size assessed 8 days after reperfusion (Lukovic *et al.*, 1993). Other workers have elaborated the structure of vitamin E to produce antioxidants like 1 (Battioni *et al.*, 1991), 2 (Aizawa *et al.*, 1990) and OPC14117. OPC14117 penetrates brain and is protective in rodent models of cerebral ischaemia. Recently, OPC14117 was shown to scavenge superoxide in aprotic solvents more effectively than α-tocopherol (Jinno *et al.*, 1991).

6-Hydroxydopamine (6-OHDA) is a neurotoxin that destroys catecholaminergic neurons in the brain. This toxicity is believed to be related to the production of ROS by the neurotoxin. Rats were fed chronically with vitamin E and then challenged with 6-OHDA. The usual depletion of SOD and reduced glutathione (GSH) in most brain regions was attenuated by the vitamin E pretreatment. The authors attributed this success to scavenging by the augmented brain levels of vitamin E (Perumal *et al.*, 1992).

A series of antioxidants that contain the backbone structure of butylated hydroxytoluene (BHT), a known antioxidant, have been evaluated in animal models of cerebral ischaemia and of inflammation. LY178002 is a compound that inhibits both iron-dependent lipid peroxidation and key enzymes of the arachidonic acid cascade (5-lipoxygenase and cyclooxygenase), and is effective in reducing damage in the hippocampal CA_1 layer and in the corpus striatum in rats subjected to 30 min of four-vessel occlusion (Clemens *et al.*, 1991a). In the Freund's complete adjuvant-induced arthritis model in rats, LY178002 was found to inhibit soft tissue swelling as well as bone damage (Panetta *et al.*, 1989). LY213829, an analogue of LY178002, has been shown to be effective in animal models of inflammatory bowel disease including an acetic acid-induced colitis model in rats (Albee *et al.*, 1991) and a formalin-immune-mediated rabbit colitis model (Albee *et al.*, 1992). It is currently being evaluated in Phase Ib clinical trials for efficacy in ulcerative colitis.

More recently, another series of di-*t*-butyl phenol antioxidants represented by LY231617 has been developed. These compounds inhibit iron-dependent lipid peroxidation and antagonize hydrogen peroxide-induced cortical neuronal injury [at 5 μM LY231617 increased neuron viability from 20% (untreated) to 70%]. Interestingly, LY231617 does not inhibit the key enzymes of the arachidonic acid cascade (5-lipoxygenase, PLA_2 cyclooxygenase). Intravenous LY231617 treatment 30 min after the onset of four-vessel occlusion resulted in a significant reduction in CA_1 hippocampal damage and striatal damage (Clemens *et al.*, 1993). LY231617 treatment was also found to preserve the electrophysiological functional integrity of the septal hippocampal circuit after four-vessel occlusion (Clemens *et al.*, 1991b).

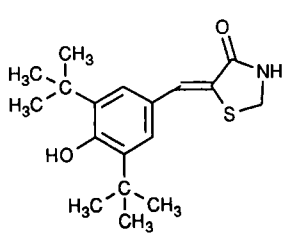
Probucol, another di-*t*-butyl phenol, is an antiatherosclerotic agent that can suppress the oxidation of low-density lipoprotein (LDL) in addition to lowering cholesterol levels. The antioxidant activity of probucol was measured, using EPR, with oxidation of methyl linoleate that was encapsulated in liposomal membranes or dissolved in hexane. Probucol suppressed free-radical-mediated oxidation. Its antioxidant activity was 17-fold less than that of tocopherol. This difference was less in liposomes than in hexane solution. Probucol suppressed the oxidation of LDL as efficiently as tocopherol. This work implies that physical factors as well as chemical reactivity are important in determining overall lipid peroxidation inhibition activity (Gotoh *et al.*, 1992).

Carvediol is a vasodilator with beta-adrenergic antagonist activity. It has cardioprotective activity in animal models. The antioxidant effect of carvediol was compared with five other beta blockers in iron-initiated lipid peroxidation, where it inhibited TBARs formation and protected membrane-bound tocopherol in rat brain homogenate (Yue *et al.*, 1992a). The *ortho*-substituted phenoxylethyl-amine is responsible for the improved antioxidant activity.

Idebenone, an inhibitor of lipid peroxidation, was shown to prolong survival time and delay the onset of ischaemic seizures in a bilateral carotid occlusion model in rats. It is marketed in Japan as a therapy to improve cerebral metabolism and performance after a stroke (Suno and Nagaoka, 1984). Cerebral protective effects after an ischaemic insult in dogs and rabbits have been seen with the hydroxyl radical scavenger, mannitol (Meyer *et al.*, 1987).

4.3 SPIN TRAPS

Historically, spin traps have been used as an analytical tool to determine the nature of free radicals being generated in reactions (Janzen, 1971). By design, they react with free radicals to form a stable radical product. They



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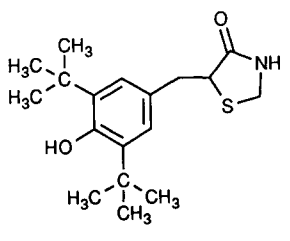

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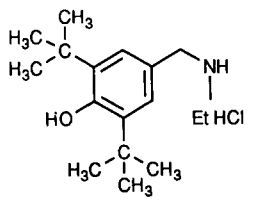

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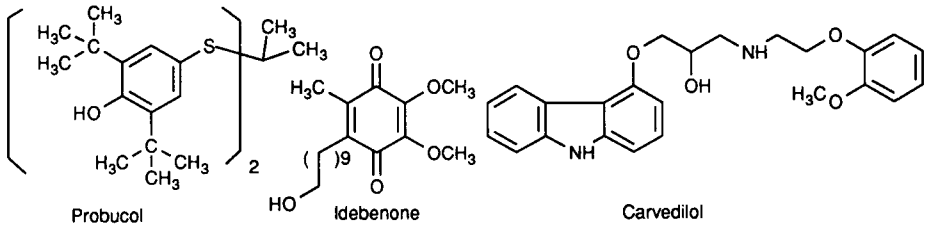


are, thus, lipid chain-reaction terminators as well as inhibitors of initiation. More recently, spin traps, such as PBN, have been used to study and characterize oxygen-derived free radicals in biological systems (Lai *et al.*, 1986) and have even been evaluated as potential therapeutic agents. PBN reduced neuronal damage and forebrain oedema in gerbils subjected to forebrain ischaemia when administered either pre- or post-occlusion (Yue *et al.*, 1992b). Endotoxin-induced mortality in rats could be attenuated with PBN treatment (Hamburger and McCay, 1989). Greater recovery of myocardial function was reported in dogs pretreated with PBN before a regional ischaemic insult (Bolli *et al.*, 1988).

Recently, PBN, α-4-pyridyl-oxide-*N*-*t*-butyl nitrone (POBN) or 5-5,dimethyl-1, pyrroline-*N*-oxide (DMPO) were evaluated in models of experimental shock (endotoxic, traumatic and mesenteric artery occlusion in rats). All three nitrones, when given prior to the insult intraperitoneally, were protective. When the nitrone's spin trapping ability was inactivated by exposure to solar light and air, they were no longer efficacious (Novelli, 1992).

4.4 ANTIOXIDANT HETEROCYCLES

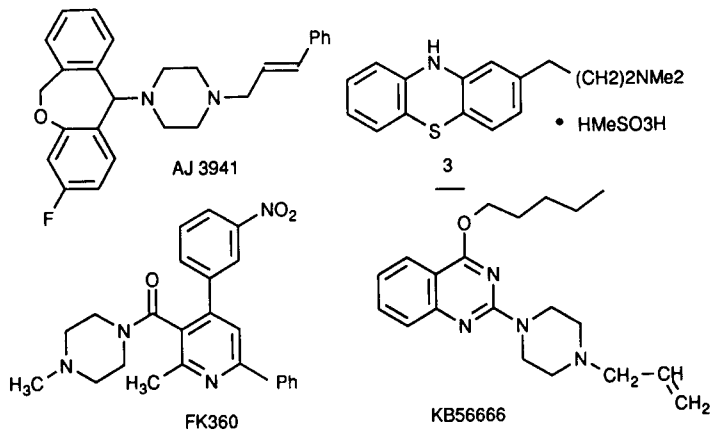
Many amino- and hydroxy-substituted heterocycles have reasonably low oxidation potentials and, as such, can act as lipid-peroxidation inhibitors. We have already discussed triaminopyrimidine examples (tirilazad and U78517) and several ascorbic-acid analogues. This section discusses a few more examples.

A series of dihydrodibenzoxepines, represented by AJ3941, was tested in animal models of global ischaemia and hypoxia, and found to be protective. AJ3941 is an inhibitor of lipid peroxidation (Kurakawa *et al.*, 1991). A novel quinazoline fumarate (KB56666, was found to inhibit lipid peroxidation in rat brain homogenates and isolated mitochondria. In a rat focal stroke model, KB56666 prevented brain oedema and neuronal damage in the ischaemic zone (Hara *et al.*, 1991).

The phenothiazines, chlorpromazine and promethazine, have been described as inhibitors of CCl_4-induced lipid peroxidation at relatively high concentrations in rat liver microsomes (Slater, 1968). Structural modifications of chlorpromazine were undertaken to try to increase antioxidant activity and maintain molecular lipophilicity. The 2-*N*-*N*-dimethyl ethanamine methanesulphonate-substituted phenothiazine (3) was found to be a potent inhibitor of iron-dependent lipid peroxidation. It was also found to block Cu^{2+}-catalysed oxidation of LDL more effectively than probucol and to protect primary cultures of rat hippocampal neurons against hydrogen peroxide-induced toxicity *in vitro* (Yu *et al.*, 1992).

A series of 4-arylpyrimidines that are amine substituted at pyrimidine C-2 was prepared. FK360 was most effective from this group on both arachidonate-induced cerebral oedema in rats and as an *in vitro* inhibitor of lipid peroxidation. The authors link effects of FK360 to the arachidonic acid cascade (Kuno *et al.*, 1992). This is an unusual structure.

Another pyrimidine derivative, RA-642, has been found to inhibit ferrous-induced lipid peroxidation in cell



membranes isolated from rat liver, brain, kidney, lung and heart. The activity of this and analogous compounds may well be due to iron binding by the heterocycle (Bellido *et al.*, 1991).

Ebselen is a seleno-organic that mimics glutathione peroxidase and inhibits iron-stimulated lipid peroxidation. It significantly reduced both infarct size and oedema progression in the middle cerebral artery exclusion (MCAO) focal model of stroke in rats (Matsui *et al.*, 1990). Ebselen has also been shown to be an effective anti-inflammatory agent in a H_2O_2-dependent inflammation model in rats (Parnham *et al.*, 1991).

Kassell (Takahashi *et al.*, 1993) has described the activity of a novel tropolone U88999E in a rabbit model of cerebral vasospasm. U88999E inhibits lipid peroxidation and acts as a calcium antagonist. Kassell showed that the compound relaxed preconstricted arterial rings *in vitro* (potency slightly less than flunarizine or diltiazem) and that it reduced vasospasm of basilar arteries after rabbit subarachnoid haemorrhage (Takahashi *et al.*, 1993).

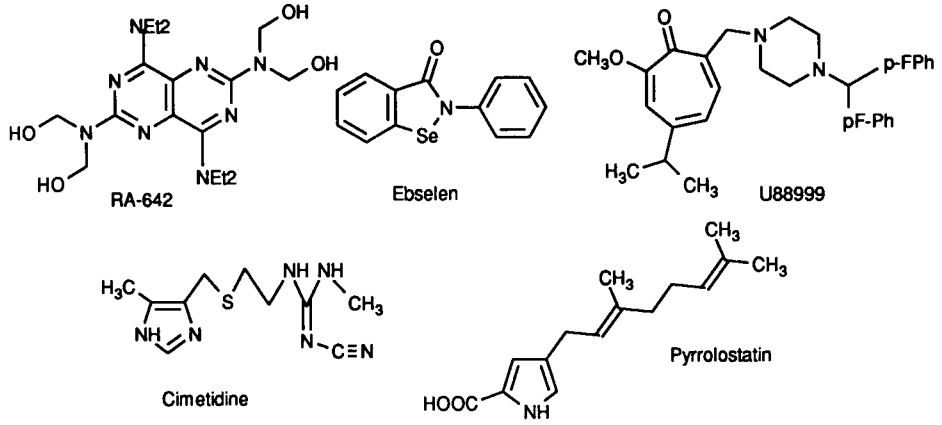
Cimetidine and other imidazole-containing H_2 receptor antagonists scavenge hydroxyl radicals at a faster rate than mannitol. This activity was attributed to the methyl imidazole moiety rather than the guanidine of cimetidine (Ching *et al.*, 1993).

The antiulcer agent rebamipide ((2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl]propionic acid) dose-dependently decreased hydroxyl radical signal generated by the Fenton reaction in an e.s.r. study. Rebamipide is active as a hydroxyl radical scavenger and inhibitor of superoxide production by neutrophils (Yoshikawa *et al.*, 1993).

Pyrrolostatin is a pyrrole-2-carboxylic acid that inhibits lipid peroxidation in rat brain homogenate (Kato *et al.*, 1993). This, like a few other compounds described in this report, looks like a compound that should have a high oxidation potential and may owe its inhibitor activity to physicochemical effects.

4.5 IRON REDOX CYCLE MODIFIERS

Iron plays a crucial role in the lipid peroxidation cascade. Iron is nearly completely complexed in plasma with the protein transferrin (Weinberg, 1992). Transferrin carries iron into cells where iron is released. The free protein, apotransferrin, leaves the cell. Iron is either included by the cell in iron-containing proteins or stored in the protein ferritin. Superoxide anion or lipid hydroperoxides react with ferritin, the innocuous storage site for iron, to release the reactive Fe(II) (Bolann and Ulvik, 1990). Fe(II) refers to cationic iron with an oxidation state of plus 2. Free iron is also released by hydrogen peroxide degradation of haemoglobin and myoglobin (Puppo and Halliwell, 1988). Fe(II) can convert hydrogen peroxide to hydroxyl radical; it is oxidized to Fe(III) in the process (the Fenton reaction) (Halliwell and Gutteridge, 1990). Fe(III) can be reduced to Fe(II) by superoxide anion. The sum of these processes is the iron-catalysed Haber–Weiss reaction. Lipid hydroperoxides (LOOH) can react with Fe(II) to form lipid alkoxides (LO$^{\cdot}$). Iron–oxygen complexes are probably important initiators of lipid peroxidation. The product of Fe(II) and hydrogen peroxide produces a ferryl intermediate that decomposes and yields hydroxyl radical (Auroma *et al.*, 1991). Fe(III) and superoxide anion form an intermediate called perferryl that is a weaker oxidant than ferryl and is probably most significant as an intermediate in the conversion of Fe(III) to Fe(II) by superoxide anion. The important role of iron in lipid peroxidation has been recently reviewed (Halliwell, 1992). Halliwell makes the salient point that the damage during ischaemic injury from excess hydrogen peroxide and superoxide anion will be affected by the location, and amount of iron and copper catalyst ions. Damage will be limited if metal ions are not available. This highlights the role of transition metals in converting relatively unreactive species like superoxide anion, hydrogen peroxide and lipid hydroperoxides to more reactive, more destructive



species such as hydroxyl radical and ferryl. Unfortunately, during injury, transition metals become available because reactive oxygen frees iron from transport and storage proteins, because lysed cells release their intracellular iron and because vascular injury releases haemoglobin at the damage site.

Chelators of transition metals like iron can remove free iron from the injury cascade. The iron chelator, desferrioxamine, prevented the accumulation of lipid peroxides in 15 min cardiac arrest model in dogs followed by 2 h of resuscitation (Komara *et al.*, 1986).

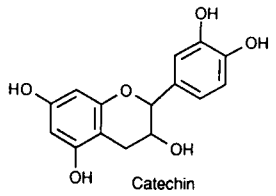
Desferrioxamine has been reported to be efficacious in a variety of animal models of inflammation (Blake *et al.*, 1983); however, early clinical trials of desferrioxamine given subcutaneously were halted due to severe cerebral and ocular toxicity (Blake *et al.*, 1985). A series of α-keto-hydroxypyridines have been developed as iron chelators to treat iron-overloaded syndromes. Clinical studies have shown 1,2-dimethyl-3-hydroxy-pyrid-4-one to be as effective as desferrioxamine in promoting urinary excretion of iron (Olivieri *et al.*, 1990) It will be interesting to see if this new generation of iron chelators will be effective in models of ischaemia–reperfusion injury. Desferrioxamine conjugated with hydroxyethyl starch inhibits iron-mediated lipid peroxidation and has been shown to be effective in a canine model of occlusion–reperfusion stunned myocardium. This benefit was attributed to iron binding. Plasma concentrations of the conjugate reached a higher peak level and had a longer half-life than regular desferrioxamine, which was not effective in the model (Panter *et al.*, 1992).

Morel *et al.* (1993) have reported that three flavanoids (catechin, quercetin and diosmetin) are cytoprotective on iron-loaded hepatocyte cultures. Their cytoprotective activity (catechin > quercetin > diosmetin) correlated with their iron-chelating ability (Morel *et al.*, 1993). These compounds should also be good phenolic antioxidants so iron chelation may only be part of the story.

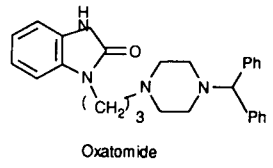
5. *Inflammatory Cascade*

Inflammatory cell phenomenon are also contributors to lipid peroxidation. Activated neutrophils may adhere to damaged endothelium and amplify traumatic, ischaemic or ischaemia–reperfusion injury. Many cyclooxygenase products of the metabolism of arachidonic acid modulate the inflammatory responses of cells. Macrophages, neutrophils and microglia are important sources of reactive oxygen at the injury site. When activated, they produce a respiratory burst that is traced to activated nicotinamide adenine dinucleotide (NADPH/NADH) oxidase.

Lymphocyte response and effector function has recently been reviewed with respect to lymphocyte receptors, lymphocyte activation, T cell effector functions and lymphocyte physiology. The role of cell surface



Catechin



Oxatomide

molecules on lymphocytes and their partner cells were also reviewed (Janeway and Golstein, 1993). In addition, the role of adhesion molecules in asthma has recently been specifically reviewed (Calderon and Lockey, 1992).

An anti-intracellular adhesion molecule-1 (anti-ICAM-1) antibody reduced neurological damage in a rabbit embolic model of stroke followed by thrombolysis with tissue-type plasminogen activator (tPA). When thrombolysis was delayed for 3 h following embolism, neither tPA nor the tPA/ICAM combination reduced neurological damage.

HOCl is one of the oxidants that activated phagocytes have in their arsenal. Hu *et al.* (1993) have shown that plasma albumin sulphydryl and ascorbic acid both protect against oxidant injury from HOCl. Neutrophils, interestingly, contain about 25 times more ascorbic acid than plasma. This suggests an endogenous self-protection role. This work is important because of the biological emphasis of reactive chlorine by the neutrophil.

Oxatomide (1-{3-[4-(diphenylmethyl)-1-piperazinyl] propyl}-1,3-dihydro-2H-benzimidazol-2-one) is an anti-allergy drug. Akamatsu has reported that oxatomide decreases neutrophil-generated superoxide anion and hydrogen peroxide formation in a dose-dependent manner. The authors hypothesize that the drug is inhibiting NADPH-dependent oxygen metabolism within the neutrophil (Akamatsu *et al.*, 1993).

6. *Conclusion*

The injury cascade that Fig. 17.1 depicts is complex. The components work in concert. We have concentrated on reactive oxygen as central to this cascade. Clearly, research in this area is active and chemically varied. Likewise, the human maladies that have been linked to reactive oxygen are diverse. Head and spinal cord injury, ischaemic and haemorrhagic stroke, septic and haemorrhagic shock, hyperoxic injury, burn progression, transplant rejection and myocardial ischaemic injury have all been linked to reactive oxygen. Many believe that chronic diseases like asthma, rheumatoid arthritis, Crohn's disease, ALS, Parkinson's disease and more are also tied to reactive oxygen. Our increased understanding of oxygen-mediated injury and the success of a variety of compounds in credible animal models promises an exciting future for this area of research.

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Glossary

Note: This glossary is up to date for the current volume only and will be supplemented with each subsequent volume.

α_1, α_2 receptors Adrenoceptor subtypes
α_1-ACT α_1-Antichymotrypsin
α_1-AP α_1-antiproteinase *also known as* α_1-antitrypsin and α_1-proteinase inhibitor
α_1-AT α_1-Antitrypsin inhibitor *also known as* α_1-antiproteinase and α_1 proteinase inhibitor
α_1-PI α_1-Proteinase inhibitor *also known as* α_1-antitrypsin and α_1-antiproteinase
α_2-M α_2-macroglobulin
A Absorbance
AI, AII Angiotensin I, II
Å Angstrom
AA Arachidonic acid
aa Amino acids
AAb Autoantibody
ABAP 2′,2′-azobis-2-amidino propane
Ab Antibody
Ab1 Idiotype antibody
Ab2 Anti-idiotype antibody
Ab2α Anti-idiotype antibody which binds outside the antigen binding region
Ab2β Anti-idiotype antibody which binds to the antigen binding region
Ab3 Anti-anti-idiotype antibody
Abcc Antibody dependent cellular cytotoxicity
ABA-L-GAT Arsanilic acid conjugated with the synthetic polypeptide L-GAT
AC Adenylate cyclase
ACAT Acyl-co-enzyme-A acyltransferase
ACAID Anterior chamber-associated immune deviation
ACE Angiotensin-converting enzyme
ACh Acetylcholine
ACTH Adrenocorticotrophin hormone
ADH Alcohol dehydrogenase
Ado Adenosine
ADP Adenosine diphosphate
ADPRT Adenosine diphosphate ribosyl transferase
AES Anti-eosinophil serum
Ag Antigen
AGE Advanced glycosylation end-product
AGEPC 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine; *also known as* PAF and APRL
AH Acetylhydrolase
AID Autoimmune disease
AIDS Acquired immune deficiency syndrome
A/J A Jackson inbred mouse strain
ALP Anti-leukoprotease
ALS Amyotrophic lateral sclerosis
cAMP Cyclic adenosine monophosphate *also known as* adenosine 3′, 5′-phosphate
AM Alveolar macrophage
AML Acute myelogenous leukaemia
AMP Adenosine monophosphate
AMVN 2,2′-azobis (2,4-dimethylvaleronitrile)
ANAb Anti-nuclear antibodies
ANCA Anti-neutrophil cytoplasmic auto antibodies
cANCA Cytoplasmic ANCA
pANCA Perinuclear ANCA
AND Anaphylactic degranulation
ANF Atrial natriuretic factor
ANP Atrial natriuretic peptide
Anti-I-A, Anti-I-E Antibody against class II MHC molecule encoded by I-A locus, I-E locus.
anti-Ig Antibody against an immunoglobulin
anti-RTE Anti-tubular epithelium
AP-1 Activator protein-1
APA B-azaprostanoic acid
APAS Antiplatelet antiserum
APC Antigen-presenting cell
APD Action potential duration
apo-B Apolipoprotein B
APRL Anti-hypertensive polar renal lipid *also known as* PAF
APUD Amine precursor uptake and decarboxylation
AR Aldose reductase
AR-CGD Autosomal recessive form of chronic granulomatous disease
ARDS Adult respiratory distress syndrome
AS Ankylosing spondylitis
ASA Acetylsalicylic acid *also known as* aspirin
4-ASA, 5-ASA 4-, 5-aminosalicylic acid
ATHERO-ELAM A monocyte adhesion molecule
ATL Adult T cell leukaemia
ATP Adenosine triphosphate
ATPase Adenosine triphosphatase
ATPγs Adenosine 3′ thiotriphosphate
AITP Autoimmune thrombocytopenic purpura
AUC Area under curve
AVP Arginine vasopressin

β_1, β_2 receptors Adrenoceptor subtypes
β_2 (CD18) A leucocyte integrin
β_2M β_2-Microglobulin
β-TG β-Thromboglobulin
B_7/BB_1 *Known to be* expressed on B cell blasts and immunostimulatory dendritic cells
BAF Basophil-activating factor
BAL Bronchoalveolar lavage
BALF Bronchoalveolar lavage fluid
BALT Bronchus-associated lymphoid tissue
B cell Bone marrow-derived lymphocyte
BCF Basophil chemotactic factor
B-CFC Basophil colony-forming cell
BCG Bacillus Calmette-Guérin
BCNU 1,3-bis(2-chloroethyl)-1-nitrosourea
bFGF Basic fibroblast growth factor
Bg Birbeck granules
BHR Bronchial hyperresponsiveness
BHT Butylated hydroxyroluene
b.i.d. *Bis in die* (twice a day)
Bk Bradykinin
Bk_1, Bk_2 receptors Bradykinin receptor subtypes *also known as* B_1 and B_2 receptors
Bk_2 receptor Bradykinin receptor subtype
Bl-CFC Blast colony-forming cells

B-lymphocyte Bursa-derived lymphocyte
BM Bone marrow
BMCMC Bone marrow cultured mast cell
BMMC Bone marrow mast cell
BOC-FMLP Butoxycarbonyl-FMLP
bp Base pair
BPB Para-bromophenacyl bromide
BPI Bacterial permeability-increasing protein
BSA Bovine serum albumin
BSS Bernard-Soulier Syndrome

^{51}Cr Chromium^{51}
C1, C2...C9 The 9 main components of complement
C1 inhibitor A serine protease inhibitor which inactivates C1r/C1s
C1q Complement fragment 1q
C1qR Receptor for C1w; facilitates attachment of immune complexes to mononuclear leucocytes and endothelium
C3a Complement fragment 3a (anaphylatoxin)
C3a$_{72-77}$ A synthetic carboxyterminal peptide C3a analogue
C3aR Receptor for anaphylatoxins, C3a, C4a, C5a
C3b Complement fragment 3b (anaphylatoxin)
C3bi Inactivated form of C3b fragment of complement
C4b Complement fragment 4b (anaphylatoxin)
C4BP C4 binding protein; plasma protein which acts as co-factor to factor I inactivate C3 convertase
C5a Complement fragment 5a (anaphylatoxin)
C5aR Receptor for anaphylatoxins C3a, C4a and C5a
C5b Complement fragment 5b (anaphylatoxin)
C_ε2, C_ε3, C_ε4 Heavy chain of immunoglobulin E: domains 2, 3 and 4
Ca *The chemical symbol for* calcium
$[Ca^{2+}]_i$ Intracellular free calcium concentration
CAH Chronic active hepatitis
CALLA Common lymphoblastic leukaemia antigen
CALT Conjunctival associated lymphoid tissue
CaM Calmodulin
cAMP Cyclic adenosine monophosphate *also known as* adenosine 3′, 5′-phosphate
CAM Cell adhesion molecule
CAP57 Cationic protein from neutrophils
CAT Catalase
CatG Cathepsin G
CB Cytochalasin B
CBH Cutaneous basophil hypersensitivity
CBP Cromolyn-binding protein
CCK Cholecystokinin
CCR Creatinine clearance rate
CD Cluster of differentiation (a system of nomenclature for surface molecules on cells of the immune system); cluster determinant
CD1 Cluster of differentiation 1 *also known as* MHC class I-like surface glycoprotein
CD1a Isoform a *also known as* non-classical MHC class I-like surface antigen; present on thymocytes and dendritic cells
CD1b *Known to be* present on thymocytes and dendritic cells
CD1c Isoform c *also known as* non-classical MHC class I-like surface antigen; present on thymocytes
CD2 Defines T cells involved in antigen non-specific cell activation
CD3 *Also known as* T cell receptor-associated surface glycoprotein on T cells
CD4 Defines MHC class II-restricted T cell subsets
CD5 *Known to be* present on T cells and a subset of B cells; *also known as* Lyt 1 in mouse
CD7 Cluster of differentiation 7; present on most T cells and NK cells
CD8 Defines MHC class I-restricted T cell subset; present on NK cells
CD10 *Known to be* common acute leukaemia antigen
CD11a *Known to be* an α chain of LFA-1 (leucocyte function antigen-1) present on several types of leucocyte and which mediates adhesion
CD11c *Known to be* a complement receptor 4 α chain.
CD13 Aminopeptidase N; present on myeloid cells
CD14 *Known to be* a lipid-anchored glycoprotein; present on monocytes
CD15 *Known to be* Lewis X, fucosyl-*N*-acetyllactosamine
CD16 *Known to be* Fcγ receptor III
CD16-1, CD16-2 Isoforms of CD16
CD19 Recognizes B cells and follicular dendritic cells
CD20 *Known to be* a pan B cell
CD21 C3d receptor
CD23 Low affinity FcεR
CD25 Low affinity receptor for interleukin-2
CD27 Present on T cells and plasma cells
CD28 Present on resting and activated T cells and plasma cells
CD30 Present on activated B and T cells
CD31 *Known to be* on platelets, monocytes, macrophages, granulocytes, B-cells and endothelial cells; *also known as* PECAM
CD32 Fcγ receptor II
CD33$^+$ *Known to be* a monocyte and stem cell marker
CD34$^-$ *Known to be* a stem cell marker
CD35 C3b receptor
CD36 *Known to be* a macrophage thrombospondin receptor
CD40 Present on B cells and follicular dendritic cells
CD41 *Known to be* a platelet glycoprotein
CD44 *Known to be* a leucocyte adhesion molecule; *also known as* hyaluronic acid cell adhesion molecule (H-CAM), Hermes antigen, extracellular matrix receptor III (ECMIII); present on polymorphonuclear leucocytes
CD45 *Known to be* a pan leucocyte marker
CD45RO *Known to be* the isoform of leukosialin present on memory T cells
CD46 *Known to be* a membrane cofactor protein
CD49 Cluster of differentiation 49
CD51 *Known to be* vitronectin receptor alpha chain
CD54 *Known to be* Intercellular adhesion molecule-1 *also known as* ICAM-1
CD57 Present on T cells and NK subsets
CD58 A leucocyte function-associated antigen-3, *also known to be* as a member of the β-2 integrin family of cell adhesion molecules
CD59 *Known to be* a low molecular weight HRf present to many haematopoetic and non-haematopoetic cells
CD62 *Known to be present on* activated platelets and endothelial cells; *also known as* P-selectin
CD64 *Known to be* Fcγ receptor I
CD65 *Known to be* fucoganglioside
CD68 Present on macrophages
CD69 *Known to be* an activation inducer molecule; present on activated lymphocytes
CD72 Present on B-lineage cells
CD74 An invariant chain of class II B cells
CDC Complement-dependent cytotoxicity
cDNA Complementary DNA
CDP Choline diphosphate
CDR Complementary-determining region

CD_{xx} Common determinant *xx*
CEA Carcinoembryonic antigen
CETAF Corneal epithelial T cell activating factor
CF Cystic fibrosis
Cf Cationized ferritin
CFA Complete Freund's adjuvant
CFC Colony-forming cell
CFU Colony-forming unit
CFU-Mk Megakaryocyte progenitors
CFU-S Colony-forming unit, spleen
CGD Chronic granulomatous disease
cGMP Cyclic guanosine monophosphate *also known as* guanosine 3′, 5′-phosphate
CGRP Calcitonin gene-related peptide
CH2 Hinge region of human immunoglobulin
CHO Chinese hamster ovary
CI Chemical ionization
CIBD Chronic inflammatory bowel disease
CK Creatine phosphokinase
CKMB The myocardial-specific isoenzyme of creatine phosphokinase
Cl *The chemical symbol for* chloride
CL Chemiluminescent
CLA Cutaneous lymphocyte antigen
CL18/6 Anti-ICAM-1 monoclonal antibody
CLC Charcot-Leyden crystal
CMC Critical micellar concentration
CMI Cell mediated immunity
CML Chronic myeloid leukaemia
CMV Cytomegalovirus
CNS Central nervous system
CO Cyclooxygenase
CoA Coenzyme A
CoA-IT Coenzyme A – independent transacylase
Con A Concanavalin A
COPD Chronic obstructive pulmonary disease
COS Fibroblast-like kidney cell line established from simian cells
CoVF Cobra venom
CP Creatine phosphate
Cp Caeruloplasmin
c.p.m. Counts per minute
CPJ Cartilage/pannus junction
Cr *The chemical symbol for* chromium
CR Complement receptor
CR1, CR2 & CR4 Complement receptor types 1, 2 and 4
CR3-α Complement receptor type 3-α
CRF Corticotrophin-releasing factor
CRH Corticotrophin-releasing hormone
CRI Cross-reactive idiotype
CRP C-reactive protein
CSA Cyclosporin A
CSF Colony-stimulating factor
CSS Churg-Strauss syndrome
CT Computed tomography
CTAP-III Connective tissue-activating peptide
CTD Connective tissue diseases
C terminus Carboxy terminus of peptide
CThp Cytotoxic T lymphocyte precursors
CTL Cytotoxic T lymphocyte
CTLA-4 *Known to be* co-expressed with CD20 on activated T cells
CTMC Connective tissue mast cell
CVF Cobra venom factor

2D Second derivative
Da Dalton (the unit of relative molecular mass)
DAF Decay-accelerating factor
DAG Diacylglycerol
DAO Diamine oxidase
D-Arg D-Arginine
DArg-[Hyp^3,DPhe^7]-BK A bradykinin B_2 receptor antagonist. Peptide derivative of bradykinin
DArg-[Hyp^3,Thi^5,DTic^7,Tic^8]-BK A bradykinin B_2 receptor antagonist. Peptide derivative of bradykinin
DBNBS 3,5-dibromo-4-nitroso-benzenesulphonate
DC Dendritic cell
DCF Oxidized DCFH
DCFH 2′,7′-dichlorofluorescin
DEC Diethylcarbamazine
DEM Diethylmaleate
desArg^9-BK Carboxypeptidase N product of bradykinin
desArg^{10}KD Carboxypeptidase N product of kallidin
DETAPAC Diethylenetriaminepentaacetic acid
DFMO α1-Difluoromethyl ornithine
DFP Diisopropyl fluorophosphate
DFX Desferrioxamine
DGLA Dihomo-γ-linolenic acid
DH Delayed hypersensitivity
DHA Docosahexaenoic acid
DHBA Dihydroxybenzoic acid
DHR Delayed hypersensitivity reaction
DIC Disseminated intravascular coagulation
DL-CFU Dendritic cell/Langerhans cell colony forming
DLE Discoid lupus erythematosus
DMARD Disease-modifying anti-rheumatic drug
DMF *N*,*N*-dimethylformamide
DMPO 5,5-dimethyl-l-pyrroline *N*-oxide
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
D-NAME D-Nitroarginine methyl ester
DNase Deoxyribonuclease
DNCB Dinitrochlorobenzene
DNP Dinitrophenol
Dpt4 *Dermatophagoides pteronyssinus* allergen 4
DGW2, DR3, DR7 HLA phenotypes
DREG-56 (Antigen) L-selectin
DREG-200 A monoclonal antibody against L-selectin
ds Double-stranded
DSCG Disodium cromoglycate
DST Donor-specific transfusion
DTH Delayed-type hypersensitivity
DTPA Diethylenetriamine pentaacetate
DTT Dithiothreitol
dv/dt Rate of change of voltage within time

ε Molar absorption coefficient
EA Egg albumin
EACA Epsilon-amino-caproic acid
EAE Experimental autoimmune encephalomyelitis
EAF Eosinophil-activating factor
EAR Early phase asthmatic reaction
EAT Experimental autoimmune thyroiditis
EBV Epstein–Barr virus
EC Endothelial cell
ECD Electron capture detector
ECE Endothelin-converting enzyme
E-CEF Eosinophil cytotoxicity enhancing factor
ECF-A Eosinophil chemotactic factor of anaphylaxis
ECG Electrocardiogram
ECGF Endothelial cell growth factor
ECGS Endothelial cell growth supplement
E. coli *Escherichia coli*
ECP Eosinophil cationic protein
EC-SOD Extracellular superoxide dismutase
EC-SOD C Extracellular superoxide dismutase C
ED_{35} Effective dose producing 35% maximum response
ED_{50} Effective dose producing 50% maximum response
EDF Eosinophil differentiation factor
EDL Extensor digitorum longus
EDN Eosinophil-derived neurotoxin
EDRF Endothelium-derived relaxing factor
EDTA Ethylenediamine tetraacetic acid *also known as* etidronic acid
EE Eosinophilic eosinophils
EEG Electroencephalogram
EET Epoxyeicosatrienoic acid
EFA Essential fatty acid
EFS Electrical field stimulation
EG1 Monoclonal antibody specific for the cleaved form of eosinophil cationic peptide

EGF Epidermal growth factor
EGTA Ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid
EHNA Erythro-9-(2-hydroxy-3-nonyl)-adenine
EI Electron impact
EIB Exercise-induced bronchoconstriction
eIF-2 Subunit of protein synthesis initiation factor
ELAM-1 Endothelial leucocyte adhesion molecule-1
ELF Respiratory epithelium lung fluid
ELISA Enzyme-linked immunosorbent assay
EMS Eosinophilia-myalgia syndrome
ENS Enteric nervous system
EO Eosinophil
EO-CFC Eosinophil colony-forming cell
EOR Early onset reaction *also known as* EAR
EPA Eicosapentaenoic acid
EpDIF Epithelial-derived inhibitory factor *also known as* epithelium*f*derived relaxant factor
EPO Eosinophil peroxidase
EPOR Erythropoietin receptor
EPR Effector cell protease
EPX Eosinophil protein X
ER Endoplasmic reticulum
ERCP Endoscopic retrograde cholangiopancreatography
E-selectin Endothelial selectin *formerly known as* endothelial leucocyte adhesion molecule-1 (ELAM-1)
ESP Eosinophil stimulation promoter
ESR Erythrocyte sedimentation rate
e.s.r. Electron spin resonance
ET, ET-1 Endothelin, -1
ETYA Eicosatetraynoic acid

FA Fatty acid
FAB Fast-electron bombardment
Fab Antigen binding fragment
F(ab′)2 Fragment of an immunoglobulin produced pepsin treatment
FACS Flow activated cell sorter
factor B Serine protease in the C3 converting enzyme of the alternative pathway
factor D Serine protease which cleaves factor B
factor H Plasma protein which acts as a co-factor to factor I
factor I Hydrolyses C3 converting enzymes with the help of factor H
FAD Flavine adenine dinucleotide
FapyAde 5-formamido-4,6-diamino-pyrimidine
FapyGua 2,6-diamino-4-hydroxy-5-formamidopyrimidine
FBR Fluorescence photobleaching recovery
Fc Crystallizable fraction of immunoglobulin molecule
Fcγ Receptor for Fc portion of IgG
FcγRI Ig Fc receptor I *also known as* CD64
FcγRII Ig Fc receptor II *also known as* CD32
FcγRIII Ig Fc receptor III *also known as* CD16
Fc$_\epsilon$RI High affinity receptor for IgE
Fc$_\epsilon$RII Low affinity receptor for IgE
FcR Receptor for Fc region of antibody
FCS Foetal calf (bovine) serum
FEV$_1$ Forced expiratory volume in 1 second
Fe-TPAA Fe(III)-tris[*N*-(2-pyridylmethyl)-2-aminoethyl]amine
Fe-TPEN Fe(II)-tetrakis-*N,N,N′,N′*-(2-pyridyl methyl-2-aminoethyl)amine
FFA Free fatty acids
FGF Fibroblast growth factor
FID Flame ionization detector
FITC Fluorescein isothiocyanate
FKBP FK506-binding protein
FLAP 5-lipoxygenase-activating protein
FMLP *N*-Formyl-methionyl-leucyl-phenylalanine
FNLP Formyl-norleucyl-leucyl-phenylalanine
FOC Follicular dendritic cell
FPLC Fast protein liquid chromatography
FPR Formyl peptide receptor
FS cell Folliculo-stellate cell
FSG Focal sequential glomerulosclerosis
FSH Follicle stimulating hormone
FX Ferrioxamine
5-FU 5-fluorouracil

Ga G-protein
G6PD Glucose 6-phosphate dehydrogenase
GABA γ-aminobutyric acid
GAG Glycosaminoglycan
GALT Gut-associated lymphoid tissue
GAP GTPase-activating protein
GBM Glomerular basement membrane
GC Guanylate cyclase
GC-MS Gas chromatography mass spectroscopy
G-CSF Granulocyte colony-stimulating factor
GDP Guanosine 5′-diphosphate
GEC Glomerular epithelial cell
GF-1 An insulin-like growth factor
GFR Glomerular filtration rate
GH Growth hormone
GH-RF Growth hormone-releasing factor
Gi Family of pertussis toxin sensitive G-proteins
GI Gastrointestinal
GIP Granulocyte inhibitory protein
GlyCam-1 Glycosylation-dependent cell adhesion molecule-1
GMC Gastric mast cell
GM-CFC Granulocyte-macrophage colony-forming cell
GM-CSF Granulocyte-macrophage colony-stimulating factor
GMP Guanosine monophosphate (guanosine 5′-phosphate)
Go Family of pertussis toxin sensitive G-proteins
GP Glycoprotein
gp45-70 Membrane co-factor protein
gp90^{MEL} 90 kD glycoprotein recognized by monoclonal antibody MEL-14; *also known as* L-selectin
GPIIb-IIIa Glycoprotein IIb-IIIa *known to be* a platelet membrane antigen
GppCH$_2$P Guanyl-methylene diphosphanate *also known as* a stable GTP analogue
GppNHp Guanylyl-imidiodiphosphate *also known as* a stable GTP analogue
GRGDSP Glycine–arginine–glycine–aspartic acid serine–proline
Gro Growth-related oncogene
GRP Gastrin-related peptide
Gs Stimulatory G protein
GSH Glutathione (reduced)
GSHPx Glutathione peroxidase
GSSG Glutathione (oxidized)
GT Glanzmann Thrombasthenia
GTP Guanosine triphosphate
GTP-γ-S Guanarine 5′*O*-(3-thiotriphosphate)
GTPase Guanidine triphosphatase
GVHD Graft-versus-host-disease
GVHR Graft-versus-host-reaction

H Histamine
H$_1$, H$_2$, H$_3$ Histamine receptor types 1, 2 and 3
H$_2$O$_2$ *The chemical symbol for* hydrogen peroxide
Hag Haemagglutinin
Hag-1, Hag-2 Cleaved haemagglutinin subunits-1, -2
H & E Haematoxylin and eosin
hIL Human interleukin
Hb Haemoglobin
HBBS Hank's balanced salt solution
HCA Hypertonic citrate
H-CAM Hyaluronic acid cell adhesion molecule
HDC Histidine decarboxylase

HDL High-density lipoprotein
HEL Hen egg white lysozyme
HEPE Hydroxyeicosapentanoic acid
HEPES *N*-2-Hydroxylethylpiperazine-*N'*-2-ethane sulphonic acid
HES Hypereosinophilic syndrome
HETE 5,8,9,11,12 and 15 Hydroxyeicosatetraenoic acid
5(S)HETE A stereo isomer of 5-HETE
HETrE Hydroxyeicosatrienoic acid
HEV High endothelial venule
HFN Human fibronectin
HGF Hepatocyte growth factor
HHTrE 12(*S*)-Hydroxy-5,8,10-heptadecatrienoic acid
HIV Human immunodeficiency virus
HL60 Human promyelocytic leukaemia cell line
HLA Human leucocyte antigen
HLA-DR2 Human histocompatability antigen class II
HMG CoA Hydroxylmethylglutaryl coenzyme A
HMW High molecular weight
HMT Histidine methyltransferase
HMVEC Human microvascular endothelial cell
HNC Human neutrophil collagenase (MMP-8)
HNE Human neutrophil elastase
HNG Human neutrophil gelatinase (MMP-9)
HODE Hydroxyoctadecanoic acid
HO· Hydroxyl radical
HO_2· Perhydroxyl radical
HPETE, 5-HPETE & 15-HPETE 5 and 15 Hydroperoxyeicosatetraenoic acid
HPETrE Hydroperoxytrienoic acid
HPODE Hydroperoxyoctadecanoic acid
HPLC High-performance liquid chromatography
HRA Histamine-releasing activity
HRAN Neutrophil-derived histamine-releasing activity
HRf Homologous-restriction factor
HRF Histamine-releasing factor
HRP Horseradish peroxidase
HSA Human serum albumin
HSP Heat-shock protein
HS-PG Heparan sulphate proteoglycan
HSV, HSV-1 Herpes simplex virus, -1
^{3}HTdR Tritiated thymidine
5-HT 5-Hydroxytryptamine *also known as* Serotonin
HTLV-1 Human T-cell leukaemia virus-1
HUVEC Human umbilical vein endothelial cell
[Hyp^3]-BK Hydroxyproline derivative of bradykinin
[Hyp^4]-KD Hydroxyproline derivative of kallidin

^{111}In Indium^{111}
Ia Immune reaction-associated antigen
Ia+ Murine class II major histocompatibility complex antigen
IB4 Anti-CD18 monoclonal antibody
IBD Inflammatory bowel disease
IBMX 3-isobutyl-1-methylxanthine
IBS Inflammatory bowel syndrome
iC3 Inactivated C3
iC4 Inactivated C4
IC_{50} Concentration producing 50% inhibition
ICAM Intercellular adhesion molecules
ICAM-1, ICAM-2, ICAM-3 Intercellular adhesion molecules-1, -2, -3
cICAM-1 Circulating form of ICAM-1
ICE IL-1β-converting enzyme
i.d. Intradermal
IDC Interdigitating cell
IDD Insulin-dependent (type 1) diabetes
IEL Intraepithelial leucocyte
IELym Intraepithelial lymphocytes
IFA Incomplete Freund's adjuvant
IFN Interferon
IFNα, IFNβ, IFNγ Interferons α, β, γ
Ig Immunoglobulin
IgA, IgE, IgG, IgM Immunoglobulins A, E, G, M
IgG1 Immunoglobulin G class 1
IgG_{2a} Immunoglobulin G class 2a
IGF-1 Insulin-like growth factor
Ig-SF Immunoglobulin supergene family
IGSS Immuno-gold silver stain
IHC Immunohistochemistry
IHES Idiopathic hypereosinophilic syndrome
IκB NFκB inhibitor protein
IL Interleukin
IL-1, Il-2...IL-8 Interleukins-1, 2...-8
IL-1α. IL-1β Interleukin-1α, -1β
ILR Interleukin receptor
IL-1R, IL-2R; IL-3R – IL-6R Interleukin-1 – 6 receptors
IL-1Ra Interleukin-1 receptor antagonist
IL-2Rβ Interleukin-2 receptor β
IMF Integrin modulating factor
IMMC Intestinal mucosal mast cell
i.p. Intraperitoneally
IP_1 Inositol monophosphate
IP_2 Inositol biphosphate
IP_3 Inositol 1,4,5-trisphosphate
IP_4 Inositol tetrakisphosphate
IPF Idiopathic pulmonary fibrosis
IPO Intestinal peroxidase
IpOCOCq Isopropylidene OCOCq
I/R Ischaemia-reperfusion
IRAP IL-1 receptor antagonist protein
IRF-1 Interferon regulatory factor 1
I_{sc} Short-circuit current
ISCOM Immune-stimulating complexes
ISGF3 Interferon-stimulated gene Factor 3
ISGF3α, ISGFγ α, γ subunits of ISGF3
IT Immunotherapy
ITP Idiopathic thrombocytopenic purpura
i.v. Intravenous

K *The chemical symbol for* potassium
K_a Association constant
kb Kilobase
20KDHRF A homologous restriction factor; binds to C8
65KDHRF A homologous restriction factor, also known as C8 binding protein; interferes with cell membrane pore-formation by C5b-C8 complex
Kcat Catalytic constant; a measure of the catalytic potential of an enzyme
K_d Equilibrium dissociation constant
kD Kilodalton
K_D Dissociation constant
KD Kallidin
Ki Antagonist binding affinity
Ki67 Nuclear membrane antigen
KLH Keyhole limpet haemocyanin
K_m Michaelis constant
KOS KOS strain of herpes simplex virus

λ_{max} Wavelength of maximum absorbance
LAD Leucocyte adhesion deficiency
LAK Lymphocyte-activated killer (cell)
LAM, LAM-1 Leucocyte adhesion molecule, -1
LAR Late-phase asthmatic reaction
L-Arg L-Arginine
LBP LPS binding protein
LC Langerhans cell
LCF Lymphocyte chemoattractant factor
LCR Locus control region
LDH Lactate dehydrogenase
LDL Low-density lipoprotein
LDV Laser Doppler velocimetry
Le^x(Lewis X) Leucocyte ligand for selectin
LFA Leucocyte function-associated antigen
LFA-1 Leucocyte function-associated antigen-1; *also known to be* a member of the β-2 integrin family of cell adhesion molecules

LG β-Lactoglobulin
LGL Large granular lymphocyte
LH Luteinizing hormone
LHRH Luteinizing hormone-releasing hormone
LI Labelling index
LIS Lateral intercellular spaces
LMP Low molecular mass polypeptide
LMW Low molecular weight
L-NOARG L-Nitroarginine
LO Lipoxygenase
5-LO, 12-LO, 15-LO 5-, 12-, 15-Lipoxygenases
LP(a) Lipoprotein(a)
LPS Lipopolysaccharide
L-selectin Leucocte selectin, *formerly known as* monoclonal antibody that recognizes murine L-selectin (MEL-14 antigen), leucocyte cell adhesion molecule-1 (LeuCAM-1), lectin cell adhesion molecule-1 (LeCAM-1 or LecCAM-1), leucocyte adhesion molecule-1 (LAM-1)
LT Leukotriene
LTA_4, LTB_4, LTC_4, LTD_4, LTE_4 Leukotrienes A_4, B_4, C_4, D_4 and E_4
L_y-1$^+$ (Cell line)
LX Lipoxin
LXA_4, LXB_4, LXC_4, LXD_4, LXE_4 Lipoxins A_4, B_4, C_4, D_4 and E_4

M Monocyte
M3 Receptor Muscarinic receptor subtype 3
M-540 Merocyanine-540
mAb Monoclonal antibody
mAb IB4, mAb PB1.3, mAb R 3.1, mAb R 3.3, mAb 6.5, mAb 60.3 Monoclonal antibodies IB4, PB1.3, R 3.1, R 3.3, 6.5, 60.3
MABP Mean arterial blood pressure
MAC Membrane attack molecule
Mac Macrophage (also abbreviated to MΦ)
Mac- Macrophage-1 antigen; a member of the β-2 integrin family of cell adhesion molecules (also abbreviated to MΦ1), *also known as* monocyte antigen-1 (M-1), complement receptor-3 (CR3), CD11b/CD18
MAF Macrophage-activating factor
MAO Monoamine oxidase
MAP Monophasic action potential
MAPTAM An intracellular Ca^{2+} chelator
MARCKS Myristolated, alanine-rich C kinase substrate; specific protein kinase C substrate
MBP Major basic protein
MBSA Methylated bovine serum albumin
MC Mesangial cells
MCAO Middle cerebral artery occlusion
M cell Microfold or membranous cell of Peyer's patch epithelium
MCP Membrane co-factor protein
MCP-1 Monocyte chemotactic protein-1
M-CSF Monocyte/macrophage colony-stimulating factor
MC_T Tryptase-containing mast cell
MC_{TC} Tryptase- and chymase-containing mast cell
MDA Malondialdehyde
MDGF Macrophage-derived growth factor
MDP Muramyl dipeptide
MEA Mast cell growth-enhancing activity
MEL Metabolic equivalent level
MEM Minimal essential medium
MG Myasthenia gravis
MGSA Melanoma-growth-stimulatory activity
MHC Major histocompatibility complex
MI Myocardial ischaemia
MIF Migration inhibition factor
mIL Mouse interleukin
MI/R Myocardial ischaemia/reperfusion
MIRL Membrane inhibitor of reactive lysis
mix-CFC Colony-forming cell mix
Mk Megakaryocyte
MLC Mixed lymphocyte culture
MLymR Mixed lymphocyte reaction
MLR Mixed leucocyte reaction
mmLDL Minimally modified low-density lipoprotein
MMC Mucosal mast cell
MMCP Mouse mast cell protease
MMP, MMP1 Matrix metalloproteinase, -1
MNA 6-Methoxy-2-napthylacetic acid
MNC Mononuclear cells
MΦ Macrophage (also abbreviated to Mac)
MPG *N*-(2-mercaptopropionyl)-glycine
MPO Myeloperoxidase
MPSS Methyl prednisolone
MPTP *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI Magnetic resonance imaging
mRNA Messenger ribonucleic acid
MS Mass spectrometry
MSS Methylprednisolone sodium succinate
MT Malignant tumour
MW Molecular weight

Na *The chemical symbol for* sodium
NA Noradrenaline *also known as* norepinephrine
NAAb Natural autoantibody
NAb Natural antibody
NAC *N*-acetylcysteine
NADH Reduced nicotinamide adenine dinucleotide
NADP Nicotinamide adenine diphosphate
NADPH Reduced nicotinamide adenine dinucleotide phosphate
NAF Neutrophil activating factor
L-NAME L-Nitroarginine methyl ester
NANC Non-adrenergic, non-cholinergic
NAP Neutrophil-activating peptide
NAPQI *N*-acetyl-*p*-benzoquinone imine
NAP-1, NAP-2 Neutrophil-activating peptides -1 and -2
NBT Nitro-blue tetrazolium
NC1 Non-collagen 1
N-CAM Neural cell adhesion molecule
NCEH Neutral cholesteryl ester hydrolase
NCF Neutrophil chemotactic factor
NDGA Nordihydroguaretic acid
NDP Nucleoside diphosphate
Neca 5′-(*N*-ethyl carboxamido)-adenosine
NED Nedocromil sodium
NEP Neutral endopeptidase (EC 3.4.24.11)
NF-AT Nuclear factor of activated T lymphocytes
NF-κB Nuclear factor-κB
NgCAM Neural-glial cell adhesion molecule
NGF Nerve growth factor
NGPS Normal guinea-pig serum
NIH 3T3 (fibroblasts) National Institute of Health 3T3-Swiss albino mouse fibroblast
NIMA Non-inherited maternal antigens
NIRS Near infrared spectroscopy
Nk Neurokinin
NK Natural killer
Nk-1, Nk2, NK-3 Neurokinin receptor subtypes 1,2 and 3
NkA Neurokinin A
NkB Neurokinin B
NLS Nuclear location sequence
NMDA *N*-methyl-D-aspartic acid
L-NMMA L-Nitromonomethyl arginine
NMR Nuclear magnetic resonance
NO *The chemical symbol for* nitric oxide
NOD Non-obese diabetic
NOS Nitric oxide synthase
c-NOS Ca^{2+}-dependent constitutive form of NOS
i-NOS Inducible form of NOS
NPK Neuropeptide K

NPY Neuropeptide Y
NRS Normal rabbit serum
NSAID Non-steroidal anti-inflammatory drug
NSE Nerve-specific enolase
NT Neurotensin
N terminus Amino terminus of peptide

$^1\Delta O_2$ Singlet Oxygen (Delta form)
$^1\Sigma O_2$ Singlet Oxygen (Sigma form)
O_2^- *The chemical symbol for* the superoxide anion radical
OA Osteoarthritis
OAG Oleoyl acetyl glycerol
OD Optical density
ODC Ornithine decarboxylase
ODFR Oxygen-derived free radical
ODS Octadecylsilyl
OH^- *The chemical symbol for* hydroxyl ion
•**OH** *The chemical symbol for* hydroxyl radical
8-OH-Ade 8-hydroxyadenine
6-OHDA 6-hydroxyguanine
8-OH-dG 8-hydroxydeoxyguanosine *also known as* 7,8-dihydro-8-oxo-2′-deoxyguanosine
8-OH-Gua 8-hydroxyguanine
OHNE Hydroxynonenal
4-OHNE 4-hydroxynonenal
OT Oxytocin
OVA Ovalbumin
ox-LDL Oxidized low-density lipoprotein

Ψa Apical membrane potential
P Probability
P Phosphate
P_aO_2 Arterial oxygen pressure
P_i Inorganic phosphate
p150,95 A member of the β-2-integrin family of cell adhesion molecules; *also known as* CD11c
PA Phosphatidic acid
pA_2 Negative logarithm of the antagonist dissociation constant
PAF Platelet-activating factor *also known as* APRL and AGEPC
PAGE Polyacrylamide gel electrophoresis
PAI Plasminogen activator inhibitor
PA-IgG Platelet associated immunoglobulin G
PAM Pulmonary alveolar macrophages
PAS Periodic acid–Schiff reagent
PBA Polyclonal B cell activators
PBC Primary biliary cirrhosis
PBL Peripheral blood lymphocytes
PBMC Peripheral blood mononuclear cells
PBN *N-tert*-butyl-α-phenylnitrone
PBS Phosphate-buffered saline
PC Phosphatidylcholine
PCA Passive cutaneous anaphylaxis
pCDM8 Eukaryotic expression vector
PCNA Proliferating cell nuclear antigen
PCR Polymerase chain reaction
PCT Porphyria cutanea tarda
p.d. Potential difference
PDBu 4α-phorbol 12,13-dibutyrate
PDE Phosphodiesterase
PDGF Platelet-derived growth factor
PDGFR Platelet-derived growth factor receptor
PE Phosphatidylethanolamine
PECAM-1 Platelet endothelial cell adhesion molecule-1; *also known as* CD31
PEG Polyethylene glycol
PET Positron emission tomography
PEt Phosphatidylethanolamine
PF_4 Platelet factor 4
PG Prostaglandin
PGAS Polyglandular autoimmune syndrome
PGD_2 Prostaglandin D_2
PGE1, PGE_2, PGF_2, $PGF_{2\alpha}$, PGG_2, PGH_2 Prostaglandins E_1, E_2, F_2, $F_{2\alpha}$, F_2, H_2
PGF, PGH Prostaglandins F and H
PGI_2 Prostaglandin I_2 *also known as* prostacyclin
P_aO_2 Arterial oxygen pressure
PGP Protein gene-related peptide
Ph^1 Philadelphia (chromosome)
PHA Phytohaemagglutinin
PHD PHD[8(1-hydroxy-3-oxo-propyl)-9,12-dihydroxy-5,10 heptadecadienic acid]
PHI Peptide histidine isoleucine
PHM Peptide histidine methionine
P_i Inorganic phosphate
PI Phosphatidylinositol
PI-3,4-P2 Phosphatidylinositol 3, 4-biphosphate
PI-3,4,5-P3 Phosphatidylinositol 3, 4, 5-trisphosphate
PI-3-kinase Phosphatidylinositol-3-kinase
PI-4-kinase Phosphatidylinositol-4-kinase
PI-3-P Phosphatidylinositol-3-phosphate
PI-4-P Phosphatidylinositol-4-phosphate
PI-4,5-P2 Phosphatidylinositol 4,5-biphosphate
PIP Phosphatidylinositol monophosphate
PIP_2 Phosphatidylinositol biphosphate
PK Protein kinase
PKA, PKC Protein kinases A and C
PKG cGMP-dependent protein kinase, protein kinase G
PL Phospholipase
PLA, PLA_2, PLC, PLD Phospholipases A, A_2, C and D
PLN Peripheral lymph node
PLNHEV Peripheral lymph node HEV
PLP Proteolipid protein
PLT Primed lymphocyte typing
PMA Phorbol myristate acetate
PMC Peritoneal mast cell
PMN Polymorphonuclear neutrophil
PMSF Phenylmethylsulphonyl fluoride
PNAd Peripheral lymph node vascular addressin
PNH Paroxysmal nocturnal hemoglobinuria
PNU Protein nitrogen unit
p.o. *Per os* (by mouth)
POBN α-4-pyridyl-oxide-*N-t*-butyl nitrone
PPD Purified protein derivative
PPME Polymeric polysaccharide rich in mannose-6-phosphate moieties
PRA Percentage reactive activity
PRD, PRDII Positive regulatory domain, -II
PR3 Proteinase-3
PRBC Parasitized red blood cell
proET-1 Proendothelin-1
PRL Prolactin
PRP Platelet-rich plasma
PS Phosphatidylserine
P-selectin Platelet selectin *formerly known as* platelet adctivation-dependent granule external membrane protein (PADGEM), granule membrane protein of MW 140 kD (GMP–140)
PT Pertussis toxin
PTCA Percutaneous transluminal coronary angioplasty
PTCR Percutaneous transluminal coronary recanalization
Pte-H_4 Tetrahydropteridine
PUFA Polyunsaturated fatty acid
PUMP-1 Punctuated metalloproteinase *also known as* matrilysin
PWM Pokeweed mitogen
Pyran divinylether maleic acid

q.i.d. *Quater in die* (four times a day)
QRS Segment of electrocardiogram

•**R** Free radical
R15.7 Anti-CD18 monoclonal antibody
RA Rheumatoid arthritis
RANTES A member of the IL8 supergene family (*R*egulated on *a*ctivation, *n*ormal *T e*xpressed and *s*ecreted)
RAST Radioallergosorbent test
RBC Red blood cell

RBF Renal blood flow
RBL Rat basophilic leukaemia
RC Respiratory chain
RE RE strain of herpes simplex virus type 1
REA Reactive arthritis
REM Relative electrophoretic mobility
RER Rough endoplasmic reticulum
RF Rheumatoid factor
RFL-6 Rat foetal lung-6
RFLP Restriction fragment length polymorphism
RGD Arginine–glycine–asparagine
rh- Recombinant human – (prefix usually referring to peptides)
RIA Radioimmunoassay
RMCP, RMCPII Rat mast cell protease, -II
RNA Ribonucleic acid
RNase Ribonuclease
RNHCl *N*-Chloramine
RNL Regional lymph nodes
ROM Reactive oxygen metabolite
RO· *The chemical symbol for* alkoxyl radical
ROO· *The chemical symbol for* peroxy radical
ROP Retinopathy of prematurity
ROS Reactive oxygen species
R-PIA *R*-(1-methyl-1-phenyltheyl)-adenosine
RPMI 1640 Roswell Park Memorial Institute 1640 medium
RS Reiter's syndrome
RSV Rous sarcoma virus
RTE Rabbit tubular epithelium
RTE-a-5 Rat tubular epithelium antigen a-5
r-tPA Recombinant tissue-type plasminogen activator
RW Ragweed

S Svedberg (unit of sedimentation density)
SALT Skin-associated lymphoid tissue
SAZ Sulphasalazine
SC Secretory component
SCF Stem cell factor
SCFA Short-chain fatty acid
SCG Sodium cromoglycate *also known as* DSCG
SCID Severe combined immunodeficiency syndrome
sCR1 Soluble type–1 complement receptors
SCW Streptococcal cell wall
SD Standard deviation
SDS Sodium dodecyl sulphate
SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM Standard error of the mean
SGAW Specific airway conductance
SHR Spontaneously hypertensive rat
SIM Selected ion monitoring
SIRS Soluble immune response suppressor
SIV Simian immunodeficiency virus
SK Streptokinase
SLE Systemic lupus erythematosus
SLe^x Sialyl Lewis X antigen
SLO Streptolysin-O
SLPI Secretory leucocyte protease inhibitor
SM Sphingomyelin
SNAP *S*-Nitroso-*N*-acetylpenicillamine
SNP Sodium nitroprusside
SOD Superoxide dismutase
SOM Somatostatin *also known as* somatotrophin release-inhibiting factor
SOZ Serum-opsonized zymosan
SP Sulphapyridine
SR Systemic reaction
sr Sarcoplasmic reticulum
SRBC Sheep red blood cells
SRS Slow-reacting substance
SRS-A Slow-reacting substance of anaphylaxis
STZ Streptozotocin
Sub P Substance P

T Thymus-derived
α-TOC α-tocopherol
$t_{1/2}$ Half-life
T84 Human intestinal epithelial cell line
TauNHCl Taurine monochloramine
TBA Thiobarbituric acid
TBAR Thiobarbituric acid-reactive product
TBM Tubular basement membrane
TBN di-*tert*-butyl nitroxide
tBOOH *tert*-butylhydroperoxide
TCA Trichloroacetic acid
T cell Thymus-derived lymphocyte
TCR T cell receptor α/β or γ/δ heterodimeric forms
TDI Toluene diisocyanate
TEC Tubular epithelial cell
TF Tissue factor
Tg Thyroglobulin
TGF Transforming growth factor
TGFα, TGFβ, TGFβ$_1$ Transforming growth factors α, β, and β_1
T_H T helper cell
T_H0 T Helper o
T_Hp T helper precursor
T_H0, T_H1, T_H2 Subsets of helper T cells
THP-1 Human monocytic leukaemia
Thy 1+ Murine T cell antigen
t.i.d. Ter in die (three times a day)
TIL Tumour-infiltrating lymphocytes
TIMP Tissue inhibitors of metalloproteinase
TIMP-1, TIMP-2 Tissue inhibitors of metalloproteinases 1 and 2
Tla Thymus leukaemia antigen
TLC Thin-layer chromatography
TLCK Tosyl-lysyl-CH_2Cl
TLP Tumour-like proliferation
Tm T memory
TNF, TNF-α Tumour necrosis factor, -α
tPA Tissue-type plasminogen activator
TPA 12-*O*-tetradeconylphorbol-13-acetate
TPCK Tosyl-phenyl-CH_2Cl
TPK Tyrosine protein kinases
TPP Transpulmonary pressure
TRAP Thrombospondin related anomalous protein
Tris Tris(hydroxymethyl)aminomethane
TSH Thyroid-stimulating hormone
TSP Thrombospondin
TTX Tetrodotoxin
TX Thromboxane
TXA_2, TXB_2 Thromboxane A_2, B_2
Tyk2 Tyrosine kinase

U937 (cells) Histiocytic lymphoma, human
UC Ulcerative colitis
UDP Uridine diphosphate
UPA Urokinase-type plasminogen activator
UTP Uridine triphosphate
UV Ultraviolet
UVA Ultraviolet A
UVB Ultraviolet B
UVR Ultraviolet irradiation
UW University of Wisconsin (preserving solution)

VAP Viral attachment protein
VC Veiled cells
VCAM, VCAM-1 Vascular cell adhesion molecule, -1, *also known as* inducible cell adhesion molecule MW 110 kD (INCAM–110)
VF Ventricular fibrillation
VIP Vasoactive intestinal peptide
VLA Very late activation antigen beta chain; *also known as* CD29
VLA α2 Very late activation antigen alpha 2 chain; *also known as* CD49b
VLA α4 Very late activation antigen alpha 4 chain; *also known as* CD49d
VLA α6 Very late activation antigen alpha 6 chain; *also known as* CD49f
VLDL Very low-density lipoprotein
***V* max** Maximal velocity
***V* min** Minimal velocity
VN Vitronectin
VO_4 *The chemical symbol for* vanadate
vp Viral protein
VP Vasopressin
VPB Ventricular premature beat

VT Ventricular tachycardia
vWF von Willebrand factor

W Murine dominant white spotting mutation
WBC White blood cell
WGA Wheat germ agglutinin
WI Warm ischaemia

XD Xanthine dehydrogenase
XO Xanthine oxidase

Y1/82A A monoclonal antibody detecting a cytoplasmic antigen in human macrophages

ZA Zonulae adherens
ZAS Zymosan-activated serum
zLYCK Carboxybenzyl-Leu-Tyr-CH_2Cl
ZO Zonulae occludentes

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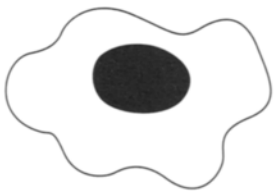
Key to Illustrations

Helper lymphocyte

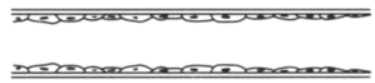
Suppressor lymphocyte

Killer lymphocyte

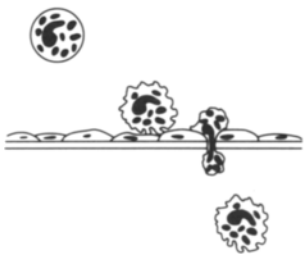
Plasma cell



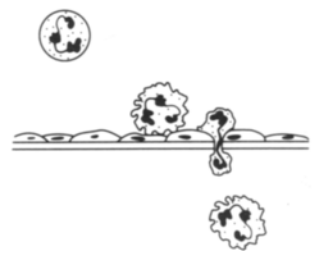
Bacterial or Tumour cell



Blood vessel lumen



Eosinophil passing through vessel wall



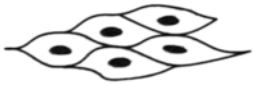
Neutrophil passing through vessel wall

Resting neutrophil

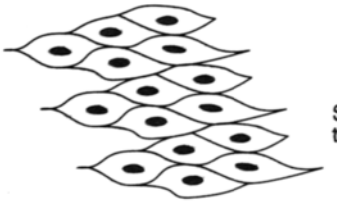
Activated neutrophil

Resting eosinophil

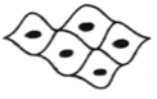
Activated eosinophil



Smooth muscle



Smooth muscle thickening



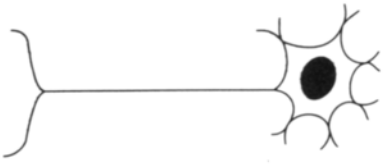
Smooth muscle contraction

Normal blood vessel

Endothelial cell permeability

Resting macrophage

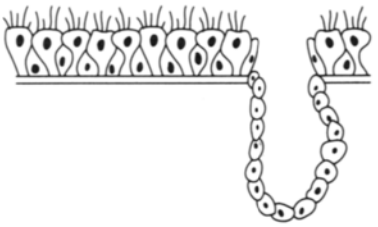
Activated macrophage



Nerve

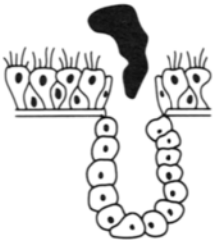
Intact epithelium

Damaged epithelium

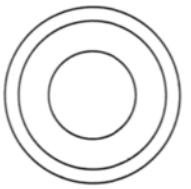


Intact epithelium with submucosal gland

Normal submucosal gland



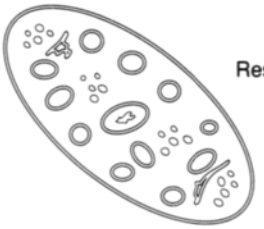
Hypersecreting submucosal gland



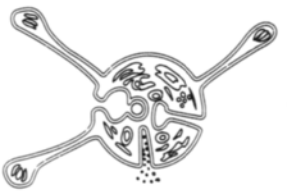
Normal airway

Oedema

Bronchospasm

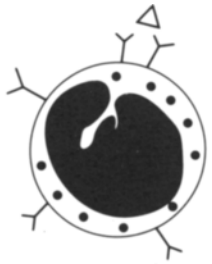


Resting platelet



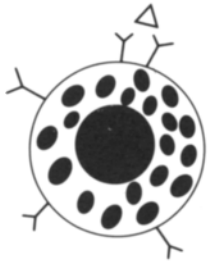
Activated platelet

Airway hypersecreting mucus



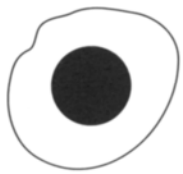
Resting basophil

Activated basophil

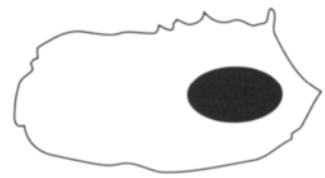


Resting mast cell

Activated mast cell



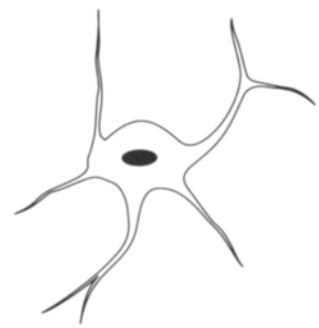
Resting chondrocyte



Activated chondrocyte

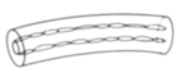
Cartilage

Fibroblast

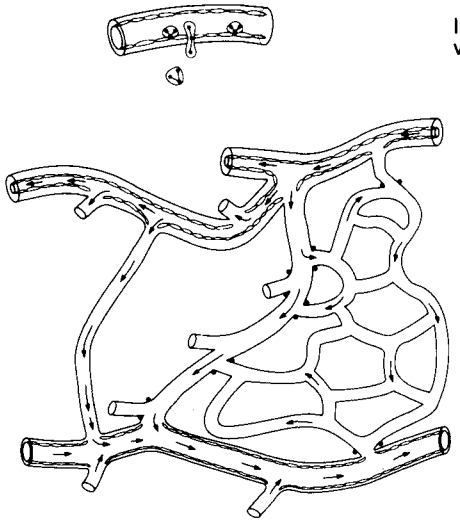


Dendritic cell/ Langerhans cell

Arteriole



Venule


Inflamed
venule
Microcirculatory
system

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Index

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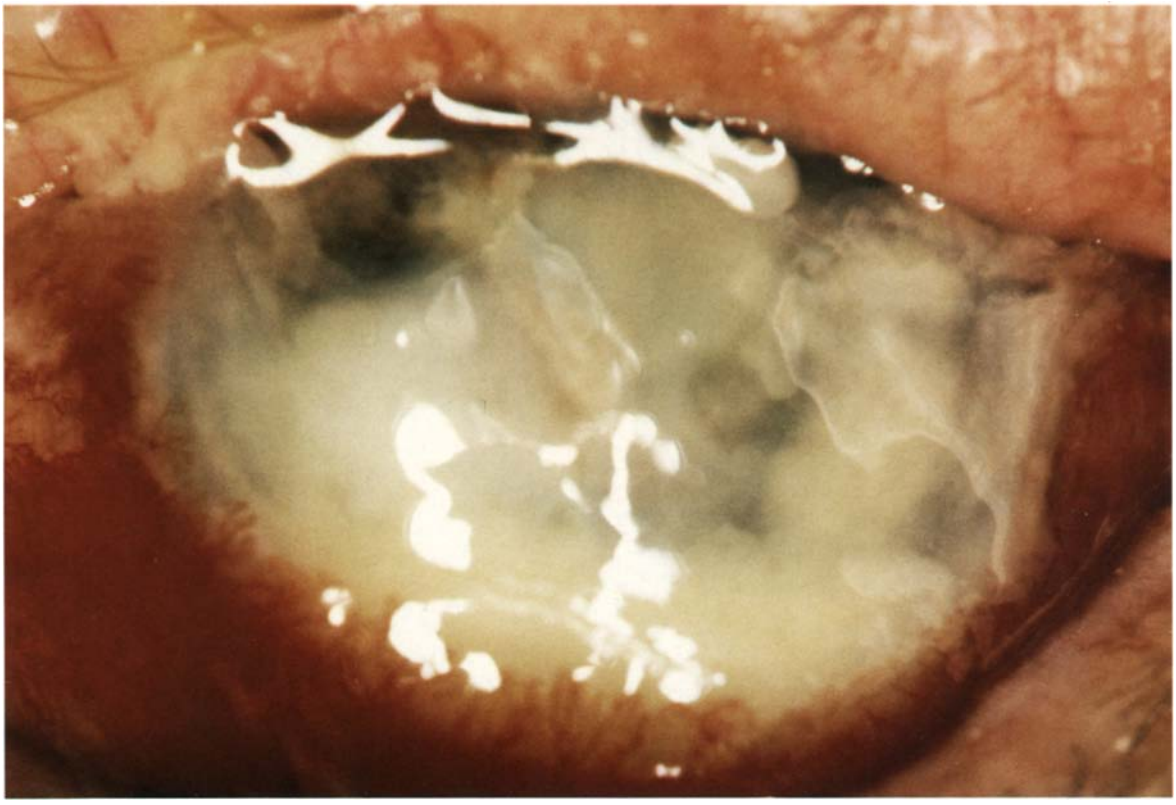

Plate 1 Inflammation of the cornea caused by severe bacterial infection, in this case, *Pseudomonas*.

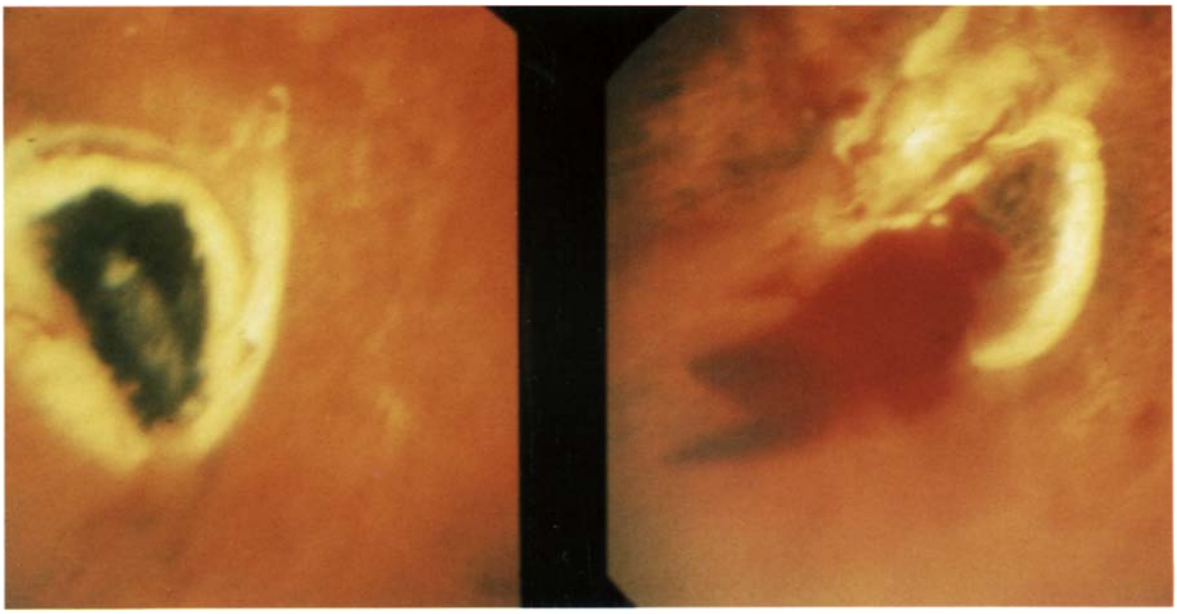

Plate 2 Iron foreign body embedded in the retina (left) and subsequent haemorrhage (right).

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