REVIEW

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Oxidative Stress in Microorganisms – I

Microbial *vs.* Higher Cells – Damage and Defenses in Relation to Cell Aging and Death

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ABSTRACT. Oxidative stress in microbial cells shares many similarities with other cell types but it has its specific features which may differ in prokaryotic and eukaryotic cells. We survey here the properties and actions of primary sources of oxidative stress, the role of transition metals in oxidative stress and cell protective machinery of microbial cells, and compare them with analogous features of other cell types. Other features to be compared are the action of reactive oxygen species (ROS) on cell constituents, secondary lipid- or protein-based radicals and other stress products. Repair of oxidative injury by microorganisms and proteolytic removal of irreparable cell constituents are briefly described. Oxidative damage of aerobically growing microbial cells by endogenously formed ROS mostly does not induce changes similar to the aging of multiplying mammalian cells. Rapid growth of bacteria and yeast prevents accumulation of impaired macromolecules which are repaired, diluted or eliminated. During growth some simple fungi, such as yeast or *Podospora* spp., exhibit aging whose primary cause seems to be fragmentation of the nucleolus or impairment of mitochondrial DNA integrity. Yeast cell aging seems to be accelerated by endogenous oxidative stress. Unlike most growing microbial cells, stationary-phase cells gradually lose their viability because of a continuous oxidative stress, in spite of an increased synthesis of antioxidant enzymes. Unlike in most microorganisms, in plant and animal cells a severe oxidative stress induces a specific programmed death pathway – apoptosis. The scant data on the microbial death mechanisms induced by oxidative stress indicate that in bacteria cell death can result from activation of autolytic enzymes (similarly to the programmed mother-cell death at the end of bacillar sporulation). Yeast and other simple eukaryotes contain components of a proapoptotic pathway which are silent under normal conditions but can be activated by oxidative stress or by manifestation of mammalian death genes, such as *bak* or *bax*. Other aspects, such as regulation of oxidative-stress response, role of defense enzymes and their control, acquisition of stress tolerance, stress signaling and its role in stress response, as well as cross-talk between different stress factors, will be the subject of a subsequent review.

CONTENTS

Abbreviations

EPR	electron paramagnetic resonance	LOO•, POO•	lipid or protein peroxyl radicals
FRAP	ferric reducing ability of plasma	LO•	alkoxyl radicals
GLR	glutathione reductase	MPT	mitochondrial permeability transition
GPx	glutathione peroxidase	P•	protein carbon-centered radicals
GSH	reduced glutathione	RNS	reactive nitrogen species
GSSG	oxidized dimeric glutathione	ROS	reactive oxygen species
LOOH, POOH	lipid or protein peroxides	SOD	superoxide dismutase

1 OXIDATIVE STRESS AS A BIOLOGICAL PHENOMENON

1.1 Introduction

In this review we try to characterize the effect of oxidative stress on microbial cells, both prokaryotic and eukaryotic, and compare the ways in which different microorganisms respond to oxidative damage. Because of the vast diversity of microbial species we concentrate only on the basic features of the process. To achieve this, we cannot avoid mentioning briefly the role of oxidative stress as a general biological phenomenon and discussing some aspects of its participation in physiological and pathophysiological processes in multicellular organisms, including the man. Microorganisms are useful models for studying various aspects of oxidative stress at biochemical, molecular biological and cellular levels because the nature of stress factors, and the damage caused by the oxidative stress to nucleic acids, proteins, lipids and other cell components are very similar in all types of organisms. Also the principles of cell defense against oxidative stress, *e.g.*, the nature and role of enzymes participating in decreasing ROS level, repair of damaged macromolecules (primarily DNA), and elimination of irreparable proteins are basically similar at all levels of cell organization. On the other hand, several aspects of the effect of oxidative stress on animal and plant cells differ from those found in microbial cells. This holds mainly for the regulation of the response to ROS through the synthesis of corresponding enzymes which involves different mechanisms of gene expression control. Also the senescence phenomena which are considered to be one of the consequences of a continuous endogenous oxidative stress on animal cells, are absent or proceed in a different way in microorganisms. In addition, the death pathways eliminating irreparably damaged microbial cells, and those of multicellular organisms, are not alike. Comparison of these aspects is therefore the subject of one chapter of this review.

The notion that oxygen exerts toxic effects through the generation of free radicals was very probably proposed for the first time by Gershman *et al.* (1954) and by Harman (1956) (*see* Sohal and Allen 1990). The term "oxidative stress" was coined in 1978 by Fridovich (Krems *et al.* 1996). It was defined as a disturbance in the cell or organism prooxidant–antioxidant balance in favor of the former (Sies 1993). Many external oxidative stresses, such as hyperbaric oxygen, γ-radiation, near-UV radiation, trioxygen (ozone), peroxides and redox-cycling drugs (Imlay and Linn 1988) have toxic consequences for both prokaryotic and eukaryotic cells.

1.2 Primary and secondary oxidative stress factors

Oxidative stress differs from many other stresses in that its primary effectors, the reactive oxygen species (ROS), can arise largely in the course of normal cell metabolism. Over a life span of 70 years, an average human consumes about 17 Mg (*i.e.* 17 tons) of oxygen, out of which a non-negligible part is converted into oxygen radicals (Esterbauer 1993). In the course of O_2 reduction to 2 H_2O and oxidation of organic substances to CO_2 during the cell energy-yielding reactions, up to 2–3 % of the oxygen molecules are reduced only partially, giving rise to different reactive oxygen species (Ames *et al.* 1993). Cells of aerobic organisms therefore cannot avoid continuous exposure to ROS, and hence a continuing damage to their components by oxidative stress during their life. Since the properties of ROS are crucial for the type, course and outcome of oxidative stress, *Chapter 2* describes the physical and chemical characteristics of basic ROS in some detail. Primary oxidants, successively formed as by-products of energetic metabolism, are the superoxide radical, hydrogen peroxide and hydroxyl radical. Hydroperoxyl radical (HOO•) is formed from superoxide under acidic conditions (Farr and Kogoma 1991) (Fig. 1). All these primary oxidative stressors can generate additional secondary reactive oxygen metabolites which also cause extensive oxidative damage to cell organelles, such as mitochondria, cell membranes or nuclei, and also to both soluble and bound enzymes (Stadtman 1992; Ames *et al.* 1993; Sohal and Weindruch 1996). Many secondary radicals (hydroperoxides, alkoxyl and peroxyl radicals, epoxides or aldehydes) are the products of lipid peroxidation (Sies 1986; Rikans and Hornbrook 1997) (*Chapter 6.2*) and protein oxidation (*Chapter 6.3*).

Hydrogen peroxide is also generated as a by-product in peroxisomes during the β-oxidation of fatty acids. Different ROS can also be produced by higher plants, *e.g.*, during detoxication of man-made agrochemicals which act as xenobiotics. Production of ROS can also be induced by some anticancer drugs (Simizu *et al.* 1998).

The most prominent among exocellular oxidant stressors, which can also cause oxidative damage, is probably trioxygen (ozone; O_3), which is decomposed to HO• and HOO• radicals and can react with organic compounds to produce ozonides (cyclic peroxides) which can further decompose to, *e.g.*, R^1–CH(O•)–CH(OO•)–R^2 radicals or aldehydes (Halliwell and Gutteridge 1989). Also nitric oxide, NO, which is involved in signal transfer, and hypochlorous acid (HClO), used in disinfection, induce oxidative damage and can therefore be included among oxidative stress factors (Dukan *et al.* 1996; Mohr *et al.* 1997).

The main targets of oxygen radicals are DNA, lipids and proteins. Oxidative damage of DNA involves single strand breaks and base alterations which can induce mutations. The number of oxidative hits per day is high – 10^4/d in humans and 10^5/d in rats – because the metabolic activity of rat cells is substantially higher (Ames *et al.* 1993). Mitochondrial DNA damage which may result in degradation of the whole macromolecule is much greater than that of nuclear DNA because mitochondria themselves are generators of oxygen radicals. Protein oxidation converts several amino acid residues into their carbonyl derivatives, induces S–S and Tyr–Tyr cross-linking (Stadtman 1992) – *for details see Chapter 6.3.* In aged animals up to 30 % of proteins can suffer oxidative damage (Smith and Pereira-Smith 1996).

Peroxidation of lipids generates secondary oxygen radicals which multiply the toxic effect of the primary stress source. The secondary ROS affect mainly proteins in the proximity of lipids.

2 FORMATION, INACTIVATION AND DETERMINATION OF ROS

2.1 Primary ROS

The term reactive oxygen species (ROS) covers oxygen molecules in different reduction and/or excitation states, and compounds of oxygen with hydrogen, chlorine and nitrogen. The generation of the most common primary ROS by one-electron reduction of oxygen is shown schematically in Fig. 1 (*for more details see, e.g.*, Halliwell and Gutteridge 1989; Gille and Sigler 1995; Khan and Wilson 1995). Oxidants are formed as normal products of aerobic metabolism but can be produced at elevated levels under pathophysiological conditions (Sies 1997). They are also generated by different types of radiation – X-radiation generates the hydroxyl radical (HO•) while UV-irradiation produces a high-energy (and therefore highly reactive though nonradical) form of oxygen called singlet oxygen ($^1O_2$; *see below*)*. Ultrasound and microwave radiation, and even shear stress can also produce ROS.

Ordinary oxygen, which is energetically in the ground triplet state ($^3O_2$; cf. Halliwell and Gutteridge 1989), does not react very avidly with normal biomolecules but reacts readily with radicals. On acquiring energy from chemical or photochemical reactions it becomes singlet oxygen $^1O_2$, (*reaction 1* in Fig. 1). $^1O_2$ has a lifetime of about 2 μs in water and it interacts with target molecules either by transferring its excitation energy, or chemically. It reacts very rapidly and selectively with unsaturated biomolecules (oligounsaturated fatty acids or guanine bases in DNA), yielding labile peroxides and hydroperoxides. It is generated, *e.g.*, in the reaction of H_2O_2 or other peroxides with peroxynitrite (Di Mascio *et al.* 1994), with O_2^-• (Khan and Kasha 1994), or with hypochlorite, and this production forms the basis of the killing of bacteria or viruses by activated macrophages and neutrophils. It is efficiently quenched by water and by superoxide, and also by vitamin C and superoxide dismutase (SOD), by vitamin E (acting at the polar–nonpolar interface) and β-carotene (in the lipid phase).

The first one-electron reduction of oxygen (*reaction 2* in Fig. 1) yields the superoxide radical, O_2^-•, a weak base with low reactivity with most biomolecules and a weak oxidant at neutral pH. O_2^-• does not pass through membranes and does not peroxidate lipids in lipophilic cell constituents, but it can reduce transition metal ions such as Fe^{3+}. The reduction is mediated by enzymes such as NADPH oxidase or xanthine oxidase, or by constituents of the respiratory chain. In mitochondria, O_2^-• is formed by reduction of dioxygen by ubisemiquinone (in complex III) and by NADH dehydrogenase (complex I). Ubisemiquinone seems to serve as the major electron donor responsible for 80 % of the O_2^-•, the rest being due to the NADH dehydrogenase flavoprotein (Liu 1997). Under physiological conditions the daily yield of O_2^-• may reach some 3×10^7 per

*It should be noted that the symbols $^1O_2$ and $^3O_2$ are combinations of the common chemical nomenclature and term symbols for atoms introduced by Russel and Saunders (West 1956).

mitochondrion but its scavenging by Mn-SOD and other enzymes brings its steady-state *in vivo* concentration to 10 pmol/L.

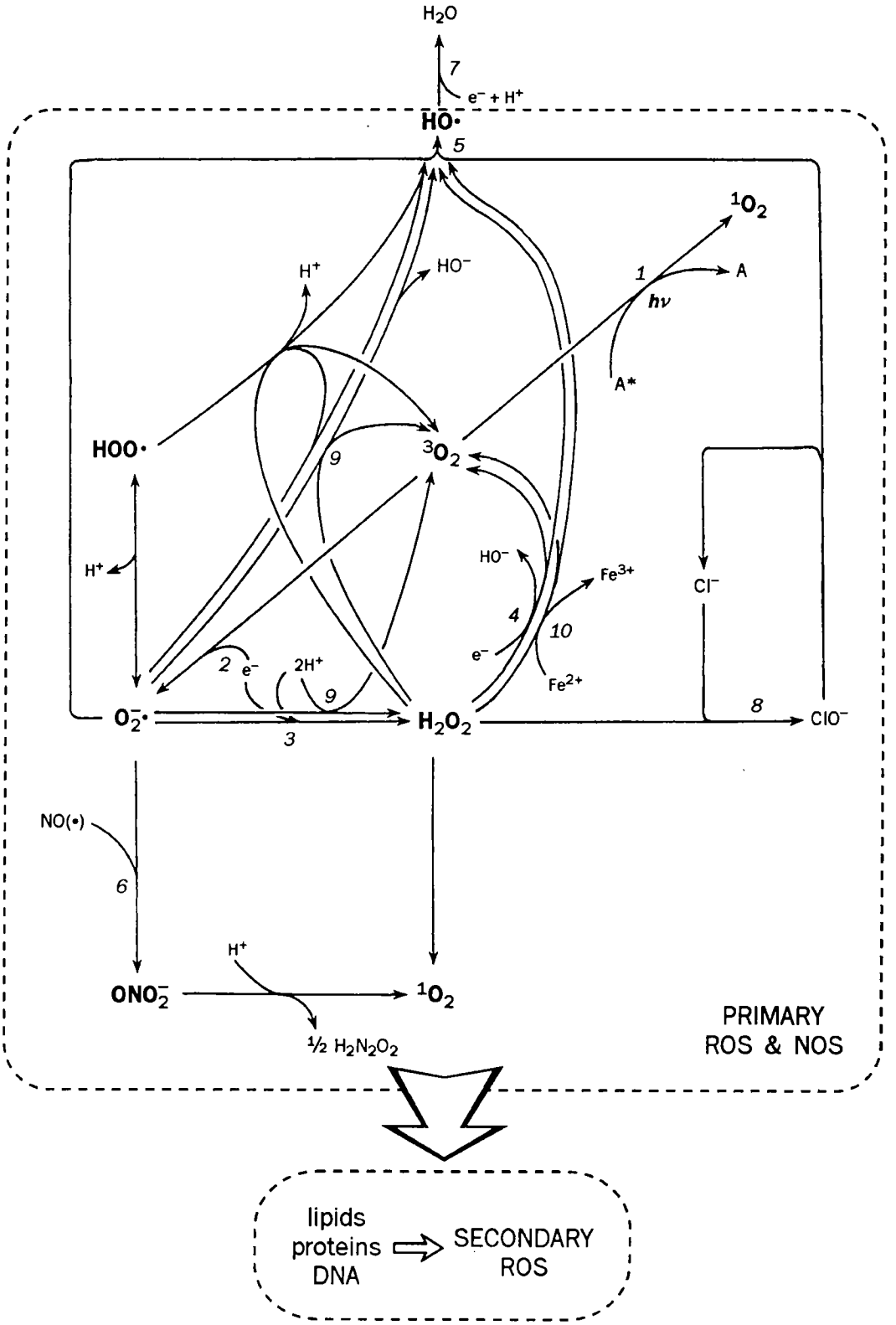


Fig. 1. Some reactions yielding reactive oxygen (ROS) and nitrogen (RNS); reactions *1–8 see text*; reaction *9* – Haber–Weiss chemistry; reaction *10* – Fenton chemistry; adapted from Farr and Kogoma (1991); Ames *et al.* (1993); Khan and Wilson (1995); Sohal (1996); Henle and Linn (1997); Saran *et al.* (1998).

The superoxide radical is also generated by autoxidation of intracellular compounds such as ubiquinols, catechols, flavins and others. At pH below 4.8, it is present as protonated hydroperoxyl radical (HOO•). Its spontaneous dismutation in water, which yields H_2O_2 and also $^1O_2$, proceeds at a very high rate, yet in the cell the dismutation is additionally catalyzed by SOD. This rapid removal of O_2^-• is very important for the cell because superoxide can give rise to very reactive ROS, such as H_2O_2, HO• or singlet oxygen; the peroxynitrite anion ($ONOO^-$) is formed when O_2^-• reacts with NO. Despite its low reactivity, O_2^-• has been shown to inhibit antioxidant enzymes, such as glutathione peroxidase, and partially also catalase (Halliwell and Gutteridge 1986). It also inhibits mitochondrial enzymes, such as NADH dehydrogenase, NADH oxidase and mitochondrial ATPase (Zhang *et al.* 1990), and may be involved in cyanide-resistant respiration – a branch of respiratory chain not associated with ATP formation at the level of the ubiquinone pool, found in plant and animal mitochondria, and also in yeasts (*e.g.*, *Rhodotorula glutinis*; Janda and Tauchová 1982).

Superoxide can react directly with iron–sulfur clusters in enzymes, such as aconitase and other hydrolyase enzymes containing 4Fe–4S clusters (Valentine *et al.* 1998 *and references therein*). The reaction of O_2^-• with these centers inactivates such enzymes both *in vitro* and *in vivo* and leads to increased levels of intracellular free iron conducive to ROS-induced cell injury. It thus represents, together with O_2^-• reaction with NO, mechanisms of ROS generation independent of the Fenton reaction (*see below*; Winterbourn 1995).

Superoxide dismutation yields hydrogen peroxide (*reaction 3* in Fig. 1). The basic form of hydrogen peroxide, HO_2^-, arises by dismutation of superoxide and the hydroperoxyl radical (HOO•). At neutral or acidic pH, hydrogen peroxide exists predominantly as fully protonated H_2O_2. Its molecule therefore carries no charge and can freely penetrate membranes and diffuse throughout the cell. This long-range action is increased by its ability to form adducts with various cell constituents. Hydrogen peroxide is a better oxidant than O_2^-• and HOO• and has been suggested to act also as a signaling molecule (Khan and Wilson 1995). In cells under physiological conditions, H_2O_2 levels are kept low (1–100 nmol/L) through the action of catalase and the nonheme, non-free-radical-forming glutathione peroxidase.

The oxidative damage caused by H_2O_2 has been attributed to its reactions with transition metals (*see below*) or with heme proteins, such as cytochrome *c*. These reactions may involve formation of protein-centered radicals and ferryl radicals (*see below*), which then catalyze oxidative processes, such as lipid peroxidation (Barr *et al.* 1996). H_2O_2 reacts very fast with various peroxidases and the reaction gives rise to free radicals derived from the other peroxidase substrate (Mason 1997). As stated above, H_2O_2 may act relatively far from the site of its production owing to its ability to form adducts (hydrogen-bonded chelate structures) with many biomolecules, such as histidine, alanine, glycine, aspartic acid, succinic acid or DNA bases, which act as H_2O_2 carriers (Schubert and Wilmer 1991). These adducts facilitate the penetration of H_2O_2 through membranes and considerably delay its decomposition, thereby enhancing its oxidative effects.

The highly reactive hydroxyl radical (HO•) is produced *in vivo* by high-energy (X-ray) irradiation of water or UV-irradiation of H_2O_2 (Stahl and Sies 1997). It is also generated in several biologically important reactions. Thus, activated neutrophils, which release hypochlorite and superoxide, may exert their cytotoxic effects on infecting microorganisms by forming HO• in the following rapid reaction (Candeias *et al.* 1993):

$$O_2^-\bullet + HOCl \rightarrow HO\bullet + O_2 + Cl^- \text{ (\textit{reaction 5} in Fig. 1).}$$

Reaction of hydrogen peroxide with transition metal ions such as Fe^{2+}, known as the Fenton reaction, also yields (HO•):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\bullet \text{, or}$$

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + H_2O + HO\bullet \text{ (\textit{reaction 4} in Fig. 1)}$$

Although the rate of the reaction is low (<100 L mol^{-1} s^{-1}), the reactivity of the ensuing HO• is such that it will react very fast (10^9–10^{10} L mol^{-1} s^{-1}) with practically every biological molecule. Its lifetime is thus very short (<1 ns). It acts either by abstracting hydrogen or adding to a double bond, and often initiates chain reactions. Its propensity to add to aromatic rings makes aromatic amino acids of proteins its prime targets. Some of the products, *e.g.*, *o*-tyrosine arising by (HO•)-induced hydroxylation of phenylalanine, can be used for its detection. It also adds to double bonds of DNA bases, producing other biomarkers of oxidative stress – thymine glycol (Karam *et al.* 1991; *see* p. 602) or 8-hydroxy-2′-deoxyguanosine (Kaneko *et al.* 1996).

When iron is complexed to a biological ligand L, the reaction with H_2O_2 may have the form

$$L\text{-}Fe^{2+} + H_2O_2 \rightarrow L\text{-}Fe(H_2O_2)^{2+},$$

$$L\text{-}Fe(H_2O_2)^{2+} \rightarrow L\text{-}Fe^{3+} + HO\bullet + OH^- \text{ or, alternatively,}$$

$$L\text{-}Fe(H_2O_2)^{2+} \rightarrow L\text{-}Fe^{4+} + 2\ OH^- + R \rightarrow L\text{-}Fe^{3+} + HRO\bullet + OH^-$$

$Fe(H_2O_2)^{2+}$ and Fe^{4+} are regarded as iron(IV) or ferryl species (Winterbourn 1995), which is charge-equivalent to an Fe^{3+}-complexed HO•, or $(FeO)^{2+}$. The nature of the chelator is the major determinant of the reaction product (HO•, L-Fe^{4+} or HRO•). The ferryl ion arises also in the reaction of some heme proteins with H_2O_2 (Catalano *et al.* 1989).

Reaction of O_2^-• with NO (*reaction 6* in Fig. 1) yields peroxynitrite $ONOO^-$, a very strong oxidant, stable under physiological conditions, with a long lifetime. It induces lipid peroxidation in lipoproteins, may impair phosphorylation and interfere with cellular signaling by nitrating tyrosine residues in proteins (Stahl and Sies 1997 *and references therein*). On decomposing it may yield $^1O_2$.

The strong oxidant hypochlorite (ClO^-) is generated from H_2O_2 and Cl^- by enzymes such as myeloperoxidase (*reaction 8* in Fig. 1). It peroxidizes and/or chlorinates many biological molecules. Hypochlorous acid generated by, *e.g.*, activated neutrophils may also be a source of hydroxyl radicals (Candeias *et al.* 1994):

$$HClO + Fe^{2+} \rightarrow HO\bullet + Cl^- + Fe^{3+}.$$

2.2 *Secondary radicals and stress-inducing species*

The reaction of primary ROS with major cell constituents – DNA, lipids and proteins – gives rise to a large number of secondary radicals that can further damage the cell (cf. *Chapter 6*). However, one should realize that even low-molar mass substances such as metabolic substrates, buffer components, *etc.*, can succumb to oxidative changes and yield products that may injure the cell. Thus glucose, the most widely used metabolic substrate in microorganisms, is known as radical scavenger (Gutteridge 1984). However, under physiological conditions, glucose can undergo autoxidation to yield free radicals (Donnini *et al.* 1996 *and references therein*). The resulting cell damage may be especially noticeable in cells with decreased GSH levels, as the GSH redox cycle is a key step in the cellular scavenging of glucose-generated free radicals (cf. *Chapter 4.1*).

Ascorbate, which at high concentrations can act as an antioxidant, behaves as a pro-oxidant at low concentrations (Nordmann *et al.* 1990). The pK_a of ascorbic acid is 4.2 and at pH 7.4 and under physiological conditions it is present predominantly as a mono-anion (Noguchi *et al.* 1997). In the presence of transition metal ions, it takes part in redox cycling giving rise to ROS (Haberland *et al.* 1996). Likewise, uric acid, an end product of purine metabolism in mammals, which is considered a natural physiological antioxidant, causes unspecific DNA breaks in the presence of transition metals; this action is enhanced by visible light (Shamsi and Hadi 1995).

Under certain conditions (during the autoxidation of iron), radicals are formed from the piperazine ring-based buffers Hepes, Epps and Pipes but not from Mes which contains a morpholine ring (Grady *et al.* 1988). At the same time, Hepes has been found to be an effective hydroxyl radical scavenger.

2.3 *Radical-forming compounds*

Paraquat and other redox active drugs, *e.g.*, menadione (2-methyl-1,4-naphthoquinone), its derivative plumbagin and ubiquinone (Fig. 2) are used in model experiments to produce nonenzymically the superoxide anion.

Iron(II)–ascorbate mixture is often used to induce lipid peroxidation. The optimum rate of *in vitro* lipid peroxidation was found to decrease with decreasing ambient pH (Haberland *et al.* 1996). Owing to the insensitivity of the reaction to superoxide dismutase, catalase, and radical scavengers, such as mannitol or α-tocopherol, and difficulties in EPR detection the nature of the radicals formed by the reaction and causing the lipid peroxidation is not known.

Among other systems used to experimentally induce oxidative stress are, *e.g.*, xanthine–xanthine oxidase, glucose–glucose oxidase, transient metals–chelators, transient metals–H_2O_2 mixtures, paraquat, t-butylated hydrogen peroxide, cumene hydroperoxide (Haberland *et al.* 1996) and others.

A highly promising group of compounds that have lately been often used for generating radicals in both the aqueous and the lipid phase of the cell comprises azo compounds (diazenes), which decompose unimolecularly to yield nitrogen and two carbon-centered radicals R• (Niki 1990):

$$R\text{–}N{=}N\text{–}R \rightarrow 2\ R\bullet + N_2.$$

Some undergo recombination but many of them diffuse apart and react rapidly with O_2 to yield peroxyl radicals. Formation of R• by azos is rapid so that the peroxyl radical HROO• is usually taken to be the initiating species of lipid peroxidation. The rate of decomposition of azo compounds is given primarily by

temperature, to some extent by medium pH, and the efficiency of R• generation depends also on medium viscosity.

At 37 °C and neutral pH the half-life of the best known azo compound AAPH (*see* Fig. 2) is about 175 h and the rate of R• generation is thus practically constant for the first several hours. The R• generated from AAPH induce chain oxidation of lipids in micelles, liposomes, or membranes. Azos are useful for studying quantitatively the (R•)-induced damage. The radicals can be generated at a constant rate at a specific site, the rate of R• formation can be measured and controlled. The azo compound used for eliciting lipid peroxidation may be chosen according to the desired purpose (Hanlon and Seybert 1997; Noguchi *et al.* 1997).

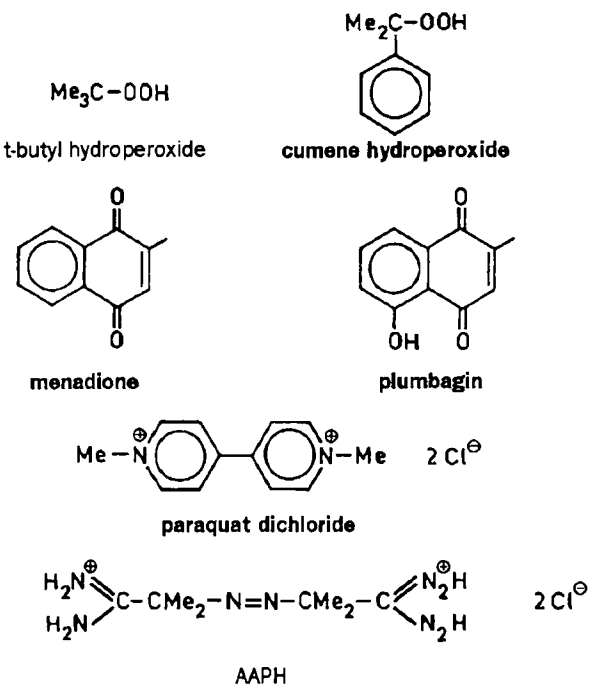


Fig. 2. Some compounds used as ROS generators.

Among agents used originally for other purposes but exhibiting pro-oxidant activities on simple eukaryotes, such as yeasts, are, *e.g.*, some disinfectants (Fujita *et al.* 1995) or tumor inducers, such as 7,12-dimethylbenz[*a*]anthracene (Frenkel *et al.* 1995).

3 ROLE OF TRANSITION METALS AND THEIR CHELATES IN ROS FORMATION

All oxygen reactions, whether slow (autoxidations of organic materials) or fast (combustion), have to be initiated by some catalytic, activating step. In practice, virtually all oxidation reactions proceed through metal catalysis (Saran and Bors 1991), most often mediated by transition metal ions (*for review see* Greenwood and Earnshaw 1993). The most important ones in terms of ROS generation are Fe and Cu, and the heavy metal Cd.

The radical-forming ability of transition metals in the presence of, *e.g.*, H_2O_2, varies depending on their complexation or chelation which may prevent rapid autoxidation of transition metal ions, such as Fe^{2+} and, at physiological pH, may increase the solubility of the metal ion (Cohen 1985). However, if the chelation lowers the oxidation potential of the ion, the ability of the resulting chelate to form radicals is higher than that of unchelated ion. Thus the Fenton reaction in the presence of the Fe-EDTA complex runs much faster than with iron alone, whereas the chelation of Cu with EDTA prevents the reaction (which is normally 70 times faster than with iron). The catalytic efficiency of metal chelates depends on the metal–chelator concentration ratio and the type of binding. When, *e.g.*, the chelate leaves one of the coordination sites of the metal free and accessible to binding of water, the catalytic activity is usually enhanced, whereas complexation that leaves no coordination bonds free usually lowers it (iron chelated by deferroxamine; *e.g.*, Klebanoff *et al.* 1989). As stated above (*Chapter 2.1*), the nature of the ligand may also affect the mechanism of reaction of the metal center with H_2O_2 (Luzzatto *et al.* 1995).

Many cell constituents can form chelates with iron. Iron ions can be loosely attached to phosphate compounds, such as ATP, saccharides and organic acids (citrate, picolinate), DNA or lipids, and can be tightly bound to nonheme proteins, ferritin, hemosiderin, transferrin, and to heme proteins, such as cytochrome *c*. Transferrin, the iron-binding and -transporting protein of animal cells, is a 80-kDa glycoprotein with two high-affinity binding sites for Fe^{3+}. Ferritin, a storage protein, has a molar mass of about 450 kDa and is composed of 24 equivalent subunits arranged around an inner core. One molecule of ferritin can store up to 4500 iron atoms as ferric hydroxide–phosphate inside the core (Galey 1997). Bacterioferritin, the iron storage protein of bacteria, is also a multimeric protein shell sequestering iron in its core together with phosphate. A ferritin-like molecule was purified also from iron-loaded *S. cerevisiae*, but its iron content was very low (Raguzzi *et al.* 1988).

3.1 Metal transport and storage, metal-induced oxidative injury

As stated above, in the presence of dissolved oxygen the iron present in cells and tissues can cause oxidative modifications of cell proteins and bring about oxidative stress (Taborsky 1973; Jimenez Del Rio *et al.* 1993). For this reason, the level of Fe^{2+} (or Cu^{2+}) in cells is carefully controlled and excess amounts of these ions are sequestered, chelated, expelled or otherwise rendered harmless. The transport and storage of these metal ions is therefore extremely important for the maintenance of cell integrity. Microbial cells have a large variety of mechanisms ensuring transition metal homeostasis, which will be discussed here in some detail.

An important step in the homeostasis is the control of metal uptake. The free equilibrium concentration of Fe^{3+} at neutral pH is very low (Adjimani and Owusu 1997 *and references therein*) and microorganisms have to find ways to scavenge iron from the external medium even at very low concentrations.

Bacteria under iron-deficient conditions produce siderophores, specific iron chelators that solubilize and transport Fe^{3+} in aqueous media (Galey 1997). Most siderophores are hydroxamates (*e.g.*, ferrichrome, deferrioxamine, rhodotorulic acid, pseudobactin and mycobactins) or catecholates (*e.g.*, enterobactin and agrobactin; *for review see* Albrecht-Gary and Crumbliss 1998). A major natural antioxidant in plants, phytic acid, has also been found to act as iron chelator (Galey 1997 *and references therein*).

Many bacteria including *E. coli* take up Fe^{3+} as ferric siderophore complexes (Pressler *et al.* 1988; Kim *et al.* 1997). The synthesis of the siderophores and the related iron transport systems in *E. coli* and other bacteria (Daniel *et al.* 1999) are controlled by the Fur (ferric uptake regulation) regulatory protein, which suppresses iron uptake (Bagg and Neilands 1987). The transport of iron-siderophore complexes across the outer membrane of *E. coli* and related species is energy-dependent, involves the TonB, ExbB and ExbD inner membrane proteins (which may also transport vitamin B_{12} and the antibacterial proteins colicins) and requires highly specific receptors. In contrast, further translocation of these siderophores is less specific and is mediated by a single system following a periplasmic binding protein (PBP)-dependent action of ABC-type ATPases (Higgins 1992).

Pathogenic bacteria have developed highly sophisticated iron assimilation systems in response to iron-limiting conditions encountered in the host's body fluids. In mammalian cells, iron is sequestered by Fe-binding proteins, such as transferrin and lactoferrin, and bacteria possess a variety of mechanisms for acquiring Fe from the host. The mechanisms often involve the action of siderophores secreted from the cells and capable of binding Fe^{3+} with high affinity (Moore *et al.* 1995; Brickman and Armstrong 1996; Fouz *et al.* 1997). Some bacteria may obtain Fe directly from transferrin or lactoferrin in a siderophore-mediated process; other bacteria do not synthesize soluble siderophores, but they can utilize transferrin and lactoferrin, but also hemin, hemoglobin, hemoglobin–haptoglobin complexes and some siderophores produced by other bacteria as iron sources for growth (McKenna *et al.* 1988; Chen *et al.* 1996; Biswas *et al.* 1997).

In many Gram-negative species ferric siderophores are internalized with the aid of specific outer membrane receptors that facilitate Fe transport through the otherwise impermeable outer membrane. Ligand-dependent gated porins are the entities transporting the iron-siderophore complexes, which cannot traverse the outer membrane, *e.g.*, *via* the open channels of general porins. Many bacteria are also capable of taking up ferric siderophore complexes secreted by other organisms (Beall and Sanden 1995).

Some bacteria excrete pyoverdin-type siderophores, which are not universally recognized, but their assimilation requires a specific recognition by an iron-repressible outer membrane protein (IROMP) (Castignetti 1997 *and references therein*).

The removal of ferric ion from the siderophores can involve esterase-catalyzed iron removal, exchange of the metal for another ligand, reductive removal catalyzed by ferrisiderophore reductases which use NAD(P)H as reducing agents, or the involvement of a stronger iron chelator (Adjimani and Owusu 1997).

The iron acquisition systems of Gram-positive bacteria are less known. Iron uptake systems have been described in, *e.g.*, *Staphylococcus aureus*, *Bacillus subtilis*, *B. megaterium* and *Corynebacterium diphtheriae*. *Streptococcus* species do not produce siderophores while *Listeria* species use an inducible citrate uptake mechanism and a surface-bound reductase (Coulanges *et al.* 1998 *and references therein*).

Yeasts such as *S. cerevisiae* do not excrete siderophores although they were also found to release metal-complexing substances (*see below*) and *S. cerevisiae* can also use siderophores excreted by other microorganisms, such as ferrioxamine B (Lessuise *et al.* 1998).

S. cerevisiae has two major systems for iron uptake. The high-affinity uptake system includes plasma membrane Fe^{3+} reductases Fre1p and Fre2p which reduce extracellular Fe^{3+} to Fe^{2+}; a multicopper ferroxidase Fet3p, which facilitates iron uptake by catalyzing oxidation of the reductase-generated Fe^{2+} to Fe^{3+} by O_2; and iron transporter Ftr1p which actually transports the iron into the cell. The expression of these functions depends on the degree of cell aerobiosis (Hassett *et al.* 1998). The low-affinity iron-uptake

system requires the integral membrane Fet4 protein that appears to be the actual Fe^{2+} transporter (Dix *et al.* 1997). The iron reduction and uptake may be facilitated by reducing and/or chelating compounds (anthranilic acid, 3-hydroxyanthranilic acid) excreted by the cells into the medium (Lesuisse *et al.* 1992). Once inside the cell, the iron is distributed among the vacuole as a storage compartment, the mitochondria (Li and Kaplan 1997) and the cytosol. Yeasts do not produce ferritin, which is the most common iron storage protein in most eukaryotes (Eide 1998). In some regulatory proteins, iron is bound in Fe–S clusters, which have been suggested to act as sensors for oxygen, superoxide, nitric oxide or other oxidants (Hidalgo *et al.* 1997).

Thus iron, which has to be reduced before its uptake, finally enters the cells as Fe^{3+}. Iron toxicity in the cell thus may depend not only on the amount of the metal taken up, but also on the redox status of the cell. In the presence of oxygen, Fe^{2+} may undergo autoxidation

$$2\,Fe^{2+} + O_2 + 2\,H^+ \rightarrow 2\,Fe^{3+} + H_2O_2\,.$$

Reaction of the resulting hydrogen peroxide with another Fe^{2+} then yields HO• (*see* Fenton reaction in *Chapter 2.1*). The intracellular Fe^{3+} may react with O_2^-• generated during metabolic processes,

$$Fe^{3+} + O_2^-\bullet \rightarrow Fe^{2+} + O_2\,.$$

The net result is a redox iron cycling that produces ROS (Wiśnicka *et al.* 1998).

Copper transport is tightly intertwined with iron transport. The copper that is taken up by the cell is bound by cytosolic copper molecular chaperones that deliver it to the cytosolic CuZn-SOD, to mitochondria or to a vesicular copper transporter, which effects the copper loading of Fet3p (Askwith and Kaplan 1998).

Heavy metal ions, such as Cd^{2+}or Cu^{2+}, strongly affect cell and organelle membranes. In aerobic *S. cerevisiae* cultures, Cu^{2+} causes a strong increase in cytoplasmic CuZn-SOD but not in mitochondrial Mn-SOD (Lee *et al.* 1996). Copper also elicits a rapid permeabilization of the plasma membrane that causes release of amino acids and cell K^+, while the permeability of the vacuolar membrane is not altered (Ohsumi *et al.* 1988). This effect was ascribed to a heavy-metal-induced lipid peroxidation (Howlett and Avery 1997). Cd^{2+}, although not redox-active, also induces strong K^+ efflux and membrane fluidization in *Schizosaccharomyces pombe*, and also affects the function of plasma membrane H^+-ATPase (Aßmann *et al.* 1996). These effects may be due to induction of oxidative stress (Brennan and Schiestl 1996).

3.2 Metal detoxication

Metal ion detoxication, which is one of the primary means of protection of cells against metal-catalyzed generation of ROS, is closely linked with metal-ion homeostasis. The uptake of transient metals in bacteria is under strict control. In *E. coli*, the induction of oxidative stress response is combined with suppression of Fe^{3+} uptake (Zheng *et al.* 1999) and the resistance of bacteria to toxic metals is based on their efflux from cells (Xu *et al.* 1998). In yeasts, maintenance of transition- and heavy-metal homeostasis includes chelation by GSH, small metal-binding peptides phytochelatins $(\gamma\text{-Glu–Cys})_n$–Gly and the small cysteine-rich proteins metallothioneins which serve both for removing excess ions and as their reservoir. The GSH–metal chelates can be transported out of the yeast cells by multidrug resistance pumps, such as the YCF1 gene product of *S. cerevisiae*. In *Schizosaccharomyces pombe*, toxic metals are sequestered or chelated by phytochelatins, which are not produced by RNA translation but arise by GSH polymerization catalyzed by phytochelatin synthase. Both metals and phytochelatin-metal complexes can again be expelled from the cells by ABC-type ATPases (Perego and Howell 1997). Other yeasts such as *Candida glabrata* seem to share these mechanisms.

4 PRINCIPAL ANTIOXIDANTS

A biological antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge 1995). Antioxidants prevent the generation of ROS, *e.g.*, by metal chelation or enzyme-catalyzed removal of a potential oxidant, or by reacting with the ROS instead of the "substrate". Some substances, such as polyols, may shield oxidation-prone groups of peptides or proteins, such as glutathione, thioredoxin and ferredoxin, from inactivation by ROS (Shen *et al.* 1997). Important in the antioxidant defense are also the tyrosyl (Lupo *et al.* 1997) and methionyl residues of proteins. The latter can be oxidized to methionine sulfoxide without affecting the function of the respective protein. The modified proteins can be subsequently repaired by methionine sulfoxide reductase (Levine *et al.* 1999). It should be noted, however,

that every antioxidant is in fact a redox agent that may lend protection against ROS in some circumstances while promoting ROS generation in others (Herbert 1996).

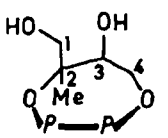
4.1 Hydrophilic antioxidants

Glutathione (GSH) is an abundant thiol tripeptide which takes part in many cell processes including amino acid transport, synthesis of proteins and nucleic acids, modulation of enzyme activity and metabolism of xenobiotics and ROS. Proteins in bacterial cytoplasm do not generally contain disulfide bonds and the maintenance of the reductive milieu is therefore important for their physiological activities. This is achieved by glutathione (GSH) and thioredoxin (Stewart *et al.* 1998). The concentration of GSH in *E. coli* cells may exceed 10 mmol/L (Fergusson and Booth 1998). The reduced form of GSH in bacteria is maintained by glutathione oxidoreductase (Becker-Hapack and Eisenstark 1995; Patel *et al.* 1998). The GSH level in unstressed wild-type yeast cells is about 40 μmol per 10^6 cells, and exposure to H_2O_2 causes its drop (Stephen and Jamieson 1996). Fluctuations in GSH levels also occur on treatment with heat, ethanol, aldehydes or fungicides. Products of lipid peroxidation, such as 4-hydroxy-2-nonenal, completely but reversibly deplete GSH in exponential *S. cerevisiae* cells. Recent findings (Żądziński *et al.* 1998) indicate that in *S. cerevisiae* glutathione may form conjugates with redox-active drugs, such as menadione, which are then exported from the cells by ABC-type ATPases. In *S. cerevisiae*, GSH alone is transported by two systems, a high-affinity ATP-driven GSH-P1 and a low-affinity GSH-P2 (Miyake *et al.* 1998).

Glutathione can directly react with HO•, reducing it to H_2O. Oxidation of GSH by ROS yields its disulfide form GSSG, which is converted back to reduced GSH in a reaction mediated by NADPH and catalyzed by GSH reductase (GLR). The NADPH acting as reductant comes from the pentose-phosphate pathway *via* reactions catalyzed by glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase (Grant and Dawes 1996).

In addition to GSH, thioredoxin, glutaredoxin and the corresponding reductases also participate in maintaining the reductive milieu in the cytoplasm of bacteria and eukaryotic microorganisms (Aslund and Beckwith 1999). Thioredoxin and glutaredoxin are small proteins containing an active site with a redox-active disulfide. They function in electron transfer *via* the reversible oxidation of two vicinal SH-groups to an –S–S– bridge. Thioredoxin acts as a hydrogen donor for ribonucleotide reductase that provides deoxyribonucleotides for DNA replication (Holmgren 1989 *and references therein*). The thioredoxin system, which is composed of NADPH, glutathione reductase, GSH and glutaredoxin, is a general disulfide reductase and catalyzes NADPH-dependent reductions of exposed –S–S– bridges in proteins. Glutaredoxin is a general GSH-disulfide oxidoreductase. It is a protein containing about 100 amino acids, with the active site Cys–Pro–Tyr–Cys. Thioredoxin is about the same size, with the active site Cys–Gly–Pro–Cys. Apart from its antioxidant effects, thioredoxin appears to play a role in vacuole inheritance in *S. cerevisiae* (Xu and Wickner 1996).

Although it is not produced by yeast cells (Wiśnicka *et al.* 1998), ascorbate is a major antioxidant in plants where the ascorbate–glutathione cycle protects plants against oxidative stress. Ascorbate is also a cofactor of some plant hydrolase enzymes. Ascorbate occurs in the cell wall where it forms the first line of defense against trioxygen (Smirnoff 1996). On interacting with ROS, ascorbate is oxidized to dehydroascorbate *via* the intermediate ascorbyl free radical. Dehydroascorbate is recycled back to ascorbate by dehydroascorbate reductase. As ROS scavenger, ascorbate is effective against $O_2^{\bullet-}$, H_2O_2, HO• and $^1O_2$. Ascorbate is capable of regenerating tocopherol from the tocopheroxyl radical that is formed in the lipid peroxidation chain-breaking reaction of vitamin E (Stahl and Sies 1997). While preventing H_2O_2-induced lipid peroxidation and protecting cells against oxidant-induced apoptosis (Deutsch 1998 *and references therein*), in the presence of transition metals ascorbate may give rise to HO• and start lipid peroxidation (*see Chapter 2.3*).



2-C-methyl-D-erythritol-2,4-diphosphate

A derivative of ascorbic acid, D-*erythro*-ascorbic acid, has been found to play an important antioxidant role in *S. cerevisiae* (Lee and Kang 1999). Ascorbate plays also some role in the prevention of oxidative damage in bacteria although its effect seems to be of minor importance as compared with that of α-tocopherol (Fuentes and Amabile-Cuevas 1998).

Some bacterial cells exposed to oxidative stress accumulate special protective substances. Thus *Corynebacterium ammoniagenes* accumulates 2-*C*-methyl-D-erythritol-2,4-diphosphate (Diomina *et al.* 1995).

4.2 Lipophilic antioxidants

The lipophilic antioxidant α-tocopherol (vitamin E), which is highly efficient in inhibiting lipid peroxidation in membranes or lipoprotein particles, scavenges lipid peroxyl radicals LOO• to yield lipid hydroperoxides. The ensuing tocopheroxyl radical has a lower reactivity than LOO• and breaks the chain reaction. The tocopheroxyl radical can be reduced by ascorbate or glutathione, or further oxidized to the quinone. It also quenches $^1O_2$, both by physical quenching and by chemical reactions, and reacts with $ONOO^-$. Tocopherol efficiency may derive from its optimal positioning in the membrane by its phytyl side chain (Sies 1997).

Carotenoids are isoprenoid membrane-protective antioxidant pigments that efficiently scavenge $^1O_2$ and peroxyl radicals, although they are 10–30 times less reactive toward ROS than α-tocopherol is (Stahl and Sies 1997; Tsuchihashi *et al.* 1995) (Fig. 3); their antioxidative efficiency is apparently related to their structure. Unsaturated hydrocarbons, such as β-carotene and lycopene, are situated within the hydrophobic membrane core, displaying certain mobility whereas xanthophylls are firmly anchored in the polar lipid headgroups *via* their two distal polar groups. They may thus have better contact with ROS arising on, or entering, the membrane periphery (Rice-Evans *et al.* 1997). The best antioxidant appears to be astaxanthin whereas the antioxidative efficiency of the most popular carotenoid, β-carotene, has recently been questioned (Liebler *et al.* 1997). In yeasts, carotenoids, in particular astaxanthin, have been found to play the role of important antioxidants during aging in *Phaffia rhodozyma* (Schroeder and Johnson 1993). In the pigmented yeast *Rhodotorula mucilaginosa*, carotenoids were found to prevent cytotoxicity induced by ROS other than singlet oxygen (*e.g.*, superoxide and hydrogen peroxide) (Moore *et al.* 1989).

Flavonoids are polyphenolic compounds that act as efficient lipophilic antioxidants (*see* Fig. 3); they are often found in fruits, vegetables and other plants. Flavonoids, such as phloretin, kæmpferol, apigenin and others possess both excellent iron-chelating and ROS-scavenging properties, scavenging HO• and O_2^-• (van Acker *et al.* 1996).

Retinol (vitamin A) is an isoprenoid compound containing a carbocyclic ring and a side chain. Apart from its role in the vision of vertebrates it has antioxidant effects. In model systems, α-tocopherol and all-*trans*-retinol were found to act synergistically against lipid peroxidation (Tesoriere *et al.* 1996).

Ubiquinone or coenzyme Q, a reversibly reducible quinone with a long isoprenoid side chain that plays a crucial role in the electron transfer chain of mitochondria, has several forms differing in the side chain length. The plasma membrane of *S. cerevisiae* contains ubiquinone-30 (CoQ_6) which apparently aids in protecting the membrane against ROS (Santos-Ocaña *et al.* 1998).

4.3 Other cell defenses

Antioxidant action may be seen to include prevention of ROS formation, interception of ROS once formed and repair of ROS-induced damage. Preventive antioxidation has been proposed to involve prevention by diversion, *i.e.* channeling the attacking ROS into a less harmful product (Sies 1997). Interception of ROS includes deactivation (formation of a low-reactivity secondary radical incapable of chain reaction) or transfer of the oxidizing species from the lipophilic to the hydrophilic phase.

Cells are equipped with several lines of antioxidant defense, both enzymic and nonenzymic, preventing or minimizing generation of ROS. The defense relies on systems for detoxication of transition metals (ferritin, transferrin, ceruloplasmin or metallothioneins – *Chapter 3.2*), or regulation of their entry into the cells (Fur in bacteria), hydrophilic radical scavengers, such as glutathione, ascorbate and urate, and lipophilic scavengers, such as tocopherols, carotenoids, retinol, flavonoids and ubiquinol (*Chapters 4.1* and *4.2*), and also on a number of enzyme systems. The enzymes preventing the toxic action of ROS will be dealt with in detail in a subsequent review which will describe their function and regulation. At this place, let us state that these enzymes include the enzymic scavengers catalase, superoxide dismutase, cytochrome-*c* peroxidase, glutathione peroxidase or thioredoxin peroxidase, which react directly with ROS. Other enzymes are involved in the reduction of oxidized low-molar-mass antioxidants (glutathione reductase) or in the maintenance of protein thiols (thioredoxin reductase). Still other enzymes maintain reducing environment inside the cell (glucose-6-phosphate dehydrogenase and other pentose-phosphate cycle enzymes regenerating NADPH). Proteolytic systems (proteinases and peptidases) that prevent the accumulation of oxidatively damaged proteins can be taken as secondary antioxidant defenses (Davies 1986).

Other means of prevention of oxidative stress include, *e.g.*, the ingenious design of some of the enzymes that might generate ROS. Thus cytochrome-*c* oxidase which carries out most of the cell oxygen reduction, does not release any ROS although it contains copper and iron (Sies 1997). Moreover, intrinsic slips in its function, *i.e.* uncoupling between the flow of electrons and proton translocation, have been suggested to play a role in antioxidant defense (Papa *et al.* 1997). Cytochrome *c* itself rapidly scavenges O_2^-• *in vitro* and is present in mitochondria at local concentrations of 0.5–5 mmol/L (Beckman and Ames 1998).

In addition to the scavenging effect, which can help to rescue cells which have the chance to survive the ROS attack, cytochrome *c* released from ROS-damaged mitochondria activates the proteolytic cascade, directing

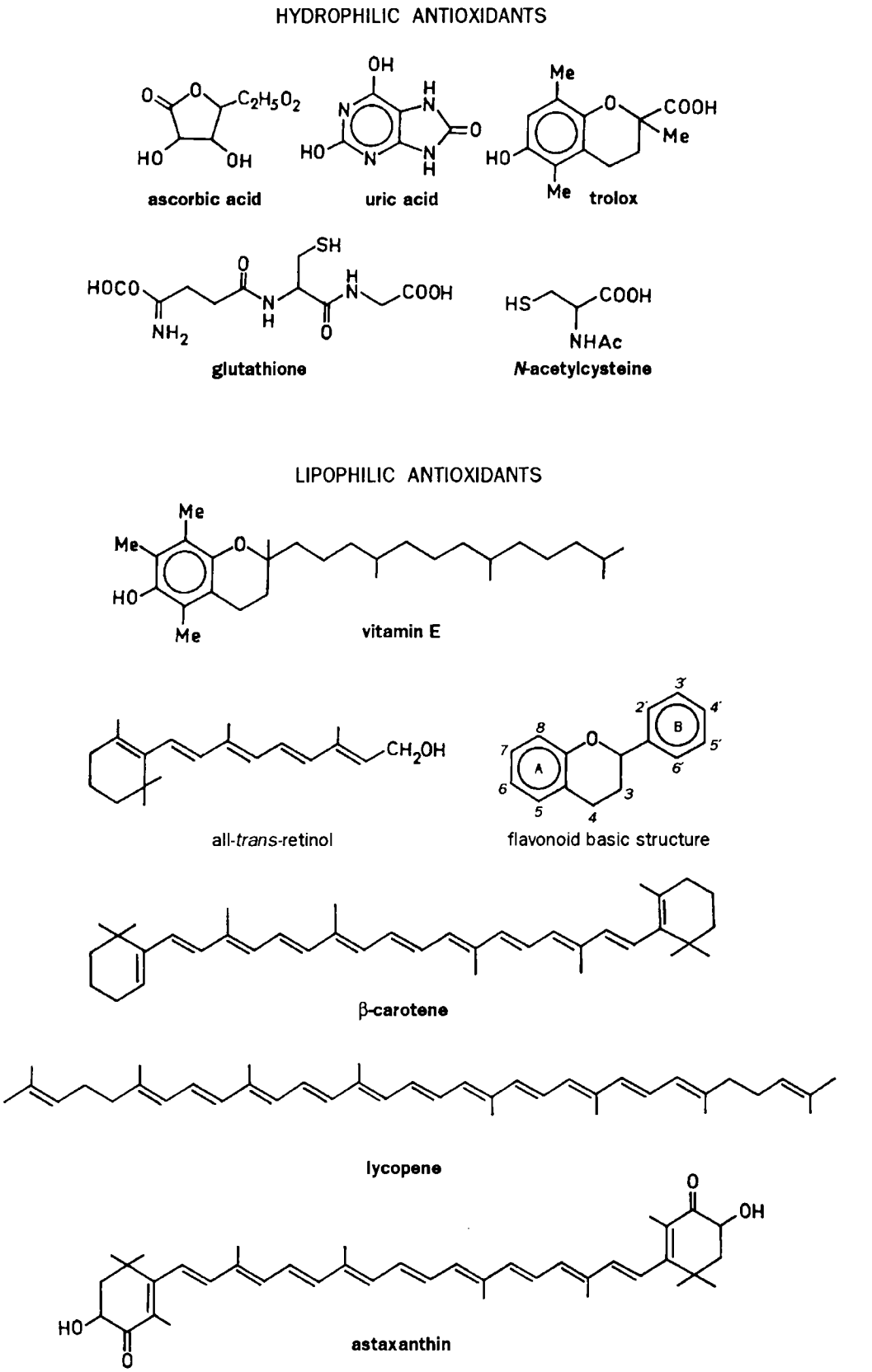


Fig. 3. Some hydrophilic and lipophilic antioxidants; adapted from Stahl and Sies (1996); Rice-Evans *et al.* (1997) and van Acker *et al.* (1996).

the severely damaged cell to apoptosis (*see Chapter 7.1*). It cannot be excluded that the rapid decrease of oxygen radicals by cytochrome *c* participates in the activation of caspases (cysteine aspartases), components of the cascade.

The yeast *S. cerevisiae* produces two catalase hemoproteins, the peroxisomal catalase A and the cytosolic catalase T, which mediate the splitting of H_2O_2 to oxygen and water (Skoneczny *et al.* 1988; Belazzi *et al.* 1991). Most bacteria contain also two catalases, one induced by oxidative stress, the other formed during the stationary phase.

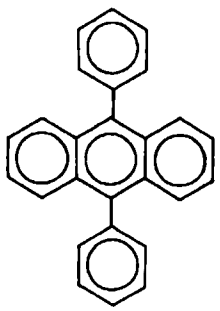
Among SODs, which catalyze the disproportionation of $O_2^{-}\bullet$ to dioxygen and H_2O_2, are the well-known cytoplasmic CuZn-SOD of eukaryotic cells, the bacterial or mitochondrial Mn-SOD, the recently identified Ni-SOD from *Streptomyces* and a monomeric CuZn-SOD found in the periplasm of *E. coli* (Valentine *et al.* 1998 *and references therein*).

5 DETECTION OF OXIDATIVE STRESS

5.1 Methods for detecting and measuring ROS

A number of methods are available for detecting and measuring ROS generation. Spectroscopic methods are used to detect and identify the unpaired electrons present in free radicals. EPR spectroscopy with spin trapping makes use of the primary ROS to react with a diamagnetic nitrone or nitroso compound (spin trap) such as phenyl-t-butylnitrone (PBN), α-2,4,6-trimethoxy-PBN, α-(4-pyridyl-1-oxide)-*N*-t-butylnitrone (POBN) or 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), yielding more stable and detectable radical adducts (Mason 1996).

$^1O_2$ can be detected by its emission at 1270 nm (Khan and Wilson 1995). Methods using chemical traps are based on given ROS forming a readily identifiable product with the trap substance. They have been used for identifying $^1O_2$ (reaction with 9,10-diphenylanthracene) or $O_2^{-}\bullet$ (reaction with the bioluminescent protein polynoidin; Colepicolo *et al.* 1990). Methods for measuring oxidative stress and/or antioxidant activity include the use of specific fluorescent probes reporting on oxidative stress, detection of oxidatively altered DNA bases and lipid oxidation products, measurement of activities of antioxidant enzymes, glutathione levels, coenzyme Q_{10}, or the use of caged compounds. The methods have been described in detail in a number of methodological handbooks (*see, e.g.*, Greenwald 1985; Armstrong 1998).



9,10-diphenylanthracene

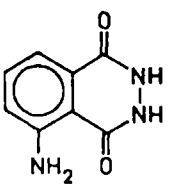
Radical quenchers, such as N-acetyl-L-cysteine, tocopherols, retinol and carotenoids, are also used for detecting the presence of ROS. The principle of the method is again the reaction of HO• or other radicals with known scavengers, and identification of the final products of the ROS-scavenger reaction by gas chromatography, HPLC, absorption spectroscopy, and other methods. The reactions of ROS with the target scavenger compounds include, *e.g.*, formation of thiobarbituric acid-reactive substances by deoxyribose oxidation (Halliwell and Gutteridge 1981), hydroxylation of benzoate, salicylate or D-phenylalanine (Bailey *et al.* 1997), ethylene production from 2-oxo-4-thiomethylbutyric acid (KMB) (Lawrence 1985), or reduction of Fe^{3+} to Fe^{2+} in cytochrome *c* by $O_2^{-}\bullet$ (Khan and Wilson 1995). HO• can also be detected by its immediate reaction with some dyes and by competition experiments with other substrates such as ethanol, which react at known rates with the HO • (Imlay and Linn 1988 *and references therein*; Zhao and Jung 1995). A recently described method for determining trace amounts of ROS is based on fluorescently derivatized nitroxides that react rapidly with carbon-centered radicals to form stable O-alkylhydroxylamines (radical adducts). These are then separated by HPLC and quantified fluorimetrically (Li *et al.* 1999).

There are a number of methods for H_2O_2 determination making use of, *e.g.*, oxidation of dimethylurea, radiolabeled methanol or formate, cytochrome-*c* peroxidase or horseradish peroxidase (Frew *et al.* 1983), O_2-electrode or some reactions of catalase (Halliwell and Gutteridge 1989). Spectrophotometric methods using agents such as dichlorophenol-indophenol or ferrous oxidation of xylenol orange (Bleau *et al.* 1998) have been described (*see also, e.g.*, Gockeritz *et al.* 1995).

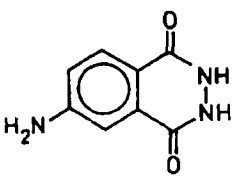
Chemiluminescence (CL) methods, especially enhanced or amplified CL, make use of agents, such as luminol (3-amino-phthalhydrazide) or isoluminol (4-amino isomer). These molecules react with the oxygen species generated in the ROS-yielding reaction, and produce excited-state intermediates that emit chemiluminescence upon relaxation to the ground state. The luminol molecule can pass through biological

membranes and ROS released from the cells as well as those present in cells can thus be measured (*e.g.*, Lundquist and Dahlgren 1996).

Lipid peroxidation can be measured by the loss of oligounsaturated fatty acids, loss of antioxidants, oxygen consumption, level of lipid hydroperoxides and conjugated dienes, levels of malondialdehyde and other aldehydes (the TBA test and other methods), concentration of products of lipid hydroperoxide degradation, formation of fluorescent lipid aldehydes and detection of lipid radicals.



luminol

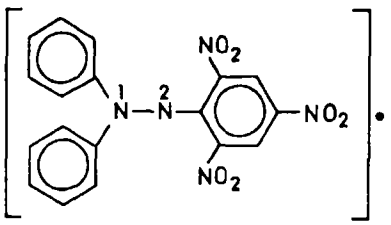


isoluminol

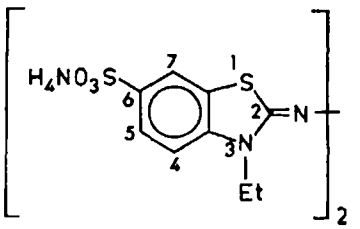
5.2 Methods for assessing oxidative damage

Techniques used for assaying and quantifying the production of ROS are also used for assessing the overall extent of oxidative stress and its effect on individual cell components or whole cells.

The cumulative effect of oxidative stress on whole cells may bring about depletion of antioxidant defenses, and increase the level of oxidized molecules in the cell. It can thus be reflected in an altered "redox status" of the cell. There are a number of methods for assessing the overall or total redox status of cells. One of the methods consists in measuring the ability of biological materials to scavenge the free radical DPPH (1,1′-diphenyl-2-picrylhydrazyl; Santiago *et al.* 1991; Santiago and Mori 1993). Another test is based on inhibition by cell homogenates of formation of the radical cation of ABTS (diammonium [2,2′-azino(3-ethylbenzothiazoline-6-sulfonate)]; Lapshina *et al.* 1995).



DPPH



ABTS

Still other assays are based on using oxidant-sensitive fluorescent probes, such as 2,7-dichloro-3,6-di-*O*-acetylfluorescein (DCAF), which is widely used for determination of H_2O_2, hydroperoxides and nitric oxide, and for studies of oxidative stress at cell level. Yeast cells take up DCAF into the cytosol by passive diffusion, where it is deacetylated by cellular esterases to 2,7-dichlorofluorescein (DCFH), which is assumed to be trapped within the cells. Once deacetylated, DCFH becomes susceptible to attack by free radicals and becomes oxidized to the highly fluorescent 2,7-dichlorofluorescin 9-lactone (DCFL) (Fig. 4). The fluorescence of the supernatant is then measured fluorimetrically (Brennan and Schiestl 1997). In fact, fluorescein and carboxyfluorescein have been found to be expelled from *S. cerevisiae* by $\Delta\psi$- or ΔpH-driven transporters (Breeuwer *et al.* 1994, 1995). DCFL was found to be released into the extracellular medium at a rate that increased with increasing pH_{out} and on addition of oxidants. Measurement of extracellular level of DCFL was thus recommended as a valid measure of oxidation processes taking place inside the cells (Jakubowski and Bartosz 1997).

Chemiluminescence techniques for measuring the antioxidant potential of cells are based on estimating the ability of the biological material to inhibit the luminol-enhanced chemiluminescence induced by oxidants (Jakubowski *et al.* 1998).

According to Benzie and Strain (1996), antioxidant power may under certain assumptions be equated to reducing ability, and a method using reductants in a redox-linked reaction employing an easily reduced oxidant in stoichiometric excess could offer a simple way of assessing this ability. The ferric reducing ability of plasma (FRAP) method is based on the fact that, at low pH, ferric-2,4,6-tripyridyl-1,3,5-triazine (Fe^{III}-TPTZ) complex is reduced to the Fe^{2+}-form that forms an intense blue color with an absorption maximum at 593 nm. The reaction is nonspecific, and any reaction, which has a less-positive redox potential than the Fe^{3+}/Fe^{2+}-TPTZ reaction, will drive the Fe^{3+}-TPTZ reduction. In the FRAP assay, excess Fe^{3+} is used and the rate-limiting factor of color formation is the reducing ability of the sample. FRAP assay offers a putative index of cell antioxidant defense.

Indices of oxidative damage of proteins include, *e.g.*, increase in the content of reactive carbonyl groups, decrease in thiol groups, increase in –S–S– bonds, loss of amino groups, formation of bityrosine (*see*

p. 604), destruction of tryptophan and formation of *N*-formylkynurenine (Rice-Evans *et al.* 1991). Oxidative damage to membrane proteins can be detected, *e.g.*, by changes in the number of membrane protein SH-groups that are accessible to the Ellman reagent (the most commonly used thiol reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; Soszyński and Bartosz 1997) or the reaction of carbonyl groups of oxidized proteins with 2,4-dinitrophenylhydrazine (Levine *et al.* 1990).

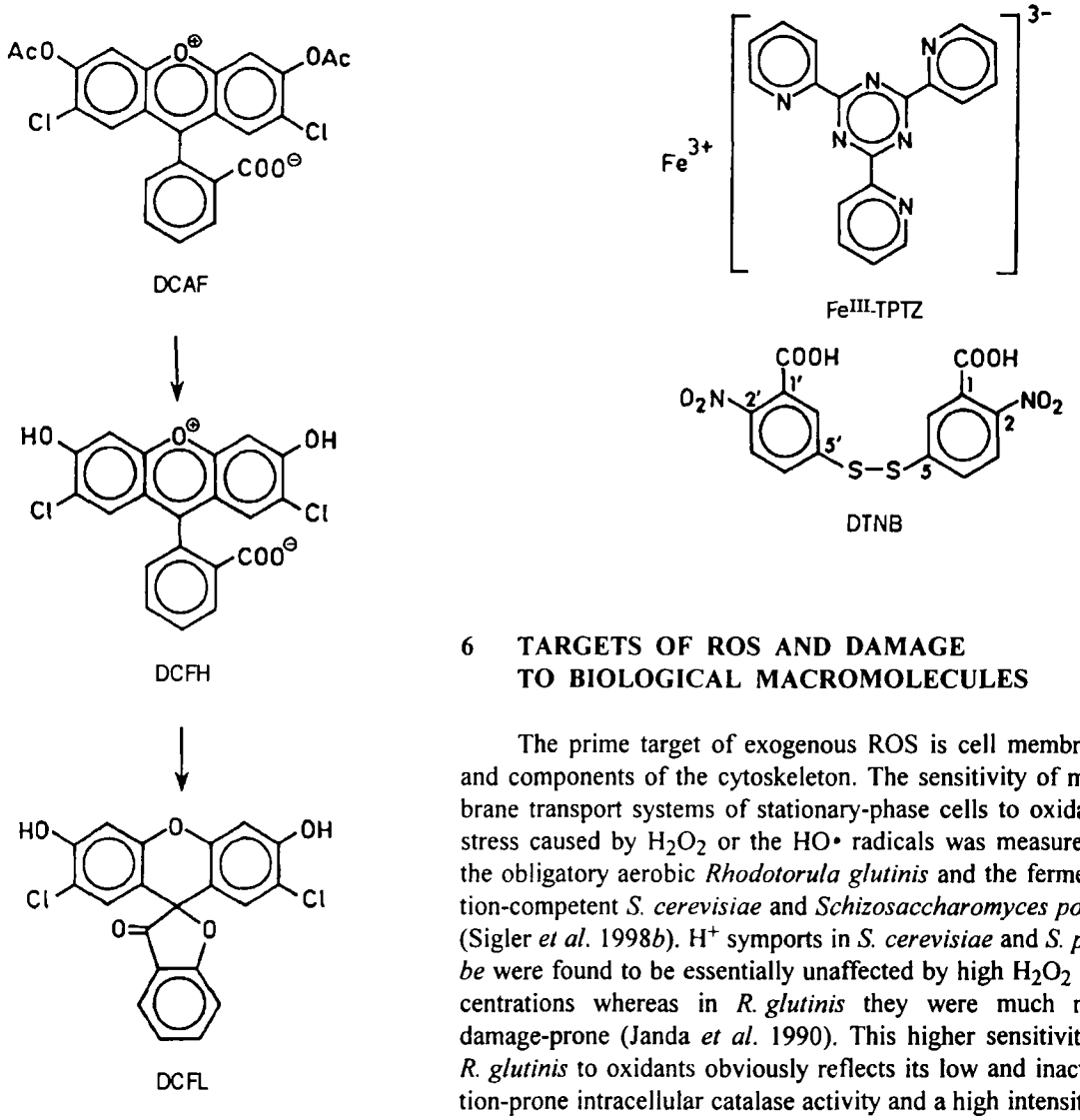


Fig. 4. Reactions of 2,7-dichloro-3,6-di-O-acetylfluorescein (DCAF) used as a measure of oxidation processes within cells

6 TARGETS OF ROS AND DAMAGE TO BIOLOGICAL MACROMOLECULES

The prime target of exogenous ROS is cell membranes and components of the cytoskeleton. The sensitivity of membrane transport systems of stationary-phase cells to oxidative stress caused by H_2O_2 or the HO• radicals was measured in the obligatory aerobic *Rhodotorula glutinis* and the fermentation-competent *S. cerevisiae* and *Schizosaccharomyces pombe* (Sigler *et al.* 1998*b*). H^+ symports in *S. cerevisiae* and *S. pombe* were found to be essentially unaffected by high H_2O_2 concentrations whereas in *R. glutinis* they were much more damage-prone (Janda *et al.* 1990). This higher sensitivity of *R. glutinis* to oxidants obviously reflects its low and inactivation-prone intracellular catalase activity and a high intensity of lipid peroxidation in the plasma membrane. In contrast to H^+-symports, the plasma membrane H^+-ATPases of all three yeast species have a low and comparable sensitivity to peroxide. These vitally important yeast P-type ATPases respond to oxidative assault by exhibiting decreased K_m for Mg-ATP and decreased v_{lim} ('v_{max}') of the ATPase reaction (Sigler *et al.* 1998*a*,*b*). Another feature of the response of the plasma membrane H^+-ATPase to oxidants, determined so far in *S. cerevisiae*, is the protection of the enzyme from the inactivating action of H_2O_2 in the presence of ATP. The target site(s) for oxidants in the ATPase appear to be several cysteine residues in the vicinity of the ATP-binding site. Other enzymes attacked by oxidative agents show, *e.g.*, altered K_m for substrate and altered interaction with ions (glycerol dehydrogenase from *Klebsiella pneumoniae*; Johnson *et al.* 1985), while ion-transporting animal systems, such as Na,K-ATPase, may show a change in kinetic cooperativity and uncoupling of ion fluxes from ATP hydrolysis (Garner *et al.* 1983, 1984). Lipid peroxidation alters Na,K-ATPase by modification at specific active sites in a selective manner, rather than through a nonspecific process (Mishra *et al.* 1989).

H_2O_2 produced by polymorphonuclear leukocytes during inflammation was found to provoke cell death by disarranging filamentous (polymerized) actin (F-actin) through oxidizing actin thiol groups. Steady-state actin polymers made up from oxidized G-actin monomers are more fragmented than control polymers

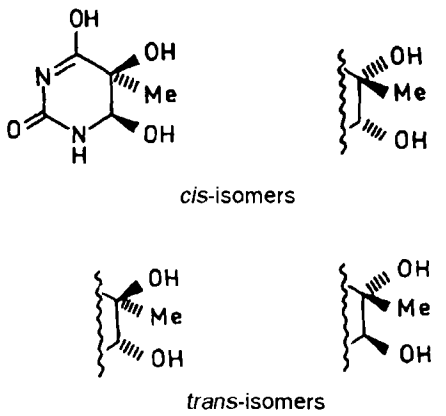
(DalleDonne *et al.* 1995). Oxidative damage strongly affects calmodulin, which loses its ability to bind and activate the plasma membrane Ca-ATPase. Oxidatively modified calmodulin binds to the autoinhibitory sites of the Ca-ATPase in an altered conformation, affecting the calcium homeostasis of the cell (Yao *et al.* 1996).

Despite the adverse effects of oxidants on cell proteins, probably the most dangerous for the cell and its progeny is the damage of DNA.

6.1 DNA

Among the many deleterious environmental factors, the attack by ROS is considered to be the most harmful source of spontaneous damage of DNA. Ames and Shigenaga (1992) have estimated that approximately 2×10^4 oxidative DNA lesions occur per human genome every day. ROS produce a wide spectrum of types of DNA damage. Approximately 100 different free radical-induced lesions have been identified (Dizdaroglu 1992). Identified oxidation products include both primary lesions which are often unstable and the breakdown products of these lesions arising from hydrolysis and rearrangement reactions.

Base damage: DNA base modifications represent approximately half of the lesions generated by hydroxyl radicals, whereas they constitute the majority of lesions induced by singlet oxygen (Epe 1995).



A major family of thymine oxidation products, collectively called thymine glycols, consists of four isomers of 5,6-dihydroxy-5,6-dihydrothymine, in which the vicinal hydroxyl groups may be either *cis* or *trans* with respect to each other. Thymine glycols undergo alkali-catalyzed decomposition which gives rise to various fragmentation products and yields urea residues N-linked to deoxyribose (Demple and Harrison 1994). Production of thymine glycols in a single-stranded DNA template results predominantly in T–C mutations. When present in double-stranded DNA, thymine glycols and their breakdown product urea are not mutagenic; they have a toxic effect because they function as a strong block to replication (Basu *et al.* 1989). Thus thymine glycols are supposed to be lethal lesions with moderate mutagenic properties.

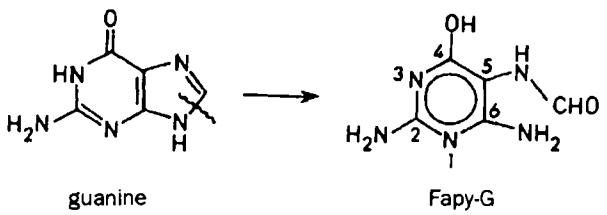
Most oxidation products of cytosine are analogous to those detected for thymine. However, cytosine glycols can undergo a secondary reaction which does not occur with thymine, *viz.* deamination (Friedberg *et al.* 1995). Deamination of cytosine hydrates produces uracil derivatives that form base pairs preferentially with adenine, leading probably to enhancement of the mutagenic effects of cytosine hydrates relative to thymine glycols which form CG→TA transitions.

Purines can undergo oxidation of ring atoms to form various products. Among these, 8-oxoguanine (8-oxoG, denoted also as 8-hydroxyguanine, the other tautomeric form), is the best characterized oxidation-induced base damage product. 8-oxoG is strongly mutagenic both *in vitro* and *in vivo* (Michaels and Miller 1992; Grollman and Moriya 1993). It does not block DNA synthesis catalyzed by prokaryotic and eukaryotic DNA polymerases. It is detected in DNA in increased amounts after exposure to a variety of agents that produce ROS, including H_2O_2, ionizing radiation, the carcinogens 4-nitroquinoline oxide and 2-nitropropane, as well as $^1O_2$ and Fe^{2+} or Cu^{2+} in combination with H_2O_2. The majority of mutations so induced were found to be targeted at the position of 8-oxoG and more than 95 % of these mutations were CG→TA transversions. From this it follows that 8-oxoG is a highly mutagenic lesion with little, if any, lethal action.

Another guanine residue generated by oxidative stress is 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G). This guanine derivative with opened imidazole ring is one of the major adducts formed in DNA by hydrolysis. The properties of Fapy-G are derived from its methylated form (Me-Fapy-G). It was shown that Me-Fapy-G is an inhibitor of DNA synthesis by *E. coli* DNA polymerase I and phage T4 DNA polymerase I (Boiteux 1993). The results show that both Fapy-G and Me-Fapy-G are lethal lesions in DNA with marginal mutagenic potential. Fapy residues (derived from guanines or adenines) can arise also as secondary products of N^7-alkylated guanines or adenines.

Deoxyribose damage: Besides base damage, ionizing radiation and free radical attack on DNA generate lesions in deoxyribose (Demple and Harrison 1994). This damage can remove and displace bases, thus creating abasic (AP) sites. It can oxidize and fragment the deoxyribose moiety. The resulting strand breaks

are typical structural alterations caused by oxidative damaging agents, such as ionizing radiation or hydrogen peroxide, and all of them can cause a complete loss of genetic information in the affected strand.



Cross-links: Ionizing radiation generates cross-links between DNA and proteins. Small amounts of interstrand cross-links can also be formed by radical attack. The repair of such lesions is mediated by recombinational repair system, as is also the case with some chemical or photochemical cross-links (West 1992).

6.2 Lipids

Oxygen radicals can damage both cell membrane phospholipids and proteins but lipid oxidation is thought to be more dangerous for the cell. It not only inflicts damage on the membrane as such, but multiplies the toxic effect of the primary stressors by generating a burst of secondary stressors, *i.e.* lipid peroxyl or alkoxyl radicals (Fig. 5). Both the primary and the secondary stressors can degrade molecules of fatty acids composing the lipid bilayer by cleaving the C–C bonds to shorter molecules.

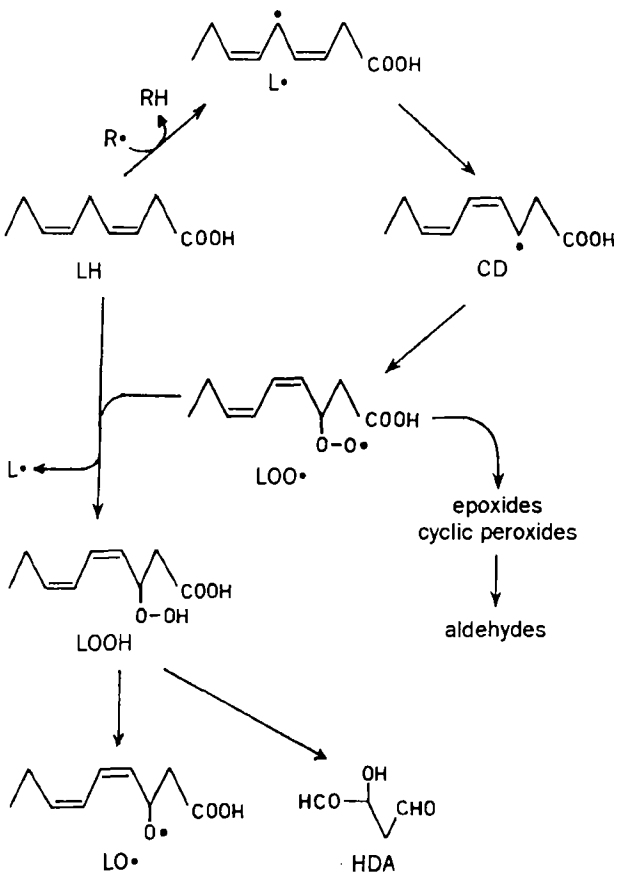


Fig. 5. Some reactions and products of lipid peroxidation; CD – conjugated diene; adapted from Valentine *et al.* (1997), Mlakar and Spiteller (1997).

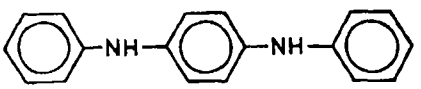
In the course of this radical-based chain reaction, lipid peroxidation generates alkanes, ketones, oxiranes and aldehydes, which increase the number of polar groups in fatty acid chains. Membrane fluidity thus changes and the cell is no more able to fully control its permeability. The membrane loses its structural

integrity and the transmembrane electrochemical gradient of protons is partially or completely dissipated. This ultimately leads to cell death. Another dangerous consequence of lipid peroxidation is the generation of secondary oxygen radicals attacking the DNA (*see above*). The secondary reactive radicals produced during the reaction of primary ROS with cell lipids then react with membrane-sited as well as cytoplasmic proteins, further compromising cell integrity.

Lipid peroxidation produces peroxyl radicals (LOO•), a relatively long-lived species (half-lives of the order of seconds) with a considerable diffusion path in cells. Further products generated in lipid peroxidation are lipid hydroperoxides (LOOH), which may undergo rearrangement to yield endoperoxides that are cleaved to aldehydes, or react with Fe^{2+} to produce alkoxyl radicals (LO•) (Mlakar and Spiteller 1997). Degradation of lipid hydroperoxides by β-cleavage yields a variety of aldehydes including alkanals, 2-alkenals, 2,4-alkadienals and 4-hydroxyalkenals (Esterbauer *et al.* 1991). The major aldehydes are often hexanal and 4-hydroxynonenal (HNE) produced by oxidation of ω-6 fatty acids. A sequential multiple oxygenation of LOOH can yield 2-hydroxydialdehydes (HDA) (Mlakar and Spiteller 1997). These and other aldehydes have toxic effects and have been suggested to act as "second toxic messengers" of oxygen radicals and lipid peroxidation (Esterbauer 1996). They are prone to polymerize and the polymerization products are fluorescent, enabling their detection (Halliwell and Gutteridge 1989). The reaction of aldehydes with amine groups of peptides and proteins is involved in the modification of lipoproteins (Stahl and Sies 1997).

The reactions of lipid alkoxyl (LO•) and peroxyl (LOO•) radicals are slower but more specific than those of HO•. Because of the close proximity of lipids to proteins and DNA in the cell, lipid radicals may be more efficient than HO• in damaging other cell components (Yang and Schaich 1996).

According to Nordmann *et al.* (1990) one of the consequences of the peroxidation of a biomembrane is a decrease in the fluidity of the membrane lipid phase, *i.e.* membrane rigidization. Membrane rigidization has indeed been found in cells of several yeast species (*S. cerevisiae*, *Schizosaccharomyces pombe*, *Rhodotorula glutinis*) on exposure to hydrogen peroxide (Gille *et al.* 1993).

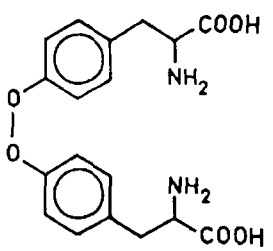


DPPD

Lipid peroxidation has been suggested to be the mechanism of toxic action of not only redox active drugs and heavy metals, such as Cd^{2+} or Cu^{2+} (*see Chapter 3.1*), but also of mycotoxins produced by filamentous fungi (as found in *Kluyveromyces marxianus*; Hoehler *et al.* 1998). It can be prevented by pretreatment of the lipid with lipophilic antioxidants such as *N,N'*-diphenyl-1,4-benzenediamine (*N,N'*-diphenyl-1,4-phenylenediamine; DPPD) (Kirkland 1991).

6.3 Proteins

Proteins – whether structural or free cytoplasmic ones – are probably the main target of externally induced ROS in animal as well as bacterial cells (Gebicky 1997). They are attacked either directly or indirectly *via* oxidized lipids. Two recent important publications – a monograph by Davies and Dean (1997) and a review by Dean *et al.* (1997) – extensively analyze the data on protein modifications by various forms of oxygen radicals and bring a detailed information on this topic. Mechanisms of oxidative protein damage and its repair or elimination are also discussed in an older review (Visick and Clarke 1995). We therefore summarize here only the most important data on this problem and supplement them with recently published findings.



bityrosine

Probably all amino acids can be modified by ROS or RNS. Amino acid residues are either oxidized to peroxidized forms, *e.g.*, tyrosine to tyrosine hydroperoxide, histidine to 2-oxohistidine; they can be transformed to another amino acid, *e.g.*, proline to glutamic acid or to an organic acid, *e.g.*, leucine to 2-oxoisocaproic acid or isovaleric acid. Methionine is oxidized to its sulfoxide and cysteine to cystine, which causes its dimerization. Other amino acids can also be dimerized, *e.g.*, tyrosine to bityrosine ("dityrosine"). The oxidative defect on one amino acid residue can also be transferred to a neighbor amino acid.

Amino acids in proteins can be oxidized either on α-carbon which forms the protein backbone, or on their aliphatic side-chains (Fig. 6). The damage of the carbon backbone, proceeding preferentially at proline or glycine residues, may result in fragmentation of the protein molecule.

The pathways of ROS-mediated protein oxidation are numerous. Leucyl and other amino acid residues can be modified by the Fenton reaction (combined effect of transition metal ions and H_2O_2), many other amino acids can be oxidized by the hydroperoxyl radicals HOO• or by superoxide. The latter can be converted to more active radicals such as peroxyl (ROO•), alkoxyl (RO•) and hydroxyl (HO•) radicals. The oxidative modification often results in formation of amino acid hydroperoxides or carbonyl compounds. Especially sensitive targets for oxidation of enzymes are the metal-binding sites in their prosthetic groups.

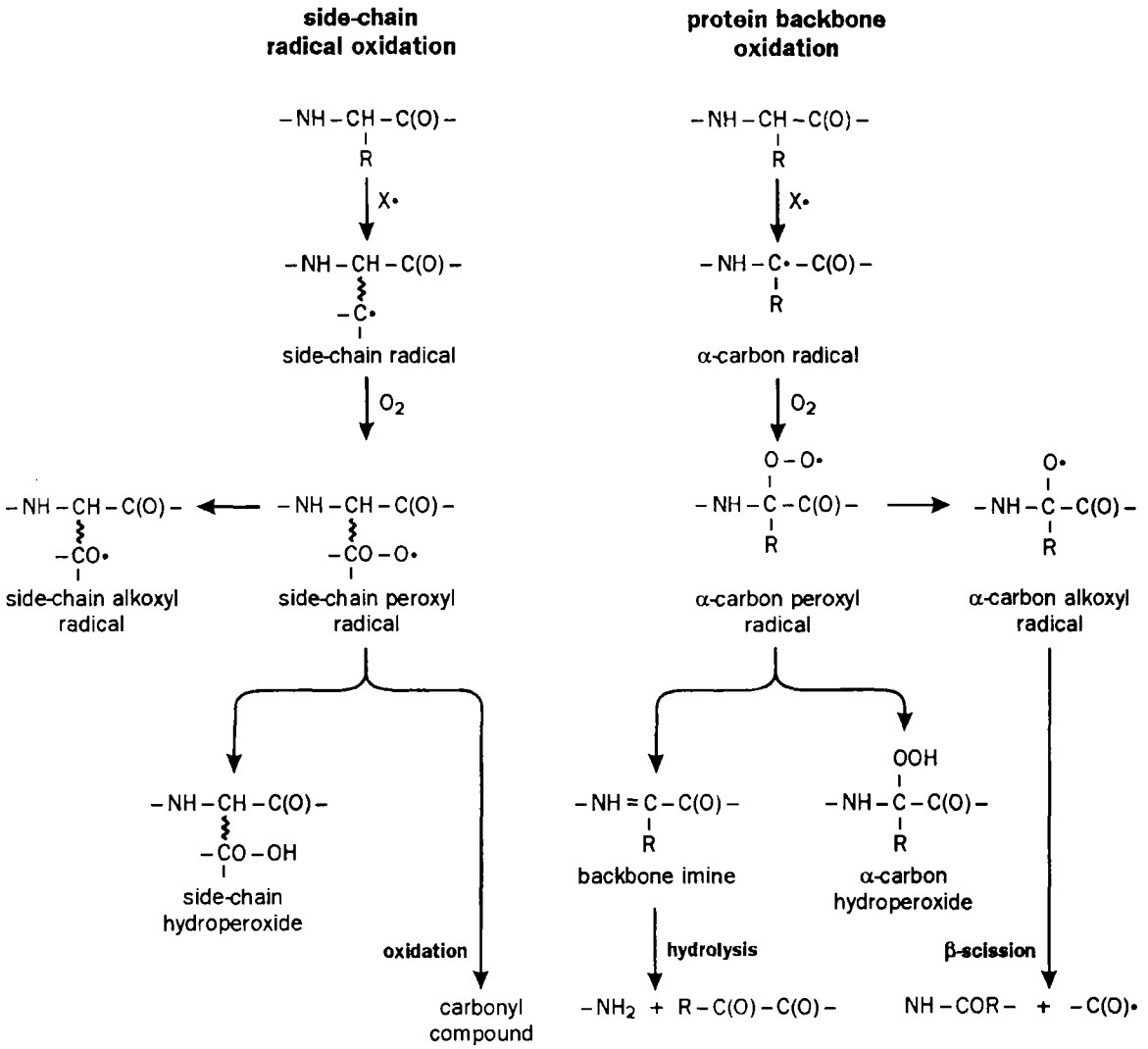


Fig. 6. Main steps in protein oxidation by ROS; based on Dean *et al.* (1997), simplified.

Oxidative damage to one kind of biomolecule can affect another one. Thus the reactive aldehyde groups of lipids can react with amino groups in proteins to form Schiff bases, or ROS can induce formation of cross-links between proteins and DNA (Stadtman 1986; Dean *et al.* 1997; Davies and Dean 1997).

A detailed analysis of oxidative protein modifications induced by different ROS in *Escherichia coli* was published by Tamarit *et al.* (1998). They analyzed the distribution of carbonyl groups in proteins separated by 1-D gel electrophoresis, and found that the most attacked were the β-subunit of the F_0F_1-ATPase, enzymes of energy metabolism and the DnaK molecular chaperone. However, they could not find any clear-cut correlation between the degree of protein modification and loss of viability.

In bacteria, oxidative stress damages proteins not only in vegetative cells but also in bacillar spores. Here, methionyl residues in small acid-soluble proteins have been found to be oxidized to methionine sulfoxide, which decreased the interaction of the proteins with DNA (Hayes *et al.* 1998). Also enzymes participating in energy-yielding reactions in spores can be inactivated by ROS (Palop *et al.* 1998). Hydrogen peroxide was found to be capable of attacking proteins directly and oxidizing their SH-groups. This was found to be true, *e.g.*, with creatine kinase from rabbit muscle (Suzuki *et al.* 1992) and also with membrane

proteins such as the plasma membrane H^+-ATPase from *Schizosaccharomyces pombe* that has a very low level of lipid peroxidation (Sigler *et al.* 1998*a*). H_2O_2 also partially inhibits mitochondrial enzymes (NADH dehydrogenase, NADH oxidase and cytochrome-*c* oxidase), and this inhibition again appears not to be related to lipid peroxidation (Zhang *et al.* 1990).

Modifications or splitting of protein molecules dramatically change the protein conformation, inactivate enzymes and often cause their denaturation. Such altered proteins are then a suitable target for proteolytic attack (Levine *et al.* 1981; Gardner and Fridovich 1991).

Partially unfolded proteins, which may exist during the first stage of oxidative damage, can be protected by molecular chaperones against irreversible denaturation and subsequent proteolysis (Craig *et al.* 1994; Fenton and Horwich 1997). However, the possibility to repair oxidatively modified proteins is limited, although the disulfides or methionine sulfoxides can be reduced back to cysteine and methionine residues under participation of thioredoxin and thioredoxin reductase (Farr and Kogoma 1991).

We shall briefly mention the fate of irreparable oxidatively damaged proteins though this topic will be a subject of a subsequent review. Most damaged proteins including nonfunctional enzymes must be eliminated by proteolysis and replaced with new ones to keep cell viability. Like proteins containing amino acid analogs or treated by a supraoptimal temperature, oxidatively damaged proteins are prone to denaturation. They are more sensitive to hydrolysis by proteolytic enzymes than most normal proteins (Goldberg and St. John 1976; Davies 1987; Gardner and Fridovich 1991). However, some oxidized proteins, especially those forming protein aggregates, are not accessible to proteolytic enzymes. Higher concentrations of ROS or of amino acid analogues or a higher temperature may therefore cause accumulation of denatured nonfunctional proteins in cells (Chopra *et al.* 1986; Davies and Lin 1988*a*; Grune *et al.* 1995).

Oxidatively modified proteins are degraded mainly by proteolytic enzymes involved in the hydrolysis of short-lived proteins, *e.g.*, by proteasomes in eukaryotic cells, but proteolytic enzymes able to recognize and degrade oxidatively modified proteins are present also in mitochondria (Marcillat *et al.* 1988). A special proteolytic system may be involved in the degradation of oxidatively modified proteins in bacteria (Davies and Lin 1988*b*) but more data are needed to characterize it. We recently showed that the Ca^{2+}-dependent serine proteinase ISP1 whose synthesis or activation in *B. megaterium* is increased by heat or osmotic stress (Váchová *et al.* 1994; Kučerová and Chaloupka 1995) is promoted also by oxidative stress (Váchová *et al.*, *to be published*). The participation of this proteinase in degradation of defective proteins has not been fully elucidated.

6.4 Saccharides and polysaccharides

The data on oxidative damage to saccharides and its consequence for the cell are much less abundant than those for DNA, lipids or proteins. Monosaccharides can enolize and thereby reduce dioxygen under physiological conditions, yielding 2-oxoaldehydes, H_2O_2 and free radical intermediates. The process could form the basis of slow nonenzymic glycosylation of proteins. The extent of this nonenzymic protein glycosylation could reflect the extent of oxidative stress (Wolff and Dean 1987).

Free radicals have been found to depolymerize polysaccharides, such as β-cyclodextrin, pectin, alginate, dextran and pullulan (Uchida and Kawakishi 1986*a*,*b*; Lahiri *et al.* 1992), and also depolymerize chitosan (D-glucosamine polymer). Oxidative chitosan depolymerization is transition-metal-catalyzed, and involves the action of HO•, but not H_2O_2, O_2^{-}• or $^1O_2$ (Tanioka *et al.* 1996). The degree of radical-induced polysaccharide depolymerization depends on the conformational state of the molecules, in particular their strandedness (Christensen *et al.* 1996). The radical-induced attacks on the polysaccharide chain are nonspecific. The depolymerization of multiple-stranded polysaccharides such as xanthan or scleroglucan differs from that of single-stranded polysaccharides (Hjerde *et al.* 1998).

7 BIOLOGICAL ASPECTS OF OXIDATIVE STRESS ON MICROBIAL, PLANT AND ANIMAL CELLS

This chapter is intended as a comparison of the effect of oxidative stress on plant and animal cells with its influence on bacteria and eukaryotic microorganisms. The main concern is with the endogenously induced stress because it affects all aerobically living organisms. However, endogenously formed ROS, such as H_2O_2, can be excreted by animal and plant cells as a defense tool in their struggle against pathogenic microorganisms, and their effect cannot therefore be neglected. ROS can also originate through natural causes (*see Chapter 1.2*) or can be used as disinfectants or experimental tools for unraveling the nature and regulation of cell response to oxidative stress as such. In all types of cells, the main biological consequence

of a severe oxidative stress is an inhibition of the cell cycle (Lowett *et al.* 1994; Wang 1998). This delay in cell cycle progression enables the cell either to decrease the ROS concentration, to repair the damage caused by oxidative stress or induce mechanisms eliminating irreparable cells. Whereas the former process is similar in all cells, the latter effect of oxidative stress proceeds differently in different cells. Cells in multicellular organisms developed sophisticated mechanisms resulting in their programmed death. The death of damaged cells thus prevents fixation of pernicious mutations, *e.g.*, those leading to malignant transformation, which would be dangerous for the whole organism. Microbial cells have not developed a universal death mechanism or only a very rudimentary one. Also the biological consequences of a milder but continuous endogenous oxidative stress in multicellular and unicellular organisms are different. Whereas the unrepaired changes in multiplying animal cells participate in their gradual aging, such changes in microbial cells are absent or rare. These problems are analyzed in the following sections.

7.1 Oxidative stress in animal and plant cell aging and death and in development of diseases

Mitochondria, key organelles producing endogenous oxidative stress, play a part in damaging other cell organelles by the ROS, which results in aging, but can also participate in induction of apoptosis – an executive pathway of programmed cell death.

Apoptosis, a genetically regulated death pathway, can be induced in animal cells either by physiological effectors, such as hormones, or by a severe cell damage caused, *e.g.*, by heat or oxidative stress (Petit *et al.* 1996; Jacobson 1996; Wyllie 1997) (Fig. 7). Physiologically induced apoptosis is involved in the elimination of cells or tissues in the course of development of the organism (Ellis *et al.* 1991; Jacobson *et al.* 1997); stress-induced apoptosis participates in cell defense by eliminating damaged cells that can no longer be properly repaired (Gabai *et al.* 1997; Hetts 1998). The main event in the process of induction of apoptosis in animals is the sensing of stress-induced DNA damage by the sensor protein p53 (Enoch and Norbury 1995; Yin *et al.* 1998). This protein is the product of the p53 antioncogene and activates a proteolytic cascade which triggers further steps in the apoptotic pathway (Hetts 1998). Protein p53 belongs among transcriptional factors regulating the expression of genes that direct the cell to the arrest of the cell cycle, differentiation or death.

A crucial role in the apoptotic pathway is played by caspases. The proteolytic cascade includes ten or more caspases and is triggered *via* several pathways. The main function of the proteolytic cascade in apoptosis is to degrade proteins, such as lamins which stabilize the nuclear structure, and enzymes participating in DNA repair, *e.g.*, poly(ADP-ribose) polymerase. The cascade also activates endonucleases (DNAases) which accomplish the terminal steps in the death pathway, by inactivating their inhibitors. The proteolytic enzymes participate also in apoptosis indirectly by degrading the mitochondrial membrane. The internal contents of mitochondria are then released into the cell. Among their components are the soluble proteinaceous factor AIF, cytochrome *c* and Ca^{2+}, which promote the apoptotic pathway (Skulachev 1996; Jacobson 1996; Cai and Jones 1998). An important factor in this process is the release of Ca^{2+} ions from mitochondria and other cell structures; these ions then activate the Ca^{2+}-Mg^{2+} endonuclease activity. The cytochrome *c* released from mitochondria participates in the activation of the proteolytic cascade (Allen *et al.* 1998; Green and Kroemer 1998; Skulachev 1998). An oncoprotein Bcl-2, which is located in the mitochondrial outer membrane, can temporarily delay or suppress the process by increasing $\Delta\psi_m$ and stabilizing the mitochondrial permeability. Although the apoptotic cell shrinks, its cell membrane functions remain almost unchanged until the end of the process.

Another factor involved in the regulation of apoptosis is the sphingolipids ceramides, the synthesis of which increases in stress conditions. Ceramides can be considered as generalized mediators of apoptosis because of their function in signal transmission. They also participate in mobilization of Ca^{2+} reserves and might thus affect the activity of nucleases (Pushkareva *et al.* 1995; Sakagami *et al.* 1996; Dickson 1998).

Another apoptotic pathway, which proceeds in parallel to that induced by p53, is triggered by accumulation of oxidatively damaged proteins (Gabai *et al.* 1997; Jäättelä *et al.* 1998; Meriin *et al.* 1999). Oxidatively modified proteins are denatured at physiological temperatures and interact with stress proteins belonging to the Hsp70 family. These stress proteins either renature the damaged proteins or make them accessible to intracellular proteolytic enzymes. This interaction depletes the pool of free Hsp70 proteins and this causes unblocking of c-Jun (an apoptosis-stimulating factor) function.

Unlike the relatively mild oxidative stress resulting usually from endogenously produced ROS, which induce apoptosis, a more severe endogenous oxidative stress or moderate concentrations of exogenous ROS kill cells by necrosis (a non-programmed cell death; Sen 1992). Necrosis proceeds as an almost immediate breakdown of the cell membrane and release of the cell contents into the environment. The decisive factor directing a damaged cell either to apoptosis or to necrosis seems to be the ATP level. Its sudden drop

induced by impairment of cell membrane or mitochondria drives the cell to necrosis. Maintaining satisfactory ATP level enables the cell to continue its metabolic activities and directs the damaged cell on the pathway to apoptosis (Tsujimoto 1997).

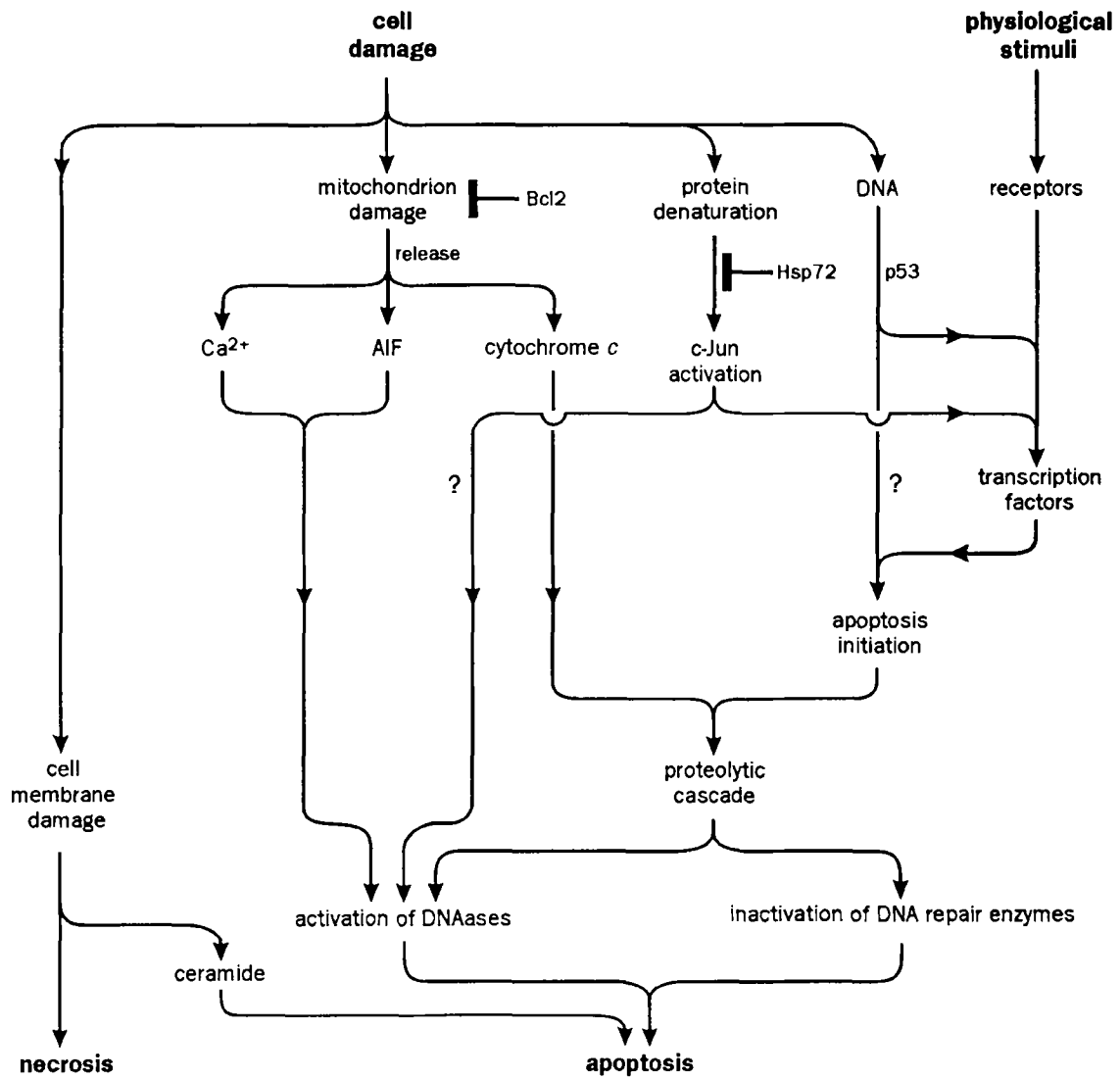


Fig. 7. Main features of death pathways in animal cells. Cell death can be triggered by two interconnected pathways: by physiological stimuli (*e.g.*, Fas antigen and tumor necrosis factor TNF) or by stress factors (*e.g.*, oxidative or heat stress) causing cell damage. Both affect transcription and trigger activation of the proteolytic cascade. An important role is played by mitochondria, which augment the death pathway by an increased production of ROS. Also, mitochondrial damage leads to the release of the apoptosis-inducing factor AIF and cytochrome *c*, which activates some compounds of the proteolytic cascade. The apoptotic pathway is stimulated by the sphingolipid ceramide whose synthesis is increased in stress condition. Serious damage to the cell membrane can direct the cell to necrosis whereas a milder impairment of cell structures stimulates the apoptotic pathway. Based on Willie (1997); Kroemer *et al.* (1997); Meriin *et al.* (1999).

A continuous influence of a relatively mild oxidative stress is one of the main causes of cell aging. Cell aging differs from apoptosis; it is a long-term biological process which leads to a continuous deterioration of cell functions, decrease of cells' reproductive activity and eventually cell death. It is controlled on the one hand by genetic regulation, and on the other it is affected by accumulation of defects caused by continuous oxidative stress (Lin *et al.* 1998). A close connection exists between cell aging and the development of degenerative diseases. The main protein targets damaged by oxidative stress are respiratory enzymes which lose their activity, and components of the cell-defense systems, such as superoxide dismutase and glutathione reductase (Oliver *et al.* 1987; Wei 1998). This brings about a decline of many cell functions, including stress and immunological response. DNA and protein damage impairs the function of the nervous system which results in degenerative diseases such as Parkinson's disease and lateral sclerosis (Ames *et al.* 1993; Hetts

1998). There is also evidence that oxidative damage to DNA is a causative factor in human cancer, and may play a role in hereditary syndromes with proclivity to malignancy, such as ataxia telangiectasia, Bloom's syndrome and Fanconi's anemia. Oxidative damage to DNA is also assumed to participate in the inception of many other diseases, including atherosclerosis, stroke and auto-immune syndromes (*for review see, e.g.*, Dreher and Junod 1996; Newcomb and Loeb 1998).

Oxidative stress also plays a role in infectious diseases, because the products of several viruses or pathogenic bacteria damage the infected tissue by inducing ROS generation (Edeas *et al.* 1997; Hahn *et al.* 1998; Akaike *et al.* 1998; Kaur *et al.* 1998). It should be noted, however, that superoxide and NO also participate in the defense of organisms against bacterial and viral infections by killing the infectious agent (Irani and Goldsmith-Clermont 1998; Okada *et al.* 1998). The role of ROS in cellular immunity is ambiguous. On the one hand, ROS and RNS produced by macrophages kill infecting bacteria and viruses and, on the other hand, their accumulation in inflammatory regions can damage the host tissue by increasing the local concentration of ROS (Ginsburg 1998; Akaike *et al.* 1998). A continuous local irritation at the place of infection can even promote the process of malignization (Vile *et al.* 1998).

A similar type of defense against pathogenic bacteria and fungi – the so-called hypersensitive response – is used also by plants. The ROS produced by infected host cells kill the invader but simultaneously induce the death of the infected tissue. The dead cells are rapidly desiccated and cannot thus be utilized as a source of nutrients for the reproduction of pathogenic bacteria or fungi that had survived the oxidative injury (Levine *et al.* 1994; Greenberg 1996; Dangl and Holub 1997).

7.2 Effect of endogenously formed ROS on growing microbial cells

Aerobically growing cells are continuously attacked by internally produced ROS induced by aerobic respiration. Multiplying aerobically growing bacterial populations do not exhibit any evident signs of aging, probably with the exception of filamentous or budding bacteria (Mason *et al.* 1986). Aerobic bacteria are equipped with an efficient enzyme machinery (*e.g.*, catalases and superoxide dismutases) to decrease the level of ROS and repair oxidative damage to biomolecules (Demple 1986; Iuchi and Weiner 1996; Storz *et al.* 1990; Farr and Kogoma 1991). In addition, the rapid growth and high biosynthetic activity of microbial cells bring about dilution of possibly nonrepaired lipids or proteins by new and functional ones. A reproducing bacterial cell divides into two similar or identical daughter cells and this prevents possible accumulation of damaged macromolecules in the mother cell (Nyström 1998). Nevertheless, because the total number of cells in a multiplying bacterial culture is usually larger than the amount of cells able to form colonies, and their ratio is affected by cultivation conditions (Decamp *et al.* 1997; Sakamoto *et al.* 1998), the problem of aging and death of bacteria in a growing population cannot be resolved definitively. Also the terms "viability" and "culturability" need not be equated because of the recent finding of "viable-but-nonculturable" bacteria (Kell *et al.* 1998). Unlike aerobically growing bacteria, which are equipped with enzymes decreasing the danger of oxidative damage, anaerobically growing bacteria contain much less of these enzymes and can be killed by endogenously produced hydroperoxides whose formation is triggered by Fe^{2+} *via* the Fenton reaction (Dunning *et al.* 1998).

Growing yeast cells exposed to endogenously produced ROS in aerated media are equipped with similar defense mechanisms as bacteria (Jamieson 1995). The action of antioxidative enzymes can be demonstrated only after their inactivation (Longo *et al.* 1996, 1997, 1999). However, unlike bacteria, multiplying cells of *S. cerevisiae* display signs of aging or so-called replicative senescence known from tissue cultures of multicellular organisms (Hayflick 1979; Campisi 1996; Faragher and Kipling 1998). This term implies that every differentiated tissue is endowed with only a limited reproductive capacity and, after several cell divisions, ceases to multiply and dies. Yeasts divide during the cell cycle into two morphologically and physiologically different cells: a larger mother cell, and a smaller daughter (virgin) cell. The mother cell can be recognized by its larger size and by the presence of bud scars remaining after separation of every daughter cell (Streiblová 1970; Jazwinski 1989). A mother cell can produce up to 40 daughter cells, then it stops multiplying and dies. This process resembles the phenomenon of replicative senescence of mammalian cells (Jazwinski *et al.* 1989; Jazwinski 1993). Yeast cell culture as such, however, does not age, because it contains one half of just separated young daughter cells and the proportion of senescent mother cells with 20 or more bud scars is negligible. Instructions for aging are encoded in the yeast genome, because mutations can cause either prolongation or shortening of the mother cell life span (Kennedy *et al.* 1995; Jazwinski 1996).

Recent data suggest that yeast aging may result from nucleolar fragmentation (Sinclair *et al.* 1998). The yeast nucleolus contains 100–200 tandem copies of rDNA units separated by nontranscribed sequences as spacers. Each spacer contains three autonomously replicating sequences (ARS). As the cell ages, one or several rDNA copies can be excised from the extrachromosomal rDNA (nucleolar DNA) tandem as circular

molecules (ERC) and, because of the presence of ARS, can replicate. These ERC molecules mostly accumulate in maternal nucleoli during cell division. After 15 or more generations the total DNA content in ERCs may be equal to that of the total yeast genome and the size of the nucleolus therefore enlarges. Still later the nucleolus breaks down and the cell dies (Fig. 8). Two recent publications suggest that oxidative stress could be involved in yeast aging. Ashrafi *et al.* (1999) found that the life-span of mother cells decreases by a prolonged incubation in the stationary phase, *i.e.* under the condition of a continuous cell exposure to endogenous oxidative stress. Moreover, Barker *et al.* (1999) proved that the function of the SOD gene is necessary to ensure the mother cell longevity.

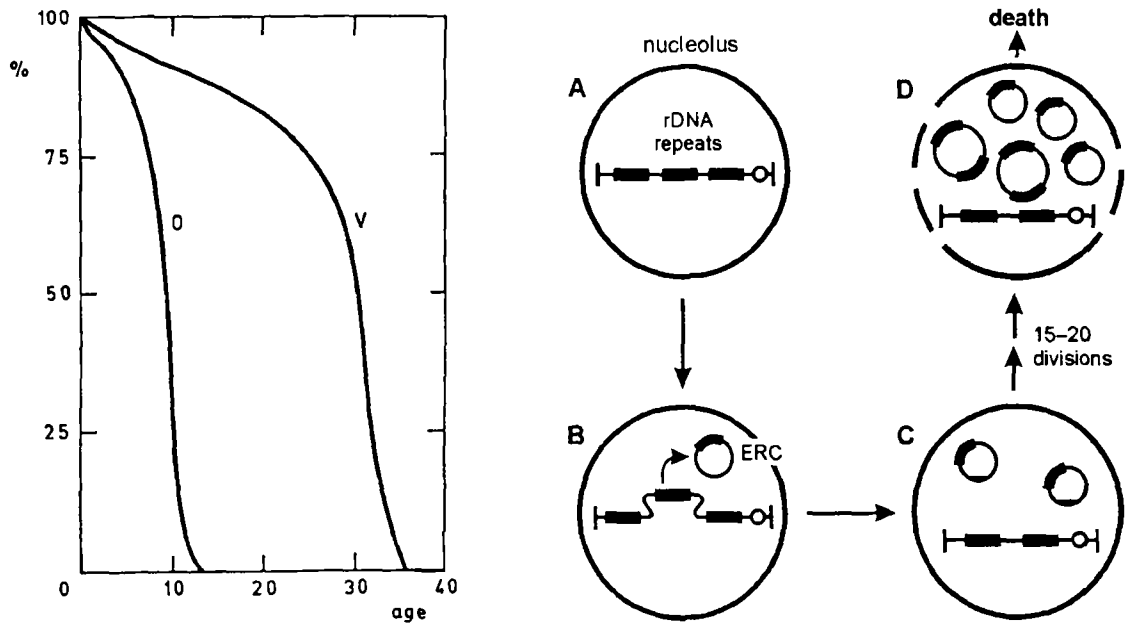


Fig. 8. Aging of yeast cells. *Left*: life span of virgin (V, daughter) cells and old (O, 18th generation) cells; *right*: aging and death of a yeast cell as a consequence of the nucleolar development. **A**, nucleolus contains 100–200 tandem copies of rDNA (*squares*) separated by spacers containing autonomously replicating sequences (ARS; *thin lines*); **B**, excision/inheritance of extrachromosomal rDNA circles (ERC) composed of rDNA and ARS; **C**, replication of ERC remaining in the nucleolus (asymmetric segregation); **D**, enlargement of the nucleolus followed by its fragmentation; based on Jazwinski *et al.* (1989); Sinclair *et al.* (1998).

Another example of aging in lower eukaryotes is the senescence and death of the filamentous fungus *Podospora anserina* (Cummings 1984; Ossiewacz 1997). In this case the senescence is caused by the loss of integrity of mitochondrial DNA, followed by release of its fragments in the form of circular plasmids. Mitochondria thus lose their function as energy-generating machines and the organism ultimately dies. However, it is only a matter of speculation whether the splitting of mitochondrial DNA is a mere consequence of its intrinsic instability or whether it can be ascribed also to the action of continuously produced ROS.

Unlike the merely hypothetical role of ROS in the aging of yeast and *Podospora* spp., oxygen radicals are without any doubt involved in the differentiation of another filamentous fungus, *Neurospora crassa* (Hansberg *et al.* 1993). Exposure of the mycelial mat of this lower fungus to air triggers adhesion of the hyphae, development of aerial hyphae and formation of conidia. The differentiation is accompanied by an increase in the number of carbonyl groups in proteins followed by their degradation. Protein degradation supplies amino acids necessary for the synthesis of new proteins under starvation conditions, which in microorganisms is usually accompanied by differentiation (Toledo and Hansberg 1990; Toledo *et al.* 1994).

7.3 Effect of endogenously formed ROS on nongrowing microbial cells

Nongrowing microbial populations in the stationary phase are exposed to permanently produced ROS, which attack the same cell all the time. It is therefore probable that injuries caused by the oxidative damage will accumulate in these cells. However, two factors decrease this danger: an extensive protein turnover which permanently eliminates defective proteins (Goldberg and St. John 1976; Liu *et al.* 1984; Davies 1986; Gottesman *et al.* 1997) and the development of defense mechanisms, *e.g.*, synthesis of catalases and superoxide dismutases, which decrease the ROS level, along with enzymes repairing DNA and

proteins which stabilize DNA (Storz *et al.* 1990; Farr and Kogoma 1991; Pahl and Bauerle 1994; Marquis *et al.* 1994). This defense apparatus is more efficient than that operating in growing cells, as the resistance of nongrowing bacterial and yeast cells against exogenous ROS is greater (Dowds *et al.* 1987; Siegele and Kolter 1992; Hengge-Aronis 1993; Flattery O'Brian *et al.* 1997).

For instance, stationary-phase cells of *Kluyveromyces lactis* are extremely resistant to both H_2O_2 and $O_2^{-\bullet}$ (Billard *et al.* 1997). Likewise, *S. cerevisiae* cells nearing the stationary phase of growth are considerably more resistant towards oxidants than exponentially growing cells (Jamieson 1992). Stationary-phase cells with lowered intracellular level of glutathione are still more resistant to H_2O_2 and $O_2^{-\bullet}$ than exponentially growing ones, suggesting that GSH level is not crucial in conferring resistance towards oxidants in stationary-phase cells (Stephen and Jamieson 1996). A similar situation was found with heat stress: exponentially growing *S. cerevisiae* cells exposed to nonlethal heat stress induce the synthesis of stress proteins and acquire tolerance to lethal heat shock in a process called acquired thermotolerance, whereas stationary cells are tolerant to heat shock without preceding heat stress, displaying the so-called intrinsic thermotolerance (Zähringer *et al.* 1997). Also, the synthesis of defense enzymes protecting growing bacteria against oxidative stress can be promoted by externally added H_2O_2 or menadione, whereas the development of the corresponding enzymes in nongrowing cells is induced by the mere entry of the population into the stationary phase (Nyström 1998; Altuvia *et al.* 1994; Kawasaki *et al.* 1997). In spite of that the viability of the nongrowing population gradually decreases (Postgate and Hunter 1962). Nevertheless, the proportion of dead cells would be much greater without the "buffering" action of the defense apparatus directed against the toxic effect of endogenous oxidative stress (Eisenstark *et al.* 1992).

Death of bacteria in the stationary phase is probably not caused only by a metabolic disorder triggered by starvation or a continuous oxidative stress. Recent data suggest that it can result also from a genetically directed process which has the features of programmed cell death. It seems to be regulated by a mutual interplay of two protein molecules – the toxin and the antidote, analogous to similar molecules involved in programmed cell death of some bacteria induced by plasmid elimination (Jensen and Gerdes 1995; Chaloupka and Vinter 1996; Nyström 1998). Unlike the suicide genes on plasmids, the genes responsible for killing bacteria in the stationary phase are localized on bacterial chromosomes (Aizenman *et al.* 1996). The genes encoding the toxin remain silent during growth or their products are inactivated by the antidote. However, starvation either induces their activation or relieves their inactivation by the antidote molecules which are more labile than the toxin and are degraded by the proteolytic apparatus. The biological role of this suicide system seems to supply degradation products of dead cells (mainly amino acids) to the surviving part of the population in order to give it a better chance for adaptation to starvation conditions.

7.4 Effect of exogenous ROS

Exogenous oxygen radicals or their inducers can be produced by different cells, *e.g.*, H_2O_2 or superoxide by leukocytes during bacterial infection (Ginsburg 1998; Irani and Goldschmidt-Clermont 1998). ROS are also present in the environment in various forms, *e.g.*, trioxygen, or originate through the activity of UV- or ionizing radiation (*see Chapter 1.2*). They can also be added in various forms (H_2O_2, paraquat, menadione, azo compounds, *etc.*) to the microorganisms by a researcher as a tool in the study of the oxidative stress as such. Their application has been instrumental in revealing the mechanisms of the response of microorganisms to ROS, as well as of the regulatory circuits involved in their control (Storz *et al.* 1990; Farr and Kogoma 1991).*

Exogenously produced ROS behave in the same way as other stress factors, *e.g.*, elevated temperature or osmotic shock. Their lower concentration added to a growing population promotes the synthesis of enzymes and other proteins that protect the organism against the toxic effect of a second dose or a high concentration of the respective stress factor (Storz *et al.* 1990). However, the use of H_2O_2 as a primary stress factor induces in some cases an increased resistance only to H_2O_2 but not to, *e.g.*, menadione, or *vice versa* (Storz *et al.* 1990; Farr and Kogoma 1991).

As mentioned earlier, the tolerance of growing bacteria and yeast not pretreated with a low dose of H_2O_2 to oxidative stress is lower than that of nongrowing cells in the stationary phase (Dowds *et al.* 1987; Jamieson 1995). An enhanced tolerance of stationary cells of bacilli to external ROS is independent of their ability to differentiate. Thus a mutation in the key sporulation-regulating gene *spo0A*, which prevents sporulation, does not decrease the resistance of nongrowing cells to an elevated concentration of peroxide (Dowds *et al.* 1987). Paradoxically, this mutation decreases their sensitivity to the oxidative stress during the exponential phase. This implies that the *spo0A* gene, whose protein product is phosphorylated at the beginning of

*A more detailed analysis of this topic will form part of a subsequent review.

the stationary phase and in this form triggers sporulation (Errington 1993; Stephens 1998), can in the non-phosphorylated form somehow promote the tolerance of growing bacilli to oxidative stress.

Induction of endogenous ROS by many chemical disinfectants is also the principle underlying the killing of vegetative bacteria and bacillary spores (Stewart and Olson 1992; Bloomfield and Arthur 1992). Also some antibiotics and chemotherapeutics kill bacteria by generating oxidative stress (Deretic *et al.* 1995; Gutteridge *et al.* 1998).

In anaerobic bacteria, oxygen plays the role of an exogenous stressor in addition to typical ROS such as H_2O_2. The first defense line against oxygen toxicity in anaerobes represents the negative aerotaxis which enables the bacterial population to move away from oxygen (Zhulin *et al.* 1997). Medium composition has also been shown to play a certain role in affecting the ROS toxicity, complex media increasing the tolerance (Leke *et al.* 1999).

Strict anaerobes (*e.g.*, *Prevotella melaninogenica*) are substantially more sensitive to the presence of oxygen or H_2O_2 than, *e.g.*, the aerotolerant anaerobe *Bacteroides fragilis* or the facultative anaerobe *Salmonella typhimurium*, as detected by oxidative DNA damage or by viability assay (Takeuchi *et al.* 1999). Anaerobic bacteria, including such strict anaerobes as clostridia, contain at least one of the ROS-scavenging enzymes, such as catalases, superoxide dismutases or thioredoxin reductases, which represent the main defense enzymes in all microorganisms (Roche *et al.* 1996; Cortez *et al.* 1998; Lynch and Kuramitsu 1999; Harms *et al.* 1998). Their concentration or activity in strict anaerobes is not sufficient for ROS detoxication in aerated cultures but permits the aerotolerant species to grow in the presence of low oxygen concentrations. In some cases, a classical defense enzyme is replaced by another one with similar enzymic activity, such as the *Clostridium perfringens* rubererythrin with superoxide dismutase activity (Lehmann *et al.* 1996).

Studies of oxidative stress and its control in anaerobes have in recent years been often carried out using the aerotolerant *Bacteroides fragilis* (Rocha *et al.* 1996; Rocha and Smith 1998). This bacterium was established to contain a fairly low concentration of catalase B mRNA during the exponential phase of anaerobic growth, while its level sharply rises during the post-exponential or at the beginning of the stationary phase (Rocha and Smith 1997).

Hyperthermophilic archaebacteria represent a special group of anaerobes (Adams 1999). They also contain ROS-scavenging enzymes such as catalase-peroxidase (Cannac-Caffrey *et al.* 1998) or superoxide dismutase (Yamano and Maruyama 1999).

It thus seems that the main defense principles coming into play in cells coping with oxidative stress are common to aerobic and anaerobic bacteria and the critical factor in the tolerance to O_2 or H_2O_2 is only the level or activity of the respective enzymes.

7.5 Cues on the mechanisms of cell death induced by ROS

Before exploring the cell death mechanisms in microorganisms it must be stated that the criteria of their death are not sufficiently defined (*see also an older review of* Mason *et al.* 1986). The mere loss of colony-forming ability is not a sufficient criterion. On the one hand, a portion of nonreproducing population can be classified as "viable but not culturable cells" and the oxidative stress may increase the size of this portion (Hochman 1997). On the other hand, differentiating cells such as bacilli lose the ability to grow in the irreversible sporulation phase (Frehel and Ryter 1969; Kretschmer 1972) in spite of their very intense metabolic activity directed to spore development (Chaloupka and Vinter 1996). A better criterion of death is the loss of cell membrane potential and an increase of membrane permeability (Niven and Mulholland 1998; Nebe-von Caron *et al.* 1998). This is usually followed by autolysis, which is the best evidence of cell death. Unfortunately, most data concerning cell death of microorganisms are based only on the loss of colony-forming ability.

There is no sufficient evidence concerning the death pathways in microorganisms induced by oxidative stress because much more effort has concentrated on the elucidation of their adaptation to oxidative stress than on the death pathways of irreparably damaged cells. We shall therefore analyze data on death mechanisms induced by other factors and speculate whether they could also take place in death triggered by ROS.

In bacteria, death often results from an impaired integrity of the cell membrane or the cell wall by lysins or autolytic enzymes. This holds for the "enforced suicide" caused by elimination of plasmids coding for a toxin and its antidote, as well as for lysis of an infected bacterium by phage lysins. Also some drugs, *e.g.*, penicillin derivatives, trigger cell death by preventing the maintenance of the cell-wall integrity either by inhibiting insertion of new peptidoglycan components into the cell wall weakened by its turnover, or by activating autolytic enzymes (Doyle *et al.* 1988). During sporulation of bacilli the mother compartment of the sporangium undergoes programmed death at the end of the developmental cycle (Chaloupka and Vinter

1996). Its autolysis is preceded by an extensive degradation of proteins prelabeled during growth or during the reversible sporulation phase (Strnadová *et al.* 1992). It is possible that the proteinases participating in this process can damage the cytoplasmic membrane or inactivate some energy-yielding enzymes which would cause activation of autolytic enzymes.

Oxidative stress injures the cytoplasmic membrane by oxidizing its lipids and proteins (*see Chapter 6*) through the action of either extracellular or intracellularly generated ROS. This holds mainly for bacteria because the bacterial cytoplasmic membrane plays the role of mitochondria in energy-yielding reactions. It seems therefore a plausible speculation that the damaged membrane can either break down and release cell components, which results in immediate death, or it loses its biochemical function, *i.e.* the generation and maintenance of the proton gradient, and this results in activation of autolytic enzymes (Kemper and Doyle 1993; Kemper *et al.* 1993). The terminal phases of bacterial cell death in this case would be more similar to necrosis than to apoptosis.

On the other hand, oxidative stress damages also DNA, impairing thus chromosome reproduction. It is therefore possible that such DNA damage may induce changes similar to thymineless death. This results from inhibition of DNA replication of bacteria and yeast and brings about single- or double-strand breaks, the former reparable, the latter irreparable, and drives the cell to death (Ahmad *et al.* 1998). The absence of thymine that stops DNA reproduction inhibits the normal course of the cell cycle. The cells can grow for some time and then they suddenly lose viability but do not autolyze (Smith and Hanawalt 1968). The mechanism of terminal steps of the thymineless death pathway has not yet been elucidated in spite of a fairly intense research.

It is also necessary to mention the existence of "suicide genes" on chromosomes of some bacteria. They can be activated during starvation (Aizenman *et al.* 1996) or by an as yet unknown signal (Feng *et al.* 1994; Pedersen and Gerdes 1999) and kill bacteria by different mechanisms. It is not excluded that oxidative or another stress could be involved in their activation.

The ultrastructure of yeast cell and of cells of other unicellular eukaryotic microorganisms is similar to animal cells. A question therefore can be raised whether, in eukaryotic microorganisms, oxidative stress can trigger death pathways similar to apoptosis. Apoptosis of animal or plant cells, whether induced by development or by the oxidative or other kind of stress, proceeds in identical or similar steps for most of its pathway (Fig. 7). The key point in its course is the activation of the proteolytic cascade, although a caspase-independent mode of apoptosis may also be possible (Shaham 1998). Scant data are available on the death mechanism in simple eukaryotes caused by oxidative stress and analysis of the apoptosis-like steps in their development could therefore shed light also on their death pathway under pathological conditions.

Programmed cell death is part of the developmental cycle of several eukaryotic microorganisms, *e.g.*, of parasitic protozoa and saprophytic fungi (*Dictyostelium* spp.). Its manifestations resemble apoptosis, especially as regards chromatin condensation, fragmentation and DNA degradation (Ameisen 1996; Welburn *et al.* 1997; Olie *et al.* 1998). It, however, proceeds by pathways different from apoptosis in multicellular organisms, because the key regulators of typical apoptosis of mammalian cells, *i.e.* p53, Bcl-2 family proteins and caspases, are absent in microorganisms (Shaham *et al.* 1998; Olie *et al.* 1998).

Yeast cells do not exhibit any signs of typical apoptosis during their development. Their replicative senescence, which involves disintegration of the nucleolus (Sinclair *et al.* 1998), is accelerated by inactivation of SOD, which implies the role of ROS in the process (Barker *et al.* 1999).

Despite the lack of typical signs of apoptosis, some components of the apoptotic (or proapoptotic) pathway do exist in yeasts and some fungi because inactivation of the *CDC48* gene regulating the cell cycle in *S. cerevisiae* (Madeo *et al.* 1997) or inhibition of the development of the fungus *Mucor racemosus* (Rose and Linz 1998) induce apoptosis-like changes in chromatin of their cells. Yeast also contains a derivative of ceramide, a signal molecule participating in apoptosis induction (Dickson 1998) and the mitochondrial F_0F_1-ATPase, which is suspected to play a role in the apoptotic pathway (Shaham *et al.* 1998). As shown recently, the ROS or a decreased glutathione level induce an apoptotic phenotype in yeast, which is characterized by chromatin condensation and DNA degradation, whereas the membrane function is preserved (Madeo *et al.* 1999). Also some protozoa (*e.g.*, *Trypanosoma brucei*) exhibit an apoptotic phenotype induced by ROS (Ridgley *et al.* 1999). Also here the death pathway is accompanied by DNA degradation while the permeability barrier remains intact.

Yeasts are often used as model organisms for the study of mechanisms of action of foreign apoptotic genes. Thus the transcription of key human apoptotic genes (*bak* or *bax*) transferred into yeast cells promotes chromatin condensation and fragmentation, typical signs of the terminal phase of apoptosis (Ink *et al.* 1997; Shaham *et al.* 1998). In addition, simultaneous expression of the *bak* or *bax* and *bcl-2* genes in yeast blocks the *bak* and *bax* activity as in animal cells (Tao *et al.* 1997; Ligr *et al.* 1998). The fact that the Bcl-2 protein increases the viability of yeast cells whose defense against the oxidative stress was impaired (Longo *et al.*

1996, 1997; Shaham 1998) suggests that this antiapoptotic protein could display an antioxidant activity in all eukaryotes. On the other hand, caspase-3, a key member of the proteolytic cascade, inhibits the growth of *S. cerevisiae* without causing cell death (Wright *et al.* 1999).

Also in *Trypanosoma brucei* the apoptotic process proceeds without participation of caspases or other proteolytic enzymes (Ridgley *et al.* 1999). Surprisingly, the apoptosis-triggering protein Bax kills the bacterium *E. coli*, probably by increasing the endogenous ROS formation (Asoh *et al.* 1998).

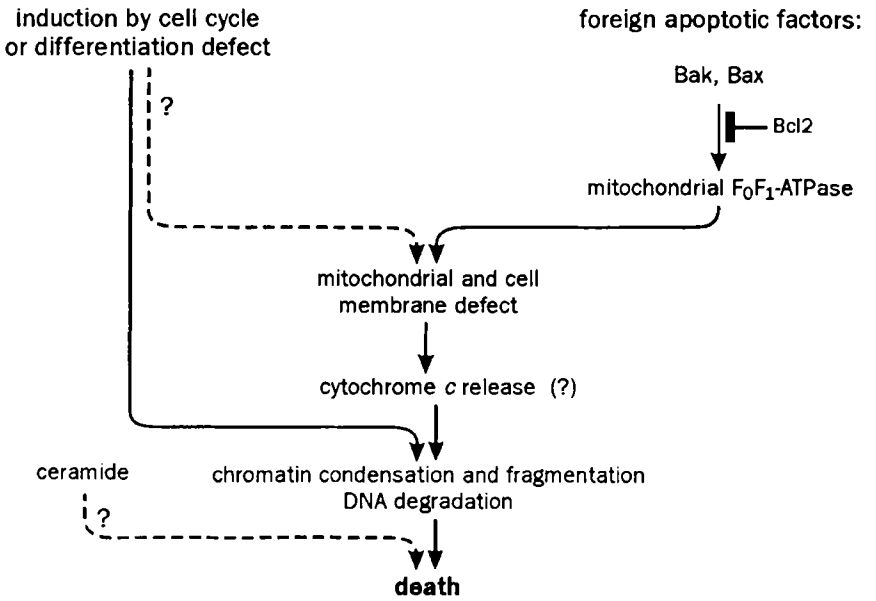


Fig. 9. A tentative scheme of the apoptotic pathway in yeast and fungi.

It thus seems that yeast and probably also other eukaryotic microorganisms contain several components of the apoptotic pathway participating in a caspase-independent mechanism of apoptosis (Sha and Reed 1997; Madeo *et al.* 1997; Ink *et al.* 1997; Tao *et al.* 1997; Ligr *et al.* 1998). Yeasts are of course equipped with a nucleolytic enzyme system involved in the repair of damaged DNA (Eide *et al.* 1996; Flattery-O'Brien 1998; Wang 1998) and in DNA degradation in the terminal phase of apoptosis. This proapoptotic system is silent under normal conditions but is activated either by foreign apoptotic factors (*e.g.*, Bak and Bax) or by internal signals triggered by interruption of the cell cycle or cytodifferentiation (Fig. 9). All this supports the idea of the existence of a part of the apoptotic death pathway in simple eukaryotes, which is characterized by chromatin condensation, DNA degradation and ROS involvement. The participation of a specific proteolytic apparatus and its involvement in this process probably developed much later.

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