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# Biologically Relevant Small Radicals

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*Abstract:* Biologically relevant small radicals are at the focus of the working group 4 (WG4) of the COST Action CM0603 (Free Radicals in Chemical Biology, CHEMBIORADICAL). This article surveys the areas of research being undertaken by the partners in WG4. The character of the radicals is described together with experimental techniques utilized to follow their structure and reactivity. Specifically, C-, S-, N- and O-centered radicals of small size, and their interaction with different biomolecules are described. Processes at the molecular level exemplifying important biological signaling and damaging pathways are introduced.

**Keywords:** Antioxidants · Reactive carbon species · Reactive oxygen species · Reactive nitrogen species · Reactive sulfur species · Signaling pathways

## 1. Introduction

Scientists, as well as the general public, sometimes turn to *www.wikipedia.org* for succinct introductions to topics of interest, and using this source and the search term 'free radical' reminds us both of the historical but now completely obsolete use of 'radical' in a chemical context as a functional group or substituent, *e.g.* methyl in methyl alcohol, and the much more widespread use, usually with the adjective 'free', reflecting the definition in this source of free radicals as 'atomic or molecular species with unpaired electrons…'. The next sentence continues: 'These unpaired electrons are usually highly reactive…', and here succinctness has triumphed over accuracy. Thus the common chemicals, oxygen and nitric oxide, have unpaired electrons but both have a rather limited spectrum of highly reactive partners in chemistry. However, it is true that many free radicals are short-lived in solution and specialized techniques are needed to monitor their reactions.

Free radicals were regarded as somewhat esoteric chemical species of interest only to a few specialists studying radiation effects until 1968 when it was discovered that the superoxide ion radical ($O_2^{\bullet-}$) is a normal product of the biological reduction of molecular oxygen.[1] The reduction of molecular oxygen to $O_2^{\bullet-}$ is arguably the commonest biological route to free radicals, and interest in the chemical biology of $O_2^{\bullet-}$ was greatly stimulated by the discovery that all living cells contain an unusual family of enzymes, the superoxide dismutases (SODs), which protect against the deleterious actions of this radical by catalyzing its dismutation to hydrogen peroxide and oxygen.[2] The discovery of the endogenous formation of nitric oxide ($^{\bullet}NO$)[3,4] led also to an explosion in research centering on the diverse role of this radical in biological systems. As Fig. 1 shows, there was almost no biological interest in $O_2^{\bullet-}$ until the discovery of SOD in 1969 and in $^{\bullet}NO$ until the discovery that it is an endothelial-derived relaxing factor in 1987.

Scheme 1 (adapted from refs [5,6]) shows some pathways by which other biologically important free radicals can be produced, either *via* $H_2O_2$ from $O_2^{\bullet-}$ or consequent upon peroxynitrite ($ONOO^-$) formation from $O_2^{\bullet-}$ and $^{\bullet}NO$.[7] Note the importance of carbon dioxide as a physiological *reactant* rather than buffer.[8] Inset to the scheme are paths by which thiols, often considered as radical protectants, can stimulate superoxide formation or transfer radical damage to ascorbate as the ultimate 'sink'. The multiplicity of species in Scheme 1 demonstrates how inadequate as descriptor the term 'reactive oxygen species' is, particularly since key biological radicals such as lipid- and protein-based peroxyl radicals, and 'downstream' species such as thiyl peroxyl radicals, are not shown for simplicity.

Free radicals are part of the chemistry of life. Many cells in the body are producing around 300,000 free radicals (>$10^{17}$ radicals $kg^{-1}$ $s^{-1}$); estimates have been made of the rate of production of hydrogen peroxide in the liver, arising from the disproportionation of two superoxide radicals, in excess of 1 μM $s^{-1}$.[9] Viagra is used to overcome a deficiency in the production of $^{\bullet}NO$ in the right place at the right time. The geometry of unsaturation of fatty acids in foodstuffs is a feature even of product marketing, isomerization from *cis* to *trans* being a consequence of free-radical exposure, as is butter going rancid because of the free-radical chain reactions of lipid peroxidation.

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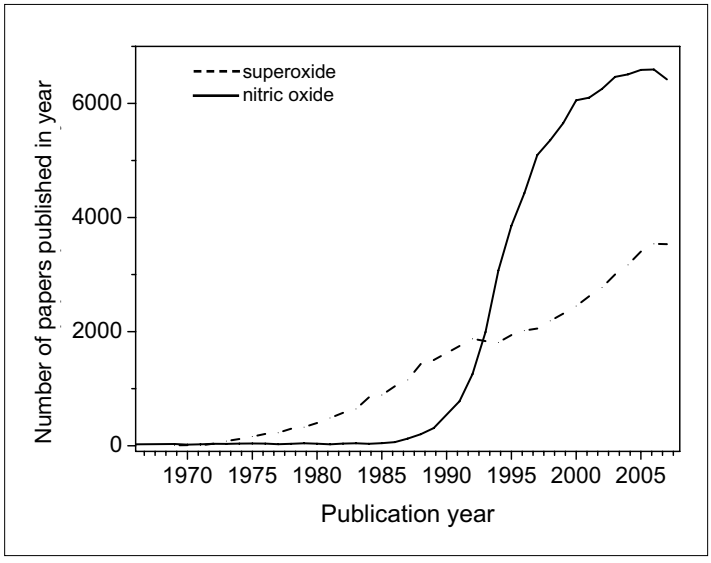
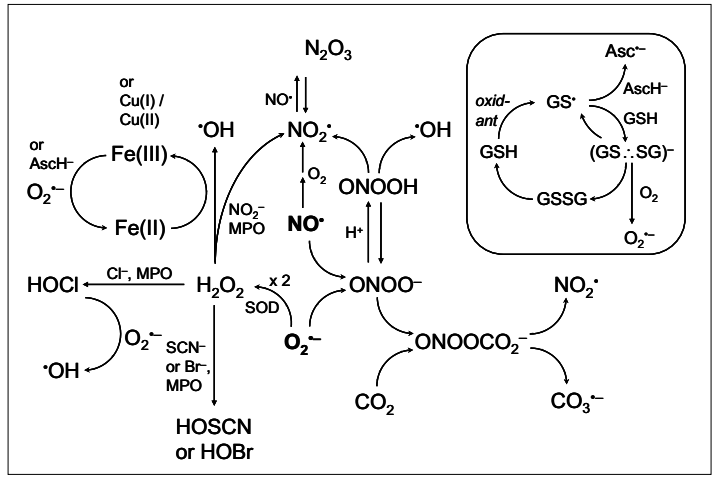



Fig. 1. Growth of research on free radicals in biology, as illustrated by the numbers of papers published each year and indexed in the PubMed database using the search terms indicated




Scheme 1. Pathways of interaction of superoxide and nitric oxide in biology. SOD = superoxide dismutase; MPO = myeloperoxidase; AscH$^-$ = ascorbate. Inset: pathways in which thiols such as glutathione (GSH) are oxidized to thiyl radicals (including hydrogen donation to carbon-centred or peroxyl radicals) and the resulting formation of superoxide or ascorbate radicals.

By definition, radiation therapy of cancer is free-radical therapy, but in the last 30 years the diverse roles of free radicals in numerous pathological conditions and diseases, and in the modes of action of some drugs, have become increasingly evident.

This article surveys the areas of research being undertaken by the partners in the fourth working group. Research focuses on the generation and reactivity of small C-, S-, N- and O-centered radicals. These radicals interact with multiple biomolecules and participate in important biological signaling and damaging pathways.

## 2. Survey of the Main Species

### 2.1. *Reactive Oxygen Species (ROS)*

From four billion years ago the earth has possessed an oxidative atmosphere due to the presence of molecular oxygen ($^3O_2$; triplet dioxygen), which plays an immense role in biological processes.[10] It is a biradical with two unpaired electrons and so its direct reaction with organic substrates is restricted.[11] By virtue of its biradical nature, it readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as reactive oxygen species (ROS). These include $O_2^{\bullet-}$, $H_2O_2$ and the hydroxyl radical ($^\bullet$OH). Singlet oxygen ($^1O_2$) is produced as a result of natural biological reactions and by photosensitization, *i.e.* the absorption of light energy by triplet dioxygen. $^1O_2$ has a pair of electrons with opposite spins; though not a free radical it is highly reactive. According to rules of physical chemistry, the 'relaxation' (excess energy loss) of singlet oxygen back to the triplet state is 'spin forbidden' and thus singlet oxygen has a long lifetime for an energetically excited molecule, and must transfer its excess energy to another molecule in order to relax to the triplet state.

ROS are formed during normal metabolism, in pathophysiological processes as well as by UV light or ionizing radiation. These species are very reactive towards DNA, proteins, and lipids, and can trigger various illnesses and enhance aging processes.[12,13] There are, however, defence mechanisms to ameliorate the injurious effect of ROS, such as SOD,[2] catalase,[14] and various quenchers of these reactive molecules (antioxidants).

Carbonate radical ($CO_3^{\bullet-}$) is also considered as a reactive oxygen species because some unpaired spin density is on the O atoms. This radical was practically ignored in biology up to the 1990s when its formation was discovered in biological systems during the decomposition of the peroxynitrite in the presence of bicarbonate[8,15] (Scheme 1), peroxidase activity of Cu,Zn-SOD[16–18] and xanthine oxidase turnover in the presence of bicarbonate.[19] The carbonate radical is less reactive than hydroxyl radical, but it is more selective. For example, $CO_3^{\bullet-}$ oxidizes selectively amino acids in proteins (most notably tryptophan, tyrosine, and cysteine)[20] and may be a mediator of protein modification in cellular environments under conditions of oxidative stress such as ageing and neurodegenerative and inflammatory processes.[21–25] Reactions of $CO_3^{\bullet-}$ with prosthetic transition metal ions in metalloproteins are also predicted but they have been little studied to date.[26]

Peroxyl (ROO$^\bullet$) and alkoxyl (RO$^\bullet$) radicals are formed *via* the reaction of carbon-centered radicals with dioxygen and are also considered as ROS. These radicals are important reactive intermediates in biological systems. For example, peroxidation has been shown to proceed *via* radical-mediated chain propagation reactions that give rise to a variety of reactive species, such as alkoxyl and peroxyl radicals, and secondary breakdown products: carbonyl groups, alcohols and hydroperoxides[27,28] (see also Section 2.4.3).

### 2.2. *Reactive Nitrogen Species (RNS)*

Nitric oxide ($^\bullet$NO) was considered until 1987 as a toxic environmental pollutant that destroys ozone, causes acid rain and possibly has carcinogenic properties. In 1987, it was discovered that nitric oxide is formed enzymatically by a variety of mammalian cells.[4] Since then $^\bullet$NO has become one of the most studied and fascinating entities in biological chemistry (see Fig. 1), and in 1992 was chosen as 'molecule of the year' by *Science*.[29] The production of $^\bullet$NO at low physiological levels is mainly involved in homeostatic biochemical and physiological processes such as signal transduction, neurotransmission, smooth muscle relaxation, peristalsis, inhibition of platelet aggregation, blood pressure modulation, immune system control, learning, and memory.[30,31] However, excess production of $^\bullet$NO can cause tissue injury, and this radical has been shown to be involved in the pathogenesis of disease states such as endotoxin shock and inflammation.[32,33]

Part of the toxicity of $^\bullet$NO is believed to be due to its fast reaction with $O_2^{\bullet-}$ forming peroxynitrite,[34,35] which oxidizes and nitrates a large variety of biomolecules through complex mechanisms.[36] The oxidation may take place directly through the reaction of peroxynitrite with the substrate, through intermediates formed during the decomposition of ONOOH or through the reaction of $ONOO^-$ with $CO_2$. ONOOH decomposes to nitrate through homolysis of the weak O–O bond forming $^\bullet$OH and $^\bullet NO_2$, whereas the reaction of $ONOO^-$ with $CO_2$ forms a short-lived adduct, $ONOOC(O)O^-$, which decomposes either into nitrate and $CO_2$ or

through the formation of $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ as oxidizing intermediates (see Scheme 1). The concentration of $CO_2$ in biological systems is relatively high due to high levels of bicarbonate in intracellular (12 mM) and interstitial fluids (30 mM). This suggests that the reaction of peroxynitrite with $CO_2$ must be the predominant pathway for peroxynitrite disappearance in biological systems. In any case $^{\bullet}NO_2$ is formed, which is a mild and selective oxidant.

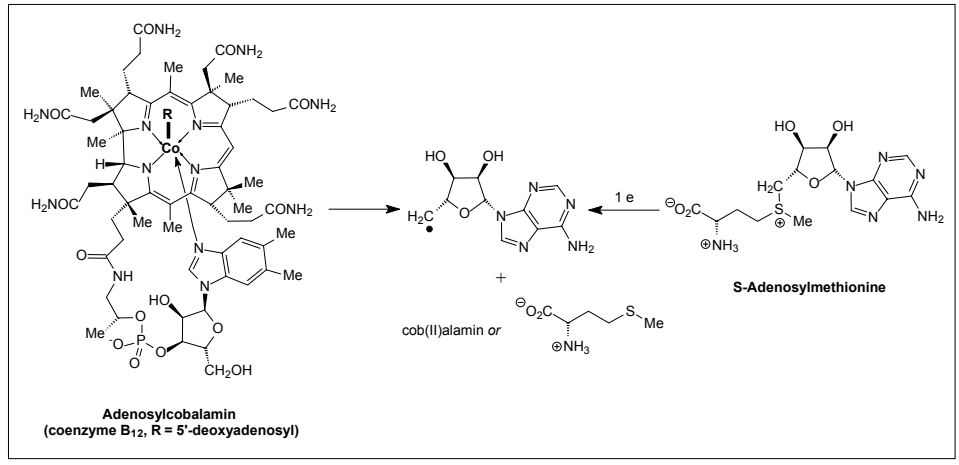
In many cases the evidence that implicates $ONOO^-$ in a large variety of diseases is the detection of 3-nitrotyrosine in the injured tissues.[37–39] However, nitration of tyrosine can also take place when $^{\bullet}NO_2$ is formed *via* other processes, *e.g.* from nitrite reacting with peroxidases,[40] or during autoxidation of $^{\bullet}NO$[41] (see Scheme 1). The autoxidation of $^{\bullet}NO$ also produces dinitrogen trioxide ($N_2O_3$) as a nitrosating species potentially capable of causing, for example, DNA damage.[42] Whether this occurs in biology, where $^{\bullet}NO_2$ concentrations are maintained at very low levels by reaction with antioxidants such as thiols, ascorbate and urate, can be questioned.[43]

Peroxynitrate ($O_2NOO^-/O_2NOOH$) is formed *via* the fast reaction of $^{\bullet}NO_2$ with superoxide and during the decomposition of peroxynitrite in the presence of organic compounds.[44,45] Under physiological conditions it decomposes rapidly to nitrite and oxygen. This peroxo compound will be considered as a reactive nitrogen species once it is shown that its decomposition product is singlet oxygen.[36]

## 2.3. *Reactive Sulfur Species (RSS)*

As S–H bond strengths are generally weaker than those characterizing C–H bonds, thiols (RSH) tend to act as radical 'repair' agents by transferring H to radical centres. However, the rules of spin conservation require such 'repair' reactions to be accompanied by formation of sulfur-centred or thiyl radicals ($RS^{\bullet}$), and these are far from inert. Even the hydrogen-donation reactions of thiols are now recognized to be an equilibrium, and hydrogen *abstraction* by $RS^{\bullet}$ is not unknown: peptide and protein thiyl radicals may lead to the oxidation and/or epimerization of amino acids adjacent to the thiyl radicals[46] (see also Section 2.4.1). Addition/elimination of thiyl radicals to double bonds may initiate *cis*/*trans* isomerization, particularly important in routes to the formation of *trans* fatty acids, with major biological implications.[47] Hence viewing thiols simply as radical 'repair' agents is at best naïve.

A key feature of thiyl radicals is the conjugation equilibrium with thiols to form disulfide radical-anions, which involve sulfur–sulfur three-electron bonds and thus may be represented by $(RS\therefore SR)^-$.[48] This equilibrium (see also Scheme 1, inset) is important because it transforms the moderately oxidizing thiyl radical to the strongly-reducing disulfide radical-anion; in particular, it provides a route to the formation of superoxide radicals following rapid reaction of $(RS\therefore SR)^-$ with oxygen. A series of parallel/sequential reactions involving competition between oxidation of ascorbate and/or urate, and reduction of oxygen *via* $(RS\therefore SR)^-$, as well as addition of $O_2$ to $RS^{\bullet}$,[49] may ensue in cells; the outcome is very much dependent upon antioxidant status and $O_2$ levels.[50]




Scheme 2. Two routes to the 5′-deoxyadenosyl radical

While the chemistry of 'reactive sulfur species' is obviously complex, it is increasingly recognized as of major importance not only to free-radical damage but also to redox-based signaling pathways.[51] Following on with major biological roles for $NO^{\bullet}$ and even CO, $H_2S$ is the newest 'player' on the block,[52–54] with roles extending to possibilities for therapeutic use.[55] Physiological $H_2S$ levels of 50–160 μM in mammalian brain tissue and 10–100 μM in human plasma have been reported. The involvement in biology of thiyl radicals derived from $H_2S$ has been less well-studied compared to *e.g.* the glutathionyl radical, although the basic features of the chemistry of sulfhydryl radicals ($S^{\bullet-}/^{\bullet}SH$) have been characterized.[56] A biomimetic model of vesicle suspension, which mimics the aqueous and membrane compartments of a cell, has recently been used to demonstrate the potential of radicals derived from $H_2S$ to access the hydrophobic fatty acid chains and attack the double bonds.[57] The phospholipids produced in this way contained a high proportion of *trans* fatty acid residues. This model offers some insight into the chemical basis of the biological activity of $H_2S$, which has not yet been established.

## 2.4. *Reactive Carbon Species (RCS)*

Carbon radicals in biological systems are either there 'as of right' or arise as a consequence of an organism's exposure to a radical precursor. Examples of radicals that are obligatory participants in cellular processes are the 5′-deoxyadenosyl radical (Scheme 2) and the glycyl radical within a polypeptide chain (–CONH$^{\bullet}$CHCONH–). Enzymes that utilize such radicals as intermediates in their catalytic pathways are designated as 'radical enzymes'.[58] In contrast to the radicals that are essential for an organism's survival, there are radicals that are fortuitous products of an organism's exposure to some 'foreign' species (xenobiotic). The carbon radicals that participate for 'better or worse' in the cell are therefore from diverse sources.

### 2.4.1. *Amino Acid Radicals*

Abstraction of a hydrogen atom from the α-position of an amino acid by a reactive radical (*e.g.* $^{\bullet}OH$) leading to a resonance-stabilized radical is a long known process associated with the degradation of peptides and proteins under aerobic conditions.[59] For a free amino acid the preferred species is $H_2N^{\bullet}CRCO_2H$, which is a so-called captodative radical because the stabilization involves both the nitrogen lone pair as donor and the carboxyl group as electron acceptor. Nature makes use of this phenomenon in glycyl radical enzymes and for the oxidative degradation of the C-terminal glycine unit of certain peptides. It has been suggested that Alzheimer's disease is the consequence of oxidative damage to prion proteins.[60]

Glycyl radicals are found in a subunit of certain radical enzymes in which they are formed by the action of the 5′-deoxyadenosyl radical (see below) on a conserved glycine residue situated near the carboxy terminus of the polypeptide chain. The first characterized glycyl radical enzyme was pyruvate:formate lyase (PFL),[62] which is synthesized by *Escherichia coli* under anaerobic conditions. PFL catalyses the fission of pyruvate to formate and acetate (as acetyl-coenzyme A) *via* a cascade of radicals. One possible reaction pathway that is supported by the crystal structure of the enzyme[62] has the glycyl radical abstract a hydrogen atom from a cysteine thiol generating a thiyl radical (Cys–$S^{\bullet}$). Transfer of the radical centre to a neighbouring cysteine is followed by addition of the newly formed thiyl radical to

the keto carbonyl group of pyruvate to give an oxy radical that fragments to an S-acetylcysteine residue and the formate radical ($CO_2^{\bullet-}$). The formate radical is quenched by a cysteine thiol to afford formate and a thiyl radical, which reacts with the conserved glycine to restore the original glycyl radical and cysteine thiol. Finally the acetyl group is transferred from cysteine to coenzyme A with restoration of the other cysteine thiol.

Glycyl radicals are also implicated in the action of peptidylglycine α-amidating monooxygenase (PAM), which degrades a carboxy-terminal glycine residue into glyoxylic acid and a carboxamide (reaction 1).[63]

$$\text{peptide–CONHCH}_2\text{CO}_2\text{H} \rightarrow \text{peptide–CONH}_2 + \text{OCHCO}_2\text{H} \quad (1)$$

The product amides are peptide hormones with a myriad of physiological properties. The enzyme is comprised of two subunits, one of which is a monooxygenase dependent on dioxygen, $Cu^{2+}$ and ascorbate that hydroxylates the methylene group of the glycine residue *via* a glycyl radical (reactions 2 and 3).

$$\text{peptide–CONHCH}_2\text{CO}_2\text{H} \rightarrow \text{peptide–CONH}^{\bullet}\text{CHCO}_2\text{H} \quad (2)$$

$$\text{peptide–CONH}^{\bullet}\text{CHCO}_2\text{H} \rightarrow \text{peptide–CONHCHOHCO}_2\text{H} \quad (3)$$

The other is a lyase that facilitates cleavage of the intermediate α-hydroxyglycine unit (reaction 4).

$$\text{peptide–CONHCHOHCO}_2\text{H} \rightarrow \text{peptide–CONH}_2 + \text{OCHCO}_2\text{H} \quad (4)$$

The monooxygenase will also operate on certain other amino acid residues, *e.g.* alanine or vinylglycine, but not on 2,2,2-trifluoroalanine because the trifluoromethyl group destabilizes the intermediate radical by *ca.* 40 kJ $mol^{-1}$.

#### 2.4.2. *5′-Deoxyadenosyl Radical*

The 5′-deoxyadenosyl radical required for the activation of PFL is derived from cleavage of an S–C bond of S-adenosylmethionine (SAM).[64] This is a one-electron reduction mediated by ferrodoxin or flavodoxin, which achieves the selective homolysis of the S–C bond to the adenosyl group and hence the formation of methionine alongside the 5′-deoxyadenosyl radical (Scheme 2). There are numerous SAM-dependent radical enzymes in which the 5′-deoxyadenosyl radical is utilized directly for substrate activation. One of the best characterized SAM-dependent enzymes is the oxygen-sensitive lysine 2,3-aminomutase from *Clostridium subterminale*.[65] The α-lysine substrate initially forms a Schiff base with pyridoxal phosphate (PLP). Abstraction of a hydrogen atom from C(3) of the lysine chain by the 5′-deoxyadenosyl radical affords a secondary alkyl radical (α-lysine-3-yl radical bound to PLP), which rearranges to the β-lysine-2-yl radical *via* an intermediate azacyclopropylcarbinyl radical (both PLP-bound). Finally, the β-lysine-2-yl radical (PLP-bound) retrieves a hydrogen atom from 5′-deoxyadenosine and affords β-lysine after hydrolysis of the PLP imine, as well as the 5′-deoxyadenosyl radical, which can either be employed for another catalytic cycle or combine with methionine to re-form SAM (note that in most SAM-dependent enzymes SAM is consumed irreversibly). All of the radicals described are protein-bound and some have been characterized by electron spin resonance spectroscopy.

The 5′-deoxyadenosyl radical can also be derived from adenosylcobalamin (coenzyme $B_{12}$) by homolysis of the Co–C bond[58,66] (Scheme 2). In this case no one-electron reduction is needed; the cobalamin moiety takes up one electron from the Co–C bond with the formation of $d^7$ cob(II) alamin. The structural complexity of coenzyme $B_{12}$ compared to SAM caused H. A. Barker to name SAM the 'poor man's $B_{12}$'. In truth, SAM is a cofactor for hundreds of radical enzymes, whereas coenzyme $B_{12}$ has only been found as cofactor for about ten enzymes, which include glutamate mutase and diol dehydratase. In the former, (*S*)-glutamate is converted by the action of 5′-deoxyadenosyl radical to the 4-glutamyl radical ($^{-}O_2C^{\bullet}CHCH_2CHNH_2CO_2^{-}$), which fragments to acrylate and the glycyl radical ($H_2N^{\bullet}CHCO_2^{-}$). Recombination of these species gives the 3-methylene-aspartate radical {$^{-}O_2CCH(^{\bullet}CH_2)CHNH_2CO_2^{-}$} and hence the product (2*S*,3*S*)-3-methylaspartate in a similar manner to lysine 2,3-aminomutase. With diol dehydratase, activation of a substrate molecule (*e.g.* propane-1,2-diol) affords a propane-1,2-diol-1-yl radical (MeCHOH$^{\bullet}$CHOH). This species undergoes a 1,2-hydroxyl shift leading to the propane-1,1-diol-2-yl radical (Me$^{\bullet}$CHCH$(OH)_2$) and hence to propane-1,1-diol and the product propanal. In all of the coenzyme $B_{12}$-dependent enzymes carbon radicals are intermediates and many of these species are highly reactive non-stabilized methylene radicals (*e.g.* the 3-methylene-aspartate radical). Many would say that precisely how the protein and cobalamin cofactor 'tames' these radicals is far from understood.

#### 2.4.3. *Trichloromethyl Radical*

The trichloromethyl radical is formed by the action of cytochrome P450 (primarily the 2E1 isozyme) on carbon tetrachloride, an organic intermediate and solvent that is still widely used despite its potential toxicity to humans.[67] Trichloromethyl reacts with dioxygen to give the trichloromethylperoxy radical that causes lipid peroxidation with the formation of aldehydes that damage proteins and DNA. The degradation of lipids can also lead to alkyl radicals and a new round of destructive events.

## 3. Experimental Methodology

### 3.1. *Kinetics*

To study the reactions involving such short living species as small radicals, special methods have to be used. Presently the most useful methods for the studies of fast reactions in solutions, *i.e.* those occurring on the timescales faster than $10^{-4}$ s, are pulse radiolysis and laser flash photolysis. The basic principle of both is the same. The short pulse (from microseconds down to pico- or even femtoseconds) of energy, delivered to the sample either by laser light or by electrons from accelerators of various types, produces radicals or radical ions which initiate chemical processes. These processes can be monitored by the time-resolved techniques, including optical absorption or emission spectroscopy, light scattering, electrical conductivity, polarography, and electron spin resonance spectroscopy. Optical absorption spectroscopy is the simplest one and therefore most popular. An important difference between flash photolysis and pulse radiolysis has to be underlined. In the former the energy is exclusively absorbed by the given component of the solution, while in the latter the energy is absorbed by the solvent, *e.g.* water, and the radical products of its radiolysis, *i.e.* $^{\bullet}OH$, $e_{aq}^{-}$, $H^{\bullet}$, then interact rapidly with purposely added solute(s) to produce radicals of interest. Both methods allow the determination of rate constants of reactions of radicals with various compounds, the determination of radical yields, and obtaining the absorption or emission spectra of short living intermediates. In the case of complex systems, where a number of reactions have to be taken into account the chemical simulation may help to establish the overall mechanism.

When neither radicals nor the target molecules can be observed directly, a so-called reference compound may be added to the system. The reference compound reacts rapidly with the radical to give the product showing an intense absorption spectrum in the convenient spectrum region. The reference compound can be used either as a model molecule enabling the comparison of the reactivity of different radicals, or as a competing agent. In the latter case the procedure known from chemical kinetics as the 'competition method' allows the determination of the rate constant of the reaction of the radical with target molecule. It is worth noting that in the case when the product obtained in the reaction of the radical with the competitor is stable, conventional detection methods can be used. Fig. 2 shows an

example of using $ABTS^{2-}$ [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate] as a competing compound in order to extract the rate constant of the reaction of $^{\bullet}NO_2$ radical with cytochrome c (cytc).

$$^{\bullet}NO_2 + cytc \rightarrow NO_2^- + cytc^{\bullet} + H^+ \quad (5)$$

$$cytc^{\bullet} + ABTS^{2-} + H^+ \rightarrow cytc + ABTS^{\bullet-} \quad (6)$$

$$^{\bullet}NO_2 + ABTS^{2-} \rightarrow NO_2^- + ABTS^{\bullet-} \quad (7)$$

Neither $^{\bullet}NO_2$ nor $cytc^{\bullet}$ radicals can be observed spectrophotometrically, while $ABTS^{\bullet-}$, the product of reactions (6) and (7), exhibits intense absorption bands peaking at 415, 650 and 730 nm.[69] When $k_2 >> k_1$, $k_3$ and [cytc], $[ABTS^{2-}] >> [^{\bullet}NO_2]$ the kinetic considerations[43] lead to the simple exponential equation describing the formation of $ABTS^{\bullet-}$ with the observed pseudo-first order rate constant ($k_{obs}$)

$$k_{obs} = k_5[cytc] + k_7[ABTS^{2-}] \quad (8)$$

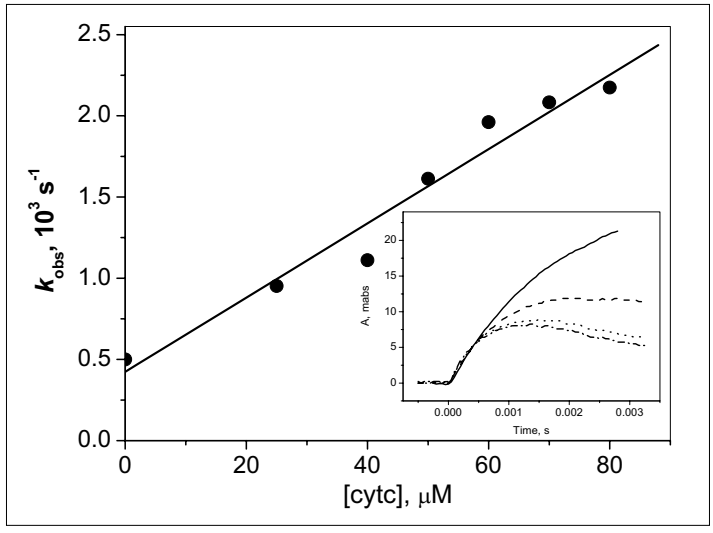



Fig. 2. Pulse radiolysis of $N_2O$ saturated solution containing 0.01 M $NaNO_2$, $2.5 \times 10^{-5}$ M $ABTS^{2-}$ and $(2.5–8.0) \times 10^{-5}$ M cytc at pH = 2.75 (0.01 M phosphate buffer) with 200 ns pulses delivering the dose of 2.2 Gy produced the curves shown in the inset. Inset: The exponential increase of $ABTS^{\bullet-}$ absorption observed at 730 nm for different concentrations of cytc ([cytc] decreases from the bottom line to the top line).[68] From the plot of $k_{obs}$ vs. [cytc] at constant $[ABTS^{2-}]$, $k_1(^{\bullet}NO_2 + cytc) = (2.5 \pm 0.2) \times 10^7$ $M^{-1}s^{-1}$ was calculated.

### 3.2. *EPR and NMR*

Several biologically relevant radicals are formed by reactions involving either very reactive paramagnetic precursors like $^{\bullet}OH$, $O_2^{\bullet-}$ or redox-active metal cations. Moreover, photo-induced processes may induce reaction sequences in which radicals participate. The formation of persistent, weakly-reactive radicals is the key feature of several natural antioxidants, *e.g.* vitamins C and E and several polyphenols (present in wines or tea).

A straightforward way for identifying a wide spectrum of radicals is EPR spectroscopy. In most cases radical reactions are observed in solution and EPR spectra detected under such reaction conditions reveal three features (Fig. 3):

i) The isotropic hyperfine coupling constants (hfc, distinguished by line distances),
ii) their multiplicities (represented by the line pattern), and
iii) the g factor (characterized by the center of the EPR spectrum).

These parameters can be interpreted in terms of the symmetry, electron distribution, and the character of the radical and provide its rather unambiguous identification. For selective generation of desired radicals, appropriate procedures have to be followed. For the formation of neutral radicals either thermally or photoinduced homolytic cleavage or abstraction reactions are utilized. Radical ions are, in most cases, formed *via* electron-transfer reactions. Oxidations and reductions can be performed by several chemical and electrochemical methods.[70,71]

As an example, the one-electron reduction of the helianthrone derivative (**1,** Fig. 4) gives[72] the EPR spectrum displayed in Fig. 5.

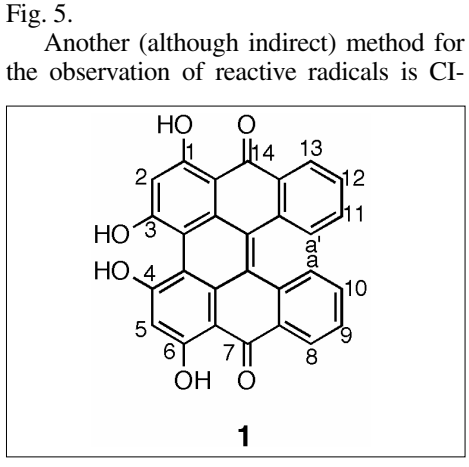



Fig. 3. A sample CW-EPR spectrum and the parameters which can be deduced from it

Another (although indirect) method for the observation of reactive radicals is CIDNP spectroscopy. This method is derived from NMR and displays products, which are exclusively formed *via* radical (ion) pairs. Analyzing the chemical shifts and the emission/enhanced-absorption pattern of CIDNP (Fig. 6) provides the photochemical pathway of the radical-forming reaction, mirrors the structure of intermediate short-lived radicals and reveals the structure of the reaction products.




Fig. 4.

These methodologies will be utilized to investigate the antioxidant properties of polyphenols, radicals formed within pathways of oxidative stress, conversions of fatty acids, light-induced processes, and radical-scavenging reactions. All these magnetic-resonance related methods are also suited for providing kinetic information at time scales

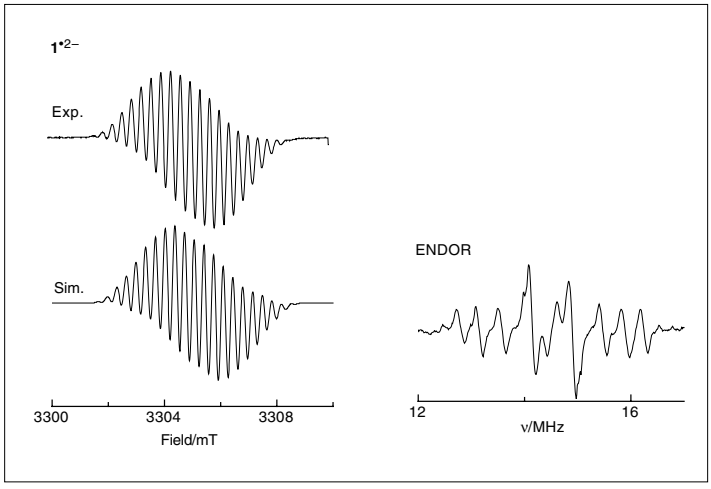



Fig. 5. EPR spectrum of $1^{\bullet 2-}$ (top left), its simulation (bottom left) and the corresponding ENDOR spectrum (bottom right). For details see [72].

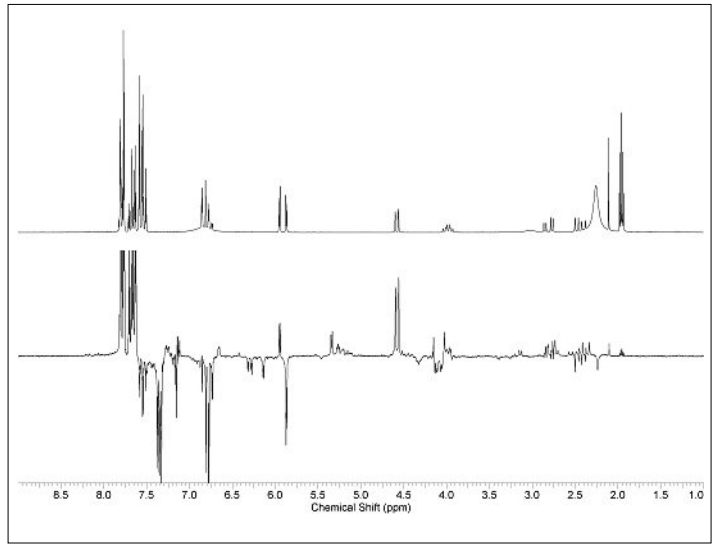



Fig. 6 NMR spectrum (upper trace) of a mixture of catechin and benzophenone in $CD_3CN/D_2O$ and the corresponding CIDNP spectrum (lower trace) obtained upon irradiation the mixture with a 200 W Hg-Xe lamp (D. Neshchadin, S. N. Bachelor, R. Lenwinn, M. Griesser, G. Gescheidt, manuscript in preparation)

ranging from 50 ns to minutes. Here real-time detection as well as competitive kinetics are applied.[73]

### 3.3. *Biomimetic Modelling*

Compartimentalization is the ability of molecular systems to create compartments, which can be obtained in aqueous medium using phospholipids able to self-organize in a bilayer fashion and give liposome suspensions. Such liposome organization is widely accepted as a model of cell membranes and is a biomimetic system that can be modulated by the choice of the phospholipid fatty acid residues, similar to those occurring in natural membranes.[74]

In the last decade, the use of liposome suspensions allowed several transformations connected to free radicals to be discovered and studied in detail, also envisaging their relevance as biologically occurring processes. The methodology of generation of radical species in liposome suspensions conveniently utilized ionizing radiation, which produce initiation from the water compartment, by the formation of primary reactive species such as $^{\bullet}OH$, $H^{\bullet}$ and $e_{aq}^{-}$. In this context, it is also possible to select further the main reactive species by the use of specific additives in the medium.

The compartmentalization afforded by liposomes allows several features of radical reactions to be studied:

i) diffusibility, because the reactive species are generated first in the water phase and can possibly produce further species able to diffuse into the lipid compartment;
ii) selectivity, because in the diffusion through the lipid compartment, the disposition of the phospholipid molecules can influence the reactivity toward the diffusing radical species; and
iii) competitivity, because the compartments separate the reaction partners, thus influencing the occurrence of competitive processes and the reactivity of the species, which are dependent on the distribution and concentration of reagents in the heterogeneous medium.

In liposome technology there are different techniques of liposome preparation, adaptable to the processes to be studied, including extrusion and injection. Using the first technique, large unilamellar vesicles (LUVET) of the desired size depending from the extrusion filter (50–1000 nm) are formed. This methodology has been used to add hydrophilic substrates, and observe the ability of radical species generated in the aqueous compartment to diffuse to the lipid bilayer, reacting with the fatty acid moieties. Also amphiphilic species can be studied, examining the equilibration of their concentration between aqueous and lipid compartments.[75] Using the second technique, unilamellar vesicles of a narrow dimensional distribution (*ca.* 100–200 nm) are formed that can be very useful for including lipophilic substrates in the lipid bilayer, as well as hydrophilic substrates inside the liposome vesicles.[76]

The groups in this Network have used these biomimetic systems of liposomes to study the behavior of a model of cell membranes and the reactivity of fatty acid residues in this context. For example, the use of unsaturated fatty acid residues forming liposomes allowed the occurrence of a *cis-trans* isomerization process catalyzed by S-centered radicals to be discovered. In this process, the naturally occurring *cis* fatty acid geometry, crucial for membrane formation and functioning, was found to be susceptible to thiyl radical leading to generate *trans* fatty acids.[47,77] Using this biomimetic system, the *cis-trans* isomerization process was studied in detail and its meaning could be extrapolated to biological systems, such as cell cultures, animals, and finally human cells, where the library of transformed lipids can be used to recognize biomarkers of radical stress.[78] These models can be fruitfully applied to study the behavior of small radicals in a biologically related context, taking into account the processes of diffusibility and reactivity of radical species, and also to reevaluate competing radical processes occurring in the different compartments of this heterogeneous system.

## 4. Antioxidants

### 4.1. *Enzymes*

Aerobic organisms have evolved antioxidant systems of defence against ROS. Antioxidants are: enzymes that metabolize ROS (SOD, catalase, peroxidases), low molecular agents that scavenge ROS (*e.g.* ascorbic acid, α-tocopherol, bilirubin, glutathione, polyphenols, uric acid), as well as proteins that prevent ROS formation by sequestering transition metal ions (*e.g.* transferrin, ferritin, ceruloplasmin).[9]

SOD catalyzes the dismutation of $O_2^{\bullet -}$ to $H_2O_2$ and $O_2$ with the rate constant in the order of $10^9$ $M^{-1}s^{-1}$.[2,79] Copper-zinc-, manganese- and iron-SODs have been described, with the last one not being found in animals cells. Dismutation of $O_2^{\bullet -}$ generates $H_2O_2$, which is removed by catalase and peroxidases. Catalase, a ferriheme enzyme, catalyzes decomposition of two molecules of $H_2O_2$ into water and $O_2$ with the rate constant in the order of $10^7$ $M^{-1}s^{-1}$.[80] Peroxidases catalyze $H_2O_2$ decomposition by using it to oxidize substrates. Selenium-containing glutathione peroxidase utilizes $H_2O_2$ to oxidize glutathi-

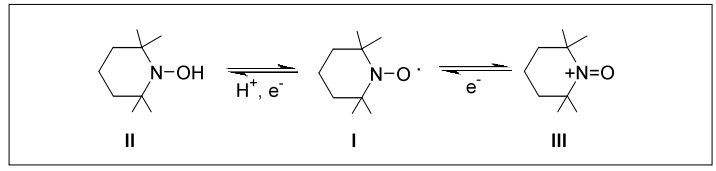
one. This enzyme, in the presence of glutathione, can also reduce other hydroperoxides, for example fatty acid hydroperoxides. Most of ferriheme peroxidases are 'non-specific', *i.e.* they oxidize several substrates. In some cases reactive species are produced. For example lactoperoxidase, found in milk and saliva oxidizes $SCN^-$ to $OSCN^-$, and neutrophil myeloperoxidase oxidizes chloride ions to hypochlorous acid. Both species are used by organisms to kill bacteria and fungi.

### 4.2. *Phenols*

Phenols are a class of compounds with O–H bond strengths that vary dramatically with substituents on the ring.[81–83] Specifically, electron-withdrawing substituents strengthen, while electron-releasing substituents weaken this bond.[82,83] Not surprisingly, the most common antioxidants contain the maximum number of alkyl and/or alkoxyl groups, all of them being electron-releasing on aromatics.[84–86] For instance, the most important biological antioxidant, vitamin E, contains three methyl groups and a six-membered alicyclic ring fused to the 3- and 4-positions with the oxygen on position 4, *i.e. para* to the O–H group. Vitamin E is thus a chromanyl derivative. Furthermore, a long alkyl chain is attached to the chromanyl, in order to make the molecule lipophilic.[84,85] The phenoxyl radicals formed are completely unreactive[87] towards $O_2$ and, due to the very weak phenolic O–H bond, abstract hydrogen from lipids very slowly.[84,85] Even so, they would eventually react and bring about some lipid damage. In order to prevent this from happening, an interaction between water-insoluble vitamin E and water-soluble vitamin C comes into play.[88] The vitamin E phenoxyl radical 'swims' to the lipid-water interphase with the $O^\bullet$-group directed towards the aqueous phase, the rest of the radical being lipophilic. Here the $O^\bullet$-group comes into contact with vitamin C and abstracts a H atom rather quickly from one of the O–H groups of vitamin C, the latter bonds being even weaker than the phenolic O–H bonds. By this mechanism the radical character is transported from lipid to water phase. The resultant vitamin C radical, a semiquinone, is then easily rereduced to vitamin C by efficient Cu-containing enzymes.[88]

### 4.3. *Cyclic Nitroxides*

Cyclic nitroxides are cell-permeable stable radicals of diverse size, charge, and lipophilicity, which effectively protect cells, tissues, organs, and whole animals from damage induced by radicals. Their protective effects have, in part, been attributed to their ability to catalyze the dismutation of $O_2^{\bullet-}$[89,90] and scavenge a large variety of deleterious species such as carbon-centered radicals,[91] $^\bullet OH$,[92] peroxyl radicals,[93] $^\bullet NO_2$,[94] $CO_3^{\bullet-}$[95,96] and thiyl radicals.[97,98]




Scheme 3.

Nitroxides (**I**) undergo one-electron redox reactions to yield the respective hydroxylamine and oxoammonium cation, as shown for 2,2,6,6,-tetramethylpiperidinoxyl (TPO) (Scheme 3).

The hydroxylamines (**II**) have extremely weak O–H bonds, and therefore the nitroxides never participate in hydrogen abstraction reactions. They are also unreactive towards molecular oxygen. However, being radicals, they react rapidly with other radicals, and in this respect the nitroxides are antioxidants. However, they are simultaneously oxidized to the corresponding oxoammonium cations (**III**). The latter may bring about oxidative damage as well. Hence, depending on conditions, nitroxides may be both anti- as well as prooxidants. However, in the presence of bioreductants, such as NADH-enzymes, the oxoammonium cation is 2-electron-reduced to the corresponding hydroxylamine[90] and the latter is an extremely efficient hydrogen donor. Thus the combination of nitroxide and reductive enzyme is a very efficient antioxidant system. One important antioxidant function of nitroxides is the catalytic dismutation of $O_2^{\bullet-}$. Here, the system oscillates between nitroxide and oxoammonium cation.[90] Nitroxides react fast with thiyl radicals as well. In this case the effect is not catalytic because the nitroxide ends up as an amine, while the thiyl radical appears ultimately to be transformed into the corresponding sulfonic acid.[98] However, this reaction protects against the possible deleterious effects of thiyl radicals, whence in this case nitroxide is seen to act as an antioxidant.

## 5. Exploring Signaling Pathways

The general public view of radicals is focussed on potential damaging and therefore detrimental effects of radicals. Even if positive effects of radicals and non-radical reactive oxygen species are considered, *e.g.* in the interaction of neutrophils with intruding bacteria, terms like 'oxidative burst' or 'oxidative stress' are usually misunderstood as indicative for a shot gun-like, non-selective action of radicals and non-radical reactive oxygen species. Without doubt, damaging effects of radicals in inflammatory diseases and multistep carcinogenesis are important and deserve further study. However, specific biological functions of radicals need to be considered much more than has been in the past, as they are fascinating on the chemical level and may have important biological functions that have been underestimated until recently.

Our COST action fosters a unique and fruitful interaction between researchers working on the chemical and the biological level of radicals. The basic work of several members of the action has established the knowledge and instruments that allow the study and modulation of signaling pathways of potential biological importance. This will be discussed here, using 'Intercellular induction of apoptosis' as one example. This biological control system is based on a complex interaction between transformed (precancerous) and non-transformed cells, leading to the specific elimination of transformed cells through apoptosis induction.[99,100] Radicals and non-radical reactive oxygen and nitrogen species are the key players that drive both the efficiency and selectivity of this process.

As shown in Fig. 7, transformed cells are characterized by oncogene-controlled generation of extracellular superoxide anions. These radicals show little aggressiveness and have a relatively short free diffusion path length. Spontaneous dismutation of superoxide anions leads to the generation of hydrogen peroxide, which is used by a novel peroxidase as substrate for HOCl synthesis. Peroxidase release from non-transformed as well as transformed cells is triggered by TGF-beta and requires the action of proteases. HOCl, in the micromolar concentration range has no direct effect on cells. However, the interaction of HOCl with superoxide anions causes the generation of hydroxyl radicals[101] that induce apoptosis through lipid peroxidation. As superoxide anions are confined to the site of their generation, *i.e.* the membrane of the transformed cell, they direct the establishment of the apoptosis-inducing effect specifically to these cells. This example illustrates how a selective action of radicals can be achieved: the interaction of wide- and short-ranging species without a damaging effect leads to the generation of damaging species (in principle unselective) with a short range and at the desired site. The same principle is used when the long-ranging $^\bullet NO$ radical interacts with the short-ranging superoxide anion radical and forms the apoptosis inducer peroxynitrite close to the membrane of the transformed target cell. Peroxynitrite is very reactive and, in addition, peroxynitrous acid may decompose

into hydroxyl radicals and nitrogen dioxide.

The HOCl and the •NO/peroxynitrite signaling pathway, as well as two additionally acting pathways based on nitryl chloride and Fenton chemistry[99] have been elucidated through the use of specific inhibitors and scavengers and have been confirmed in reconstitution experiments. These have been performed through the addition of reactive oxygen and nitrogen species or myeloperoxidase to transformed and nontransformed cells and the determination of apoptosis induction in the absence and presence of inhibitors. In this way, the potential of transformed cell-derived extracellular superoxide anions to drive both the efficiency and selectivity of the process has been verified. Recent experiments used siRNA-mediated knockdown to define the major players involved in intercellular signaling and subsequent intracellular events related to the control and execution of apoptosis through the mitochondrial pathway of apoptosis.

Interestingly, tumor progression seems to depend on establishment of resistance against intercellular induction of apoptosis through specific and powerful interference with intercellular signaling. Work in progress is elucidating multiple ways for restoring intercellular signaling in tumor cells through inhibition or destruction of the interfering system.

The signaling pathways shown in Fig. 7 represent central elements within a complex network of radical interactions, with potential relevance for the control of tumorigenesis and possibly for novel therapeutic approaches. The elucidation of this signaling system has been and will be further enhanced through the expertise present in our COST action.

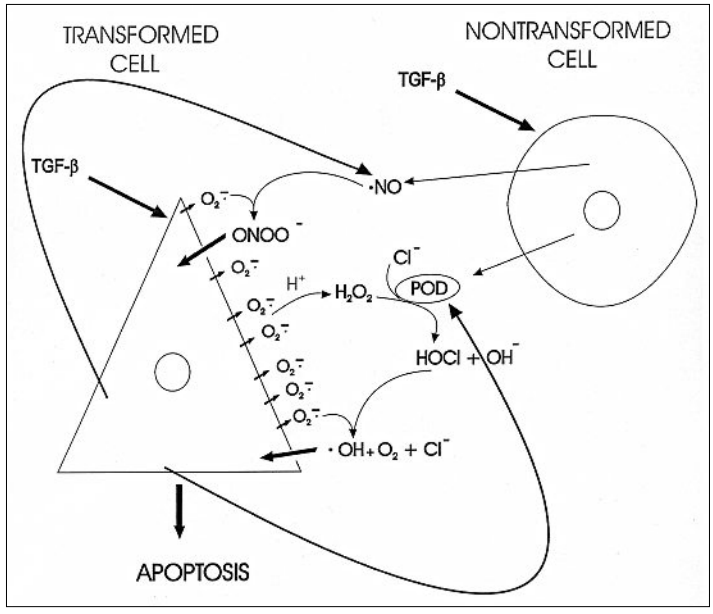



Fig. 7. Intercellular induction of apoptosis.[99,100,102,103] TGF-beta-treated nontransformed effector cells release the effector molecules •NO and a novel peroxidase. Transformed target cells generate extracellular $O_2^{\bullet-}$ through the activity of a membrane-associated NADPH oxidase. The interaction of signaling compounds from effector and target cells establishes two major signaling pathways, *i.e.* the HOCl/•OH and the •NO/peroxynitrite signaling pathway. In the HOCl/•OH pathway $O_2^{\bullet-}$ dismutates and forms $H_2O_2$, which is used by peroxidase for the synthesis of HOCl. HOCl then interacts with target cell-derived $O_2^{\bullet-}$ yielding apoptosis-inducing •OH radicals. In the •NO/peroxynitrite signaling pathway, •NO interacts with $O_2^{\bullet-}$ and forms the apoptosis inducer peroxynitrite. As $O_2^{\bullet-}$ has a relatively small free diffusion path length, the formation of apoptosis-inducing species is confined to the direct vicinity of the membrane of the target cell. The concentrations of $H_2O_2$ and HOCl reached in this system are not toxic by themselves. This interplay of different ROS leads to selective elimination of transformed cells through apoptosis induction. Transformed cells release the effector molecules •NO and peroxidase like nontransformed cells. Therefore, in parallel to intercellular induction of apoptosis mediated by neighbouring nontransformed cells, transformed cells may undergo autocrine apoptotic self-destruction based on the same signaling chemistry as described for intercellular induction of apoptosis. This effect requires a high number of transformed cells (for optimal peroxidase and •NO release) and a high local density (for optimal $H_2O_2$) generation through spontaneous dismutation.

## 6. Conclusions

Free-radical chemistry has moved from being an esoteric curiosity, mainly limited to applications in nuclear reactor chemistry and related specialities, to being a core component of numerous biological processes central to both normal and pathological conditions. While we cannot yet weigh out superoxide and make solutions as we would sodium chloride, the realization that this radical and its chemistry is commonplace and important has been an important step forward in advancing chemical biology. The same is true of nitric oxide, and as this short article has demonstrated, these two simple radicals initiate a rich chemistry.

The COST Network CHEMBIORADICAL brings together scientists from all over Europe to advance understanding in this area. Collaborations between partners have already been fruitful.[104] Many more discoveries will stem from this Action.

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