Radical Functions *in vivo*: A Critical Review of Current Concepts and Hypotheses*

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Most of the basic knowledge about radical reactions comes from radiation chemical studies *in vitro*. In view of the rapidly increasing knowledge on radical reaction *in vivo*, it is important to reconcile the fundamental physico-chemical reaction characteristics of radicals with the need to explain their alleged biological effects. Severe problems in the understanding of their *in vivo* action remain unsolved. An example is phagocytosis, which seems to be a paradigm of a 'deleterious' radical process. The exact mechanism is not clear; so it is an open question whether the intruder is eventually killed by radicals (like $^{\cdot}OH$) or by endproducts of radical reactions (like H_2O_2 and/or HOCl). It is even more difficult to understand signalling by radicals: owing to their chemical nature they are 'unspecifically' reacting species -they withdraw or add electrons- and thus their reactions are governed by redox-properties. Since all radicals have different redox characteristics and different molecular shapes, the usual key-and-keyhole picture for molecular interaction does not apply, as there, is no reactive site conceivable which has the property of reacting with radicals 'specifically.

Our intent in this article is: (i) to briefly review some fundamental characteristics of *in vitro* radical reactions, (ii) to extrapolate from this to the conditions *in vivo*, and (iii) to discuss current hypotheses concerning the redox-regulation of cellular signalling.

This leads us to the tentative conclusion that radicals *per se* must be tolerated by the cell and do not threaten its life, if they stay below a certain concentration limit. The main biological implication of radical-reactions seems to be that the cell derives signals from the balance of oxidative *versus* reductive processes and that radicals may interact with pathways of intra- and intercellular communication.

Introduction

The literature concerning speculations on the significance of radical reactions in biology and medicine is growing exponentially since the discovery of McCord and Fridovich in 1969 that an enzyme exists which catalyzes the disproportionation of the superoxide radical anion $O_2^{\cdot-}$ to oxygen and hydrogen peroxide (for a review see Fridovich, 1995). Until the discovery of superoxide dismutase (SOD), the $O_2^{\cdot-}$ radical had mainly been of interest to radiation chemists, who knew it as a long-lived species being formed when oxygenated aqueous solutions or cells are subjected to ionizing radiation (for references see Czapski, 1971).

If a water molecule is hit, an electron of the H_2O orbitals is expelled into the solution. The remaining water cation disintegrates in several ways (mainly forming $^{\cdot}OH$ and $^{\cdot}H$ radicals); the electron orients water molecules around itself and diffuses in the bulk solution as the smallest possible radical, a 'lone electron' *per se*. It may eventually add to oxygen with diffusion controlled rate forming $O_2^{\cdot-}$.

With the earlier association of radiation with something deleterious, the first years of research on radicals in biological systems centered around the idea that $O_2^{\cdot-}$ was a noxious species and that SOD served the function of a protective enzyme. Yet with the discovery that phagocytes, patrolling through the body to eliminate foreign cells, use the $O_2^{\cdot-}$ producing enzyme NADPH oxidase as a means of defense against intruders (Babior *et al.*, 1973), it became increasingly clear that radicals

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could serve some biological function aside from being just an adventitious byproduct of irradiation or of some ill-controlled autoxidation reaction. Over the years many enzymatic reactions were found to produce $O_2^{\cdot-}$ (prominent example xanthine oxidase) aside from enzymes which produce H_2O_2. The latter species, even though not being a radical, is in most instances involved in radical processes.

In the last two decades numerous radical reactions have been investigated *in vitro*, e.g. by the method of pulse radiolysis, and tables with the respective reaction rates have been collected by the National Institute of Standards (Ross *et al.*, 1994) and can be obtained via the Internet (http://allen.-rad.nd.edu).

However the central question as to whether, how and where radical reactions are of relevance *in vivo* is still to be answered. At the moment the best we can do is to extrapolate from the knowledge of fundamental traits of radical reactions under *in vitro* conditions to those aspects that might help to define radical functions in biological systems.

General Reaction Pattern of Radicals

The chemical reactivity of radicals, i.e. the avidity by which electrons are withdrawn from neighbouring molecules, is governed by the oxidation potential. At the top of the scale, having 2.3 volt positive attraction potential for electrons, stands the OH radical. At the bottom of the scale is the electron itself having -2.9 volts of 'repulsive' potential, i.e. the tendency to add to another substance. The redox potentials of all other radicals lie between these two extremes (see e.g. Buettner, 1993). Some redox couples which are relevant to the following discussion are listed in Fig. 1.

Autoxidation and the Fenton-reaction

Hydroxyl radicals as produced by irradiation may also be generated by reductive splitting of a molecule of hydrogen peroxide. This process, commonly referred to as the Fenton-reaction, describes the transfer of an electron onto H_2O_2 facilitated by metal catalysis:

$$H_2O_2 + e^- \quad >\text{metal}> \quad {}^{\cdot}OH + OH^-$$

Replacing H_2O_2 with organic hydroperoxides, one generates alkoxyl radicals, $RO^{\cdot}$, the organic equivalent of OH radicals:

$$ROOH + e^- \quad >\text{metal}> \quad RO^{\cdot} + OH^-$$

Reactions of this type, in most cases catalyzed by complex-bound metal ions rather than the free entities, are the most common chain initiation processes and thus the cause for spoilage of foodstuffs, rancidity of oils, etc. Such chain processes occur adventitiously in organic matter which is exposed to an oxygen atmosphere (and light, which often functions as chain initiator). Chelated in a complex, the metal may alter its redox potential quite markedly, e.g. iron in the reduced state transfers an electron to oxygen whereas in its oxidized form, being coordinated differently, it accepts electrons from $O_2^{\cdot-}$. Such cyclic processes

$$Fe(II)_{complexbound} + O_2 > Fe(III) + O_2^{\cdot-}$$
$$Fe(III)_{complexbound} + O_2^{\cdot-} > Fe(II) + O_2$$

in connection with chain propagating steps are collectively termed 'autoxidation', as they proceed as an ubiquitous, and in the aerobic world inevitable, by-process of life. It is easily seen that superoxide dismutase would act as a chain breaker if it interfered by scavenging the chain propagator $O_2^{\cdot-}$ and in this respect SOD may serve as a paradigm of an antioxidant.

Haber-Weiss-Fenton chemistry

The complex interaction of H_2O_2 and (cyclic) $O_2^{\cdot-}$ generation is often crudely referred to as Haber-Weiss chemistry. This notation gives credit to the researchers who in the early thirties had postulated the participation of the radical $HO_2^{\cdot}$ (the acid form of $O_2^{\cdot-}$ below pH 4.8) in the iron catalyzed decay of H_2O_2. Their reaction mechanism,

$$HO_2^{\cdot}/O_2^{\cdot-} + H_2O_2 \rightarrow {}^{\cdot}OH + O_2 + OH^-/H_2O$$

in connection with the Fenton reaction quoted above, in effect results in a metal catalyzed reductive cleavage of H_2O_2 producing OH radicals. The whole reaction sequence is extremely complex as it not only depends on the chemical characteristics of the radicals involved but also crucially in which way the catalyzing metal is chelated. As it is impossible to review this subject in a brief manner (for a recent review see e.g. Wardman and Can-

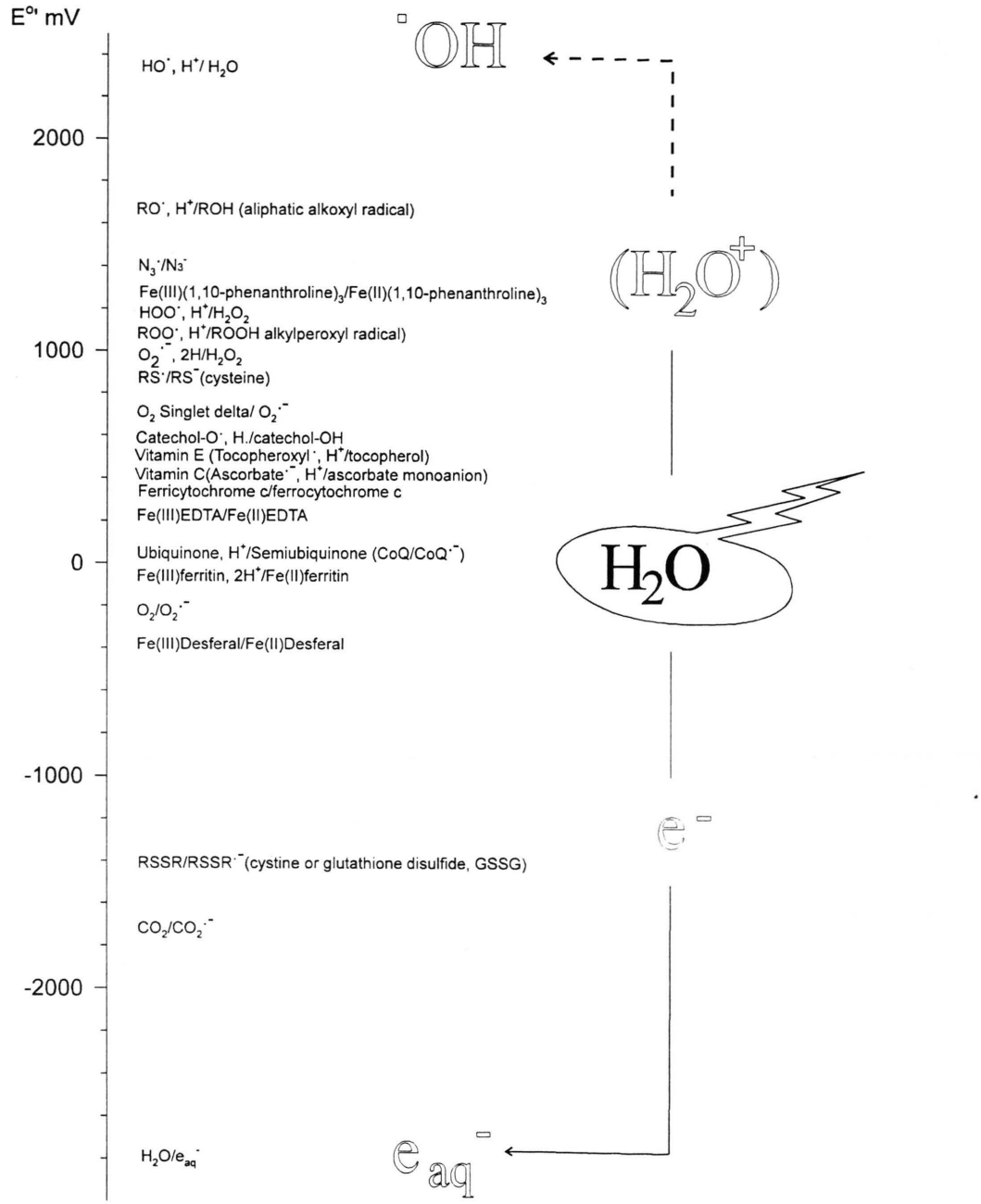

Fig. 1. Radiation induced radical reactions. A water molecule which is hit by ionizing radiation disintegrates into OH radicals and electrons (both at a yield of 280nM per Gray). Minor radiation products are H_2 and H_2O_2. ·OH is the most strongly oxidizing radical known and thus able to react with any biomolecule by one-electron oxidation. The electron in its hydrated form, being the most strongly reducing radical, may do so by one-electron reduction. The left hand scale lists the potential of some redox-couples of biological compounds which governs the 'pecking order' of radicals. In principle, chain reaction may proceed 'downhill' until they come to an end at zero potential difference between donor and acceptor.
(Adapted from Buettner, 1993).

deias, 1996) it must suffice at this point to summarize all that is known in one central thesis: *there is probably not a single radical chain process in vivo that proceeds without the participation of some metal in loose or bound form, whether as side effect or even playing a dominant role.* Figure 2 may help to illustrate this.

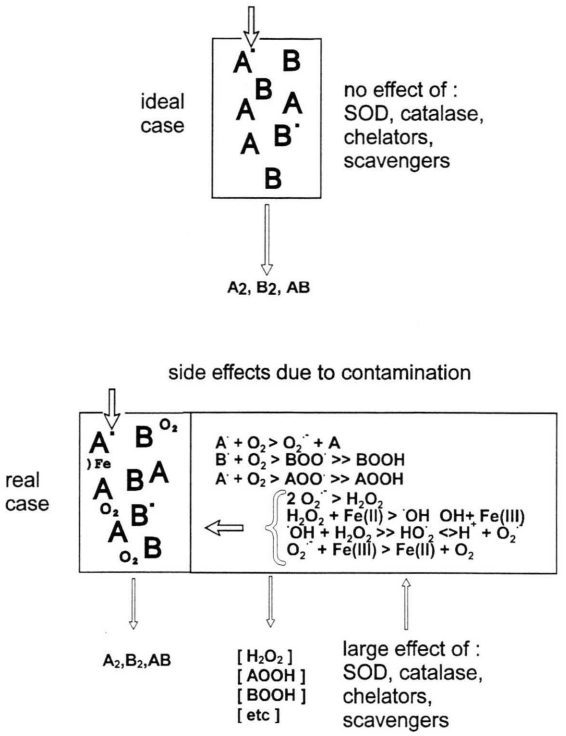

Fig. 2. Radical chains under *in vivo* conditions are usually distorted by 'Haber/Weiss/Fenton contaminants'. The upper box (ideal case) stands for the reaction of molecular species A and B in a 'clean' experimental system. Any induced radical chain will only result in dismutation products or dimers of the species A and B.
In the realistic case experimental systems are 'contaminated' by oxygen and (complex-bound) metal catalysts. The investigator, therefore, if adding SOD, catalase, a chelator, or a scavenger, will most certainly notice a modification of the product distribution of A and B. This observation, however, does not allow him to answer the question which course the chain has taken.

What radical species may be relevant in vivo?

Figure 3 briefly lists some of the most common types of radicals that could occur in organic material. In reality there may exist as many different radicals as there are different molecules in the cell. The picture is further complicated by the fact that the cell is not just a vessel containing different molecules in even distribution but rather a highly structured entity with regions of different composition being separated by compartment boundaries which are not permeable for all molecules in an equal manner. From a general viewpoint, it is thus impossible to draw a generalized picture covering all possible reaction pathways. Therefore we briefly highlight only those traits which are fundamental to understand the *differences* between cells and aqueous model systems.

What are the main characteristics of radical reactions under in vivo conditions?

1.) With respect to cellular dimensions a radical chain reaction is a rather 'local' event. This means that a radical reaction being initiated in some cellular compartment spreads over only a limited volume within this compartment: after an initiating step the radical density rises by propagation until chain-terminating dimerisations become dominant and the reaction stops. For phospholipid membranes about 5 to 10 propagating steps have been experimentally verified (Niki *et al.*, 1991). Chain termination usually implies formation of stable products (hydroperoxides) or interaction with chain-breaking antioxidants, such as alpha-tocopherol (vitamin E).

2.) The progression of a radical chain depends on 'organisational' parameters, i.e. on the order and packing density of the involved molecules. This is exemplified in Fig. 4 which contrasts chain propagation in unordered, i.e. dissolved, lipid molecules to the case when the same molecules are closely packed in structures which resemble their aggregation in a cellular membrane (Gebicki and Allen, 1969). In analogy we have to expect different results of radical interactions with DNA as well, depending on the state of order, i.e. whether the DNA is in a 'quiescent' mode or actually being processed by transcriptional or other activities.

3.) The outcome of any radical chain depends on the composition of the compartment in which the reaction proceeds. This statement is rather trivial if it only means that different cellular compartments produce different endproducts. It becomes important, however, if it implies that endproducts of different compartments *behave* differently, i.e. have subsequent pharmacological consequences. It is

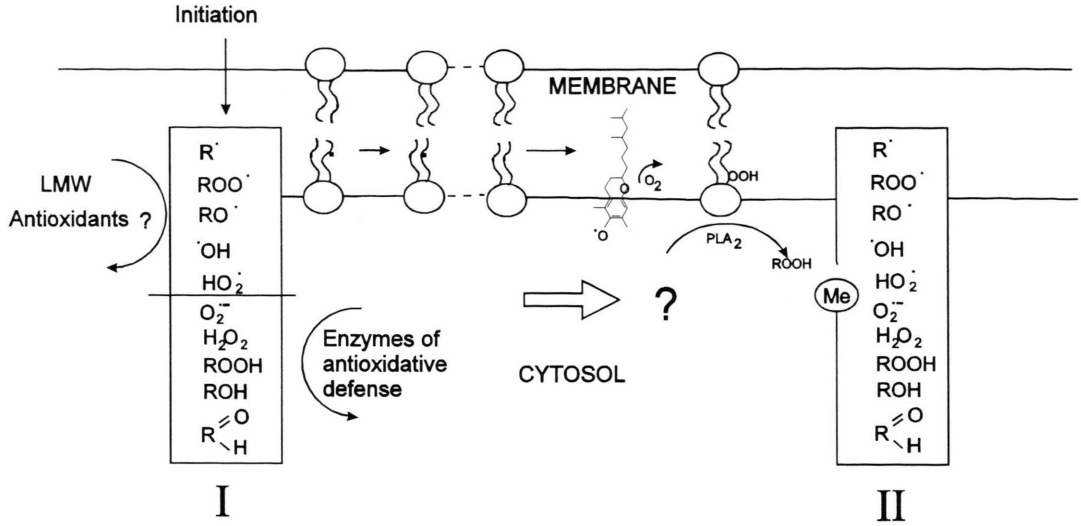

Fig. 3. Radical chains under in vivo conditions usually proceed in 'clusters'. After being initiated, a radical chain *in vivo* typically involves the species listed in the upper part of Box I (i.e. alkyl-, alkoxyl-, alkylperoxyl-, hydroperoxyl-radicals or similar). Those species listed in the lower part are able to diffuse over relevant distances in the cytosol. If not intercepted by enzymes or other reactants, some of them, mainly the peroxidic species, may induce at some suitable site (e.g. a bound metal) a secondary radical chain (Box II). It is important to note that such a mechanism allows the attribution of a long range activity to 'radicals' which by far exceeds their free diffusion pathlength. (Also depicted is a start reaction for a secondary radical chain by a 'physiological' process, i.e. the cutting out of a membrane hydroperoxide by phospholipase A2 (PLA2).
(Adapted from Saran and Bors, 1989).

easily seen that lipid hydroperoxides or aldehydes, which are typical for phospholipid membranes, may result in other metabolic consequences than aldehydes or sulfoxides which derive from cytosolic proteins even though the common root of both might have been an attack by an OH radical.

4.) The cellular results of radical reactions are usually modified/amplified by cellular responses. The cell possesses many different pathways for defending against and repairing the disturbances initiated by radicals; this most likely results in a masking of the original radical interaction. Aside from defense mechanisms based on low molecular weight antioxidants and enzymes such as SOD there are also examples for repair on a high level of complexitiy, e.g. of damaged membranes (van Kuijk *et al.*, 1987) (cf. next chapter and Fig. 5). Of particular importance is repair of damaged DNA, which responds to disturbances by SOS-repair, unscheduled DNA-synthesis, and various other modes of restoring the integrity of the genome (for references see von Sonntag, 1987).

5.) On the systemic level the outcome of radical reactions may be related with intercellular communication. Cells are not stand-alone entities but are interwoven in a dense net of intercellular communication. Thus a radical reaction which results in an alteration of one of the involved messengers (hormones, cytokines etc) may have systemic consequences that are significantly more important than a localized chemical event. Examples of such interdependencies will be discussed later.

These five theses, in combination, imply that we cannot ask questions concerning the *in vivo* fate of a single radical, e.g. ·OH, but rather have to inquire about 'the result of a reaction sequence which was originally initiated by an OH radical in the cytosol..., in the membrane... in the DNA...' or similarly. In other words this also means that the course of a radical chain *in vivo* is not precisely predictable and the investigator is left with the attempt to derive conclusions by 'asking the cell' how it experiences the entire radical chain: e.g. by investigating changes in metabolism, in the induction pattern of transcription factors, in the appearance of newly formed messenger RNA or by analyzing the defense mechanisms it raises against an insult.

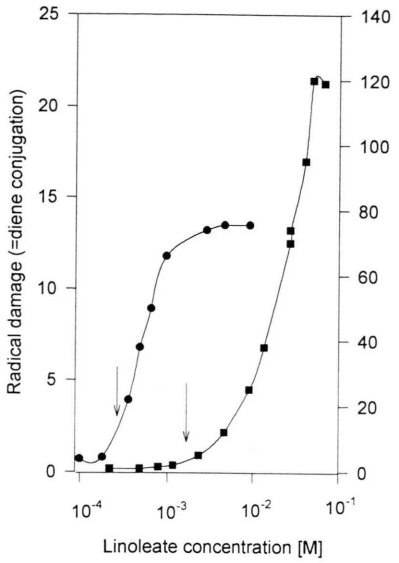

Fig. 4. Radical reactions depend on the 'degree of order' in the substrate. An example depicting radical damage to fatty acid molecules in solution (the ordinate scale represents a measure of this damage). The left curve shows a low level of damage at low concentration, i.e. when the molecules are monodispersely distributed; at higher concentration (marked by left arrow) the critical micellisation concentration (CMC) is reached; at this concentration the molecules start to aggregate into micelles. This results in a steep increase of the observed damage. The right curve shows the same phenomenon at more alkaline conditions resulting in a different arrangement of water molecules around the aggregated lipids.
(Adapted from Gebicki and Allen, 1969).

General aspects of detoxification and antioxidation

Superoxide dismutases and Catalase

A glance at Fig. 2 shows that one of the central tasks of the cell is to curtail uncontrolled chain propagating reactions by $O_2^{\cdot-}$ and H_2O_2. This can be achieved enzymatically by the enzymes superoxide dismutase and catalase. For the control of $O_2^{\cdot-}$ several SODs have evolved. The manganese containing SOD (MnSOD), localized in the mitochondrial membrane, is evidently specialized to limit damage resulting from electron leakage of the respiratory chain. It has been estimated that about 4% of all electrons being channeled from NADH via flavoproteins through quinones to cytochrome c eventually end up producing a superoxide anion (see Fridovich, 1995). Thus MnSOD serves as a bodyguard for the mitochondrion with the duty of transforming the hazard inherent in the radical $O_2^{\cdot-}$ into the more easily handled hazard of molecular H_2O_2 which can effectively be degraded by the enzyme catalase. Other superoxide-dismutating enzymes are known besides MnSOD: a Cu/Zn-containing cytosolic species, an extracellular Cu/Zn-enzyme being attached to the lining of the vascular system, and an iron containing FeSOD in procaryotes and plants (for reviews see Fridovich, 1995, Bowler *et al.* 1994). There are also examples of organisms (e.g. *Propionibacterium shermanii*) (Meier *et al.*, 1995) which are able to use either Fe or Mn ions for constructing their SOD. It is not entirely clear whether different SODs serve different teleological ends, but the fact that most SODs are inducible by enhanced oxidation states within cells (Touati, 1991, Demple, 1996) and that SODs and catalases in most cases are induced concomitantly, support the notion that both enzymes act in a concerted way together.

Peroxidases

This class of enzymes is important for radical detoxification since H_2O_2 or hydroperoxides may account for up to 80% of the endproducts produced e.g. in membranes. While most peroxidases utilize H_2O_2, GSH-dependent peroxidases are the starting point for a redox cycle, involving GSH reductases and a source of reducing equivalents. The phospholipid-hydroperoxide specific GSH-peroxidases are especially important for cellular detoxification since they act in combination with phospholipase A_2 to restore membrane integrity after oxidative damage (cf Fig. 5).

Enzymatic defense against radicals other than $O_2^{\cdot-}$?

In contrast to the cellular means to detoxify stable peroxides and the rather long-lived radical $O_2^{\cdot-}$ no specific enzymatic defense mechanisms against short-lived radicals or other common end-products like aldehydes have been convincingly demonstrated. The chemical diversity of organic radicals (like $RO^{\cdot}$, $ROO^{\cdot}$ etc) may preclude any specific scavenging mechanism and with respect to the OH radical it is obvious that a specific mechanism is not even theoretically conceivable: the OH radical is so reactive that it reacts with almost any

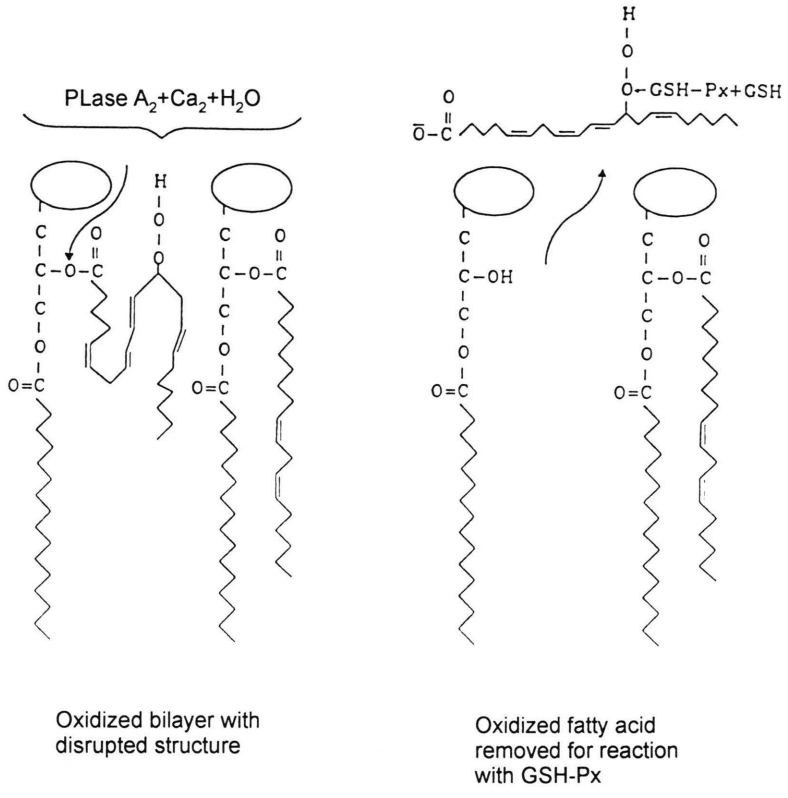

Fig. 5. Radical damage to biological structures may be repaired by enzyme systems. Damaged molecules of lipid bilayer membranes are cut out by phospholipase A_2 and digested by glutathione peroxidase. The membrane is thereafter repaired by re-acylation.
(Adapted from van Kuijk *et al.*, 1987).

molecular configuration at first encounter. This means that any enzyme with an active site to handle OH radicals would be useless since the supporting protein would be attacked more efficiently than the active site.

Low molecular weight antioxidants

On first glance it seems that the cell had no effective remedy to stop chain reactions of fast reacting radicals. Yet, one of the general explanations for the functioning of low molecular weight (LMW) antioxidants is the idea that these molecules intercept in chain processes by rapidly scavenging reactive radicals. Due to structural peculiarities they are capable to stabilize their own radical state by mesomerism and slow the chain reaction down in order to give reducing compounds such as ascorbic acid (vitamin C) the chance to heal the radical structure by one-electron donation (Bors *et al.*, 1990). To emphasize, the essential criteria for optimal antioxidants are not only effective scavenging rate constants but, even more important, sufficient stability of the antioxidant radical to prevent adventitious chain reactions.

A very intriguing mechanism along these lines has been proposed by Winterbourn and coworkers (Munday and Winterbourn, 1989) who suggested that GSH, which is one of the major cellular reductants present in very high (millimolar) concentration, could react with most oxidizing radicals to form a thiyl radical $GS^{\cdot}$. This oxidizing species reacts with excess glutathione thiolate, GS-, to the disulfide radical anion $GSSG^{\cdot-}$. The latter could then serve as a turnout transferring electrons onto oxygen producing $O_2^{\cdot-}$ and thus put SOD in the position to dismutate $O_2^{\cdot-}$ to H_2O_2,

i.e. to hand the problem over to catalase for final solution. From this viewpoint GSH in conjunction with SOD and catalase would function as a multipurpose radical defense system with broad applicability.

General Aspects of Radical Reactions Within Cells

Do cellular structures (targets) exist which are specifically sensitive to radical attack?

The concept of site-specific radical reactions

Ionizing radiation interacts 'indiscriminately' with any molecule and thus is able to start a radical chain at any site within the cell. Chain initiation by chemical reactions, in contrast, depends on energetic and kinetic parameters, i.e. radical attack may be more or less favored at one or the other accessible site. Despite such inferences for site specificity there remains one serious reservation: even assuming that a chain began at a 'specific' site, it is difficult to see how the endpoint of the chain could bear any 'direct causal relationship' to the point where the process had started, due to the inherent unpredictability of the course of radical chains *in vivo*. In other words: decisive for the future of the cell is what happens *after* the chain has ceased; since it cannot reproducibly be traced back to the initial radical interaction, we have no need to search for that site of primary action. The only instance where radical chains would reproducibly be started – and most probably be confined to a rather limited volume around this point – would be if a hydroperoxide interacted with some bound metal starting a Fenton-Haber-Weiss analogous chain reaction. Such site specific effects (Samuni *et al.*, 1983) have convincingly been demonstrated for *in vitro* model systems in radiobiology (Prütz, 1984) but definite proof for their relevance *in vivo* is still lacking. Another constellation with the characteristics of site-specificity is given when myeloperoxidase, by virtue of being a polycation, binds by electrostatic forces to negative membrane areas of bacteria and thus produces the oxidant HOCl directly at the intended site of action (Weiss, 1989).

The concept of translation and/or amplification

If the claim for site-specific actions of radicals is still lacking evidence, how then could the consequences of radical reactions *in vivo* be interpreted? It is obvious that an unspecific action could be made specific by 'translation' if we consider that any kind of radical, after entering into some black box, produced therein a whole variety of different products and one or two of them had a clearly defined action spectrum. An example might be lipid peroxidation: if, after initiation by a radical, only one of the products had pharmacological properties comparable to those of intermediates of the eicosanoid cascade then the membrane would serve as a broad band amplifier with the function of translating an unspecific action of any initiating radical into a specific cellular message. The oxidation of SH-groups may provide a second example: if this oxidation merely implied that the cell looses one or the other SH-containing molecule, the consequences might be negligible; if, however, the protein changed its conformation and hence activity by opening or closing a disulfide bridge (e.g. on an enzyme or a receptor), this could result in a tremendous biological amplification of the original radical-derived effect. Analogous arguments may be valid for the enzyme aconitase which not only functions as metabolic enzyme but under certain conditions also as iron regulatory protein. This property would make it a modifier of two different processes (Gray *et al.*, 1993) and put it in the position to regulate radical effects by two different pathways.

The concept of cellular redox status (oxidative tone)

The difficulties of defining specific action modalities for radicals together with the difficulty of finding specific reaction sites have led to a makeshift construction: the balance between the oxidative potential of reactive oxygen species (ROS such as radicals, hydrogen peroxide, peroxides, HOCl, or peroxynitrite) *versus* the reductive potential of the glutathione/glutathione reductase and other cellular reducing systems is subsumized under the term 'redox equilibrium'. This concept is now almost synonymous for 'radical reactions' and widely used to explain all sorts of *in vivo* effects. It results in two very important implications: if the oxidizing branch supersedes, *all* presumptively sensitive sites become more or less oxidized (according to their abundance), if the balance

shifts into the opposite direction, the *same* sites become reduced. This way of looking at the problem avoids the main problems discussed above: (i) we don't have to search for a specific action of a specific radical, (ii) we don't have to search for a specific site of action but instead may resort to generalized descriptions like 'the elevated redox status shifts essential sulfhydryls to a more oxidized state', 'it augments lipid peroxidation', 'it activates or deactivates transcription factors', 'it induces antioxidative enzymes', 'it is responsible for adaptive responses', etc.

Do cellular processes exist which are specifically sensitive to radicals and redox changes?

H_2O_2 and $O_2^{\cdot-}$ inducible transcription factors

Treatment of bacteria with hydrogen peroxide induces at least 30 proteins including catalase, alkyl hydroperoxide reductase, glutathione reductase. A large subset of these enzymes being induced within 10 min is controlled by the oxyR regulon which has been suggested to function as a 'sensor' for oxidative stress (Storz and Tartaglia, 1992). The oxidized form of the OxyR protein activates transcription as a consequence of conformational change; the reduced form, while still binding to the promoter, seems to repress its own expression. This type of regulation, originally observed in bacteria, has now been shown to be operative in eukaryotic cells as well. The presence of superoxide generating compounds induces a group of proteins, nine of which are controlled by the soxR regulon (Demple, 1990) including the MnSOD mentioned above, and endonuclease IV which is essential for the repair of damaged DNA (Chan and Weiss, 1987). SoxR contains an iron-sulfur center which suggests that some redox-active conformational change in the SoxR-protein may be responsible for activation of the regulon.

Redox processes and enzyme function

A host of intracellular enzymes is known to be sensitive to oxidizing conditions, among them glyceraldehyde phosphate dehydrogenase and glucose-6-phosphate dehydrogenase (reviewed by Stadtman, 1993). Calmodulin, another enzyme of central importance for metabolic control, looses its function upon oxidation of a sulfhydryl group of its protein backbone (Yao *et al.*, 1996). The subendothelial matrix, consisting of heparan sulfate proteoglycans, is known to be effectively degraded by a combination of neutrophil-derived elastase and the HOCl generating MPO system. Neither elastase alone nor the MPO system alone were able to induce proteolysis. However, when the cells were exposed to elastase some time before MPO was added, a synergistic effect was observed. As elastase is another example of a cationic protein with the pre-disposition to bind to negative sites, e.g. membranes, we may view this mechanism as further indication that processes have evolved which use synergism between enzymes and oxidants to optimize metabolic (or catabolic) processes (for review see Ginsburg and Kohen, 1995).

Analogous mechanisms also seem to operate in the extracellular space. Latent gelatinases and collagenases were shown to be activated by the MPO system of PMNs, the activation being due to a thiol-dependent intramolecular rearrangement of the protein moiety of the proteases.

Cysteine (sulfhydryl group) dependent transcription

Most prominent examples are the transcription factors NF-kB and AP-1. NF-kB, which is involved in the transcription of a wide array of agents, including tumor necrosis factor-alpha, consists of three subunits: p50, p60 and an inhibitor I-kB. After phosphorylation of I-kB the two other subunits are translocated to the nucleus for transcription. The NF-kB system seems to be redox-sensitive at two levels: a (tyrosine-dependent) phosphorylation of I-kB being activated by oxidation, and a Ref/thioredoxin-dependent binding of the p50 subunit to the DNA being governed by the reduction of an essential cysteine group in the p50 subunit (for review see Flohé *et al.*, 1997).

The transcription factor AP-1 and its equivalent YAP-1 in yeast are highly sensitive to changes in the redox status of the cell as they only bind to the respective DNA consensus sequence if two cysteines in the c-fos and c-jun subunits are reduced by the redox mediator Ref1 (Abate *et al.*, 1990).

Particularly sensitive to redox processes is the zinc-finger motif where the cysteine residues in-

volved in finger formation coordinate with Zn^{2+}. DNA-binding of this domain seems only possible when the residues are in a reduced form; an oxidative environment abrogates the binding to DNA. Analogous mechanisms apparently operate with the glucocorticoid and other members of the steroid-thyroid hormone receptor family having multicysteine zinc-finger domains; here again the DNA binding capability is abolished by oxidative formation of disulfides and restored by reduction with dithiothreitol. Another example of a redox-sensitive transcription factor is Egr1 which possesses three zinc-finger motifs and regulates, among others, c-fos and c-jun. The DNA-binding ability, which is lost upon oxidation or GSH-depletion, can be restored by reduction with dithiothreitol (DTT) or by help of the ubiquitous redox factor Ref1 (for references see Cimino *et al.*, 1997). The DNA-binding domain of the p53 cell cycle regulator only functions when the cysteine residues to which the Zn^{2+} is coordinated are in a reduced state. *In-vitro* addition of metal-chelating (phenanthroline) or oxidizing agents (diamide), or irradiation by UV (Bender *et al.*, 1997) decreases the DNA-binding efficiency and the addition of reducing agents permits the refolding of the protein and restores DNA-binding. The fact that exchange of one of the three essential cysteines by mutation greatly diminishes DNA-binding may serve as additional indication that the activity of p53 and hence control at the G1-checkpoint of the cell-cycle may depend on the redox status of the cell (for references see Johnson *et al.*, 1996).

Redox processes at the membrane: involvement of lipid products?

This field is far too broad to be dealt with in cursory form. Numerous reviews on lipid peroxidation (e.g. Marnett, 1995) and the messenger function of diacylglycerol (Potter, 1995) are available and the involvement of ceramides (Haimovitz-Friedman *et al.*, 1994) and arachidonic acid and its metabolites (Abramson *et al.*, 1993) in cellular signalling is recognized. It is further known that lysophosphatides (e.g. lysophosphatidic acid LPA) are inducers for mitogen-activated protein kinases (MAP-kinases), i.e. the same pathway that is activated by H_2O_2 (for review see Coleman, 1993). In the context of this article only two new aspects shall be briefly mentioned: the first relates to an observation of Spiteller and colleagues (Dudda *et al.*, 1996) who have identified dormant lipoxygenases which translate stress to the membrane (i.e. lesions afflicted by oxidative stress or mechanical disruption) into plasmalogen-derived messengers. The second relates to experiments of Ginsburg and colleagues who showed that the combined action of proteinases and oxidants leads to formation of membrane blebs (see Ginsburg and Kohen, 1995) which very much resemble the early steps in apoptosis.

Redox processes and apoptosis

The different pathways which are currently believed to induce programmed cell death respond by variable degrees to oxidizing conditions. For the Apo/Fas/CD95 pathway to our knowledge no clear correlation with ROS has been demonstrated thus far and the triggering sequence of the ceramide pathway (Verheij *et al.*, 1996) has not been elucidated in detail. The calcium pathway, in contrast, has been reported to be correlated with a substantial rise of ROS within the cell (Orrenius and Nicotera, 1994). In the context of this article one process, however, seems to be of special relevance: the voltage sensor of the mitochondrion is gated by redox reactions and its operation strictly depends on a reduction/oxidation of sulfhydryl groups close to the respective membrane pore (Petronilli *et al.*, 1994). As this is a very sensitive process which yields a very strong signal for the induction of apoptosis (Marchetti *et al.*, 1997), it does not seem unreasonable to suspect that induction of programmed cell death in this case may directly be related to the availability of oxidizing equivalents, i.e. reflect the metabolic state of the cell.

Effects of metal chelation

According to Fig. 2, we would expect changes in the redox balance to be intimately related to the availability of metal catalysts. In fact, the large influence of metals on radical reactions has caused the evolvement of complicated measures to control the level of free metal ions (Gutteridge, 1997). If cells are subjected to oxidative stress, the storage protein ferritin, which regulates the pool of free iron, is induced. This seems to be partly due

to transcriptional activation (Cairo *et al.,* 1995) and partly due to posttranscriptional inhibition of the iron regulatory factor (IRF) which controls ferritin mRNA translation. IRF is activated by exposure of cells to H_2O_2 (Pantopoulos *et al.,* 1997). On the other hand, $O_2^{\cdot-}$ has been shown to release iron from ferritin (Buettner *et al.,* 1987), a process which would increase the availability of catalytic metals under oxidative stress. The exact switching mechanism, by which the redox status is related to the regulation of metal concentration is not entirely clear at the moment but several models have been proposed (Pantopoulos *et al.,* 1997). Interestingly, it was shown that stimulated neutrophils are only able to induce lipid peroxidation in the presence of ferritin (Winterbourn and Kettle, 1992) and that the plasma level of free iron, due to saturation of the sequestering capacity of ferritin after bone marrow transplantation (Dürken *et al.,* 1997) causes enhanced oxidative stress.

Interrelations of redox-regulated phenomena with other stress responses

Mammalian cells respond to a wide variety of chemical and physical stresses by induction of stress proteins. At elevated temperatures, cytosolic heat shock factors (HSF-1 to HSF-4) are activated in the cytosol and translocate to the nucleus to combine there with a heat shock element HSE to activate the transcription of a variety of heat shock proteins HSPs. With the exception of HSF-2, all other HSFs contain cysteine residues in their DNA-binding domains (which closely resemble the binding domain of NF-kB) (El Yaagoubi *et al.,* 1997). Conditions which induce the heat shock response also deplete the GSH pool and increase the abundance of ROS with the consequence that the expression of SOD is induced (see Fridovich, 1995); furthermore the susceptibility to killing by heat is increased when antioxidative defense mechanisms are inhibited. All of the quoted agents have been shown to induce essentially the same pattern of kinases, i.e. those of the mitogen-activated protein kinase (MAPK) pathway (Kyriakis and Avruch, 1996). Correspondingly, the activation of MAPK by ionizing radiation can be prevented by pretreating cells with reducing agents such as N-acetylcysteine (NAC) (for review see Coleman, 1993). By screening DNA and RNA libraries close matches were found between cDNA of human fibroblasts treated with H_2O_2 and heat-shock (Keyse and Emslie, 1992), and both resembled closely the active-site sequence of protein-tyrosine phosphatases. This, in conjunction with the observation by Herrlich and coworkers (see Bender *et al.,* 1997) that UV-irradiation and hydrogen peroxide modulate the signal derived from growth-factor receptors via the oxidation of a sulfhydryl group in the respective tyrosine phosphatase, may indicate that heat shock, UV, ionizing radiation, and oxidative conditions all converge in a common pathway of cellular stress response.

Intracellular versus extracellular redox regulation?

The actively metabolizing cell is required to keep its millimolar pool of GSH in the reduced form. Almost any stress condition to which the cell is exposed leads within minutes to a temporary decrease in the pool of GSH which is rapidly restored if sufficient energy is available for the enzyme GSH-reductase to reduce the oxidation product GSSG. Yet the GSH pool may stay at very low levels for hours and the cells remain alive (Reglinski, 1988). After addition of glucose or other energy sources, the GSH-pool is restored to normal levels. This means that a resting cell, which is not exposed to external or internal oxidants (e.g. arising from actively respiring mitochondria) does not suffer a life-threatening radical stress. Furthermore, experiments with gsh- mutants of *E. coli* have shown that GSH, as a source of reduction equivalents for enzymes such as ribonucleotide reductase (RR), can be substitued by other SH-sources (thioredoxin, ergothionein) without noticeable deficiencies for the cell.

The intracellular and the extracellular GSH status do not seem to be directly correlated. Even though it has been shown that excretion of GSSG from the cell is related to the intracellular GSH/GSSG ratio (for references see Sies, 1986), it was observed that during redox-related diseases such as rheumatoid arthritis the GSH levels in the plasma were lowered while the cytosolic levels were substantially unaffected. This is in line with the observation that in healthy individuals 90% of the ascorbate in the plasma is reduced whereas during attacks of rheumatoid arthritis it is predominantly oxidized (Sharma *et al.,* 1994) leaving

the intracellular ascorbate level unchanged. The extracellular redox-balance, therefore, seems to respond to its own regulation. One of the most challenging tasks for the cell is to keep exofacial sulfhydryl groups reduced: without that, it would not have a functioning import of nutrients (via the hexose transporter), nor controllable calcium channels, nor the ability to receive extracellular signals (for details see Fig. 6). The routes for the export of intracellular reducing equivalents are not entirely clear, but the membrane-bound enzyme thioredoxin reductase, which mediates the

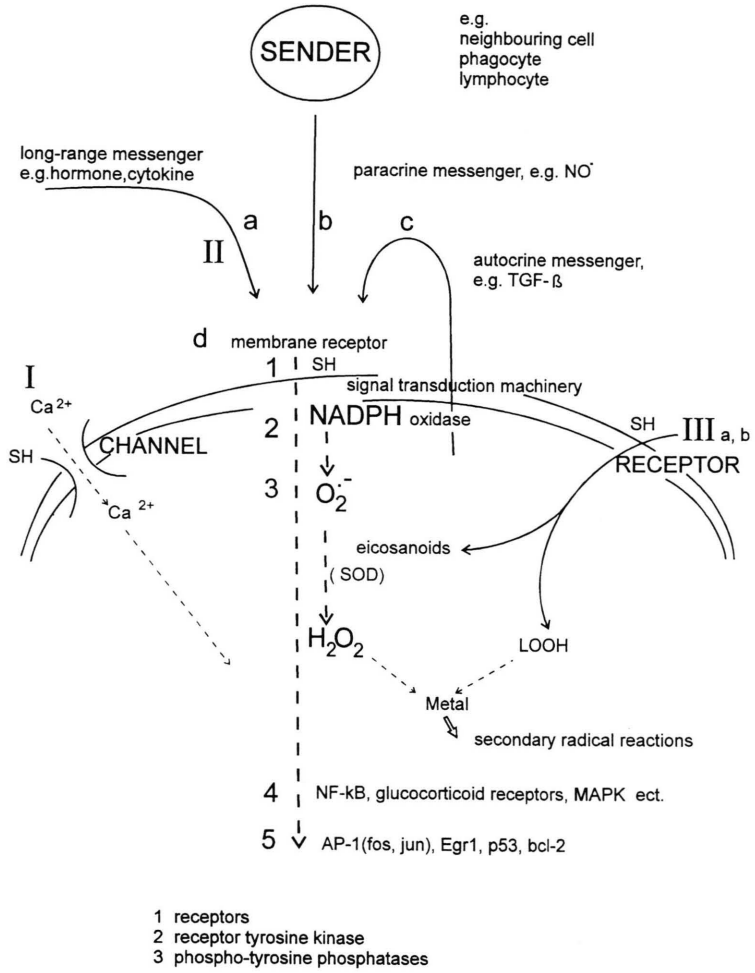

Fig. 6. Possible interrelations between intra- and extracellular signalling pathways. Along the dashed line five steps are outlined which a cellular signal of the tyrosine receptor pathway takes: a receptor (1) activates a kinase (2), a phosphatase (3) eventually regulates the signal, until it arrives at some intracellular switching device (4) which finally regulates transcription (5). Steps 3 to 5 have clearly been shown to respond to redox imbalances and there are indications that also tyrosine itself, i.e. step 2, may be modified by oxidants.
Three conceivable mechanisms for the translation of extracellular oxidative stress into intracellular consequences are depicted:
I. Oxidants may react with functional sulfhydryl groups at channel proteins. The intracellular consequences are then determined by the function of the channel (e.g. Ca^{2+} or hexose transport) and will have no 'direct' correlation with the signal.
II. Oxidants may interact with intercellular messengers (a to c) or with a membrane receptor of one of the messengers; the intracellular consequences will show an 'intermediate' level of correlation to the oxidants.
III. Oxidants may interact with a receptor that is especially tuned for translating their signal into some specific intracellular messenger; the intracellular consequences will show a high degree of correlation to the oxidants.

transfer of redox equivalents from NADPH via protein disulfide isomerase and ascorbate, was suggested to play an essential role (Holmgren, 1993). Interestingly, the inactivation of the disulfide reducing activity of thioredoxin reductase correlates with a large increase of reactive oxygen species (Arnér *et al.*, 1995).

Another observation concerning the dichotomy of extra- and intracellular phenomena was made by Wong (1995) who investigated the induction of the antioxidative enzyme MnSOD by cytokines: she supplied oxidized thioredoxin to the medium in a concentration range where no induction of MnSOD was observed; when she added thioredoxin reductase and NADPH, however, MnSOD began to be induced; when the extracellular thiols were kept in the oxidized state by diamide or alkylated by chloro-2,4-dinitrobenzene, the induction was lost again. It is tempting to speculate that the responsible *reduced* sulfhydryls were located in some membrane receptor which only functions properly in the reduced state.

At present no sufficient data are available to describe the link between extra- and intracellular redox phenomena in full detail; the observation, however (Pantopoulos *et al.*, 1997), that *extra*cellularly administered hydrogen peroxide influences the translational regulation of ferritin synthesis in a different way than *intra*cellularly generated H_2O_2, may be a promising starting point for further investigation.

Signalling by Radicals: What Mechanisms Are Theoretically Conceivable?

Two distinct mechanisms may be envisaged by which a radical could act as a messenger (Saran and Bors, 1989):

a) in a *sender-to-point* fashion, equivalent to a unique message being directly communicated from a sender to an addressee (an example could be $O_2^{\cdot -}$ diffusing from some source and interacting somewhere with a receptor which is specifically sensitive for this radical);

b) in a *sender-to-collective* fashion, equivalent to a variety of radicals emanating from a sending device and acting on e.g. sulfhydryl groups collectively thereby inducing each of these groups to do what they are built for, e.g. close a channel, interrupt some supply, interfere with a communication loop, etc.

Irrespective of which mechanism applies, however, it should meet several prerequisites:

1) the messenger must have a suitable half-life which allows it to diffuse from *a* to *b* without loosing its identity,

2) its interaction with the site of reception must evoke reproducible results, and the reaction should be at least 'specific' in the sense that no other molecule may interact with the signal path in the same way;

3) a generation system should exist for efficient production of the messenger.

Condition 1 severely limits the choice of radicals which could directly act as messengers. Estimates for free path lengths in the cellular milieu range from less than the thickness of a membrane for the $^{\cdot}OH$ to less than half a cellular diameter for the superoxide anion (Saran and Bors, 1989) to several cell diameters for nitric oxide. All other hitherto identified intermediates of *in vivo* radical chains react with cell constituents faster than $O_2^{\cdot -}$. This, in our opinion, prohibits their use as messengers and leaves $NO^{\cdot}$ and $O_2^{\cdot -}$ as the only candidates with a reasonable reach in terms of cellular dimensions (Saran and Bors, 1994).

Condition 2 is, according to present knowledge, only met by the above two radicals. The common characteristic of radicals is that of bearing a lone electron. The manifold possible structures of $R^{\cdot}$, $RO^{\cdot}$, $ROO^{\cdot}$ radicals make any key-to-keyhole matching mechanism with a unique receptor site inconceivable. Since even $NO^{\cdot}$ or $O_2^{\cdot -}$ are unlikely to operate via such a 'docking' strategy one has to assume that 'most efficient orbital overlaps' between the radicals and the responsive receptor site define the docking point. At present, we do not know to what extent one-electron coupling (Kovacic, 1996) might be of general importance in biological systems. It should not be forgotten, however, that any proposal for interaction of radicals with molecular reactants must also include an explanation of the fate of the single electron, i.e. how it is handled within the receiving molecule and how it is coupled to another single-electron state to achieve a stable molecular orbital.

For the radical $NO^{\cdot}$, which undoubtedly serves as a messenger, details of the coupling to the receptor site on soluble guanylate cyclase has only very recently been elucidated (Craven and Ignarro, 1996).

For $O_2^{\cdot-}$ we recognize at present SOD as the only receiver which fulfills the criterion of specificity. The active site of this enzyme takes advantage of the fact that $O_2^{\cdot-}$ has a defined charge and thus is conducted by electrical gradients. However, as SOD would merely translate the message borne by the radical $O_2^{\cdot-}$ into a molecule of H_2O_2 (which is an 'unspecifically acting' two-electron oxidant), such a process could not be regarded as a meaningful example of message transmission. There have been speculations that analogous receptor sites may exist to derive signals from $O_2^{\cdot-}$ (Saran and Bors, 1989). Without such sites being identified, however, the most likely alternatives are Fe-S clusters, which have been discussed for aconitase. The fact that these structural moieties are present at crucial sites in various proteins and that they are involved in critical steps of metabolic chains makes them plausible targets for radical interaction (Gardner, 1997).

Condition 3 is nicely met by $O_2^{\cdot-}$. It has been shown that the same NADPH oxidase being switched on in phagocytes upon stimulation is also present in other types of cells such as endothelial cells (Rosen and Freeman, 1984), fibroblasts (Meier *et al.*, 1991), and many others. The activation of this enzyme bears convincing analogies to other mechanisms of signal transduction resulting in second messengers such as cAMP, diacylglycerol (DG), and Ca^{2+}. Most of these mechanisms are based on the activation of GTP-binding proteins which regulate enzymes such as adenylate cyclase, phospholipases, and kinases of the tyrosine cascade. In the case of NADPH oxidase the enzyme is present in the membrane in inactive form and becomes activated by two cytosolic proteins $p47^{phox}$ and $p67^{phox}$ and then triggered by another protein $p21^{rac1}$ (Abo *et al.*, 1994). In plants, a similar enzyme has been found, whose activation pathway still needs to be elucidated. Yet its function during the 'hypersensitive response' to pathogens is to prepare a necrotic barrier and it uses $O_2^{\cdot-}$ and H_2O_2 for this purpose (for references see Baker and Orlandi, 1995).

The switch of xanthine dehydrogenase into its oxidase mode, induced by proteases after an ischemic event, has been suggested to represent a controlled source of $O_2^{\cdot-}$. This mechanism, however, since it comes along with oxidative stress at many other cellular sites (Granger and Korthuis, 1995) may not represent a typical example for an intended use of a radical for signalling purposes.

Even though it is not known at present what teleological goal the NADPH oxidase of nonphagocytic cells may serve, it is evident that this enzyme complex - with respect to being triggerable and specific for the production of a radical species – would fulfill all criteria of being a generator of a second messenger.

Signalling by Radicals: What Triggering Mechanisms Are Plausible?

If we tentatively accept that an enzyme system such as NADPH oxidase were a suitable intracellular signalling source, we must then ask how it may be set in action: is it triggered intra- or extracellularly?

Figure 6 refers to several possibilities.

Mechanism I illustrates some 'radical event' (resulting in extracellular oxidative stress) unspecifically oxidizing exofacial sulfhydryl groups. If such groups belonged to a calcium channel, the oxidative stress would converge with the normal calcium signal path with all of the cellular consequences of the latter; if the affected SH groups belonged to a hexose transporter or something similar, the intracellular effect would be 'starvation' (with intracellular consequences of energy depletion and activation of the respective cellular regulation mechanisms). In summary, mechanism I describes the lowest degree of specifity: radicals in this case would merely act as a jammer for other signalling pathways.

Mechanism II is somewhat more specific. Suggestions *a* to *c* differ by the range attributed to the extracellular messengers, case *d* represents the idea that a surface receptor of one of the three alleged messenger types might be altered. Such a mechanism would be specific in the sense that oxidative stress would merely evoke those cellular responses for which the messenger in question is programmed. To our knowledge no experimental proof for such a process has been presented.

Mechanism III is the most specific of the quoted processes. It is based on the assumption that a receptor exists which is tuned to selectively receive the signal 'oxidation' and to translate it into some defined intracellular signal. Lipid hydroperoxides or arachidonic acid derivatives are mentioned as

examples; if mechanism III would apply, i.e. if such a membrane-bound sensor with oxygen sensivity could eventually be found, we would rather expect its signal transduction machinery to resemble one of the other pathways, e.g. of the GDP- or tyrosine kinase type. Today, however, no specific sensor is known.

Concluding Remarks

In summary, our attempt to extrapolate from known physico-chemical properties of radicals to the conditions *in vivo* only partially helped to answer some pertinent questions:

Are radicals deleterious?

Judged from their *in vitro* reactivities almost all intermediates and endproducts of radical chains have the 'chemical' potential to be deleterious, i.e. to adversely affect the functioning of other molecules. This chemical potential is certainly not lost *in vivo,* but here we have to put the question of what might be deleterious in a more subtle way: 'what annoys the cell?' We believe that radicals *per se* do not necessarily pose an existential threat to the cell. Since there is no effective remedy against these species, the cell indulges in tolerating the respective radical reaction. It is forced, however, to handle the stable endproducts of the chains, i.e. H_2O_2, hydroperoxides, aldehydes, etc., with great care. Especially for (hydro)peroxides, the cell has developed a broad array of defense measures. In the case where it is overwhelmed by too many 'radical-derived products' and the countermeasures (essentially the GSH system) fail to balance the oxidant tone, a great variety of SH-sensitive transcription processes may be triggered. Only if the range of normal cellular feedback control is overrun, the cell dies.

In those special cases where a deleterious action of 'radicals' is teleologically intended, e.g. in phagocytosis, the chemicals H_2O_2, HOCl, and $O_2^{\cdot-}$ are produced by specialized enzyme systems and delivered 'on the spot', e.g. by attaching the 'ammunition factory' MPO by electrostatic binding directly to required site of action, the bacterial membrane.

Do radicals serve metabolic goals?

Thus far, well documented examples are known only in plants. Both the biosynthesis of lignin and the enzymatic degradation of this plant polymer by fungal peroxidases are paradigms for the utilization of radicals *in vivo.* While the former proceeds via stereospecific coupling of phenoxyl radicals of hydroxycinnamic alcohol precursor molecules, the latter reaction involves the intermediary formation of radical cations of aromatic ethers (see Ros Barceló, 1997). It would be intriguing to assume that polymerisation reactions in animal tissues would likewise involve radical reactions, but this remains speculative. **De**polymerisation reactions involving ROS have always been considered uncontrolled side effects.

The many instances where radicals have *mechanistic* implications in metabolism are confined to enzymes that use radical intermediates for catalytic purposes such as ribonucleotide reductases, thioredoxin reductases, cytochrome *c* oxidase, etc. But in all of these examples the radicals are 'bound'. Free radicals may interfere with metabolic processes at seemingly crucial points (e.g. aconitase/iron binding proteins, calmodulin etc) but in none of these instances it has been convincingly shown that the radical step serves a metabolic goal in a truly teleological sense.

Do radicals exert control functions?

If we use the general terms 'redox balance' or 'oxidant tone' to account for 'radical reactions', the answer may be an unqualified yes. Variations of the metabolic rate, being in pace with variations of the throughput of electrons through the mitochondrion, cause variations of the radical tone around this 'power plant' and thus directly reflect metabolic activity. We can safely assume that the ratio of oxidant to reducing conditions, i.e. the interplay between 'radical reactions' and the thiol status described above, is part of a feedback mechanism that sustains life.

Do radicals serve as signals?

This is the most intriguing question and can only be answered for individual radical species.

With the exception of $NO^{\cdot}$, radicals have very limited pathlengths within the cell and are not able

to cross compartment boundaries. Furthermore no process is presently known which shows that radicals may interact in a *specific* way with any target molecule; therefore we do not see how radicals *in general* could properly act as messengers, neither on an intracellular nor on an extracellular scale.

Irrespective of our present inability to define a convincing pathway for *direct* signalling by radicals we believe in the possibility that radicals contribute to signalling cascades which are triggered at the membrane and translated into an intracellular second messenger. Examples for such actions have been reported (Sundaresan *et al.*, 1995, Burdon, 1996). Furthermore, the possibility exists of some cross-talk between *extra*cellular signalling pathways and the *extra*cellular redox-balance. It has already been shown that intercellular communication by TGF-beta, which induces apoptosis in transformed cells (Langer *et al.*, 1996) is sensitive to changes in the oxidant tone of the cell-surrounding medium and that apoptosis may be greatly modified by antioxidants such as GSH, NAC etc. This is an area of research where we expect interesting results in the near future.

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