HANDBOOK *of* OXIDANTS *and* ANTIOXIDANTS *in* EXERCISE

Edited by CHANDAN K. SEN
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ELSEVIER

Handbook of Oxidants and Antioxidants in Exercise

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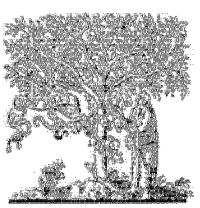

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Preface

Through the ages man has sought to harness the knowledge of exercise; interest in exercise science dates back at least to ancient Greece. Today, exercise is seen as not merely a leisure activity, but as an effective preventive and therapeutic tool in medicine. The study of exercise physiology and biochemistry has had a revolutionary overall impact on biomedical research, and exercise has been used as a model for studying the response of physiological regulatory mechanisms to stress. At the same time, advances in oxygen free radical biochemistry have lured exercise biochemists to study the effects of the increased oxygen consumption that accompanies exercise. The first reports in this field, published in the early 1970s, indicated that strenuous physical exercise might cause oxidative lipid damage in various tissues. Since then, a considerable body of research has accumulated concerning the effects of exercise, nutrition and training on indices of oxidative stress and antioxidant responses in various tissues. Most studies support the contention that during strenuous exercise, generation of reactive oxygen species (highly reactive, partially reduced metabolites of oxygen) is elevated to a level that overwhelms tissue antioxidant defence systems. The result is oxidative stress. The magnitude of the stress depends on the ability of the tissues to detoxify reactive oxygen species; i.e., antioxidant defences. Endurance training enhances such defence in various tissues, especially in skeletal muscle and heart. Antioxidants produced by the body act in concert with their exogenous (mainly dietary) counterparts to provide protection against the ravages of reactive oxygen as well as nitrogen species.

Exercise and Oxygen Toxicity was first published in 1994. The purpose of this multiauthor volume — the first of its kind — was to examine different aspects of exercise-induced oxidative stress, its management, and how reactive oxygen may affect the functional capacity of various vital organs and tissues. Remarkable interest of the readers and favourable critical reviews published in leading journals provided the impetus to put together an enlarged second edition. We started to accomplish that goal. After two years of tireless effort of many, the current volume was ready to go the press. This volume was over double in size of the original edition. Key related issues such as analytical methods, environmental factors, nutrition, aging, organ function and several pathophysiological processes were thoroughly addressed. Leading experts provided unprecedented insight into the understanding of the role of reactive species and antioxidants. The combination of these properties makes this volume an authoritative treatise.

During the course of review of the finalized manuscripts at the publishing house it was brought to our attention that the structure and contents of this volume more closely resembled a *Handbook* than just a second edition of *Exer-*

cise and Oxygen Toxicity. This view was shared by many of our colleagues and peers, and thus we decided to name this volume as the *Handbook of Oxidants and Antioxidants in Exercise.*

Since *Exercise and Oxygen Toxicity* was published, interest in exercise, reactive species and the possible role of endogenous and supplemented antioxidants has soared. Search of the PubMed database of the National Library of Medicine using a combination of the keywords "exercise" and "antioxidant" show that the number of research reports in 1997 is more than double of that in 1994. This handbook is therefore a timely publication. It is relevant to all those who have interest in biomedical sciences and is designed to be intelligible to a general scientific audience.

We are delighted to be involved in this project. The excellent editorial assistance of Dr. Savita Khanna and the outstanding contribution of authors are gratefully acknowledged. We hope that this volume will contribute to the further development of this important late-breaking field of research.

Chandan K. Sen
Lester Packer
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Part I • Chapter 1

Free radical chemistry

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1 INTRODUCTION

It is well accepted these days in the scientific community that free radicals participate in many biological processes, often in a quite decisive manner [1–8]. This clearly emerges from the still rapidly increasing number of studies on this subject and the very important fact that such investigations are not restricted to the views of just one particular group of researchers. Free radical studies in biological systems embrace practically all fields from fundamental chemistry to applied clinical studies these days and have proved to be particularly successful when scientists of the various fields engage in collaborative work and extensive interdisciplinary discussions.

In living systems, including humans, the presence of free radicals is something very natural. The decision of nature to provide the enzyme superoxide dismutase

(SOD), for example, clearly hints on the existence of the superoxide radical, $O_2^{\bullet}$, – considered to be one of the most prominent radical species in oxygenated biological systems – and it also shows the obvious need of cellular systems to regulate its concentration [9–15]. While this serves as an example that a "too much" of a particular radical species is not in the interest of a biological system there are, on the other hand, also observations where a "too little" of radicals seems equally unwanted. For example, healthy cervix tissue has been reported to contain quite a measurable radical concentration whereas a cancerous tissue is almost completely devoid of radical species [16]. In general, however, it seems that the majority of malignant disturbances is associated with an increase in free radical concentration [17]. Free radicals are undoubtedly involved in many degenerative processes including aspects of aging, cancer, cardiovascular diseases, arteriosclerosis, neural disorders, skin irritations and inflammations. Reperfusion injuries, e.g., in the aftermath of surgery, are also typically characterized by an enhanced formation of free radicals [18]. Actually, any metabolism involving redox active centers is, per se, a possible source of continuous free radical production. This certainly allows the extrapolative conclusion that any stress situation, including exercise, carries the potential of excess free radical production associated with all the chemical consequences arising thereof.

Most of the radicals relevant in biological systems are either derived from or associated with the presence of molecular oxygen, in particular, the already mentioned superoxide anion ($O_2^{\bullet -}$) and peroxyl radicals ($ROO^{\bullet}$). Other oxygen-centered and highly reactive radical species are hydroxyl ($^{\bullet}OH$) and oxyl radicals ($RO^{\bullet}$). Their biological significance is not in doubt but somewhat more in the debate than that of $O_2^{\bullet -}$ and $ROO^{\bullet}$. Of course, it is not only oxygen-centered free radicals which are of importance and interest. Many others, generated both from endogenous as well as exogenous substrates are also of significance. A particularly relevant example is the thiyl radical, $RS^{\bullet}$, which is the one-electron redox intermediate between thiols and disulfides, two vital classes among the biologically abundant substrates [19,20].

The action of free radicals is generally determined by their chemical reactivity and the availability of a suitable reaction partner in the vicinity of their production site. In some cases such a radical-molecule interaction may directly lead to a biological damage in a few or even just one reaction step [7]. Thus, an $^{\bullet}OH$ reaction with the sugar moiety of DNA can result in a strand break due to a radical specific phosphate cleavage reaction. Such a singular event does, however, not necessarily imply cell death as there are enzymatic and chemical, possibly even radical associated repair mechanisms. Accumulation of hazardous compounds as a result of free radical reactions is often the reason for long-term effects. In this case it may even be difficult to prove the free radical's responsibility. It should also be pointed out that the appearance of free radicals does not automatically imply their direct participation in the disturbance of vital biological functions. In other words, free radical reactions are not necessarily the cause of disorders but may equally well just be a consequence thereof. In this case free

radicals and their reactions may, however, serve as useful markers.

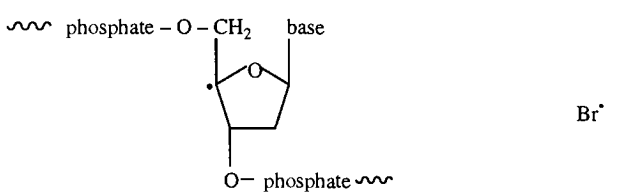
Nature and modern medicine related science have provided us with mechanisms and substrates to cope with free radicals by "deactivation". Enzymes such as the above already mentioned SOD serve this function. Another important group of compounds in this respect are the so-called antioxidants [22,23]. Most prominent representatives in this respect are vitamin E (α-tocopherol) and vitamin C (ascorbate) which are most effective free radical scavengers in the lipid cell membrane and adjacent, more aqueous compartments, respectively. The same function is assigned to many other redox-active compounds such as, for example, thiols, carotenoids, quinones, etc.

Whenever a free radical reacts with a molecular compound it loses its identity but at the same time it proliferates its general radical properties to a new radical formed in this reaction. In order to assess the action of free radicals it is, therefore, necessary to know not only the properties of the initiating but also of all subsequently generated species. The aim of the present article is, therefore, to elucidate on all these aspects and to provide an understanding for the chemical basis of free radical processes. In the first part of our essay we shall focus on the general properties of free radicals, their generation in chemical model systems, and provide some basic information on the most common methods of their detection. Later we shall focus on the identification and chemical properties of specific groups or individual free radical species which are considered to be of potential significance in biological processes. To find out whether the latter actually applies is then a challenge for the biochemist, biologist and medical researcher. It must, of course, always be remembered that a chemical "test tube" or *in vitro* experiment can only provide the basic information what a free radical can and will do chemically when meeting a suitable reaction partner. Such knowledge is an absolute prerequisite to understand a free radical's potential action. By no means can an *in vitro* experiment, however, substitute for the *in vivo* situation where many more parameters need to be taken into the consideration.

2 PROPERTIES AND DETECTION OF FREE RADICALS

2.1 What is a free radical?

A free radical is, per definition, a molecule or just a single atom with an unpaired electron, conventionally symbolized by a radical dot "$^{\bullet}$". Examples, extremes from the molecular size point of view, would be a DNA radical (4′ in sugar moiety) and a bromine atom.



Both are species of interest in certain biological processes. The DNA radical is generated, for example, by reaction of an $^{\bullet}OH$ radical with this vital macromolecule in any cellular system exposed to ionizing radiation and is a direct precursor for a potential strand lesion [7]. The bromine atom, on the other hand, is formed *en route* of reductive degradation of 1,2-dibromoethane, a well-known toxin [24–30].

What means "unpaired" electron? Typically, any electron seeks the association with another of its kind, so that they can pair up their spins in a system of lower energy. A spin is the rotational motion of an electron which can attain two directions and, viewed in a simplified picture, sort of represent a right handed (or "up") and a left handed (or "down") version. Two radicals "shaking hands", i.e., teaming up two individually provided unpaired electrons of opposite spin, form a new bond with this process:

$$\uparrow \quad + \quad \downarrow \quad \rightarrow \quad \uparrow\downarrow \qquad (1)$$

A chemical example would be the combination of two bromine atoms which results in the formation of the Br–Br σ-bond:

$$Br^{\bullet\uparrow} \quad + \quad Br^{\bullet\downarrow} \quad \rightarrow \quad Br - Br \text{ (with the spins } \uparrow\downarrow \text{ in the bond)} \qquad (2)$$

Generally, any even number of electrons in a molecule arranges in "up"-"down" spin coupled pairs, both in bonds or as nonbonding electron pairs. Usually this is an energetically quite favorable process. Sometimes, however, it may be more economic from the energy point of view that two electrons remain uncoupled, even within the same molecule. In this case the two unpaired electrons attain the same spin direction and the chemist talks about a "triplet state" or, if they are located at different atoms, a biradical. A most important example for this situation is the molecular oxygen which we generally view as a molecule with a double bond (a). The two electrons of its p-bond seem, however, to some extent separated and may thus act as if there were two centers providing one unpaired electron each (b):

$$O = O \qquad (a) \qquad\qquad ^{\uparrow\bullet}O - O^{\bullet\uparrow} \qquad (b)$$

The presence of unpaired electrons or unpaired spins has a most important consequence, namely the generally very high reactivity of such species.[1] This, of course, reflects the genuine desire of the radical species to attain the most favorable energetic state by coupling its unpaired spin with that of an opposing sign in a new bond between two spin carrying atoms. Accordingly, most radical-radical and also many radical-molecule reactions occur as soon as the two reacting species meet each other. Such a reaction is, as we say, a diffusion controlled process. The time it actually takes for completion of any chemical reaction depends on two parameters, a reaction specific rate constant (which attains its

highest value for a diffusion controlled reaction) and the concentration of the reacting species. In biological systems the free radical concentration is usually very low and rarely acquires values high enough so that a radical-radical reaction could beat a reaction of a radical with a suitable molecular partner. The latter are typically present at much higher concentration in the near-by vicinity.

In order to illustrate this, let us discuss a simple example. Carbontretrachloride, CCl_4, known as a potent liver toxin, is readily reduced by the enzymatic P-450 system of the endoplasmic reticulum [31]. In the presence of oxygen this results in the formation of $CCl_3OO^\bullet$, i.e., trichloromethylperoxyl radicals as an important intermediate in the CCl_4 degradation process (further mechanistic details on peroxyl radical decay will be presented later in this article). Its steady state concentration is, however, extremely small and only few research groups with highly sensitive equipment and great experience have been able to actually detect this radical *in vivo* [32]. It is probably even lower or, at most, comparable to the steady state radical concentration of $\leqslant 1$ pM ($\leqslant 10^{-12}$ M) prevailing in systems which are subjected to γ-irradiation in an average research ^{60}Co-source. In the absence of any suitable reaction partner the above peroxyl radicals have, indeed, no other chance than to react with each other:

$$CCl_3OO^\bullet + CCl_3OO^\bullet \rightarrow \text{products} \quad (2k = 2 \times 10^8\ M^{-1}\ s^{-1})\ [33] \quad (3)$$

The half-life for such a so-called second order process is mathematically given by an equation which includes the rate constant (for this kind of second order process defined as "2k") and the radical concentration (in molar units) [34]:

$$t_{1/2} = 1 / \{(2k) \times [CCl_3OO^\bullet]\} = 1 / \{2 \times 10^8 \times 10^{-12}\} = 5\,000\ s \quad (I)$$

On the other hand, if a scavenger such as vitamin E (α-tocopherol) was around in just nanomolar (10^{-9} M) concentration the following reaction would successfully take over:

$$CCl_3OO^\bullet + \text{Vit E} \rightarrow \text{products} \quad (k = 5 \times 10^8\ M^{-1}\ s^{-1})\ [35] \quad (4)$$

When the concentration of the molecular reaction partner (Vit E) significantly exceeds that of the radical ($CCl_3OO^\bullet$) the reaction becomes of pseudo-first order (reflecting the fact, that the concentration of the excess component hardly changes during the course of the reaction). For such a condition, the mathematical equation attains a slightly different form, the most important difference being that it contains the concentration of the molecular reaction partner and not that of the radical:

$$t_{1/2} = \ln 2 / \{k \times [\text{Vit E}]\} = 1 / \{5 \times 10^8 \times 10^{-9}\} = 1.4\ s \quad (II)$$

By comparison, these two half-lives show that the scavenging of the $CCl_3OO^\bullet$

radical by Vit E is several orders of magnitude faster, i.e., more probable to occur than the mutual radical-radical deactivation.

While this example, from the biological point of view, pertains more to the lipid phase (where Vit E is preferentially located) a similar system may be considered for the aqueous compartments. Thiyl radicals, $RS^{\bullet}$, although not oxygen-centered but nonetheless equally frequent and important as any oxygen-centered free radical in cellular systems, readily react with the water-soluble ascorbate (Vit C), as formulated in the following equation for the thiyl radical, $GS^{\bullet}$, from the probably most abundant thiol, glutathione (GSH):

$$GS^{\bullet} + \text{ascorbate } (AH^{-}) \rightarrow GS^{-} + A^{\bullet -} \qquad (5)$$

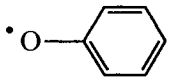
With a rate constant of $k = 6 \times 10^{8}\ M^{-1}\ s^{-1}$ [36] and an estimated Vit C concentration (e.g., in liver tissue) in the low millimolar range (10^{-3} M) the half-life of this process, calculated according to eq.(II), assumes a value of about 1 ms. For a second order thiyl radical recombination process ($2k_6 \approx 10^{9}\ M^{-1}\ s^{-1}$) [37] to compete, i.e., to occur with a comparable half-life, a $GS^{\bullet}$ concentration of about 1 mM would be necessary (calculated with eq. (I)). Such a high steady-state radical concentration is, however, pretty unrealistic in any biological system.

$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG \qquad (6)$$

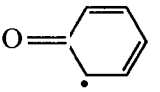
Therefore, any radical-radical reaction is extremely unlikely to occur and the discussion of radical reactions in biological systems should accordingly always be checked first with respect to possible radical-molecule processes. Even if the latter are slow they may still benefit from the usually considerably higher concentration of the molecular reaction partner as compared to the free radical concentration.

2.2 How fast are free radical reactions?

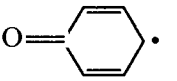
As mentioned above most radical reactions are essentially controlled just by the diffusion of the reacting species. In liquid environment this typically results in rate constants within the order of $10^{9}\ M^{-1}\ s^{-1}$ with the individual value depending on the size and structure of the respective species, on solvation effects, and also on the viscosity of the environment. One intrinsic parameter lowering this value could be an internal stabilization of the free radical, meaning delocalization of spin and electron density into the existing regular bonds or to atoms of particularly high electron affinity. Often it is feasible to characterize such radicals by resonance structures, especially when aromatic π-systems are involved. For example, phenoxyl type radicals (structurally similar to the chromanoxyl radical obtained upon oxidation of Vit E) mostly behave as oxygen-centered radicals (a) but certain reactions such as di- and oligomerizations can only be understood in terms of their carbon-centered resonance forms (b) and (c) [38]:



(a)



(b)



(c)

Sometimes such resonance structures are so stable that the radical becomes more and more persistent and may, in extreme cases, even be bottled. At the same time the reactivity towards other radicals and molecular substrates becomes lower and lower, and rate constants accordingly diminish, possibly by many orders of magnitude.

A second parameter which influences the rate of a radical reaction pertains to radical-molecule reactions:

$$R^{\bullet} + A-B \rightarrow R-B + A^{\bullet} \qquad (7)$$

In this case, the initial radical $R^{\bullet}$ succeeds in pairing up its lonesome electron, a process certainly beneficial for a high rate constant. At the same time, however, it is necessary to break the molecular A–B bond, and this is a process which requires energy and is, therefore, of adverse influence on the overall rate of reaction. In fact, the complete assessment of the kinetics of such processes must also take into account the energy contents of the product species and, in particular, of the transition state in going from the educt to the product system. This transition state, in which (with reference to the above example) $R^{\bullet}$ has associated already with A–B but $A^{\bullet}$ has not left yet (i.e., $[R...B...A]^{\bullet}$, has, per definition, always the highest energy content along the reaction trajectory. The energy difference between the educts and the transition state is the so-called activation energy, and this is essentially the parameter which decides on the overall rate constant of a reaction. For a typical diffusion controlled process the activation energy is so small that it is essentially provided by the Brownian motion of the reacting partners. However, if this activation energy becomes higher statistically fewer and fewer of the reacting species provide the necessary internal energy to, literally spoken, push the reaction over the transition state mountain. Such reactions are not any more *diffusion* but *activation energy controlled.* An example, quite relevant and important from the biological point of view, would be an oxidative cleavage of a C–H bond by, e.g., a trichloromethyl peroxyl radical:

$$-\overset{|}{\underset{|}{C}}-H + CCl_3OO^{\bullet} \longrightarrow -\overset{|}{\underset{|}{C}}{}^{\bullet} + CCl_3OOH \qquad (8)$$

Abstraction of a bisallylic hydrogen atom from linoleic acid, for example, has been reported to occur with a rate constant on the order of 10^6 M^{-1} s^{-1} [39], i.e., three orders of magnitude below the diffusion limit. This means that, despite the strong activation these hydrogen atoms receive from the allylic double bonds of this PUFA, statistically only one in about a thousand encounters actually leads

to completion of the above reaction.

An important consequence of these rate constant considerations is the following: radicals which are generated in the vicinity of substrates will definitely undergo fast and quantitative reaction with the latter whenever this reaction is a diffusion or near-diffusion controlled process. Such a situation does not allow any migration of the radicals over larger distances and must, therefore, not be considered for any direct action at distant sites or longer times after their generation.

In this respect it may, in fact, be interesting to know how far a species can actually move until it reacts. Let us consider, for example, a hydroxyl radical $^{\bullet}OH$ generated e.g., in a radiation exposed biological sample. These radicals are known to react with almost any available C–H bond with rate constants $> 10^7$ M^{-1} s^{-1} [40,41]. Assuming potential targets of this kind in a cellular fluid of at least millimolar concentration this translates into a half-life of the $^{\bullet}OH$ radical of $\leqslant 70$ μs and after ten half-lives, i.e., 700 μs, the reaction can, by all practical means, considered to be completed. The simplest mathematical equation relating time (t) with the mean displacement of a diffusing species (Δx) and the diffusion coefficient (D) is:

$$(Dx)^2 = 2\ D\ t \qquad \text{or} \qquad Dx = (2\ D\ t)^{1/2} \tag{III}$$

With a typical diffusion coefficient of about 10^{-5} cm^2 s^{-1} the linear free pathway would thus calculate to $\Delta x \leqslant (2 \times 10^{-5} \times 7 \times 10^{-4})^{1/2} \approx 10^{-4}$ cm. For diffusion controlled processes ($k \geqslant 10^9$ M^{-1} s^{-1}) the distance shrinks for another order of magnitude (because of the square root dependence) to $\approx 10^{-5}$ cm. These are clearly no macroscopically interesting distances.

Larger pathways can only be envisaged for lower concentrations of the target molecules, or for lower than the above applied rate constants. An extreme case is given when the radical cannot find a suitable molecular partner and lives until it meets another radical of its kind. As calculated for the particular example of the trichloromethyl peroxyl radical (see Eqs. 3 and I) with an estimated steady state radical concentration of 1 pM the half-life extends to about 5,000 seconds, or more than one hour. In this case, the Δx calculation becomes, however, rather meaningless since convectional motion of the cellular fluid is likely to take over with respect to mass transport.

In conclusion, all the previous discussion has hopefully shown how important it is to know the exact rate constants for the all possible radical reactions as well as the local concentrations of both the radicals and their potential molecular reaction partners. Both parameters are an essential prerequisite for any mechanistic assessment in a free radical containing biological system.

2.3 Detection of free radicals by time-resolved optical spectroscopy

As the reader may have realized from the previous chapter, free radical reactions are usually very fast and thus clearly escape any direct detection in an *in vivo*

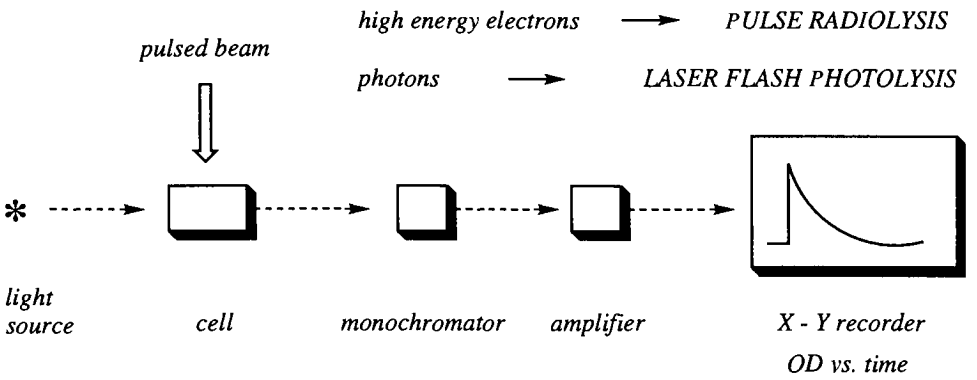
real-time experiment. Fortunately, absolute rate constants are an inherent property of any particular reactive system and may, therefore, be determined, without introducing any error, outside the biological compartment in suitable and often even much better defined *in vitro* model studies.

The basic problem one has to deal with in case of free radical reactions is the usually high rate at which most of these processes occur. The typical time window for the lifespan of a free radical, reacting in solution via a diffusion controlled process with molecular substrates which are present at μM to mM concentrations, lies in the millisecond time scale. Any technique for direct study of such fast reactions must, therefore, comprise of a correspondingly fast detection system. For quantitative kinetic analysis it is highly desirable, in fact almost mandatory, that the formation of the radical of interest is completed within a time period that is short compared to the lifetime of the radical. Only then becomes it possible to directly "see" such a species. A further prerequisite is, of course, that the radical exhibits a detectable property. A very suitable parameter, in this respect, is the optical absorption. In fact, most radicals are colored, not necessarily in the visible part of the spectrum, but IR and UV regions are equally accessible by modern time-resolving optical spectroscopy. In principle, also any other property detectable with appropriate time resolution is equally suitable [42]. The most frequently used is conductivity which helps, in particular, to study charged species, i.e., radical ions [43,44].

Two techniques have been especially successful to study fast processes and to determine absolute rate constants for free radical reactions, namely, the radiation chemical method of pulse radiolysis [42,45,46] and laser flash photolysis [6,47]. The principle idea is the same for both methods and shall briefly be introduced in the following simplified scheme. It depicts the formation and decay of a transient which is generated in the "cell", typically an all-quartz vessel of 0.5–10 cm^3 volume containing the chemical solution to be investigated. For convenience and rapid sample exchange the cell is often attached to a flow system. Irradiation is achieved by admitting short pulses of either high energy electrons (MeV range), generated in a Van de Graaff or Linear Accelerator (pulse radiolysis), or photons from a laser (laser flash photolysis). For kinetic studies the duration of these pulses is typically in the pico- to microsecond time range. The result of such a pulse is an energy deposit in the solution contained in the "cell". This, in turn, is responsible for the free radical production.

Despite the principle similarities of these two techniques the actual detailed picture includes some differing aspects. The somewhat easier to understand system is the photolytic one. Here, the incoming photons from the laser pulse interact directly with a target molecule in the "cell" compartment in a process which may lead to its excitation and ionization. A prerequisite is, however, that this target molecule absorbs light at the wavelength of the incoming photons with a sufficiently high extinction coefficient and the energy of the photon is sufficient to initiate the desired process.

In case of pulse radiolysis the energy of the incoming electrons is significantly



Scheme 1. Time-resolved optical spectroscopy.

higher compared to that of a laser photon. Considering that ionization of a molecule typically requires energies in the 5–20 eV range one might, in fact, get quite scared about such a "bombardment" with MeV electrons. Fortunately, those high energy particles lose their energy in small packages via many consecutive steps rather than at once. In simple terms, while flying through the atomic world of matter a high energy electron very rarely hits any of the atomic electrons or positively charged nuclei directly. Typically, it just passes those charged centers at more or less larger distance. Since charges can "see" each other over quite a distance (coulombic interaction) the "flying" electron becomes deflected from its original path at each of these encounters. Physics tells us that any such event is accompanied by an exchange of energy. As a consequence, all the kinetic energy is deposited in small portions which, on average, are of 50–100 eV magnitude. These energy islands (also called spurs) are more or less homogeneously distributed in the irradiated sample. This amount of energy is just sufficient for molecular excitations and one or two ionizations, i.e., events which lead directly to free radicals. There are, of course, some more particulars to it which do not, however, need to be elaborated further in this context. Certainly they do not involve any unresolved magic with respect to the chemistry initiated by radiation. Basically, everything is described in common radiation chemistry [45,48–50] or radiation biology [6,7] textbooks, for example.

When comparing the two time-resolved methods one more important feature must be pointed out. In pulse radiolysis the high energy electron, while flying through matter, does not distinguish between the various molecules since it interacts with the coulombic field of any atomic electron or nucleus it passes by. Statistically, the respective energy deposits are, therefore, preferentially located in the solvent rather than near any low concentration solute. This results, in the first instance, in solvent-derived radicals, and the free radicals of interest would then be formed in a subsequent reaction of these primary radicals with the solute. We call this an "indirect" formation of free radicals.

In principle, however, both the photolysis as well as the pulse radiolysis method

are equally suited to generate and study free radicals and their reactions. In fact, they are in many ways complementary.

Let us now return to the time-resolved experiment outlined in the above schematic drawing. Perhaps we would like to study the free radical induced oxidation of ascorbate (Vitamin C). In a pulse radiolysis experiment our irradiation cell would then probably contain an aqueous solution of typically about 100 μM ascorbate and the sample would have been saturated with nitrous oxide (N_2O). The latter serves two functions. Firstly, by purging the solution with this gas oxygen will be removed (O_2 usually disturbs because it scavenges many free radicals before they can undergo the reaction of interest). The second function of the N_2O is to establish defined chemical conditions as will become apparent in the following discussion of the general reaction scheme in irradiated aqueous solutions.

Irradiation of this ascorbate solution leads, in accordance with our above considerations, primarily to reactive species from the solvent water (Eq. 9):

$$H_2O \rightarrow e_{aq}^-, {}^\bullet OH, H^\bullet, H_{aq}^+, H_2O_2, H_2 \quad (9)$$

The two most important ones are the hydrated electrons (e_{aq}^-) and hydroxyl radicals ($^\bullet OH$) which together account for 90% of the initial free radicals. They are typically formed at about μM-concentrations per pulse.[2] However, while the hydrated electron is a strong reductant the hydroxyl radicals is a powerful oxidant.[3] This potentially disturbing problem can be overcome by the presence of nitrous oxide which converts hydrated electrons into hydroxyl radicals:

$$N_2O + e_{aq}^- + H_2O \rightarrow {}^\bullet OH + OH^- + N_2 \quad (10)$$

At N_2O saturation this process is completed within a few nanoseconds and consequently the only reactive species available for further reaction is the hydroxyl radical. In our model experiment this would then react with the ascorbate (denoted as AH^-) to generate the ascorbyl radical, $A^\bullet$:

$$^\bullet OH + AH^- \rightarrow A^\bullet + H_2O \quad (11)$$

At millimolar ascorbate concentration this reaction is completed within less than a few ms. The ascorbyl radical, now, has an important property: it absorbs light and the maximum of its absorption lies at about 360 nm [51]. Accordingly, if we shine light through the irradiation cell while the system is exposed to the pulse of high energy electrons the intensity of this light will be diminished as soon as the absorbing ascorbyl radical is generated. In order to scan this event the analyzing light beam, after having penetrated the irradiated solution, enters a monochromator which allows to select a suitable wavelength. After conversion of the optical signal into an electrical signal and amplification it is finally displayed on an X–Y-recorder in terms of a time-dependent voltage which actually represents the change in optical density at a selected wavelength as a function of time. The

signal shown on our scheme is thus a true fingerprint of a transient which is formed in a fast process and then eventually decays away again. Exactly this is the type of signal we observe in our above example on the oxidation of the ascorbate under the discussed conditions and which is attributable to the ascorbyl radical.

The intensity of such signals recorded at varying wavelengths provides the complete absorption spectrum. Kinetic analysis of both the formation and decay of the absorption yields the respective rate constants of the underlying processes, here the reaction of $^{\bullet}OH$ with ascorbate and the subsequent disproportionation of the ascorbyl radical.

Generally, such studies conveniently allow to investigate the reactivity of just any absorbing transient with other substrates. In this case we just need to add the suspected reaction partner in small quantities (small enough so that it does not react directly with one of the primary radicals) prior to irradiation. If it does indeed react with our transient this will show up as an accelerated decay of the transient signal which, furthermore, depends on the concentration of the added substrate. Such experiments are actually the general basis for the evaluation of absolute rate constants.[4]

2.4 Detection of free radicals by electron spin resonance

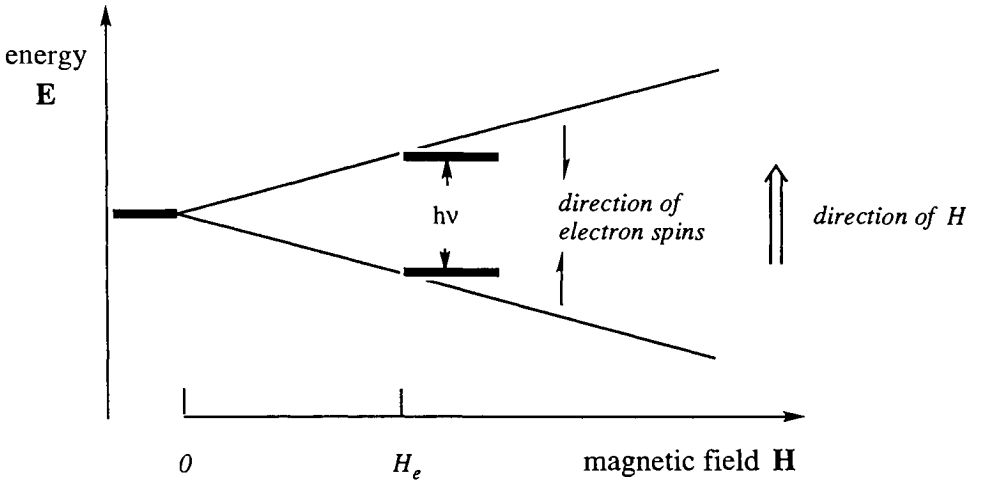
As mentioned already, the time-resolved detection techniques discussed above, as excellent as they are for relatively simple model studies, they do usually not permit investigations with more complex biological material. Whenever a direct proof of free radicals in tissue is at stake another technique, namely, electron spin resonance (ESR) is probably the better method of choice [52–54].

The principle idea behind this method is to take advantage of the spin of the radical's unpaired electron. As a "moving" charge an electron has an associated magnetic moment which can be aligned either in (parallel to) or against (antiparallel to) the direction of an external magnetic field. Now, it is important to realize that the energy level of the magnetic moment of the unpaired electron is higher for the antiparallel than for the parallel alignment and, furthermore, that the resulting energy difference, or split in energy levels, depends linearly on the external magnetic field as shown in the simple pictorial. All this is basically controlled by quantum mechanics.

The energy separation, expressed in terms of $h\nu$, increases with increasing magnetic field, here depicted for a deliberately chosen experimental field strength H_e. The energy difference between the two levels is given by

$$h\nu = g\beta H$$

where g denotes a spectroscopic splitting factor and β the Bohr magneton. While β is a constant, g varies with the nature of the free radicals. For a single free electron, which is solely characterized by the angular momentum of the spin, it



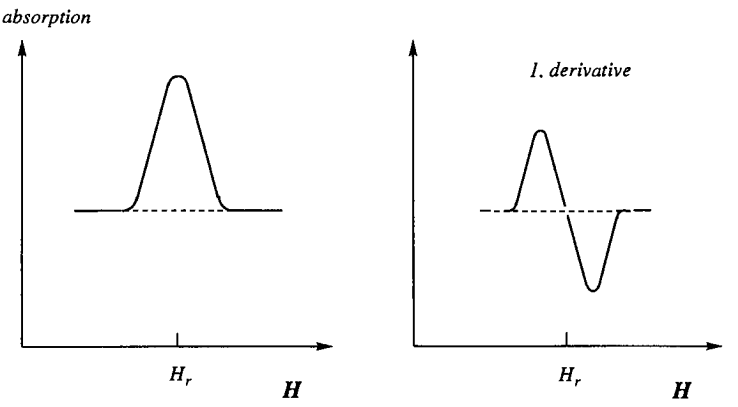
Scheme 2. Splitting of energy levels in magnetic field.

assumes a value of 2.0023. However, if this electron interacts with its surroundings this number may deviate, usually within $\leqslant \pm 0.01$. The g-factor thus provides the information on the molecular orbital of the unpaired electron and in that respect is a property of the radical as a whole [55].

How is it possible to verify this concept experimentally? We need to measure the energy level difference $h\nu$ at a given magnetic field H_e. One possibility to do this would be to offer a varying spectrum of electromagnetic energies $h\nu$ (in ESR typically within the microwave range) at constant magnetic field and see which of the frequencies is absorbed in order to promote the electron from the low (parallel) to the high (antiparallel) energy alignment. Experimentally this procedure is, however, more difficult to realize than the second possibility where the microwave frequency is kept constant (e.g., $\approx$ 9 GHz X-band or $\approx$ 36 GHz Q-band microwaves) and the probe is exposed to a varying external magnetic field. Wherever in such a scan the separation of the two energy levels matches the offered microwave energy $h\nu$ there will be a resonance absorption, i.e., energy will be transferred from the microwave field into the molecule. Generally, the absorption signal is not recorded as such but as its first derivative. Both are depicted in the following scheme.

With H_r, $h\nu$ and the natural constant β we now have all parameters to evaluate g. (For most known free radicals g values have explicitly been measured and many of them are tabulated in data collections) [56].

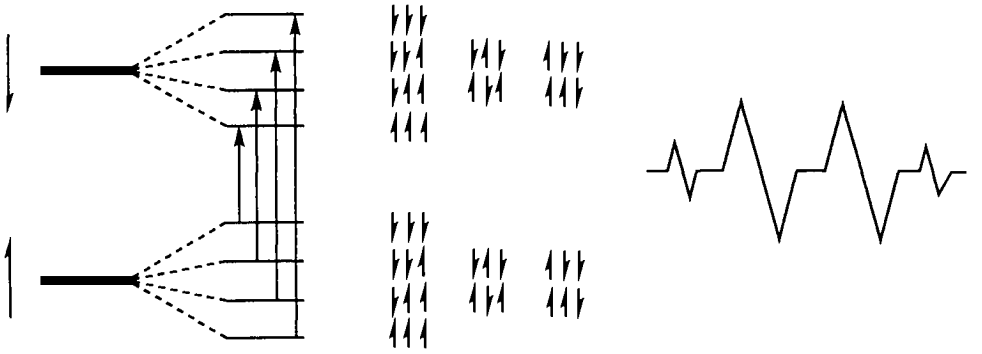
Some of the radical assignments rely pretty much on such g factor determinations, a prominent example being the superoxide anion, $O_2^{\bullet -}$. There are, however, further effects which give even more insight into the molecular structure of a free radical. Just as all the other electrons contribute to the position of the two spin energy levels with their orbital magnetism, a corresponding effect is caused



Scheme 3. Microwave absorption in magnetic field.

by the magnetic fields of nuclear spins. This influence is not very big and thus usually restricted to the atoms in the immediate vicinity of the unpaired radical electron, but this makes it actually a very specific probe for a partial structure of the free radical. One prerequisite must be met though, and that is the presence of a nuclear spin in the atom of interest. Typically, atoms with all even numbers of protons and neutrons do not have any nuclear spin. Therefore, the most abundant carbon isotope, ^{12}C, is of no use in this respect; ^{13}C, on the other hand, would be a most suitable atom since it has an unpaired neutron (nuclear spin of 1/2). Because of its low abundance this isotope is, nevertheless, only of lesser importance in ESR, but it serves a very significant role in the related field of NMR (nuclear magnetic resonance). Two other atoms with nuclear spin play, however, an important, almost decisive role in ESR, namely, nitrogen and hydrogen with nuclear spins of 1 and 1/2, respectively.

Let us briefly discuss just one simple example, a methyl radical, $^{\bullet}CH_3$. Here, the unpaired electron "sees" the magnetic fields exerted by the three protons (spin 1/2). Following quantum mechanical rules these nuclear magnetic moments will couple with that of the electron spin in either parallel or antiparallel fashion. Since there are three protons a total of four possibilities arises: all three parallel (total spin +3/2), two parallel and one antiparallel (total spin +1/2), one parallel and two antiparallel (total spin −1/2), all three antiparallel (total spin −3/2), and the statistical probability that they do so is 1:3:3:1. What is the consequence? Each of the two original spin energy levels of the free, unpaired electron will be further split into four new levels as shown in the following scheme:



Scheme 4. ESR spectrum of methyl radical.

Considering the selectivity rules, four transitions become possible and thus the ESR signal of the $^{\bullet}CH_3$ radical will consist of four lines the intensities of which reflect the above mentioned statistics.

Had there been only one proton the two possible nuclear spin directions, +1/2 and −1/2, would have caused a split into two levels and the resulting absorption spectrum would have been a doublet of equal (1:1) intensity. In case of two interacting protons nuclear spin combinations assume values of +1, 0, and −1 yielding a triplet with a 1:2:1 intensity ratio.

For larger radicals influences from even further distant groups may become visible. For example, in an ethyl radical, $^{\bullet}CH_2-CH_3$, the two energy levels of the "free" electron will be split into three levels due to the nuclear spin interaction of the two α-protons (CH_2-group), and then each of these resulting levels will be further split into four levels due to the interaction of the three β-positioned protons in the CH_3-group. This provides the possibility of a total of twelve transitions and accordingly the microwave absorption spectrum consists of twelve lines. Such secondary effects, generally referred to a hyperfine interaction, become smaller and smaller the further distant the interacting nuclei are positioned.

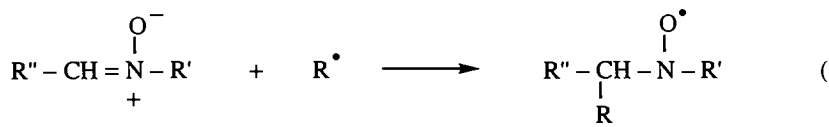
Of course, there is much more about ESR, impossible to cope with in detail in this short and anyway diversifying introductory article. This pertains, in particular, to the vast amount of structural information which may also be extracted from these hyperfine splittings, from anisotropy effects, and special techniques such as ENDOR [52,57]. However, it can hopefully be appreciated how potent, in principle, ESR is to identify free radicals and to determine their structure. It is generally less suited for kinetic studies as compared to the optical measurements although several time-resolved ESR studies have provided very useful specific kinetic data as well. Innovative research will certainly depend on both of these, in the best sense, complementary approaches.

2.5 Spin trapping

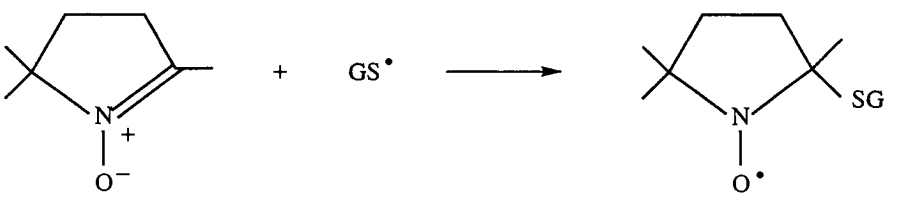
One of the problems, particularly with respect to biological samples, is the generally extremely small steady-state concentration of the free radicals, either because only few radicals are produced, or their lifetimes are too short for accumulation. It often helps to freeze the sample in order to slow down the kinetics. Furthermore, certain radicals exhibit rather unspecific spectral characteristics with perhaps just one broad line and lack of any fine structure information. In fact, four biologically quite relevant radicals belong to this group, superoxide ($O_2^{\bullet -}$), thiyl radicals ($RS^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkoxyl radicals ($RO^{\bullet}$) [52,58].

One possibility to overcome many of these problems is to trap the free radical of interest by a suitable scavenger and thus convert it into a better detectable and usually longer-lived secondary radical. This procedure is generally known as "spin trapping" [48,59].

What is a good spin trapping agent? Probably the best studied and one of the most efficient class of compounds in this respect are nitrones. They usually incorporate free radicals in fast addition processes and the resulting nitroxyl radicals are generally highly persistent and thus subject of convenient ESR investigations. The underlying chemical reaction is formulated in its general form in Eq. 12. A specific example, namely, the trapping of a glutathiyl radical which was generated as metabolite in a horseradish peroxidase induced oxidation process [60] is given in Eq. 13:



(12)



(13)

The radical of interest, $R^{\bullet}$, adds to the carbon in the C=N double bond of the nitrone, and the unpaired electron becomes located at the oxygen with some spin delocalization also to the nitrogen. An ESR investigation of such nitroxyl radicals usually allows to reveal the identity of R through the hyperfine splitting.

Spin trapping has become one of the most sensitive in situ methods for the detection of free radicals in tissue. Despite its unquestionable success a word of caution should be issued though. Whenever free radicals are suspected and spin trapping agents are introduced it is certainly necessary to confirm by independ-

ent measurements that the ultimately trapped radicals are not themselves a metabolic product of the spin trap compound, or generated because of an interference of the spin trap with a mechanism which, in the absence of the spin trap, would not produce these radicals.

2.6 Product analysis

Besides these two powerful tools for the direct study of free radicals, time-resolved spectroscopy and ESR, there is, of course, still the most classical method to prove free radicals and that is stable product analysis. Many free radical reaction routes have been revealed in thorough and often sophisticated chemical studies. For example, the formation of phosgene ($COCl_2$) as a transient, and CO_2 and HCl as stable end products upon reductive degradation of the liver toxin carbon tetrachloride can quantitatively be described by a free radical mechanism [61,62]. A corresponding statement holds for the products from one of the frequently used anaesthetics, halothane (CF_3-CHClBr), namely, CF_3-COOH, CF_2-CHCl, CF_2-CHO, CO_2, F , Cl , Br , and some others [63–65]. All these result from free radical reactions under particular conditions, such as oxidative or reductive initiation, and the presence or absence of oxygen. The identity of the products found both *in vitro* (radiation chemical and photocatalytic free radical studies) and as a result of metabolism suggests, in fact, that the biological degradation of halothane involves most likely free radicals.

While these examples leave little doubt how the final molecular products are generated and which kind of free radicals participate *en route*, again a word of caution must be given. Not always is it possible to extrapolate straight back from a final molecular product to an initiating or transient free radical generated in the course of a reaction sequence. Quite a number of highly speculative and strange mechanisms are offered once in a while, much to the amusement of a free radical chemist. Clearly, in order to unravel an unknown mechanism a certain degree of imagination is necessary. Final products should, however, better be viewed as just the ultimate proof of a proposed mechanism which ought to be sustained by a separate study of as many as possible intermediate steps. Again, it hopefully becomes apparent that the most beneficial approach is the cooperative one.

2.7 Reduction potentials

As stated in our introductory chapter the main driving force for a reaction of a free radical is its desire to retain an even number of electrons. Whenever this is achieved by either donating the unpaired or accepting an additional electron (without incorporation of any further atomic or molecular constituents) such a reaction qualifies as a classical one-electron transfer or redox process.[5]

How potent a free radical species is with respect to any oxidative or reductive electron transfer is usually quantified by the standard redox or better, by IUPAC

recommendation, *reduction* potential E°. The actual figures for E° refer to reactions of the general form

$$\text{oxidant} + n\,e \rightarrow \text{reductant} \quad (14)$$

and are denoted as standard reduction potentials E°(Ox/Red). (The term "standard" refers to unit activity, i.e., ca 1 M in concentration for liquids and solids, and unit fugacity, i.e., 1 atm at 298 K for gases). For one-electron processes either the oxidant or the reductant is a radical.

Let us now consider a chemical redox reaction which involves two pairs of redox couples, namely, $A^\bullet/A^-$ and $B^\bullet/B^-$ with the respective standard reduction potentials $E^\circ(A^\bullet/A^-)$ and $E^\circ(B^\bullet/B^-)$. ($A^\bullet$, $B^\bullet$ and A^-, B^- refer to the oxidized and reduced forms, respectively, irrespective of the actual state of charge). The deciding factor into which direction the equilibrium reaction

$$A^\bullet + B^- \rightleftharpoons A^- + B^\bullet \quad (15)$$

actually proceeds depends on the relative desire of $A^\bullet$ and $B^\bullet$ to accept an electron, in other words, on the difference in the respective reduction potentials:

$$\Delta E^\circ = E^\circ(B^\bullet/B^-) - E^\circ(A^\bullet/A^-)$$

If this figure is negative the reaction proceeds from left to right, and vice versa. (Please note: The couple with the reduced form on the left hand side of the equation appears first, here the $B^\bullet/B^-$ couple).

The difference in reduction potentials is related to the equilibrium constant, K, for reaction 15 via the standard free enthalpy G°:

$$\Delta G^\circ = -nF\Delta E^\circ = -RT \ln K$$

For a one-electron reaction (n=1) ΔE° calculates to

$$\Delta E^\circ\,[\text{mV}] = 59.1 \log K$$

If K is not known it may be determined through measurement of equilibrium concentrations or the ratio of rate constants for the forward and back reactions. In the above example $K_{15} = [A^-]\,[B^\bullet] / [A^\bullet]\,[B^-] = k_{15}/k_{-15}$. In order to achieve this experimentally within appropriate accuracy the E° values for the individual couples should not differ too much. Finally, the reduction potentials E° can be calculated from ΔE° by knowing the other E° involved, or by measurement of ΔE° against the standard (normal) hydrogen electrode (NHE) whose $E^\circ(H^+ / \frac{1}{2} H_2) = 0$ per definition.

For practical assessments just this reminder: any deviations from standard conditions affects, of course, the actual potential (E) through the influence of the

concentrations ($E = E^\circ + RT/nF \ln [Ox]/[Red]$). This is particularly relevant for all redox processes involving protons (e.g., $RS^\bullet + e^- + H^+ \rightleftharpoons RSH$) where the reduction potential becomes pH dependent. When looking up potential values attention should also be turned to the reference potential. This is not always that of the normal hydrogen electrode (NHE) but often refers to other standards. Many systems have been measured against the saturated calomel (SCE) or a Ag/AgCl electrode. These values can be converted to NHE values by adding 244 mV and 222 mV, respectively (both correction terms referring to 298 K).

Another important source of confusion is particularly relevant for one of the biologically most interesting redox couples, namely that of $O_2/O_2^\bullet$. Thus, the potential amounts to $E^\circ = -325$ mV if standardized according to the gas phase rules, namely, to one atmosphere of partial oxygen pressure. In aqueous solution this refers accordingly to saturation concentration, namely, $[O_2]_{sat} \approx 1.3$ mM. Applying, however, the liquid phase standardization rules the reference concentration is 1 M and, extrapolated to this figure, the liquid phase "standard" potential assumes quite a different value, namely, $E_{liq}^\circ = -155$ mV.

2.8 Where to find rate constants, ESR splittings, reduction potentials, etc.

Fortunately, it is not necessary anymore to determine all the rate constants, spectroscopic data, redox properties and many other physico-chemical parameters which one may need to know for a complete understanding of a free radical mechanism. In fact, a huge amount of such data have been gathered already and are now compiled in special reference books. For convenience we shall mention just a few of the most comprehensive editions.

The probably largest collection of rate constants for free radical reactions in solution is to be found in a five volume issue of the "Landolt-Börnstein" (Springer Verlag) [66], including an extensive recent supplement. A most valuable source of information is also several issues of the *Journal of Physical and Chemical Reference Data* (former NSRDS-Series) [40,68,69]. ESR data have also be compiled in a "Landolt-Börnstein" series (including recent supplements) [56]. For reduction potentials, finally, an excellent and well commented survey is given by P. Wardman in an issue of the *Journal of Physical and Chemical Reference Data Series* [70]. We like to add though that this is, nevertheless, only a very personal selection and shall not discredit other textbooks or compilations not mentioned nor any singular result in the regular scientific literature.

3 OXYGEN FREE RADICALS

In the following we shall now present and discuss the most abundant and important oxygen centered free radicals as there are:

the hydroxyl radical	$^\bullet OH$
the peroxyl radical	$ROO^\bullet$
the alkoxyl radical	$RO^\bullet$

the phenoxyl and semiquinone radicals $ArO^{\bullet}$, $HO-Ar-O^{\bullet}$
the superoxide $O_2^{\bullet -}$

In doing this, we will restrict ourselves, however, to the fundamental physicochemical and chemical properties in order to provide just the basis for an understanding of their reactivity. For illustration some specifically selected examples will be included.

3.1 The hydroxyl radical

By far the most reactive oxygen containing species is the hydroxyl radical, $^{\bullet}OH$. Whenever discussed in biological context it must, therefore, be recognized that its direct chemical action is strictly confined to the close vicinity of the site of its generation. There may, of course, be an indirect effect through an $^{\bullet}OH$-induced process at further distance but the actually active species would then always be a secondary radical or molecular product.

What makes the hydroxyl radical such a reactive species, and what means "high reactivity"? The answer to the first question lies in the versatility of this radical which can undergo three types of reactions. Thus it is a potentially powerful oxidant from the thermodynamic point of view with a reduction potential of 1.9 V for the $^{\bullet}OH/OH^-$ couple (2.7 V for $^{\bullet}OH,H^+/H_2O$). In other words, the hydroxyl radical is, in principle, very eager to accept an electron and convert to the hydroxide ion (or water). Most interestingly, this is, however, a rather rare process although, from the mere redox point of view, a large amount of molecules should be prone for such an electron transfer mechanism. One of the few reactions of this kind is the oxidation of hexacyano ferrate (II):

$$^{\bullet}OH + Fe(CN)_6^{4} \rightarrow OH^- + Fe(CN)_6^{3-} \quad (16)$$

It should perhaps be noted that this inorganic complex is electronically unique in the sense that the central Fe^{2+} ion, through interaction with the six cyano ligands, has attained completely filled s-, p-, and d-orbitals in its M shell (n = 3) and completely filled s- and p-orbitals in its outer-most N shell (n = 4). This leaves practically no chance anymore for the hydroxyl radical to enter the ligand sphere as a molecular entity or, in other words, to add on to a molecule.

Addition of $^{\bullet}OH$ to atoms and molecules is, in fact, the most preferred process this radical species undergoes. This reflects two important parameters. Firstly, the hydroxyl group is an excellent ligand and, therefore, any OH^- formed in an electron transfer process with, e.g., a metal ion, may stick to the oxidized form of the latter. An example is the $^{\bullet}OH$-induced oxidation of Tl^+ to Tl^{2+} which proceeds via a short-lived but unambiguously identifiable intermediate adduct:

$$Tl^+ + {}^{\bullet}OH \rightarrow Tl(OH)^+ \quad (17)$$

This is then involved in acid/base equilibria

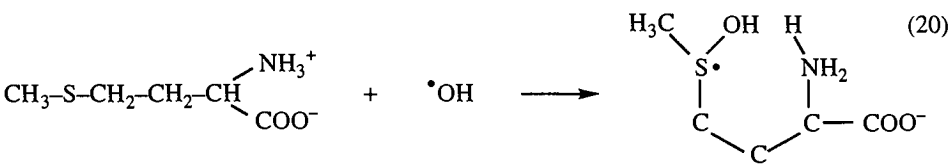
$$Tl(OH)_2 \rightleftharpoons Tl(OH)^+ + OH^- \rightleftharpoons Tl_{aq}^{2+} + 2\,OH^- \quad (18)$$

with pKs of 7.7 and 4.6, respectively [71,72]. Biologically perhaps more relevant examples refer to the corresponding oxidations of copper or iron in many vital complexes of these metals. Just recently, such a transient hydroxyl adduct has been identified in the $^{\bullet}OH$-induced oxidation of a copper-(I)-thia-crown-ether [73], a model for certain copper proteins.

A further important property of the hydroxyl radical is its pronounced electrophilic character. Thus it likes to add to centers of high electron density as they are provided by aromatic π-systems, olefinic double bonds or free (lone) electron pairs. In each case the hydroxyl clearly draws on the offered electron density but remains in close contact with the potential donor at least for some limited time. An intensively studied example is the oxidation of organic sulfides where the hydroxyl radical adds to the lone p-electron pair of sulfur [74–76]:

$$-\ddot{S}- + {}^{\bullet}OH \longrightarrow >\overset{OH}{\underset{|}{S}}{}^{\bullet} \quad (19)$$

The resulting sulfuranyl radical typically decays within less than a microsecond but in some cases it may be stabilized through hydrogen bridging and then assume considerably longer lifetimes [76]. Such an adduct constitutes, for example, an important intermediate *en route* to an oxidative decarboxylation of the essential amino acid methionine [77–79] where it is a direct precursor of a sulfur-nitrogen linked (three-electron-bonded) species which, in turn, is the key transient which transfers the reactive site from sulfur to the amino acid moiety. (For further information on three-electron bonds see chapter on "Sulfur-centered Free Radicals").



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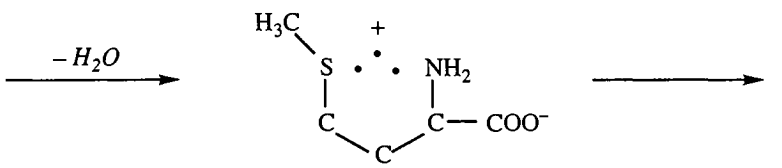

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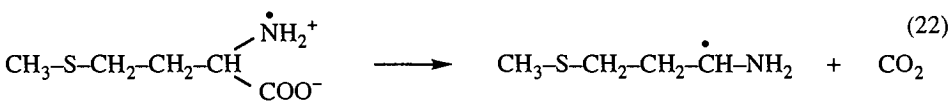

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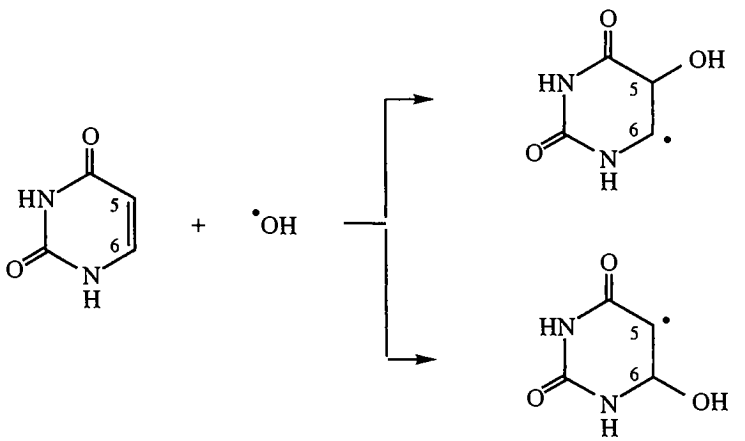
By the way, the α-amino radical, $CH_3SCH_2CH_2C^{\bullet}HNH_2$, formed in this reaction sequence is a highly reductive species and readily transfers an electron to oxygen and NAD^+, for example [80]. Considering that the initiating species was the oxidizing hydroxyl radical this constitutes a complete turnover of the redox properties along the reaction coordinate. Many more corresponding examples with other sulfur containing amino acids and peptides have been described recently [81–83].

Similar redox considerations apply to many $^{\bullet}OH$-adducts to double bonds. Biologically striking examples are the pyrimidine bases which are vital constituents of the DNA. Uracil, with its C-5/C-6 double bond, provides such a target for an electrophilic $^{\bullet}OH$-addition. Two possible radicals may be generated: a 6-yl radical, when $^{\bullet}OH$ adds to C-5, and a 5-yl radical, when it adds to C-6. Both are indeed formed, with an 82:18 relative ratio [84]. Most important, the 6-yl radical has reducing properties while the 5-yl radical, on the other hand, is an oxidant. For rationalization, please note the α-positioned amino group relative to the radical site in the 6-yl species. This, therefore, resembles the α-amino radical from methionine mentioned above. In the oxidizing 5-yl radical the unpaired spin can probably be delocalized to some extent into the carbonyl group to yield an oxyl radical resonance form.



Other pyrimidine bases show different 5-yl/6-yl ratios since other substituents affect the electron density at C-5 and C-6 differently, and may exert also some steric constraints [7,21,84]. In general, the 6-yl radical prevails.

The third type of reaction a hydroxyl radical frequently undergoes is abstraction or displacement. If, for example, an $^{\bullet}OH$ radical was formed in the vicinity of a thiol, such as cysteine or glutathione, it would most likely abstract (or displace) a hydrogen atom:

$$RS{-}H + {}^{\bullet}OH \rightarrow RS^{\bullet} + H_2O \qquad (24)$$

This reaction is fast, i.e., almost diffusion controlled [40] since the S–H bond energy is considerably smaller than that of the O–H bond energy in water.

A prime target are also C–H bonds, and in this context it must be recognized that practically all biologically relevant material contains plenty of them. The general reaction

$$-\overset{|}{\underset{|}{C}}-H \quad + \quad {}^{\bullet}OH \quad \rightarrow \quad -\overset{|}{\underset{|}{C^{\bullet}}} \quad + \quad H_2O \qquad (25)$$

usually requires some more activation energy than the $^{\bullet}OH$ reaction with thiols since C–H bonds are, on average, stronger than S–H bonds [85]. Of course, not all C–H bonds are of equal strength and, therefore, there will be preferred and less probable reaction sites in a molecule which contains various kinds of C–H bonds. Some general rules can be applied. Thus, the most difficult C–H bond to break is that in CH_3-groups (primary H-atom), while it becomes increasingly easier to displace a secondary (CH_2-group) or tertiary (CH-group) hydrogen. Particularly labile are the bisallylic hydrogens in polyunsaturated fatty acids; they can even be abstracted by thiyl radicals [86]. However, in the case of PUFAs it must be recognized that the double bonds do not only serve the function of activating the bisallylic hydrogens but are themselves a sink for $^{\bullet}OH$ via electrophilic addition [87].

Although there are differences in C–H bond energies and, as a result of that, different reaction rates with respect to hydrogen abstraction, a selective process in a larger target molecule is highly improbable. The reason is that the associated activation energies for an $^{\bullet}OH$-induced abstraction process are generally low and do not allow complete discrimination. Take the simple alcohol ethanol where three types of hydrogens (primary and secondary C–H, and O–H bonds) may be cleaved [88]:

$$^{\bullet}OH + CH_3{-}CH_2{-}OH \rightarrow \begin{array}{lll} {}^{\bullet}CH_2{-}CH_2{-}OH & (13.2\%) & (26a) \\ CH_3{-}C^{\bullet}H{-}OH & (84.3\%) & (26b) \\ CH_3{-}CH_2{-}O^{\bullet} & (2.5\%) & (26c) \end{array}$$

Such relative abstraction yields vary, of course, with the individual compound and will have to be determined experimentally for any quantitative assessment. However, quite many of them have been documented in the meantime, particularly for the sugar moiety of DNA [7].

A question arises, of course, on how to distinguish between the various possible abstraction routes. One possibility is product analysis and knowledge of the mechanism by which the initially formed radicals convert to the analyzable stable substrates. Another successful approach is time-resolved direct observation of the intermediates, e.g., through their optical absorptions. Considering the above alcohol example, a discrimination is possible on the basis of the redox properties of the three radicals formed in reaction 26. Thus, the α-hydroxyl radical (formed

at the highest, i.e., 84.3% yield) exhibits reasonably strong reducing properties; the oxyl radical (formed with 2.5% yield), on the other hand, is a strong oxidant; and the remaining β-hydroxyl radical (13.2% yield) is practically redox inert [88]. Similar considerations have been applied for the above mentioned $^{\bullet}OH$/pyrimidine adducts and show the value of free radical redox studies [7,84].

The variety of possible $^{\bullet}OH$ reactions can still be enlarged since several of the three mechanisms discussed (electron transfer, addition, abstraction) may occur simultaneously. Just one example shall briefly be mentioned, namely, the oxidation of disulfides. The reaction with simple aliphatic disulfides thus proceeds with about equal probability via a one-electron oxidation to yield the disulfide radical cation and a, pH dependent, displacement process [89]:

$$\begin{array}{lll} & & (RSSR)^{\bullet+} + OH \quad (27a) \\ RSSR + {}^{\bullet}OH & \rightarrow & RS^{\bullet} + RSOH \text{ (neutral pH)} \quad (27b) \\ & & RSH + RSO^{\bullet} \text{ (low and high pH)} \quad (27c) \end{array}$$

The high reactivity of the hydroxyl radicals is, as mentioned already at the beginning of this chapter, the reason why its reactions are usually confined to the immediate vicinity of their generation. Within this reaction sphere a great variety of final products may, nevertheless, be formed. The latter is a direct reflection of the "reactivity-selectivity" principle which, incidentally, applies for all free radical reactions: The more reactive a radical species (i.e., the lower the activation energies for potentially competing reaction routes) the less selective it becomes.

One further property of the hydroxyl radicals needs to be mentioned although this is not of any direct relevance to real biological systems. It is the acid/base equilibrium

$$^{\bullet}OH + OH^{-} \rightleftharpoons O^{\bullet} + H_2O \quad (28)$$

which the $^{\bullet}OH$ is involved in. As can be appreciated from the high pK of 11.9 the conjugate base, $O^{\bullet-}$, may at best serve a function in principle mechanistic *in vitro* studies. But it points out a general and important property of many α-functionalized free radicals, namely, the existence of acid/base equilibria. Two examples mentioned already in this article belong to this group, namely, the α-hydroxyl radical from alcohols and the α-amino radical from amines. The pKs of the particular equilibria

$$CH_3{-}C^{\bullet}H{-}OH \rightleftharpoons CH_3{-}C^{\bullet}H{-}O^{-} + H^{+} \quad (29)$$

$$CH_3S(CH_2)_2C^{\bullet}H{-}NH_3^{+} \rightleftharpoons CH_3S(CH_2)_2C^{\bullet}H{-}NH_2 + H^{+} \quad (30)$$

are 11.6 [90] and 3.85 [80], respectively, i.e., by orders of magnitude lower than for their molecular parents, ethanol and (3-methylthio)propyl amine. Could this have any general implication? Yes, because acidic and basic form often exhibit

different properties. Generally, the basic form is a stronger reductant (or weaker oxidant) than the acidic form, and these radical equilibria thus constitute a redox regulating parameter. Another possible change becomes apparent in the hydroxyl radical system where the $O^{\bullet}$, because of its negative charge, has completely lost the electrophilicity of its conjugate $^{\bullet}OH$ partner.

3.2 Peroxyl radicals

While the hydroxyl radical clearly is the most reactive among the oxygen radicals, peroxyl radicals are probably the most abundant radicals in biological systems. This conclusion emerges merely from the fact, that they are readily formed in any oxygen containing environment and generally are not as reactive as other free radical species. In principle, this group of transients would include both organic peroxyl radicals as well as superoxide. Considering their different modes of formation and chemical properties it is certainly justified, however, to deal with them in separate chapters.

Organic peroxyl radicals, generally denoted as $ROO^{\bullet}$, result from oxygen addition to practically any carbon-centered free radical ($R^{\bullet}$).

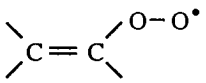
$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (31)$$

This is typically a fast, i.e., diffusion or close-to-diffusion controlled process with rate constants close or within the 10^9 M^{-1} s^{-1} range [66,69]. Variations are explained to some extent by differences in electron density at the radical site exerted by near substituents in connection with the electrophilic character of the oxygen molecule. However, this is not the only parameter. Addition of oxygen to trichloromethyl radicals, for example, – a process of great significance in the toxicology of carbontetrachloride –

$$^{\bullet}CCl_3 + O_2 \rightarrow CCl_3OO^{\bullet} \quad (32)$$

proceeds with a rate constant of 3.3×10^9 M^{-1} s^{-1}, i.e., practically as fast as that of the O_2-addition to the $^{\bullet}CH_3$ radical, despite the strong electron density withdrawing chlorine substituents [62]. A plausible explanation is provided by steric arguments. While $^{\bullet}CH_3$ is planar, $^{\bullet}CCl_3$ is pyramidal. The chlorinated radical thus offers a much more exposed site for the oxygen addition which obviously compensates for the reduced electron density.

A direct observation of organic peroxyl radicals is, in the majority of cases, difficult or impossible. ESR spectra are generally characterized by rather unspecific broad single lines, and the optical absorptions reported are usually weak and confined to the UV < 300 nm where they can easily be missed because of other radicals absorbing in this wavelength range. An interesting example with a VIS absorption near 500 nm has, however, been reported [91], namely, vinylperoxyl radicals of the general structure:



The possibility of electronic resonance with the π-systems of the vinyl double bond is undoubtedly the advantageous parameter for a shift of the optical transition energy to lower values and thus higher wavelengths.

However, for the study of most peroxyl radicals and their reactions one has to rely on indirect measurements. The rate constant for the oxygen addition to the trichloromethyl radical, for example, was derived from a reaction sequence where reaction 32 was followed by reaction 33 [62,92]:

$$CCl_3OO^{\bullet} + \text{phenothiazine (PZ)} \rightarrow PZ^{\bullet+} + CCl_3OO \quad (33)$$

The phenothiazine radical cations (PZ = promethazine, chlorpromazine, metiazinic acid, etc.), in contrast to $CCl_3OO^{\bullet}$, show very strong and thus easily detectable absorptions [93,94]. Therefore, in order to measure the rate constant for reaction 32, it was only necessary to look at the rate of $PZ^{\bullet+}$ formation at high phenothiazine concentrations, i.e., under conditions were reaction 33 became faster than reaction 32 and consequently the oxygen addition to $^{\bullet}CCl_3$ became the rate determining step.

Reaction 33 indicates already one of the chemical properties of peroxyl radicals, particularly if they are substituted with electron withdrawing atoms like halogens: they are oxidants. Reduction potentials for $ROO^{\bullet}/ROO^{-}$ couples are almost unknown and only two estimates have been reported, ca 0.6 V and > 1 V for the $CH_3OO^{\bullet}$ and $CCl_3OO^{\bullet}$ systems, respectively [95]. One difficulty for an unambiguous determination of such potentials arises from the fact that peroxyl radicals may not only get involved in true electron transfer processes but often oxidize their reaction partners via an addition-elimination mechanism. This seems to apply, for example, for the peroxyl radical induced oxidation of organic sulfides (R_2S, e.g., methionine) where the first step *en route* to a sulfide radical cation is an addition, followed by a substitution and equilibration of the transient three-electron bonded dimer radical cation to the molecular radical cation $R_2S^{\bullet+}$ [96].

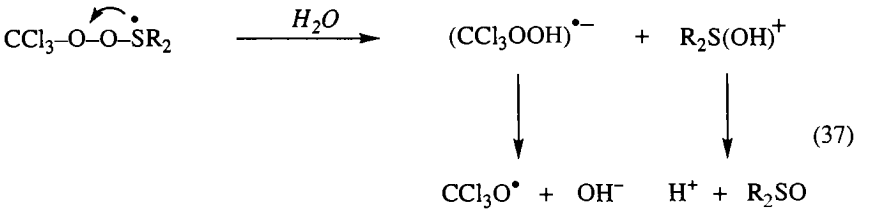
$$R_2S + CCl_3OO^{\bullet} \rightarrow CCl_3OO{-}{}^{\bullet}SR_2 \quad (34)$$

$$CCl_3OO{-}{}^{\bullet}SR_2 + R_2S \rightarrow CCl_3OO^{-} + (R_2S\therefore SR_2)^{+} \quad (35)$$

$$(R_2S\therefore SR_2)^{+} \rightleftharpoons R_2S^{\bullet+} + R_2S \quad (36)$$

It will not be difficult to appreciate that such a complex mechanism cannot be linearly related to any redox potentials. Even more so, because the adduct radical, in competition with reaction 35, enters yet another pathway in which the sulfuranyl radical electron is transferred intramolecularly to the hydroperoxide moiety

(under participation of water) to yield sulfoxide and a reduced hydroperoxide (which decays into the oxyl radical):



(37)

This latter process constitutes a most interesting mechanism in the sense that it actually describes the second step of an overall two-electron transfer initiated by a peroxyl radical. The two-electron oxidation product is the sulfoxide and the corresponding two-electron reduction counter part is the oxyl radical. Formally, this peroxyl-radical-induced sulfoxide formation via Eqs. 34 and 37, may also be viewed as oxygen atom transfer, however, studies with labeled oxygen indicate that the sulfoxide oxygen is coming from the solvent water [96]. (This does not mean that the possibility of oxygen transfer has to be dismissed in all cases; it seems to be a real possibility in the peroxyl induced oxidation of certain organic tellurides, for example) [97].

The capability of peroxyl radicals to undergo both, one-electron and two-electron processes pertains particularly for the sulfide-to-sulfoxide oxidation and would, therefore, be of special biological relevance for methionine and methionine-containing peptides and proteins. Generally, the sulfoxide yields, produced via the 2-e-mechanism, are higher than via the 1-e-mechanism [96,98] where, e.g., competing radical cation deprotonation may come into play.

Absolute rate constants for addition reactions of peroxyl radicals have hardly been measured so far but can reasonably be anticipated, at least for the above addition to sulfides, to be in the range of diffusion controlled values. Electron transfer reactions from a donor to the peroxyl show a distinct dependence on the electron density at the peroxyl radical site. The lower the latter the faster, in general, the reaction. This is nicely exemplified by a series of chloromethylperoxyl radical induced oxidations of ascorbate where the rate constant increases from 2.2×10^6 to 2.0×10^8 M^{-1} s^{-1} from $CH_3OO^\bullet$ to $CCl_3OO^\bullet$, respectively [99]. These early figures have, in the meantime, been corroborated by many more examples for 1-e-oxidation reactions of peroxyl radicals with different degrees of halogenation [69,92,95,99–102].

Besides electron transfer and addition reactions peroxyl radicals also undergo hydrogen atom abstraction reactions, and thus resemble quite similar reactivity features as $^\bullet OH$. The most important abstraction process is probably the chain carrying reaction in lipid peroxidation (with R–H = PUFA) and other autoxidation processes [1,7,13,15,17,103–106]. In principle, any carbon-centered radical, $R^\bullet$, may get involved in the following reaction sequence:

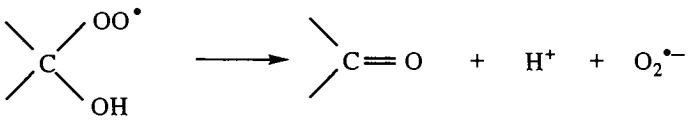
$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (38)$$

$$ROO^{\bullet} + R{-}H \rightarrow ROOH + R^{\bullet} \quad (39)$$

Reaction 39 is the slower of the two processes and thus rate controlling. Not too many rate constants are known. Published values range from the $10^{-4} - 10^{4}$ M^{-1} s^{-1} order of magnitude [66,69] and, to mention a biologically relevant example, $k_{39} = 36$ M^{-1} s^{-1} has been reported for the peroxyl radical reaction in the autoxidation of linoleic acid [106]. It should further be mentioned that the chain length involving the above two processes significantly increases when going from homogeneous solutions to micellar aggregates [107,108] and, by extrapolation, lipids. A plausible explanation for this is the restricted diffusion of the radicals and, as a result thereof, a much lower probability for radical-radical termination processes.

In all the discussion of peroxyl radical reaction kinetics, including those for the abstraction processes, it must be recognized that peroxyl radicals are highly polarizable [109] and its electron density distribution depends to a significant extent on the polarity of the solution. Therefore, absolute rate constants will vary with the nature of the environment [110,111] in which these reactions occur and caution is advised when trying to extrapolate aqueous or specific organic solvent *in vitro* figures to, for example, cellular conditions. In first approximation, it is probably safe though to operate within the given order of magnitude.

In the absence of suitable molecular reaction partners, or if the corresponding reactions are too slow, peroxyl radicals may decay via unimolecular processes [7,21,112]. The most prominent and, from the biological point of view, most significant mechanism is a superoxide elimination. Prerequisite for this process is a sufficiently large electron delocalization into the peroxyl group. This may be achieved by simultaneous stabilization of a positive, i.e., electron deficient entity as it would be the case upon liberation of a proton. Since the latter is easily cleaved from hydroxyl groups, for example, any α-hydroxyl peroxyl radical is prone for such a process [113–117].

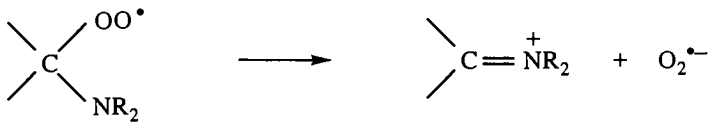


(40)

This elimination is considered to be assisted by a transient cyclic five-membered ring structure in which the hydroxyl proton interacts with the terminal peroxyl oxygen. Typical rate constants for this first-order decay are on the order of $10^{5} - 10^{6}$ s^{-1}, corresponding to half-lives in the lower microsecond region. The superoxide elimination becomes even faster in basic solutions [114,117] where the α-hydroxyl group increasingly equilibrates to its conjugate anion ($-OH \rightleftharpoons -O^{-}$).

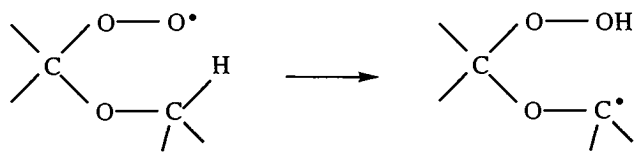
The positive charge may also be stabilized within the remaining molecule. With

a dialkylamine substituent, for example, the peroxyl radical becomes even so unstable that it actually escapes detection [118]:



(41)

Steric arguments can also be forwarded to rationalize intramolecular hydrogen abstraction or addition reactions by peroxyl groups. Five- and six-membered ring structures would thus facilitate hydroperoxide and epoxide formations (the latter via an endoperoxide radical) [21,119]. Respective examples are given in the following:



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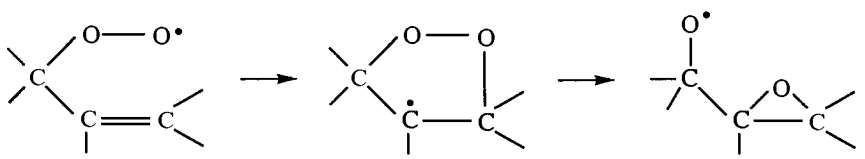


(43)

The actual rates of these processes depend very much on the electronic influence exerted by the substituents near the reactive sites.

Finally, if there are neither suitable reaction partners nor the possibility for intramolecular conversion, the peroxyl radicals will suffer the ultimate free radical fate, namely, a bimolecular radical-radical deactivation. The actual mechanism of the generalized process

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow [ROO{-}OOR] \rightarrow \text{products} \quad (44)$$

may be quite manifold but is considered, in any case, to proceed via an intermediate tetroxide. At room temperature these tetroxides are usually not stable enough for analytical detection. Their transitory existence seems beyond doubt though as emerges from low temperature studies [120,122] as well as from the following mechanistic considerations.

Generally, the bimolecular termination reactions (Eq. 44) are quite fast in terms of rate constants, and long lifetimes result mostly only because of the low steady-state concentrations of these radicals. This pertains, in particular for primary and secondary peroxyl radicals, i.e., those which still carry at least one hydrogen atom at the peroxyl carbon. Many measured bimolecular rate constants on the order of 10^9 M^{-1} s^{-1} ($2k_{40}$) indicate diffusion controlled processes

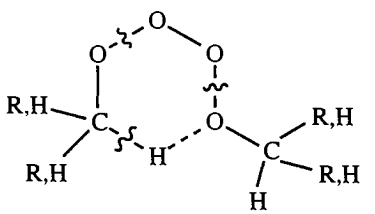
[66,69]. Deviations to lower values can mostly be rationalized in terms of steric hindrance. Tertiary peroxyl radicals, on the other hand, terminate with significantly lower rate constants, e.g., $2k = 2 \times 10^4\ M^{-1}\ s^{-1}$ for *tert*-butylperoxyl [123]. This probably does not only reflect steric constraints but also the smaller number of possible reaction channels available, as compared with primary and secondary peroxyl radicals.

Tertiary peroxyl radicals, $R_3COO^\bullet$, practically all decay via the tetroxide and by subsequent oxygen elimination to the respective oxyl radicals (the three substituents R do not need to be identical):

$$2\ R_3COO^\bullet \rightarrow [R_3COOOOCR_3] \rightarrow 2\ R_3CO^\bullet + O_2 \qquad (45)$$

The further chemistry is then that of the oxyl radical which will be discussed in detail in a later chapter.

This type of reaction may also apply to primary and secondary peroxyl radicals where at least one R=H. However, for these species additional routes become possible. The most preferred pathway is, in fact, a cyclic mechanism known as "Russell mechanism", the key element of which is considered to be a cyclic six-membered transition structure of the tetroxide [124]. Three bond ruptures and a hydrogen transfer directly yields one molecule of oxygen, an alcohol and an aldehyde or ketone. From the redox point of view this constitutes a disproportionation.



$$\longrightarrow\ >C=O + >CH\text{–}OH + O_2 \qquad (46)$$

An interesting aspect in this mechanism is the possible generation of single oxygen, $^1O_2$, which has been claimed to be formed in organic solutions [125,126]. In aqueous solutions, on the other hand, the natural triplet oxygen, $^3O_2$, seems to be cleaved which is then, however, accompanied by a triplet excited carbonyl compound [127].

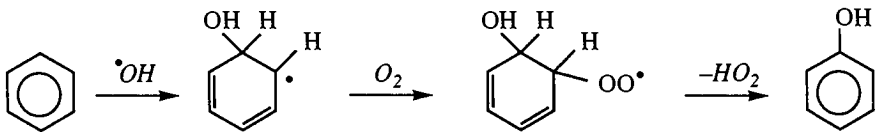
Another decay mode of the tetroxide leads to two carbonyl molecules and, instead of oxygen, to hydrogenperoxide (R = H and ≠ H):

$$[R_2HCOOOOCHR_2] \rightarrow 2\ R_2C{=}O + H_2O_2 \qquad (47)$$

This alternative pathway is also considered to involve cyclic transition structures which, in aqueous solution, probably include water molecules [21,112,128].

In addition to these general features there are further specific characteristics to certain peroxides which are mainly attributable to individual structural and elec-

tronic parameters. This is not the place to present and discuss any of them in greater detail (for more information see [21,112]), and just one group-specific example shall be mentioned here. Thus, all peroxyl radicals generated as a result of oxidative or reductive radical attack at aromatics show a high tendency to embark on reaction routes which re-establish the aromaticity. This clearly reflects thermodynamic stability criteria. A specific example is the •OH-induced oxidation of benzene in oxygenated solution for which phenol is formed as final product with about 60% efficiency via the following route [21]:



(48)

Some further words also about halogenated peroxyl radicals: As mentioned above most bimolecular peroxyl termination processes lead to alcohols and aldehydes as stable molecular products. If any of the hydrogen substituents at the hydroxyl-carrying or carbonyl carbon is a halogen atom then these compounds (α-halo-alcohols, "α-haloaldehydes" = acyl halides) assume, in fact, a higher oxidation state and, upon hydrolysis, directly convert into aldehydes/ketones and acids, respectively [65,129,130]:

>CH–OH	>CX–OH ——*(H₂O)*→	>C=O	(49)
alcohol	*α-haloalcohol*	*aldehyde/ketone*	
–CH=O	–CX=O ——*(H₂O)*→	–COOH	(50)
aldehyde	*acyl halide*	*acid*	

Any metabolic degradation of halogenated organic material, proceeding via peroxyl radicals as intermediates, would thus yield aldehydes and acids. The bad reputation of aldehydes as toxins is well known [131], and the organic acid (in combination of the inorganic acid H^+/X released in the hydrolysis processes) may exert a harmful influence due to pH changes.

3.3 Reversible oxygen addition

Addition reactions, as organic chemistry has shown in many examples, inherently seem to include the possibility to be reversed. Free radical addition to a double bond and subsequent β-scission, in principle, also fall into this category although the cleaved radical may not be identical with the incoming one:

$$R^\bullet + -C(Y) = C< \rightleftharpoons -C(R)(Y)-C^\bullet< \rightleftharpoons -C(R) = C< + Y^\bullet \quad (51)$$

The question whether a reaction is reversible or not is of principle significance.

Any irreversible process is a once-and-forever event. A reversible reaction, however, always means an equilibrium which may have a preferred side but has not closed the door yet for the back reaction. And most important, any substrate which engages a "minority" component in an irreversible reaction pulls eventually the entire equilibrium back to the "wrong side". An instructive example is a thiyl radical mediated degradation of carbontetrachloride in the presence of *iso*-propanol [132–135] where the equilibrium

$$RS^{\bullet} + (CH_3)_2CHOH \rightleftharpoons RSH + (CH_3)_2C^{\bullet}OH \qquad (52)$$

lies far on the left hand side. It may, nevertheless, completely be shifted to the right in the presence of CCl_4 which engages the iso-propanol radical in a dissociative, i.e., irreversible electron transfer reaction:

$$(CH_3)_2C^{\bullet}OH + CCl_4 \rightleftharpoons (CH_3)_2CO + H^+ + Cl^- + {}^{\bullet}CCl_3 \qquad (53)$$

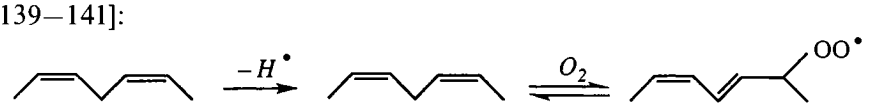
What about a possible reversibility of oxygen addition to free radicals? The answer is: Yes, it may well occur but it depends on the nature of the addition center and seems connected with the electrophilicity of the molecular oxygen [21]. Practically no addition has ever been observed, at least at room temperature, to organic oxygen-centered radicals, i.e., the possible reaction if occurring at all, must be reversible with the equilibrium way on the left hand side.

$$-O^{\bullet -} + O_2 \rightleftharpoons -O-OO^{\bullet} \qquad (54)$$

This pertains to all oxyl and peroxyl radicals. An interesting exception is the basic form of the hydroxyl radical, $O^{\bullet -}$, which in the presence of oxygen has been reported to form the ozonide radical anion, $O_3^{\bullet}$ [136].

Carbon-centered free radicals are generally prone for irreversible oxygen addition. However, there are some examples also for a reversible process. One of them is the addition of O_2 to the hydroxyhexadienyl radical formulated in Eq. 48. Here, the peroxyl radical is formed with a rate constant of $3.1 \times 10^8\ M^{-1}\ s^{-1}$ ($t_{1/2} \approx 8$ ms in air saturated solution) and the back reaction, i.e., the re-elimination of oxygen occurs with $1.2 \times 10^4\ s^{-1}$ ($t_{1/2} \approx 57$ ms [137].

A biologically most relevant example is the oxygen addition to those pentadienyl-type PUFA radicals [138,139] which result from abstraction of the particularly labile bisallyllic hydrogens by practically any kind of reactive free radical, even $RS^{\bullet}$ [86] and $ROO^{\bullet}$ [69]. An interesting feature in this context is also that oxygen preferentially does not add to the bisallylic site but to a resonance form thereof with the result of a thermodynamically favorable conjugate double bond structure [139–141]:



(55)

The peroxyl radical, incidentally, exhibits the same UV absorption (234 nm) which is characteristic for conjugated dienes, in general.

Another example for reversible oxygen addition, and also of particular biological relevance, is provided by thiyl free radicals. For quite some time and various reasons in dispute, there seems to be convincing evidence now for an equilibrium between thiyl and thioperoxyl radicals [142–145]:

$$RS^{\bullet} + O_2 \rightleftharpoons RSOO^{\bullet} \qquad (56)$$

Rate constants for the forward reaction and back reactions of about 2×10^9 M^{-1} s^{-1} and 6×10^5 s^{-1}, respectively, have been measured and they appear to be independent on the nature of R. Accordingly, an equilibrium constant of about 3×10^3 M^{-1} applies. (Measured values refer to cysteine, glutathione and 2-mercaptoethanol).

Does such a reversible oxygen addition to a sulfur-centered free radical make sense? From the electronic point of view certainly yes. The electronegativity of sulfur lies in between that of oxygen and carbon, and, as we have seen before, oxygen does not add to oxygen-centered free radicals (i.e., an "addition" is highly reversible) while most of the respective processes with carbon-centered free radicals seem irreversible.

The chemistry of thioperoxyl radicals has not yet completely been revealed and still leaves open questions particularly with respect to the redox properties of $RSOO^{\bullet}$. In part, this is probably due to the above equilibrium (Eq. 56). Further complicating factors with respect to establishing a reliable and quantitative picture arise from side reactions [145–150]. A most interesting one is a rearrangement by which the oxygen-centered is converted to a sulfur-centered sulfonyl free radical [146]. This process, depicted in Eq. 57, is of considerable significance because the sulfonyl radical can add another molecule of oxygen to yield a sulfonyl peroxyl radical [146,151] which itself is considered to be a precursor of sulfonic acids.

$$R\text{–}S\text{–}O\text{–}O^{\bullet} \xrightarrow{\bigcirc} R\text{–}\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}{}^{\bullet} \quad (RSO_2^{\bullet}) \qquad (57)$$

$$RSO_2^{\bullet} + O_2 \rightarrow RSO_2OO^{\bullet} \rightarrow \rightarrow \rightarrow RSO_3H \qquad (58)$$

3.4 Alkoxyl radicals

We have learned already that oxyl radicals may be formed *en route* of the peroxyl radical decay. One direct possibility to generate them is by hydrogen atom abstraction from a hydroxyl group, e.g., in the $^{\bullet}OH$ reaction with an alcohol:

$$R{-}OH + {}^{\bullet}OH \rightarrow R{-}O^{\bullet} + H_2O \quad (59)$$

This reaction competes, of course, with the C–H cleavage in other parts of the molecule, particularly in α-position to the hydroxyl group, as discussed already in the chapter on $^{\bullet}OH$ reactions. The yield of O–H cleavage in aliphatic compounds or, better, detectable alkoxyl radicals appears pretty small and attains, at most, ca 7% relative to the reacting $^{\bullet}OH$ in the case of methanol [88]. This figure might, however, only represent a lower limit. As we shall see later there is a fast intramolecular rearrangement (1,2-hydrogen shift) which may take place with certain oxyl radicals and thus obscure part of the initial yield.

Oxyl radicals are also generated upon reductive degradation of peroxides and hydroperoxides. The key feature of the underlying reaction is that the incoming electron is accommodated in an antibonding σ* orbital which causes an O–O bond lengthening and thus prepares it for easier rupture.

$$R{-}O{-}O{-}R(H) + e \rightarrow [R{-}O\therefore O{-}R(H)]^{-} \rightarrow RO^{\bullet} + {}^{-}OR(H) \quad (60)$$

This mechanism is, incidentally the same, as for the reduction of H_2O_2 (resulting in $^{\bullet}OH + OH^{-}$ formation) and disulfides [75,149–153] (resulting in $RS^{\bullet} + RS^{-}$ formation) (for further details on this latter process and the three-electron bond "∴" notation, see section on sulfur-centered free radicals below). The intermediate $(R{-}O \therefore O{-}R)^{-}$ radical anion is generally much too short-lived as to become of direct chemical significance or to establish a measurable equilibrium with the oxyl radical.

Reaction 60 reminds us of the well-documented Fenton-Haber-Weiss process. Whenever the reductive power of enzymatic systems may not be sufficient for an electron transfer to peroxides and hydroperoxides this function can be served by low oxidation state metal ions, particularly iron-(II) or copper-(I). The biologically most relevant reaction

$$ROOH + \text{Fe-(II)} \rightarrow RO^{\bullet} + OH^{-} + \text{Fe-(III)} \quad (61)$$

thus becomes understandable in view of the same electronic concept as outlined above for the general peroxide reduction mechanism.

The most basic chemical property of alkoxyl radicals is that they are oxidizing species and may be detected through their reaction with an electron donor, e.g., iodide [88,154]:

$$RO^{\bullet} + 2\,I^{-} \rightarrow I_2^{\bullet-} + RO^{-} \quad (62)$$

Alkoxyl radicals also engage in hydrogen atom abstraction reactions, a process which usually requires, however, considerable activation energy: