3

The Hydroxyl Radical

3.1 General Properties

The various ways of forming •OH were discussed in Chapter 2. It is a very reactive, electrophilic (ρ = -0.41; Anbar et al. 1966a) radical, and with most substrates it reacts at close to diffusion-controlled rates (for a compilation of rate constants, see Buxton et al. 1988). It undergoes mainly three types of reactions: (1) addition to C-C and C-N double bonds, (2) H-abstraction and (3) ET. Addition and H-abstraction reactions will be discussed below in some detail, because they are relevant for an •OH-attack at the nucleobases and at the sugar moiety in DNA.

Although it is of little importance at the pH values that prevail in biological systems, it must be mentioned that at high pH •OH deprotonates [equilibrium (1), pK_a(•OH) = 11.8 (Weeks and Rabani 1966), 11.54 (Poskrebyshev et al. 2002); for its temperature dependence see Elliot and McCracken (1989); for UV spectra see Czapski and Bielski (1993) and Poskrebyshev et al. (2002)].

$$^{\bullet}OH + OH^- \rightleftharpoons O^{\bullet -} + H_2O \quad (1)$$

The $O^{\bullet -}$ radical may also be generated in neutral solution (e.g., $e_{aq}^- + N_2O \rightarrow O^{\bullet -} + N_2$; $NO_3^- + h\nu \rightarrow {}^{\bullet}NO_2 + O^{\bullet -}$), but it is so rapidly protonated by water ($k_{-1} \approx 10^8$ s^{-1}) that its reaction with a given substrate can be neglected (cf. Mark et al. 1996), and only the typical •OH reactions are observed. Thus $O^{\bullet -}$ reactions only occur to a significant extent at pH >12.

The (nucleophilic) $O^{\bullet -}$ radical still undergoes H-abstraction at a high rate (for the energetics, see Henglein 1980), but its ability to add to double bonds is strongly reduced (for a compilation of rate constants, see Buxton et al. 1988). For example, in nucleosides the preference of attack at the base is shifted at high pH towards a pronounced H-abstraction from the sugar moiety (Scholes et al. 1992). An important difference between the (electrophilic) •OH and its conjugate base, the (nucleophilic) $O^{\bullet -}$, is their reactivity towards O_2. While O_2 does not react with •OH, it readily adds to the electron-rich $O^{\bullet -}$, yielding the ozonide radical anion [equilibrium (3); pK = 6.26; for details, see Elliot and McCracken 1989].

$$^{\bullet}OH + O_2 \rightarrow \text{no reaction} \quad (2)$$

$$O^{\bullet -} + O_2 \rightleftharpoons O_3^{\bullet -} \quad (3)$$

3.2 Addition to Double Bonds

The electrophilic •OH reacts readily with C-C and C-N double bonds (in purines) but not with C-O double bonds which are electron-deficient at carbon, the position where •OH would prefer to add. Although •OH reacts with C-C double bonds at close to diffusion-controlled rates, it is highly regioselective largely due to its

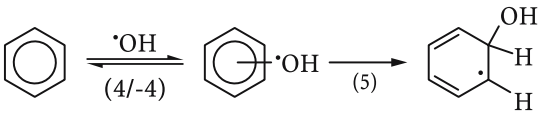
Table 3.1. Hydroxyl-radical-induced hydroxylation of some benzene derivatives in the presence of $Fe(CN)_6^{3-}$ serving as an oxidant of the •OH adducts. Yields (in %) relative to the •OH yield

Substrate	ortho	meta	para	Other products	Reference
Anisole	48	8	40	Phenol (6)	Steenken and Raghavan (1979)
Phenol[a]	48	3	36		Raghavan and Steenken (1980)
Chlorobenzene	45	22	31	HCl (0.5)	Merga et al. (1996)
Benzoic acid	28	38	20	Phenol (7)	Klein et al. (1975)
4-Methylphenol			~12		Schuler et al. (2002)

[a]Using 2,5-dimethylbenzoquinone as oxidant

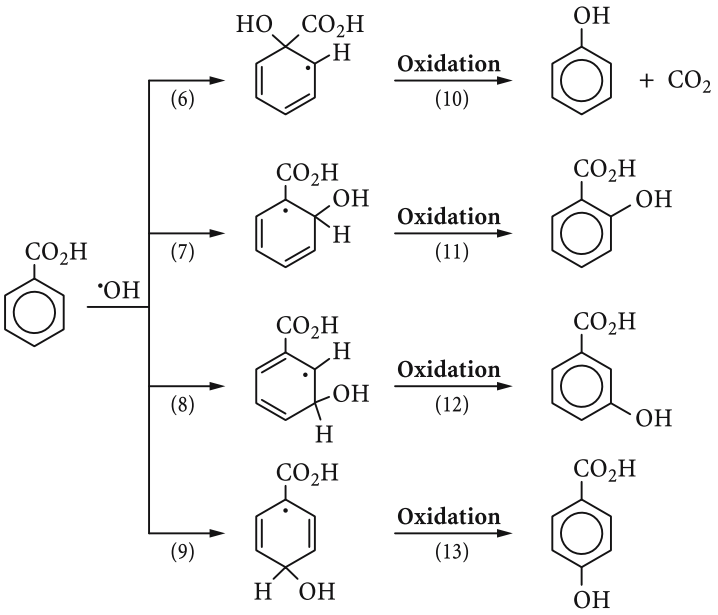
electrophilic nature. For example, the *C*(5)–*C*(6) double bond in the pyrimidines is preferentially attacked at the electron-richer *C*(5) (Chap. 10).

It has been suggested that •OH fixation at a given carbon may be preceded by a short-lived π-complex (Cercek and Ebert 1968; Volkert and Schulte-Frohlinde 1968). There is now experimental evidence from high-temperature pulse radiolysis studies that this view may be correct (Ashton et al. 1995). While the formation of the π-complex is a reversible reaction [equilibrium (4)], once the σ-complex is formed [reaction (5)] the OH group remains tightly bound. The high regioselectivity of •OH addition reactions may, in fact, occur at the transition from the π- to the σ-complex.

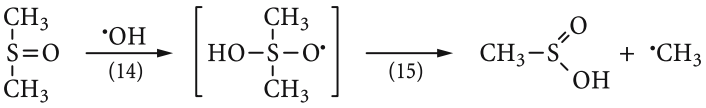


π-complex σ-complex

In benzene derivatives, electron-donating substituents direct into the *ortho*- and *para*-positions, while in the case of the electron-withdrawing substituents considerable *meta*-addition is observed (Table 3.1); otherwise a more equal distribution is established [reactions (6)–(9) and Table 3.1]. In agreement with the pronounced regioselectivity, *ipso*-addition at a bulky substituent such as the chlorine substituent in chlorobenzene is disfavored. Evidence for this is the low HCl yield in the case of chlorobenzene, the low yield of *para* adduct in 4-methylphenol (Table 3.1), or the decarboxylation in the case of benzoic acid [reactions (6) and (10)].



With sulfoxides, •OH reacts mainly by addition to the S-O double bond [DMSO: reaction (14); 92%; $k = 7 \times 10^9$ dm^3 mol^{-1} s^{-1}]. The resulting adduct has not been detected, because it decomposes very rapidly by β-fragmentation [reaction (15); Dixon et al. 1964; Norman and Gilbert 1967; Veltwisch et al. 1980].

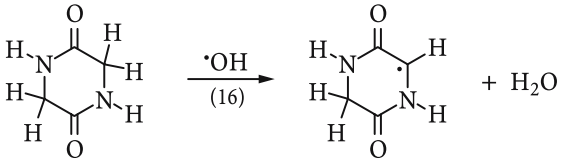


3.3 H-Abstraction

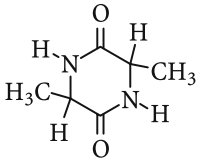
The HO-H bond dissociation energy (BDE) is 499 kJ mol^{-1}, while the C-H bonds in saturated hydrocarbons are much weaker (BDE = 376–410 kJ mol^{-1}; Berkowitz et al. 1994; for a compilation, see Chap. 6). Thus, there is a considerable driving force for H-abstraction reactions by •OH. On the other hand, vinylic hydrogens are relatively tightly bound, and an addition to the C-C double bond is always favored over an H-abstraction of vinylic or aromatic hydrogens. Hence, in the case of ethene, no vinylic radicals are formed (Söylemez and von Sonntag 1980), and with benzene and its derivatives the formation of phenyl-type radicals has never been conclusively established.

Despite the considerable driving force for the H-abstraction reaction, there is some remarkable selectivity. Primary hydrogens (-CH_3) are less likely abstracted than secondary (-CH_2–) and tertiary (-CH-) ones (Asmus et al. 1973). In addition, neighboring substituents that can stabilize the resulting radical by elec-

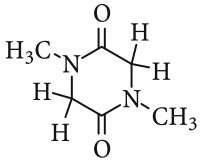
tron donation [such as -OR or -NR_2] favor abstraction of a given hydrogen atom. On the other hand, neighboring electron-withdrawing groups such as carbonyl functions decrease the rate of H-abstraction by the electrophilic •OH. Thus, its rate with acetic acid is rather low ($k \approx 10^7$ dm^3 mol^{-1} s^{-1}), while that with the somewhat electron-richer acetate ion is noticeably faster ($k \approx 10^8$ dm^3 mol^{-1} s^{-1}; Buxton et al. 1988). Here, it is worth mentioning that in the case of electrophilic/nucleophilic radicals the site of H-abstraction may be reversed, that is, C-H BDE is only one factor that governs the site of attack (Brocks et al. 1998). It has been suggested that a combination of electron donation and electron withdrawal (the push-pull effect) additionally favors H-abstraction (Viehe et al. 1985). Whether this effect or just the contribution of electrophilicity is the reason for the remarkable free-radical chemistry of amino acid anhydrides, is not yet known. In the series of amino acid anhydrides, H-abstraction by •OH occurs at the peptide carbon [cf. reaction (16)], and in alanine anhydride an H-abstraction at the methyl group is not observed. In sarcosine anhydride, however, H-abstraction at the methyl group (22%) is an important process (Mieden and von Sonntag 1989).



Glycine anhydride



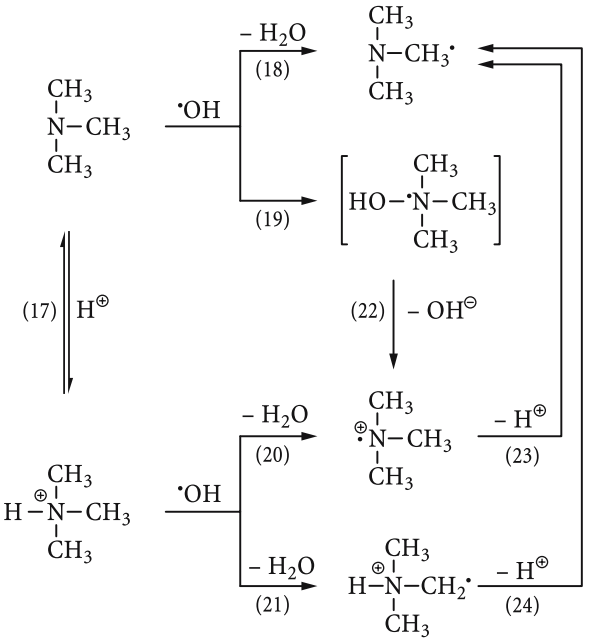
Alanine anhydride



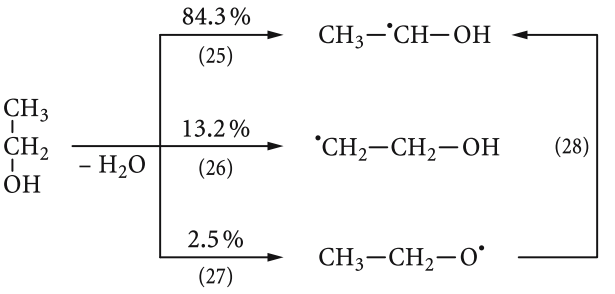
Sarcosine anhydride

Dihydrouracil, an isomer of glycine anhydride, has two kinds of carbon-bound hydrogen atoms. Those activated by the neighboring NH-group react much more readily (90%) than those next to the carbonyl function (ca. 5%; Schuchmann et al. 1984; for details, see Chap. 10). Thus, a high regioselectivity is again observed.

In the case of amines, protonation that withdraws electron density from the center of reaction lowers the rate of reaction by a factor of 30 (Das and von Sonntag 1986). Besides H-abstraction from carbon [reactions (18) and (21)], the formation of *N*-centered radical cations is observed [reactions (19)/(22) and (20); for amino acids see, e.g., Bonifacic et al. 1998; Höbel and von Sonntag 1998]. Reaction (20) is also an H-abstraction reaction. The ET reaction (19)/(22) may proceed via a (bona-fide, very short-lived) adduct (Chap. 7).



In Table 3.2, the rate constants of •OH with MeOH, EtOH, 2-PrOH and *t*BuOH and the percentages of H-abstractions at the various sites [for example, reactions (25)–(28)] are compiled.



Although the rate constants are all close to diffusion controlled, there are noticeable differences. *t*BuOH and MeOH have only primary carbon-bond hydrogens, but despite the fact that *t*BuOH carries three times as many, MeOH reacts faster due to the enhanced radical stabilization by the neighboring OH group (which is equivalent to a lower C–H BDE, Chap. 6). Although a comparison of the yields of α-hydroxyalkyl radicals formed in EtOH and 2-PrOH seems to indicate that the single tertiary hydrogen in 2-PrOH might be as reactive as the two secondary hydrogens in EtOH, a study of the reactions of •OH with D-glucose shows that this must not always be the case, and in this more complex molecule the reactivity of the primary and secondary hydrogens are very similar (Schuchmann

Table 3.2. Rate constants (unit: of $dm^3\ mol^{-1}\ s^{-1}$) of •OH with some alcohols (Buxton et al. 1988) and the position of H-abstraction (in percent). (Asmus et al. 1973)

Alcohol	Rate constant	-CH_3 (%)	-CH_2- (%)	-CH- (%)	-OH (%)
MeOH	9.7×10^8	93	-	-	7.0
EtOH	1.9×10^9	13.3	84.3	-	2.5
2-PrOH	1.9×10^9	13.3	-	85.5	1.2
*t*BuOH	6×10^8	95.7	-	-	4.3

and von Sonntag 1977). In alcohols and carbohydrates, the oxygen-bond hydrogens are quite tightly bond (BDE = 435 kJ mol^{-1}), and hence alkoxyl radicals are formed only in low yields. In water, their detection is complicated due to their ready conversion into α-hydroxyalkyl radicals by a water-assisted 1,2-shift [e.g., reaction (28): Berdnikov et al. 1972; Gilbert et al. 1976; Schuchmann and von Sonntag 1981], and hence their yields given in Table 3.2 might have been slightly underestimated.

The activation energy for H-abstraction from MeOH as measured over a very wide range, from 22–390 °C, has been found to be 13.3 kJ mol^{-1} (Feng et al. 2003). Because of the high reactivity of •OH in its H-abstraction reactions the H/D-isotope effects are rather small, e.g., $k(CH_3OH)/k(CD_3OH) = 2.5$, $k(CH_3CH_2OH)/k(CD_3CD_2OH) = 1.6$ and $k((CH_3)_2CHOH)/k((CH_3)_2CDOH) = 1.5$ (Anbar et al. 1966b). More recently, a value of 1.96 has been reported for the EtOH system (Bonifacic et al. 2003). These values are of interest in comparison with H/D-isotope effects observed for the reaction of •OH with the sugar moiety of DNA (Balasubramanian et al. 1998; Chap. 12).

The rates of •OH addition to C–C double bonds and of H-abstraction are both close to diffusion-controlled. When both reactions can be given by a substrate molecule, addition will be the generally preferred route. This even holds for molecules that have very weakly bound hydrogens. The 1,4- and 1,3-cyclohexadienes provide a good example (Pan et al. 1988). Both contain two C–C double bonds and four weakly-bound pentadienylic hydrogens. In 1,4-cyclohexadiene the two double bonds are separated, while in 1,3-cyclohexadiene the double bonds are conjugated. In the case of 1,4-cyclohexadiene, H-abstraction occurs to an extent of 50%, in the other isomer it is only 25%. Here, •OH addition mainly (50%) yields the allylic radical. Even more pronounced is the situation in the case of toluene. Although there are three weakly bound benzylic hydrogens, H-abstraction occurs with a yield of only 4% (Christensen et al. 1973). Thus, also with thymine, •OH addition to the *C*(5)–*C*(6) double bond is much preferred over an abstraction of a (weakly bound) allylic hydrogen at the *C*(5)–CH_3 group (Chap. 10). This is in marked contrast to, for example, the behavior of peroxyl radicals (Chap. 8).

3.4 Electron Transfer

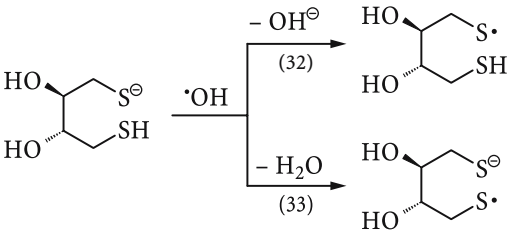
Although the redox potential of •OH is very high [E(•OH/OH^-) = +1.9 V (Kläning et al. 1985); E(•OH, H^+/H_2O) = 2.73 (Wardman 1989)], direct ET is rarely observed in •OH-reactions, and where it occurs intermediate complexes are likely to be involved. For example, in its reaction with thiocyanate, where the final product is the three-electron bonded dirhodane radical anion [reaction (31); for other three-electron bonded systems, see Chaps. 5 and 7], a similar three-electron bonded intermediate might precede ET [reactions (29) and (30)].

$$^{\bullet}OH + SCN^- \rightarrow HOSCN^{\bullet -} \quad (29)$$

$$HOSCN^{\bullet -} \rightarrow OH^- + SCN^{\bullet} \quad (30)$$

$$SCN^{\bullet} + SCN^- \leftrightarrows (SCN)_2^{\bullet -} \quad (31)$$

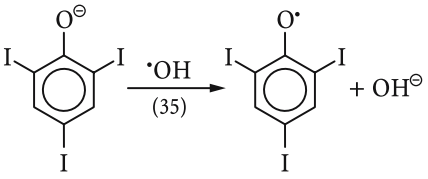
The reaction of •OH with thiolate ions, taken as an overall reaction, is an ET reaction [reaction (36)]. One must, however, again take into account that a three-electron bonded intermediate is formed in the first step (Chap. 7). In semi-deprotonated dithiothreitol reaction (32) dominates over the H-abstraction reaction (33) (Akhlaq and von Sonntag 1987), although the rate constant for the reaction of •OH with a thiol and a thiolate ion are both diffusion controlled ($k = 1.5 \times 10^{10}$ dm^3 mol^{-1} s^{-1}). This is another example of the potentially high regioselectivity of •OH reactions.



Although an ET from phenolates is highly exothermic (for reduction potentials: Lind et al. 1990; Jonsson et al. 1993) and ET is thermodynamically favored over addition (Lundqvist and Eriksson 2000), the usually preferred mode of reaction is addition rather than ET. Yet, addition and ET are in competition (Tripathi 1998), and, when the *ortho*- and the *para*-positions which are the relevant positions of addition for the electrophilic •OH are blocked by a bulky substituent [e.g., reaction (34)] ET may become dominant (Table 3.3). Thus, also for these reactions a short-lived π-complex [cf. reaction (6)] may be postulated as common precursor wherefrom the competition between addition and ET occurs.

Table 3.3. Addition vs. ET in the reactions of •OH with halogenated phenolate ions. (Fang et al. 2000)

Substrate	Addition (%)	Electron transfer (%)
Pentafluorophenolate	73	27
Pentachlorophenolate	47	53
Pentabromophenolate	27	73
2,3,5-Triiodophenolate	3	97



Again, in the oxidation of transition-metal ions, adducts have been established as intermediates [e.g., reaction (35); O'Neill and Schulte-Frohlinde 1975; Asmus et al. 1978; for the equilibrium of Tl^{2+} and •OH, see Schwarz and Dodson 1984].

$$\bullet OH + Tl^{+} \rightarrow TlOH^{+} \qquad (35)$$

$$TlOH^{+} + H^{+} \rightarrow Tl^{2+} + H_2O \qquad (36)$$

A similar situation holds for the reaction of •OH with Cu^{2+}. The reaction proceeds by the replacement of a water molecule of its solvation shell (Cohen et al. 1990) rather than by ET. In neutral solution, the intermediate formed carries zero charge [reaction (37); Barker and Fowles 1970; Asmus et al. 1978; Ulanski and von Sonntag 2000], and only in more acid solutions more positively charged species start to dominate (Fig. 3.1), but real aqua-Cu^{3+} may not to be formed to any major extent, because at below pH 3 the reaction becomes increasingly reversible [reaction (40); Meyerstein 1971; Ulanski and von Sonntag 2000].

$$\bullet OH + \text{aqua-}Cu^{2+} + 2\,H_2O \rightarrow \text{aqua-}Cu(OH)_3 + 2\,OH^{-} \qquad (37)$$

$$\text{aqua-}Cu(OH)_3 + H^{+} \leftrightarrows \text{aqua-}Cu(OH)_2^{+} + H_2O \qquad (38)$$

$$\text{aqua-}Cu(OH)_2^{+} + H^{+} \leftrightarrows \text{aqua-}Cu(OH)^{2+} + H_2O \leftrightarrows \text{aqua-}Cu^{2+} + \bullet OH \qquad (39/40)$$

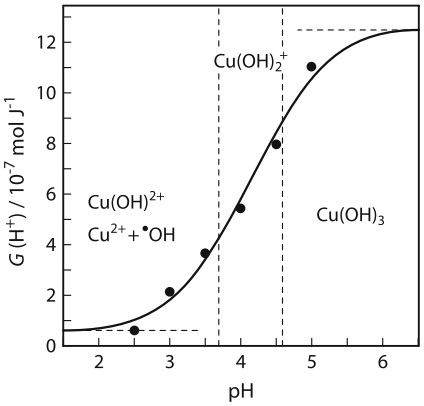


Fig. 3.1. Pulse radiolysis of N_2O-saturated aqueous solutions of Cu^{2+}. Consumption of H^+ as measured by conductometric changes after completion of the reaction. (Ulanski and von Sonntag 2000, with permission)

Thus, in most •OH-induced oxidations short-lived adducts must be considered as intermediates. A case in point in the realm of DNA free-radical chemistry is the oxidation of guanine. From the above, it is evident that •OH, despite its high reduction potential, cannot be directly used for the study of one-electron oxidation reactions. However, one can make use of its high reduction potential by producing other reactive intermediates [e.g., Tl(II); Chap. 10], which no longer undergo an addition to double bonds or H-abstraction.

3.5 Detection of OH Radicals

When one looks for methods to detect •OH, one always has two keep in mind that these radicals are very reactive, and in the presence of substrates their steady-state concentrations are extremely low even at a high rate of •OH production. The fact that •OH only absorbs far out in the UV region (Hug 1981) is thus not the reason why an optical detection of •OH is not feasible. Electron paramagnetic resonance (EPR) must also fail because of the extremely low steady-state concentrations that prevail in the presence of scavengers. The only possibility to detect their presence is by competition of a suitable •OH probe that allows the identification of a characteristic product [probe product, reaction (41)]. When this reaction is carried out in a cellular environment, the reaction with the probe is in competition with all other cellular components which also readily react with •OH [reaction (42)]. The concentration of the probe product is then given by Eq. (43), where [•OH] is the total •OH concentration that has been formed in this cellular environment and η is the yield of the probe product per •OH that has reacted with the probe.

$$^{\bullet}OH + \text{probe} \rightarrow \text{probe product} \tag{41}$$

$$^{\bullet}OH + \text{cellular components} \rightarrow \text{not detectable products} \tag{42}$$

$$[\text{Probe product}] = [^{\bullet}OH] \times \eta \frac{k_{41}[\text{probe}]}{k_{41}[\text{probe}] + k_{42}[\text{cellular components}]} \tag{43}$$

The detection of $^{\bullet}OH$ production in cells is therefore extremely difficult (if not impossible), because both probes and all cell components will react at approximately the same rate, i.e., close to diffusion-controlled (for a detailed discussion of $^{\bullet}OH$-scavenging in cells, see von Sonntag and Schuchmann 1994; von Sonntag et al. 2000). A brief calculation may exemplify this. A cell contains about 70% water and 30% organic material, and thus it is reasonable to take the substrate concentration at about 1 mol dm^{-3} (in subunits in the case of polymers such as DNA). With an averaged $^{\bullet}OH$ rate constant of 10^9 dm^3 mol^{-1} s^{-1}, this yields a scavenging capacity of 10^9 s^{-1} [a somewhat higher scavenging capacity of 3×10^9 s^{-1} was suggested by von Sonntag et al. (2000); a much lower scavenging capacity of 10^7 s^{-1} was estimated by Boveris and Cadenas (1997)]. Although the estimate of 10^9 s^{-1} could be still somewhat on the high side, the value of 10^7 s^{-1} is certainly too low. Yet even with the most reactive probes [$k(^{\bullet}OH$ + salicylate) $\approx 2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}], their scavenging capacity will not exceed 2×10^6 s^{-1} at a probe concentration of 10^{-4} mol dm^{-3} (to achieve this concentration in his body, a human would have to take ~1 g of aspirin). As a consequence, only ~0.2% of $^{\bullet}OH$ (taking 10^9 s^{-1} as the cellular scavenging capacity) that are formed within the cell are scavenged by the probe under such conditions. However, the efficiency factor η is never unity, and in the most commonly used salicylate system it is ca. 0.2 (formation of 2,3-dihydroxybenzoate). Obviously, the possibility of detecting the formation of the probe product very much depends on the sensitivity of its detection. For the salicylate system, very sensitive techniques are available, and the detection limit is given as 2×10^{-7} mol dm^{-3} (Coolen et al. 1998; Tabatabaei and Abbott 1999). Accepting that already a probe product concentration of twice its detection limit (that is, 4×10^{-7} mol dm^{-3} 2,3-dihydroxybenzoate) would give a reliable answer, a total $^{\bullet}OH$ concentration of 2×10^{-3} mol dm^{-3} would have to be formed in the cellular environment. This is quite a high concentration. To visualize this, one may convert it into an equivalent dose of ionizing radiation. Taking 70% cellular water and $G(^{\bullet}OH) = 2.8 \times 10^{-7}$ mol J^{-1}, one arrives at an equivalent dose of 10^4 Gy. This calculation may be taken as a caveat when probing $^{\bullet}OH$ formation in cells, and it really does not matter if the cellular scavenging capacity has been overestimated even by an order of magnitude in this calculation. Thus, detection of $^{\bullet}OH$ formed in cellular systems remains a severe problem (for further details see below), but may be feasible in artificial systems and in areas where the scavenging capacity is markedly lower (extracellular fluids?) and the probe concentration for some reason locally enriched (a suggestion, how this might be tested is made below).

Many detection systems are based on $^{\bullet}OH$-induced hydroxylation of salicylate (see below). Salicylate, however, inhibits some enzymatic reactions that may

be of importance in the in vivo production of •OH. It has, therefore, been suggested (Acworth et al. 1999) to reduce the salicylate concentration, not realizing that a reduction of the probe concentration also reduces its scavenging capacity (Eq. 43).

Some systems that have been proposed as suitable •OH probes (for reviews see Hageman et al. 1992; Kaur and Halliwell 1994, 1996; Loft and Poulsen 1999; von Sonntag et al. 2000), and the principles on which they are based (and, if possible, their reliability) will be discussed in the following.

3.5.1 Aromatic Hydroxylation

Aromatic hydroxylation is most commonly used for the detection of •OH. However, the primary •OH adducts must be oxidized to yield the final product(s). Disproportionation reactions produce these compounds usually only in very low yields. For this reason, an oxidant is required. Although oxygen may serve as an oxidant, the yields are not quantitative because of side reactions (Chap. 8). The addition of a one-electron oxidant, for example $Fe(CN)_6^{3-}$, may overcome this problem (Volkert and Schulte-Frohlinde 1968; Bhatia and Schuler 1974; Madhavan and Schuler 1980; Buxton et al. 1986), but in certain cases an even stronger oxidant such as $IrCl_6^{2-}$ may be required (Fang et al. 1996).

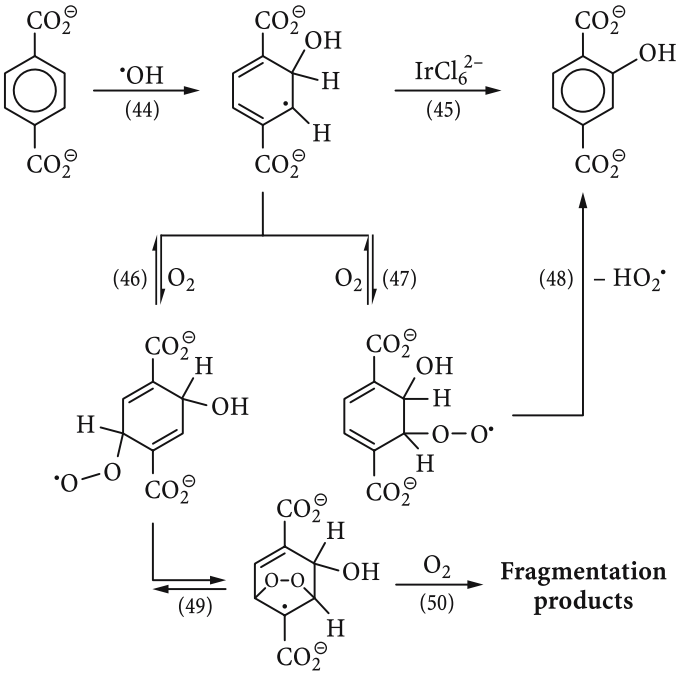
Quite a number of systems that might give rise to •OH-typical products, that is, products that are not formed in enzymatic oxidation processes, have been proposed. For example, phenylalanine (present in all proteins and thus can serve as an internal marker; Karam et al. 1984; Karam and Simic 1988a,b; Kaur et al. 1997), tyrosine (Maskos et al. 1992), terephthalic acid (Armstrong et al. 1963; Matthews 1980), salicylic acid (Ingelmann-Sundberg et al. 1991; Coudray et al. 1995; Bailey et al. 1997) and its isomer 4-hyroxybenzoic acid (Ste-Marie et al. 1996), 5-aminosalicylic acid (Kumarathasan et al. 2001), dopamine (Slivka and Cohen 1985), phthalic hydrazide (Backa et al. 1997), coumarin-3-carboxylic acid (Makrigiorgos et al. 1993, 1995; Chakrabarti et al. 1996, 1998; Manevich et al. 1997; Parker 1998), antipyrine (Coolen et al. 1997) and CO_2 formation as a by-product of the hydroxylation of benzoic acid [Lamrini et al. 1994; reaction (10)]. The very sensitive luminescence detection methods, commonly used in vitro, are not feasible in vivo (Hirayama and Yida 1997). Some of the systems, especially mechanistic implications, will be discussed below in more detail.

It was mentioned above that in aromatic hydroxylation an oxidant is required, and the product yields vary considerably with the oxidant used (for the reason why O_2 does not serve as a typical one-electron oxidant, see Chap. 8). A typical example is the formation of tyrosines from phenylalanine (Table 3.4). Their yields are especially low in the absence of an oxidant, since dimerization usually dominates over disproportionation in these systems. The determination of the products is usually done by either HPLC or GC/MS after trimethylsilylation, and the proteins have to be hydrolyzed prior to analysis. Attention has been drawn to the fact that in vivo cytochrome P-450 enzymes hydroxylate phenylalanine to *p*-tyrosine (Bailey et al. 1997).

Table 3.4. Yields of tyrosines (in % of the •OH yield) formed upon •OH-attack on phenylalamine. (Wang et al. 1993)

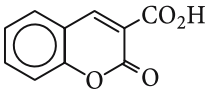
Product	No oxidant (%)	$Fe(CN)_6^{3-}$ (%)	O_2 (%)
Total tyrosine	10	80	52
o-Tyrosine	2.5	37	18
m-Tyrosine	3.5	24	16
p-Tyrosine	4.5	19	18

The terephthalate system follows the same principle. It has the advantage that the main product, 2-hydroxyterephthalate, is the only product which fluoresces and thus can be easily detected, even at low concentrations. Details of the mechanism have been elucidated (Fang et al. 1996). Hydroxyl radicals [k(•OH + terephthalate) = 3×10^9 dm^3 mol^{-1} s^{-1}] react preferentially (85%) at the 2-position [reaction (48)]. The resulting •OH adduct is much more difficult to oxidize than many other hydroxycyclohexadienyl radicals (Chap. 6), and the more powerful oxidant $IrCl_6^{2-}$ is required for a quantitative oxidation [reaction (45)]. With O_2 as the oxidant [reactions (46)–(50)], the yield of 2-hydroxyterephthalate is only

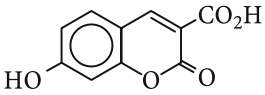


35%. Again, side reactions [reactions (49) and (50)] are the reason for its lower yields (Chap. 8). The detection limit of the fluorescing 2-hydroxyterephthalate has been given as 5×10^{-8} mol dm^{-3} (Saran and Summer 2000).

Aromatic hydroxylation is also the basis of the formation of fluorescent 7-hydroxycoumarine-3-carboxylic acid from coumarine-3-carboxylic acid. Biomolecule-conjugates with coumarin-3-carboxylic acid can be made, and this permits ex vivo probing of •OH in the vicinity of a polymeric conjugate such as proteins or DNA (Makrigiorgos et al. 1993, 1995; Parker 1998; Chakrabarti et al. 1996, 1998; for a review: Makrigiorgos 1999). However, the 7-position in is not an activated position, and hence the yield of 7-hydroxycoumarine-3-carboxylic is very low (~5% of •OH yield; von Sonntag et al. 2000). Despite this fact, this •OH-probing system rates among the most sensitive ones (detection limit 1.5×10^{-8} mol dm^{-3} •OH, taking data reported by Makrigiorgos 1999).



Coumarine-3-carboxylic acid



7-Hydroxycoumarine-3-carboxylic acid

A widely used system is salicylic acid, which was first studied by Grinstead (1960). Being a phenol, the rate of reaction of salicylic acid with •OH is faster [k(•OH + salicylate ion) = 2×10^{10} dm^3 mol^{-1} s^{-1}] than those of the other aromatic acids discussed above. Its main products, 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid, are very sensitively determined by HPLC using electrochemical detection (Coolen et al. 1998) or by LC/MS methods (detection limit near 2×10^{-7} mol dm^{-3}; Tabatabaei and Abbott 1999). Due to its phenolic function, the chemistry in the presence of O_2 is different (see below) to that of the other aromatic acids, yielding in the presence of this oxidant high yields of the desired products. The same holds for its isomer, 4-hydroxybenzoic acid. A potential disadvantage is that in a cellular environment salicylic acid may be oxidized by other oxidizing agents.

Since •OH is strongly electrophilic, the OH group directs •OH into its *ortho*- and *para*-positions [reactions (51)–(53)]. One of the *ortho*-positions is occupied by the somewhat bulky carboxylate group which renders reaction (51) less likely than reaction (52). An addition to the *meta*-position can be largely neglected. Upon oxidation of the •OH-adduct radicals, cyclohexadienones are formed [reactions (54)–(56)] which either decarboxylate [reaction (57)] or rearrange into the corresponding phenols [reactions (58) and (59); e.g., Bausch et al. 1976]. Product yields from hydroxybenzoic acids are compiled in Table 3.5; from salicylic acid in Table 3.6.

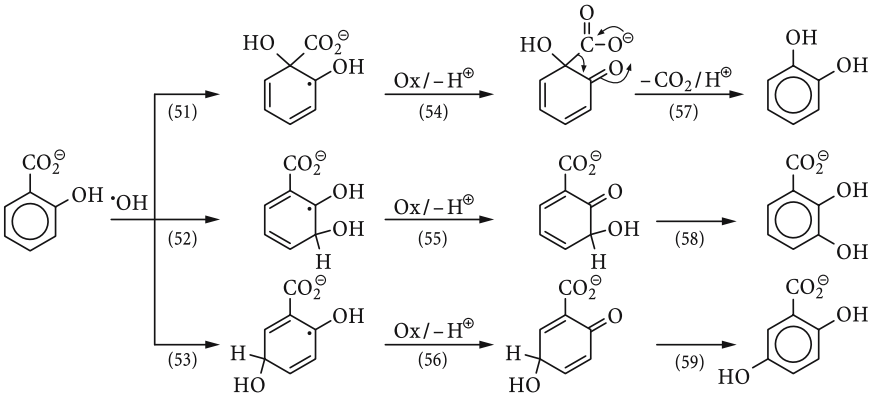
The •OH-adducts of phenols behave differently as compared to those of other aromatic compounds. The parent compound, phenol, has been investigated in quite some detail (Land and Ebert 1967; Raghavan and Steenken 1980; Roder et al. 1999; Mvula et al. 2001). Its *ortho*- and *para*-•OH adducts undergo rapid H^+/OH^--catalyzed water elimination thereby yielding the (thermodynamically

Table 3.5. γ-Radiolysis of hydroxybenzoic acids in N_2O- and air-saturated solutions. *G* values of the dihydroxybenzoic acids (Maskos et al. 1990). Values in parenthesis gives the yield relative to the total •OH yield (N_2O: 5.8×10^{-7} mol J^{-1}, air: 2.9×10^{-7} mol J^{-1})

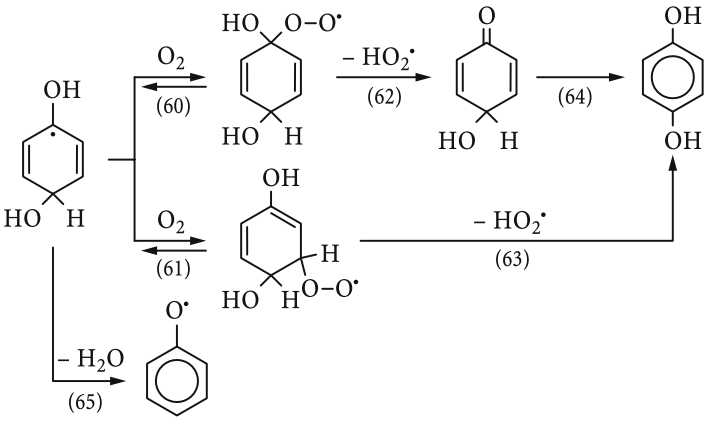
Educt	Gas	2,3-dHB	2,5-dHB	3,4-dHB	2,4-dHB	Total (% •OH)
2-Hydroxy-benzoate	N_2O	1.8	0.2	–	<0.03	2.0 (35%)
	Air	0.83	0.83	–	<0.03	1.7 (59%)
3-Hydroxy-benzoate	N_2O	0.03	0.08	1.02	–	1.13 (19%)
	Air	0.08	0.3	0.73	–	1.25 (43%)
4-Hydroxy-benzoate	N_2O	–	–	0.92	0.02	0.94 (16%)
	Air	–	–	1.33	0.02	1.35 (47%)

Table 3.6. γ-Radiolysis of salicylic acid in N_2O-containing solutions in the presence of $Fe(CN)_6^{3-}$ and O_2. *G* values (unit: 10^{-7} mol J^{-1}) of the products. In parentheses yields in percent of •OH yield. (Mark and von Sonntag 1998, unpubl.)

Product	$Fe(CN)_6^{3-}$	O_2
Catechol	0.5 (9%)	0.8 (14%)
2,3-Dihydroxybenzoic acid	1.5 (26%)	1.5 (26%)
2,5-Dihydroxybenzoic acid	1.3 (22%)	1.7 (29%)
2,4-Dihydroxybenzoic acid	0.1 (2%)	Not detected
Total	3.4 (59%)	4.0 (69%)



more stable; Lundqvist and Eriksson 2000) phenoxyl radical [e.g., reaction (69)]. Interestingly, the *para*-•OH adduct undergoes water elimination much faster than the *ortho*-•OH adduct [$k = \sim 1.7 \times 10^9$ dm^3 mol^{-1} s^{-1} vs $\sim 1.1 \times 10^8$ dm^3 mol^{-1} s^{-1}; Raghavan and Steenken 1980; Roder et al. 1999; Mvula et al. 2001; see also Raghavan and Steenken 1980]. Phosphate buffer catalyses the water elimination in weakly basic solutions with a rate constant of 5×10^7 dm^3 mol^{-1} s^{-1}. At neutral pH, water elimination occurs with $k = 1.8 \times 10^3$ s^{-1} (Raghavan and Steenken 1980; Roder et al. 1999; Mvula et al. 2001) $\sim 5 \times 10^3$ s^{-1} (Land and Ebert 1967). In neutral solutions and the absence of buffer, this reaction is slow compared to the reactions of these •OH adducts with O_2 [e.g., reactions (60) and (61); $k = 1.2 \times 10^9$ dm^3 mol^{-1} s^{-1}; with little if any reversibility, in contrast to other hydroxycyclohexadienyl radicals (Fang et al. 1996); for details see Chap. 8] and the subsequent elimination of $HO_2^\bullet$ [e.g., reactions (62) and (63); $k = 1.3 \times 10^5$ s^{-1}; Raghavan and Steenken 1980; Roder et al. 1999; Mvula et al. 2001]. This reaction is more than 100 times faster than an $HO_2^\bullet$ elimination from the peroxyl radicals of other (nonphenolic) aromatic compounds. This remarkable increase in rate is possibly due to additional routes such as reaction (62). This high rate of $HO_2^\bullet$ elimination renders cyclization and subsequent fragmentation reactions [cf. reactions (49) and (50)] less likely, and in the presence of O_2, the final yields of catechol and hydroquinone are only slightly lower than in the presence of another oxidant (Raghavan and Steenken 1980; Roder et al. 1999; Mvula et al. 2001).



The simple phenol system has been discussed here at some length, because material balance is obtained and mechanistic details are fairly well understood. However, according to the data in Tables 3.5 and 3.6, there is a very noticeable gap in the material balance in the case of the hydroxylated benzoic acids, although some aspects such as the acid-catalyzed water elimination, in salicylate also more pronounced in the case of the *para*-OH-adduct radical, are very similar (Mark and von Sonntag, unpubl.). Interestingly, addition of Fe(III) to oxidize the intermediates also did not improve the material balance (Tables 3.5 and 3.6). From this, it follows that the underlying chemistry of the salicylate and the other hydroxybenzoate systems are at present not yet adequately understood, and the

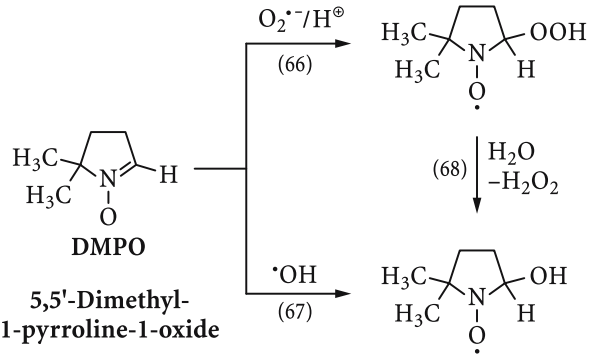
many fluctuations in the reported data may be partly due to the fact that these systems are very sensitive to variations in the reaction conditions. This, however, is not be very helpful for their use as •OH probes.

It is seen from Table 3.5 that in the 2-hydroxybenzoate system, for example, the yields of the 2,3- and 2,5-dihydroxybenzoic acids are close to 1:1 when the precursor radicals are oxidized by O_2, but in the absence of an oxidant this ratio is around 9:1. For the detection of •OH in cellular or in in vivo systems, it is hence not sufficient that these products are formed, but the second requirement is that an adequate oxidant is present (for example, a sufficiently high O_2 tension must be maintained) in order to guarantee that they are formed in a 1:1 ratio. Experiments have been carried out with rats that were given high doses of salicylic acid as a probe for •OH formation induced by the drug (Ste-Marie et al. 1996). Much more 2,5- than 2,3-dihydroxybenzoic acid was detected (cf. Table 3.8). The authors realized that the second requirement of this probe was not fulfilled and stated that there was still the possibility that the drug had induced a metabolic oxidation of salicylic acid yielding mainly 2,5-dihydroxybenzoic acid. Nevertheless, when this paper was later cited in the literature, it was taken as a proof for •OH formation under these conditions. We would like to emphasize here that both requirements, formation of the 2,3- and 2,5-dihydroxybenzoic acids and their 1:1 ratio, is necessary in order to ascertain •OH formation. It is noted that 2,5-dihydroxybenzoic acid is formed in the reaction of salicylic acid with singlet dioxygen ($O_2{}^1\Delta_g$) (Kalyanaraman et al. 1993). More importantly, it may also result from enzyme reactions (Ingelman-Sundberg et al. 1991). Especially the latter makes it difficult if not impossible to ever observe a 1:1 ratio. Whether the suggestion to only concentrate on 2,3-dihydroxybenzoic acid formation for •OH detection (Ingelman-Sundberg et al. 1991) is an adequate solution remains debatable.

3.5.2 Spin Traps

Hydroxyl-radical spin trapping (and detection by EPR) would be a direct detection method [e.g., reaction (67); $k = 2 \times 10^9$ dm^3 mol^{-1} s^{-1}]. Besides reacting with •OH, spin traps also react with $O_2{}^{\bullet-}$ [e.g., reaction (66); $k = 10$ dm^3 mol^{-1} s^{-1}] and the $HO_2{}^\bullet$ adduct to DMPO has only a short lifetime of about 8 min [reaction (68); Pou et al. 1989; Rosen et al. 1994].

Although the difference in rate between •OH and $O_2{}^{\bullet-}$ scavenging by the spin trap is eight orders of magnitude, the yield of the $O_2{}^{\bullet-}$-spin-adduct may be considerably higher, because of the usually much higher steady-state concentration of $O_2{}^{\bullet-}$. In vivo, steady-state concentrations of $O_2{}^{\bullet-}$ have been estimated at around 10^{-11} to 10^{-10} mol dm^{-3} (Boveris and Cadenas 1997) and those of •OH at some 10^{-20} mol dm^{-3} (the latter using too low a scavenger capacity in our opinion, i.e., the •OH steady-state concentration would be at least an order of magnitude lower). As a consequence, its eight orders of magnitude lower rate is more than compensated by the ten (or more) orders of magnitude higher steady-state concentration of $O_2{}^{\bullet-}$. Thus, in a biological system, spin trapping of $O_2{}^{\bullet-}$ will strongly dominate, and the decay of its adduct, reaction (68), may lead to the



erroneous assignment of $^{\bullet}OH$ formation. Moreover, this approach is not directly applicable to the in vivo study of biological oxidations (Kaur and Halliwell 1996), at least not in humans because spin traps are poisonous (Pou et al. 1989). Also, it is considered that the spin-trap method is neither sufficiently sensitive (Mason and Knecht 1994; Rosen et al. 1994), i.e., requiring the presence of micromolar concentrations of the radical species being measured (Ste-Marie et al. 1996), nor practical (Coudray et al. 1995). Thus, it remains to be seen whether a GC/MS technique (Castro et al. 1997) or electrochemical detection (Floyd et al. 1984) can improve the sensitivity situation. The problem of the competition with the $O_2^{\bullet-}$ also seems now on the way to be overcome, and *N*-oxides have been designed that are more specific for $^{\bullet}OH$ (Rosen et al. 1994), and this may be especially true for 2,2-dimethyl-4-methoxycarbonyl-2*H*-imidazole-1-oxide (Tsai et al. 1999). The detection of secondary radicals such as $^{\bullet}CH_3$ from dimethylsulfoxide has been employed to measure $^{\bullet}OH$ (Rosen et al. 1994). Complications can also arise in vivo because the spin trap (such as *N*-oxides) may undergo metabolic reduction (Belkin et al. 1987).

3.5.3 Miscellaneous

There are $^{\bullet}OH$ reactions, however, that allow the detection of the product of the reaction without a further transformation. A case in point is the formation of methanesulfinic acid from DMSO [Babbs and Gale Steiner 1990; reactions (14) and (15)], a nontoxic xenobiotic (Tsay et al. 1998). Methanesulfinic acid has been used as an $^{\bullet}OH$-marker in vivo (Tsay et al. 1998) (detected ex vivo), and ex vivo systems (Fukui et al. 1993). In this context, it may be worth mentioning that methanesulfinic acid can be readily oxidized, and in the presence of O_2 it undergoes a very efficient chain reaction, methanesulfonic acid being the product (Sehested and Holcman 1996; Flyunt et al. 2001). The other product of reaction (15) is the methyl radical. In the presence of O_2, it is converted to a large extent into formaldehyde (Schuchmann and von Sonntag 1984), and it has been suggested (Klein et al. 1981) that this product be used as a marker for $^{\bullet}OH$ in biological DMSO-containing systems.

Table 3.7. Hydroxylation of salicylic acid (5×10^{-3} mol dm^{-3}; product: 2,3-dihydroxybenzoic acid, 2,3-DHB; 2.13×10^{-5} mol dm^{-3} in the absence of a scavenger) and phenylalanine (5×10^{-3} mol dm^{-3}; product: *p*-tyrosine, 7.3×10^{-6} mol dm^{-3} in the absence of a scavenger, total tyrosines 2.21×10^{-5} mol dm^{-3} by the Fenton reagent (Fe(III) 5×10^{-6} mol dm^{-3}, EDTA 1.5×10^{-5} mol dm^{-3}, H_2O_2 5×10^{-5} mol dm^{-3}, ascorbate 5×10^{-5} mol dm^{-3}, phosphate buffer pH 7.4) (Kaur et al. 1997). Calculations are based on competition kinetics using established rate constants. (Buxton et al. 1988)

Scavenger	2,3-DHB measured (%)	2,3-DHB calculated (%)	p-Tyrosine measured (%)	p-Tyrosine calculated (%)
None	100	100	100	100
2-Deoxyribose (0.1 mol dm^{-3})	98	25	69	8
Mannitol (0.1 mol dm^{-3})	28	33	31	11
Formate (0.1 mol dm^{-3})	3	25	46	8

Deoxyribose gives rise to TBA-reactive products (Halliwell 1990; Aruoma 1994; Loft and Poulsen 1999) which can be detected spectrophotometrically or by their fluorescence (Biaglow et al. 1997). However, the test is not specific [the reaction is also given by base propenals in the case of BLM action on DNA which is not •OH-induced (Chap. 12) and can give misleading results (Gutteridge 1986); see also Draper et al. (1993)].

Moreover, the oxidation of methional to ethylene has been proposed (Beauchamp and Fridovich 1970; Biaglow et al. 1997), and the formation of allantoin (Grootveld and Halliwell 1987; Halliwell et al. 1988) from uric acid as well as the imidazolone derivative from histamine (Ching et al. 1995) have been suggested as an index of •OH action in vivo (Halliwell et al. 1988).

3.5.4 References for OH-Radical Probes

For setting up a probe system for use in biological systems, it is required that it provides •OH but also adequately behaves with respect to competition kinetics. The Fenton system seems to fulfill the first criterion in that it produces the required products in good yields but certainly not the second one. As can be seen from Table 3.7, the measured yields and the calculated ones [based on competition kinetics, Eq. (43), and established rate constants] dramatically disagree. The reason for this is not yet known, but it is evident that this system cannot be used with advantage as a reference for •OH production.

Similar effects have been observed when •OH was generated using the xanthine/xanthine oxidase system in the presence of EDTA-complexed iron, a similar Fenton-type system (Owen et al. 1996). The efficiency of suppression of the formation of the dihydroxybenzoic acids by •OH scavengers increases from

mannitol:EtOH:butanol:DMSO in the sequence 1:2:8:24, but it is recalled that their ratios of rate constants with •OH spans a much smaller range (1:1.1:2.5:3.8; Buxton et al. 1988). In addition, the ratio between the products 2,5-dihydroxybenzoate:2,3-dihydroybenzoate changes from 4.8 when the iron is complexed by EDTA to 7.1 when it is complexed by DTPA, and when the complexing agent is omitted only 2,5-dihydroxybenzoate is formed (Owen et al. 1996). In addition, the dihydroxybenzoic acids may interfere in Fenton-type systems and suppress •OH production (Capelle et al. 1992) in a manner which is very far from correlating with their •OH rate constants.

In this context, an interesting observation is worth mentioning. The hydroxylation of salicylic acid by H_2O_2 is catalyzed by fibers (e.g., asbestos and glass wool; Maples and Johnson 1992). The rate of hydroxylation increased proportionally to the H_2O_2 concentration at rather high H_2O_2 concentrations (up to 4×10^{-2} mol dm^{-3}). When the fibers had been treated with salicylate in order to leach out potential transition-metal ions and when, after filtering, this extract was treated with H_2O_2, very little hydroxylation was observed. Although this has been taken as proof that •OH are formed by these fibers, the reaction parameters are difficult to reconcile with what we know at present about Fenton-type reactions, that is, we do not know much about this heterogeneous catalysis of salicylate oxidation. Salicylate is not the only system, where probing •OH scavenging causes considerable difficulties. The luminol/Fe^{2+}/H_2O_2 system, suggested to give adequate data (Hirayama and Yida 1997), also fails when compared with established •OH rate constants.

These dramatic discrepancies mentioned above may also be of some relevance in biological systems, where these probes have been widely used (see below). If •OH were formed in biological systems by Fenton-type reactions, these probe molecules could strongly overestimate (or underestimate) the •OH production in these systems.

A more reliable means of providing a reference of •OH in a biological system may be by means of irradiation with ionizing radiation (von Sonntag et al. 2000). The action of ionizing radiation on an aqueous medium gives rise to •OH whose yield/dose relationship (G value) is known (Chap. 2). Apart from this, since biological media are concentrated solutions the formation of the indicator product, e.g., a phenol (ArOH), via the 'direct effect' [expressions (69) and (70)] must in principle be taken into account as well. It can be shown that with k_{41}[probe]/k_{42}[cellular components] above 10^{-4} the direct effect contributes less than 10% of the •OH pathway.

$$\mathrm{ArH} \rightarrow \mathrm{ArH}^{\bullet+} + \mathrm{e}^- \tag{69}$$

$$\mathrm{ArH}^{\bullet+} + \mathrm{H_2O} \rightarrow \mathrm{ArH(OH)}^{\bullet} + \mathrm{H}^+ \tag{70}$$

The various procedures mentioned above may be calibrated by conducting the incubations for the duration of equal times t and then subject the sample ex vivo to different doses D of ionizing radiation (γ, e-beam). The intrinsic rate of •OH-formation $\nu(\mathrm{OH})_{\mathrm{intrinsic}}$ without irradiation can then be calculated from a

plot of $X_{total}/X_{intrinsic}$ vs D [expression (71)], where X is the quantity that is being experimentally determined.

$$X_{total}/X_{intrinsic} = 1 + G(OH)\, D / t_{incubation} \nu(OH)_{intrinsic} \quad (71)$$

Irradiation would be carried out at the same O_2 status as in incubation, i.e., dose rates will have to be such that O_2 depletion by peroxyl radical formation (Chap. 8) is minimal.

3.5.5 Metabolic OH-Radical Production and Probing for OH Radicals in Vivo

At present, it seems that we do not yet have any reliable data on the rate of metabolic production of $^{\bullet}OH$ within the biological system. This will be proportional to the rate of O_2 uptake. In a human organism, this has been estimated to be near 15 mol day^{-1}, of which 1% is channeled into $O_2^{\bullet-}$, i.e., ~ 4×10^{-3} mol dm^{-3} day^{-1} (Halliwell and Gutteridge 1999). It is not very likely that there is a direct channel into $^{\bullet}OH$, and it is reasonable to assume that its formation requires as precursors $O_2^{\bullet-}$ and H_2O_2 (in combination with low-valent transition metal ions). A major source of H_2O_2 may be $O_2^{\bullet-}$ (cf. the role of SOD). Considering the action of catalase which keeps the cellular steady-state H_2O_2 concentration at a low level, the rate of $^{\bullet}OH$ production must be significantly lower than that of $O_2^{\bullet-}$. Hydroxyl-radical production will not be uniform within the cell, and in a more complex system such as a human it is even more likely that there are areas of low and high $^{\bullet}OH$ production. This will make the probing very difficult, because a given probe may be unevenly distributed within the body.

An approach to estimate the rate of $^{\bullet}OH$ production *in the very neighborhood of DNA* is on the basis of oxidized nucleobases excreted. From that, the total rate of 'oxidative hits' at the DNA in man is estimated at 10^4 per cell per day times 6×10^{13} cells per body (Ames and Shigenaga 1992), i.e., 1×10^{-6} mol $body^{-1}$ day^{-1} (provided that part of the oxidized bases is not recycled in the cell). Putting the ratio of DNA to the total of organic material in the body (without bones) at 100 g/35 kg, we *extrapolate* to 7×10^{-6} mol dm^{-3} day^{-1}. From an estimate of the $^{\bullet}OH$ steady-state concentration (in hepatocytes) (Boveris and Cadenas 1997), one calculates a rate of $^{\bullet}OH$ production of 1.5×10^{-5} mol dm^{-3} day^{-1}.

The two latter values are much below the value of 2×10^{-3} mol dm^{-3} day^{-1} calculated on the basis of salicylate data (Coudray et al. 1995) and the cellular scavenging capacity discussed above. This raises the question whether the salicylate assay can be taken as a reliable in vivo probe. It has been discussed above that 2,5-dihydroxybenzoate is also formed enzymatically and that this product cannot be used for monitoring $^{\bullet}OH$ production. For this reason, reference has always been made only to the other isomer, 2,3-dihydroxybenzoate. However, the question has already been raised whether there are unknown cellular reactions (besides via $^{\bullet}OH$) which produce this isomer (Halliwell and Grootveld 1987). Evidence that this may indeed be the case comes from data compiled in Table 3.8. Here, salicylate (or aspirin) has been given to either a rat or a human,

Table 3.8. Plasma levels of 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate 2 h after administering salicylic acid (rat; Ste-Marie et al. 1996) or aspirin. (man; Isobe et al. 1996)

Species	mg kg^{-1}	[2,3-DHB]/10^{-9} mol dm^{-3}	[2,5-DHB]/10^{-9} mol dm^{-3}
Rat	300	95	264
Man	10 - 20	63 ± 24	832 ± 309

in the former case at a ~15 times higher concentration. However, close to equal 2,3-dihydroxybenzoate levels were found in the rat and the human despite the fact that in the rat the scavenging capacity of salicylate was about 15 times higher (Eq. 43). Assuming an equal rate of $^{\bullet}OH$ production in these two species, the rat should thus have shown 15 times higher 2,3-dihydoxybenzoate levels. This discrepancy is even larger considering that smaller animals have typically higher metabolic rates and hence a potentially higher rate of free-radical production.

With this cautionary remark in mind, we would like to mention some studies where attempts have been made to monitor $^{\bullet}OH$ production and a variety of conditions, including stressed systems: dextran sulfate-induced colitis (Blackburn et al. 1998), diabetes patients (Ghiselli et al. 1992), obstructive jaundice of rats (Tsay et al. 1998), ischemic reperfused mycocardium (Das et al. 1989), activated human neutrophils (Kaur et al. 1988), healthy humans (Grootveld and Halliwell 1988), fecal flora (Owen et al. 1996), in chondrocytes and cartilage (Tiku et al. 1998).

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