Free Rad. Res., Vol. 33, pp. 567-579
Reprints available directly from the publisher
Photocopying permitted by license only

Arguments Against the Significance of the Fenton Reaction Contributing to Signal Pathways Under *in vivo* Conditions*

MANFRED SARAN†, CHRISTA MICHEL, KURT STETTMAIER and WOLF BORS

Institut für Strahlenbiologie, GSF-Forschungszentrum für Umwelt und Gesundheit, 85764 Neuherberg, Germany

Accepted for publication by Prof. H. Sies

(Received 22 December 1999; In revised form 03 April 2000)

One of the common explanations for oxidative stress in the physiological milieu is based on the Fenton reaction, i.e. the assumption that radical chain reactions are initiated by metal-catalyzed electron transfer to hydrogen peroxide yielding hydroxyl radicals. On the other hand – especially in the context of so-called "iron switches" – it is postulated that cellular signaling pathways originate from the interaction of reduced iron with hydrogen peroxide.

Using fluorescence detection and EPR for identification of radical intermediates, we determined the rate of iron complexation by physiological buffer together with the reaction rate of concomitant hydroxylations of aromatic compounds under aerobic and anaerobic conditions. With the obtained overall reaction rate of 1,700 $M^{-1}s^{-1}$ for the buffer-dependent reactions and the known rates for Fenton reactions, we derive estimates for the relative reaction probabilities of both processes.

As a consequence we suggest that under *in vivo* conditions initiation of chain reactions by hydroxyl radicals generated by the Fenton reaction is of minor importance and hence metal-dependent oxidative stress must be rather independent of the so-called "peroxide tone". Furthermore, it is proposed that – in the low (subtoxic) concentration range – hydroxylated compounds derived from reactions of "non-free" (crypto) OH radicals are better candidates for iron-dependent sensing of redox-states and for explaining the origin of cellular signals than the generation of "free" hydroxyl radicals.

Keywords: Oxidative stress; ROS (reactive oxygen species); free radicals; Fenton reaction; iron switch; hydrogen peroxide; ferryl/perferryl species

INTRODUCTION

The question how radicals are formed *in vivo* and what biological consequences their generation may have is still a matter of debate. The Fenton reaction, as one of the preferred explanations for hydroxyl radical generation, has been reviewed repeatedly.[1–6] Prerequisite for this reaction to proceed is that a reduced metal state is created, e.g. under pathophysiological conditions of metal overload[7], after degradation of heme proteins, liberation of iron from ferritin stores, disintegration of iron-sulfur centers, or similar processes, with concomitant reduction of the normally prevailing oxidized form by

* this paper is dedicated to Professor Ulrich Hagen on the occasion of his 75th birthday in recognition of his intellectual curiosity as a radiation biologist and in gratitude for his continuous interest in our work during his years as the Director of our Institute.

† Address for correspondence: Tel.: +49/89/3187-2458; FAX: +49/89/3187-3378; Email: saran@gsf.de

reductants such as ascorbate or thiols. The reduced metal may then react with peroxide and initiate chain reactions via formation of hydroxyl radicals (in the case of H_2O_2) or alkoxyl radicals (in the case of organic peroxides ROOH). Therefore the main prerequisite for the Fenton reaction to occur is that sufficiently high levels of peroxide molecules are present in the biological milieu to allow for their encounter with the metal reaction partner with reasonable probability. Alternatively, some process could be visualized that generates hydrogen peroxide right at the location of the metal thereby rendering the process of hydroxyl radical generation "specific for the site" where the metal is bound, but independent of an *in toto* elevated level of hydrogen peroxide.

On the other hand there exists a host of biological effects that resembles those of hydroxyl radicals but which cannot be inhibited by common hydroxyl radical scavengers. This has led to the postulation of "crypto-OH" complexes[8] which in certain aspects behave differently from the freely diffusible hydroxyl radicals known from radiation chemistry or those OH-species that originate from the Fenton reaction.

Fe(II) ions are rather stable at acidic pH but rapidly autoxidize to Fe(III) in alkaline solution or at neutral pH. This autoxidation can be followed by observing the absorption spectrum of iron(III)[9]. However, at neutral pH – and more so under alkaline conditions – Fe(III) rapidly precipitates in form of insoluble hydrates unless this is prevented by complexation. *In vivo* the molecules of foremost interest with respect to complexation are obviously bicarbonate and phosphate anions since they are the most abundant components of the physiological buffer system, being present at about 25 mM and 1 mM concentration, respectively. Buffer concentrations of that magnitude are capable of keeping up to 20μM of iron(III) in solution. The complex of iron with pyrophosphate

$$[(P_2O_7)_2Fe^{III}(H_2O)]^{5-} \Longleftrightarrow [(P_2O_7)_2Fe^{III}OH]^{6-} + H^+,\ \text{pK } 9.6$$

has been described in detail[10], whereas structures of other carbonato/bicarbonato- and phosphato-complexes are not exactly known.[11] Some of them, however, are assumed to contain iron in the ferryl-state Fe(IV)[12] thus resembling intermediate states of hydroxylating metalloenzymes such as cytochrome P-450[13] and others.[14]

The ability to hydroxylate aromatic compounds is not reserved to enzyme intermediates but also occurs with low-molecular inorganic complexes.[15,16,17] As will be shown below, however, such hydroxylations are not promoted by free OH radicals but proceed via iron/buffer-complexes which behave differently. Taking into account the relative abundance of phosphate and bicarbonate ions in cellular systems, which is several orders of magnitude above the steady state concentration of hydrogen peroxide, we expect that reactions of the metal with buffer dominate over the reaction of Fe(II) with H_2O_2 and hence may be more important for explaining the observable biological endpoints.

Such an interpretation would have consequences for understanding "redox sensing" under *in vivo* conditions. (i) For the initiation of a radical chain eventually producing hydroxylated or otherwise oxidized (oxygenated) biomolecules no "pre-existing" H_2O_2 would be necessary; (ii) *initiation* of such radical reactions would not be inhibitable by scavengers of free OH radicals, (iii) new mechanistic explanations for interdependencies between metal catalysis and molecules contributing to redox-dependent signalling pathways would have to be sought.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest commercially available purity and used as supplied. Solutions

were prepared with water from a Millipore milli-Q system^{R}. Gases were at least of 99.999% quality (Linde, Germany).

The gas content of experimental solutions was varied by bubbling the solutions through stainless steel injection needles for at least 20 minutes before and during the whole experiment with the respective gas stream (which, in case of gas mixtures, was kept at constant mixing ratio and overall flow rate by use of precision flowmeters (Kobold, Germany)).

Experimental procedures

For fluorescence and absorption measurements a flow-through set-up was used: reagent glasses of different size (2 to 50 ml), supplied with stainless steel needles for extraction of liquid and gas supply, were connected via PTFE-tubes to the flow-through cell (3µl active volume) of a fluorescence detector (LS30, Perkin-Elmer, Germany) and/or to a half-micro cuvette (10 mm pathlength) connected via light guides to a diode-array spectrophotometer (X-DAP, Polytec, Germany). After the passage through both detection devices the solution was recycled by means of a peristaltic pump (Minipuls, Gilson, USA) into the original reactor. Transfer time from the reaction vessel to the detection cuvettes could be varied from about 3 seconds to 60 seconds by adjusting the pump speed, the total transfer volume (including cuvettes and hoses) was kept to less than 5% of the total reaction vessel. Pulse radiolysis experiments (using 1.8 MeV electrons from a FEBETRON accelerator) were conducted as previously described.[18] Long-term steady state radiolysis was carried out with ^{60}Co gamma radiation using a Gamma Cell (Atomic Energy of Canada Ltd, Canada) delivering a dose rate of 11 Gy/min. For fluorometric detection of hydroxyl radicals the terephthalate dosimeter[19,20,21,22] was used which depends on the hydroxylation of non-fluorescent terephthalic acid (TA) yielding 2-hydroxy-terephthalate (TA-OH) which strongly fluoresces around 435 nm when excited at 323 nm. This method has originally been thought to be specific for OH radicals. However, as has recently been shown[23] and will be elaborated in more detail below, TA-OH may also result from hydroxylations by buffer-coordinated iron complexes. Measurements to differentiate between free OH and the crypto-species were carried out with an ESP 300 Spectrometer by Bruker, Germany. The spin-trap DMPO (5,5-dimethyl-1-pyrroline N-oxide) was supplied by Sigma and purified further by charcoal filtration under nitrogen in the dark.[24]

RESULTS

Upon injection of a bolus of $FeSO_4$-solution into phosphate or bicarbonate buffer, Fe^{2+} starts to associate with phosphate or bicarbonate anions yielding a buffer-coordinated iron complex.[15,25] When terephthalic acid is present, the immediate rise of the fluorescence of TA-OH provides evidence that this complex is able to hydroxylate the aromatic ring of TA. Figures 1 and 2 briefly summarize the basic features of the experimental system:

(i) TA hydroxylation depends on the concentration of iron(II) (Figure 1a);

(ii) The TA hydroxylating capacity depends on phosphate (Figure 1b); in pure water there is no reaction at all; at the physiological concentration of 1 mM phosphate the yield of TA-OH is close to one third of the maximum;

(iii) the process is sensitive to the presence of dissolved oxygen (Figure 2a). It should be noted, however, that even at "zero" oxygen, i.e. the lowest oxygen concentration that is attainable by continuously purging the solution with pure nitrogen or argon, the yield of TA-OH is not zero. As can be seen in panel b of Figure 2, the overall reaction just becomes slower in the absence of oxygen. Figure 3 shows that the ability to hydroxylate tereph-

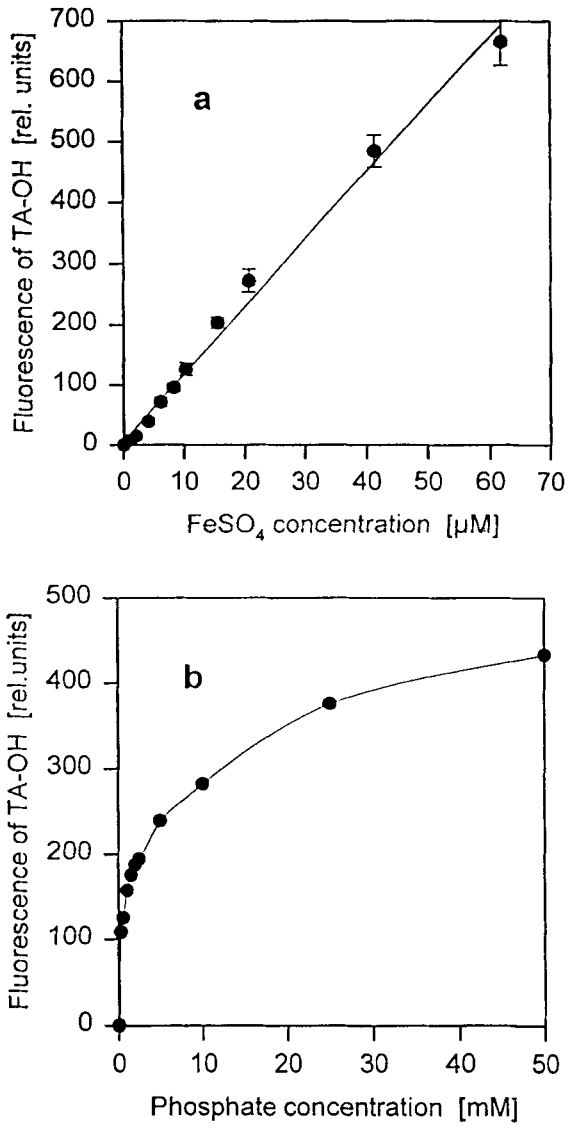


FIGURE 1 The hydroxylation of terephthalate to form TA-OH depends on the presence of Fe(II) (panel *a*) and phosphate (panel *b*). Boli of $FeSO_4$-solution were injected into the respective buffer (adjusted to pH 7 by mixing Na_2HPO_4 with NaH_2PO_4). For panel a buffer concentration was 40mM. TA concentration was 400 μM and fluorescence of TA-OH was read after the reaction had run to completion. The solutions were constantly bubbled with oxygen

thalate is not solely a feature of iron-phosphate complexes but also proceeds when boli of $FeSO_4$ are injected into an aqueous solution of bicarbonate.

An indication that hydroxylations due to the buffer complex differ from those by "free" hydroxyl radicals generated by radiation comes from Figure 4. The pH profiles are completely different: for the free OH-reaction almost no pH dependence is seen between pH 4.85 and 8.5; hydroxylation by the phosphate complex, in contrast, has a distinct pH optimum around pH 6.3.

To corroborate the results of Reinke et al.[15,26] and our own experiments reported earlier[23] that hydroxylations by radiolytic OH, "true" Fenton-OH radicals, and those of buffer complexes are mechanistically different, the series of ESR experiments shown in Figure 5 was conducted. The main panel shows the time traces for the development of TA-fluorecence when boli of $FeSO_4$ are injected into phosphate buffer alone (triangles) or phosphate buffer containing hydrogen peroxide (circles). As expected, the solution with H_2O_2 – being the classical Fenton-system – produces free OH which is verified by the appearance of an EPR-spectrum typical for the hydroxyl adduct of the spin-trap DMPO (spectrum a). Surprisingly, however, the solution without H_2O_2, even though hydroxylating terephthalate as well as the H_2O_2-containing solution, does not produce any DMPO signal at all (trace b) indicating that the observed TA hydroxylation must have been due to something else than a free OH radical. When ethanol is added to the iron/phosphate system, the signal of an ethanol-derived radical appears (spectrum b'); when ethanol is added to the Fenton-system, the EPR spectra of the hydroxyl- and the ethanol-radical are superimposed (trace a').

One should point out that the experiments of Figure 5, for the sake of clarity, were conducted at high phosphate concentrations (which are only observed in working muscle cells); the same effects, however, could also be verified at the much lower physiological concentration of around 1 mM of phosphate which pertain to blood plasma and interstitial fluids (data not shown).

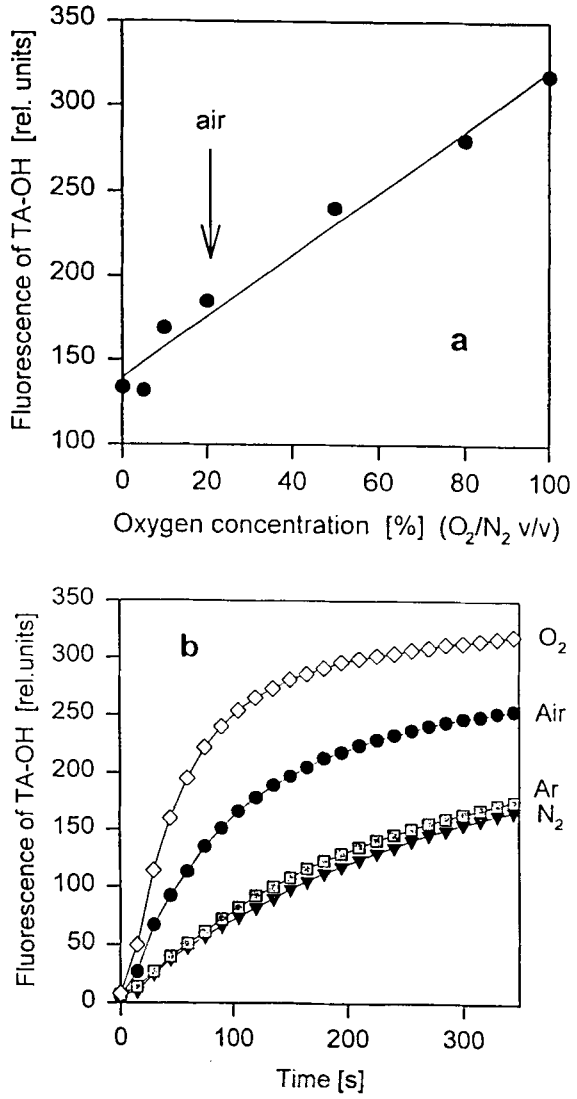


FIGURE 2 The overall yield (panel *a*) and the kinetics (panel *b*) of terephthalate hydroxylation are affected by the presence of oxygen. For panel *a* experiments were carried out by mixing O_2 and N_2-saturated 400μM TA solution (in 40mM PB) in various ratios and subjecting it to a 50 ns pulse of 1.8 MeV electrons producing 10 μM of hydroxyl radicals. (Note that according to the ordinate value at zero oxygen, TA-OH is also efficiently produced without oxygen). Panel *b* shows the kinetics of the rise of fluorescence after injection of a bolus of $FeSO_4$ into 50 mM phosphate buffer at pH = 7, resulting in a final Fe(II)-concentration of 40 μM. The solutions were constantly bubbled with the respective gases

The data clearly demonstrate that the iron/phosphate-derived species is "cryptic" in the sense that it does not generate "free" hydroxyl radicals, whereas its oxidation potential evidently suffices to oxidize ethanol to the corresponding DMPO-identifiable species.

DISCUSSION

Reviewing the importance of metal-dependent reactions in biological systems Halliwell and Gutteridge[27] stated: "However, there has been repeated controversy as to whether OH is formed at all in Fenton reactions at physiological pH values. Challenges to the existence of OH seem to erupt every few years and then subside, leaving us none the wiser". An example for this is a dispute in Accounts of Chemical Research after Sawyer and coworkers proposed Fenton reactions to proceed via "bound" OH radicals[28] and this suggestion was rebutted by MacFaul et al.[29] and Walling[30] who raised arguments in favor of the occurrence of truly "free" OH. The present investigation, however, was not aimed at renewing the discussion about the structure of Fenton complexes, which, in view of the available literature and the especially careful investigations of the chemistry of iron in hypervalent oxidation states by Bielski[9–12] and others[31] also seems superfluous. Since it is known, however, that oxygen activation by iron complexes strongly depends on the nature of the ligands[32,33] and on pH[34] and – already in the case of rather "simple" complexes such as Fe-EDTA and others -[35] proceeds by complicated multistep-mechanisms[36,37] an explanation of the significance of such complexes under *in vivo* conditions and the question where the oxygen atom in TA-OH comes from needs a more detailed discussion.

Mechanistic aspects of TA-hydroxylation

Radiolysis experiments have established that according to the pathway outlined in equations

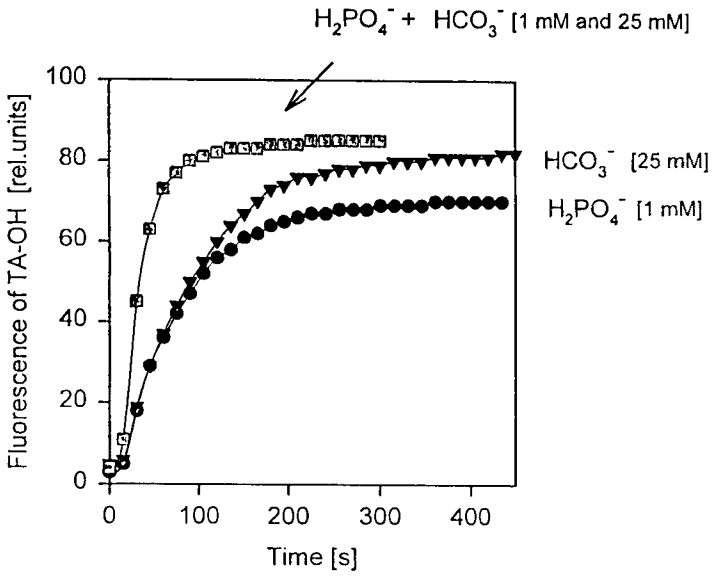


FIGURE 3 Phosphate and bicarbonate buffers possess about equal hydroxylating efficiency at "physiological" concentration. Boli of $FeSO_4$ (resulting in a final iron concentration of 20 μM) were injected into the respective buffer at pH 7.4 (which, in the case of bicarbonate, was kept constant by purging the solution with a 95/5% v/v mixture of air and CO_2)

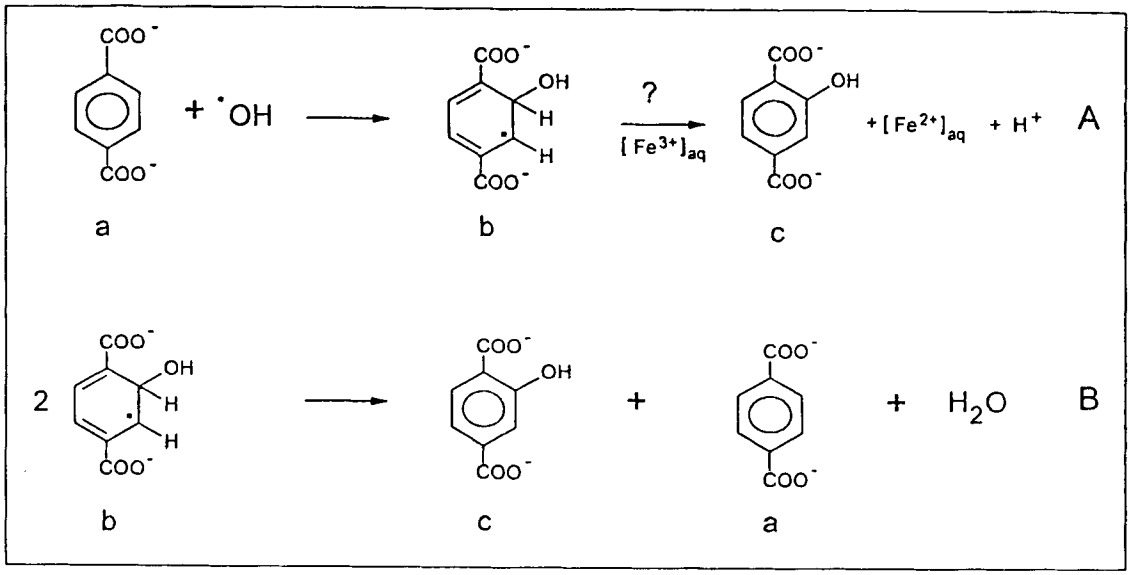
A and B of Scheme 1[20,23] free OH is able to hydroxylate TA without the involvement of dissolved molecular oxygen. This could explain the "basic" yield of TA-OH in the radiolysis system at "zero" oxygen concentration (Figure 2a). The rise in the yield of TA-OH (Figure 2a), and the acceleration observed in Figure 2b with increasing oxygen concentration, could then be due to additional TA-hydroxylation by contribution of equations C and D of Scheme 1.

As it is impossible to obtain *entirely* oxygen-free solutions by bubbling with gases like N_2 or Ar, we cannot exclude that the "zero oxygen" conditions in Figure 2 still contained sufficient amounts of oxygen to allow for the formation of some hypervalent >Fe=O species or some other peroxidic intermediate accounting for the observed yield of TA-OH under those presumably anoxic conditions. Additionally, the mechanism outlined in Scheme 1 would only explain the results in the buffer system if the latter would generate "free" hydroxyl radicals. This, however, is not the case. In the beginning, when the TA reaction was thought to be specific for free OH, the fluorescence of TA-OH was expected to be a direct proof for reactions involving this species. Comparison of the fluorescence data and the ESR measurements made under identical conditions clearly showed that TA-OH formation must have accounted for the presence of free as *well as* of some hitherto unidentified species, which DMPO could not detect. On the other hand, DMPO detected the presence of that crypto species, when the oxidative capacity of the latter was "translated" by alcohol or formate into the corresponding ethyl- or formate spin-adducts. Knowing that an oxidation potential of about 1.2 Volts is needed to abstract hydrogen atoms from alcohols we may take an oxidation potential of that magnitude as an estimate for the lower limit of that of the postulated crypto-OH. If TA-hydroxylation can not – or at least not exclusively – be due to hydroxyl radical generation by some mechanism analogous to the simplified equation

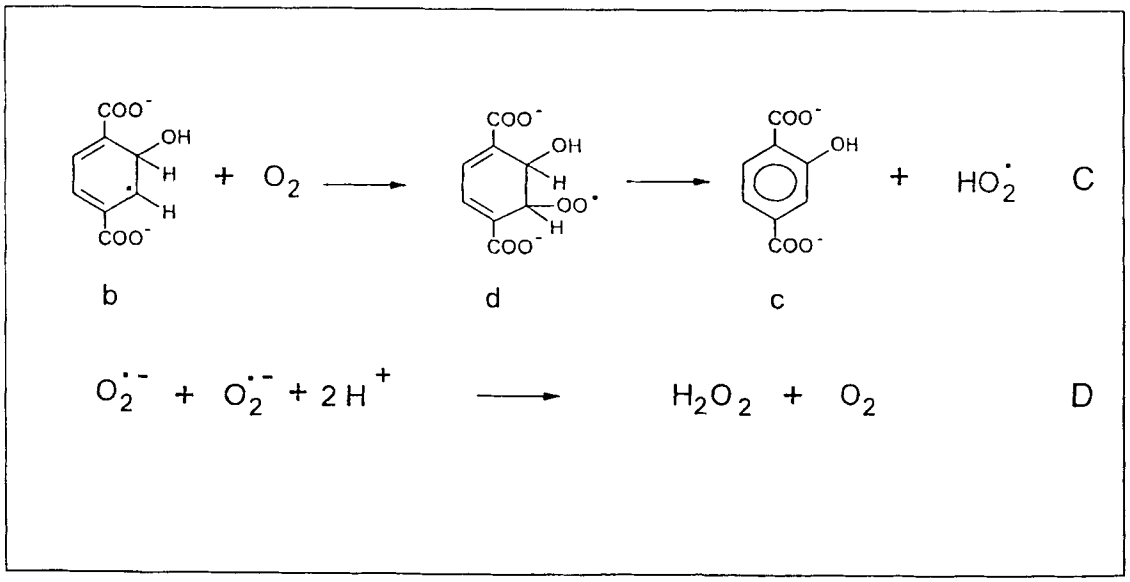
$$L\bullet Fe^{II}H_2O + H_2O_2 \;>\; L\bullet Fe^{III}H_2O + {}^{\bullet}OH + OH^-$$

other explanations have to be sought. One of the possibilities left is that TA hydroxylation by the

Anaerobic conditions



Aerobic conditions



SCHEME 1 Reaction pathways of terephthalate hydroxylation by "free" OH. Under anaerobic conditions TA-OH (*c*) may be formed via intermediary hydroxy-cyclohexadienyl radicals (*b*) by direct oxidation by complexed metal (reaction A), or *via* disproportionation of two hydroxy-cyclohexadienyl radicals (reaction B). Under aerobic conditions intermediary peroxyl radicals (*d*) may directly yield TA-OH after elimination of a superoxide radical. The latter could induce a cyclic redox-reaction by reducing iron(III), or, after dismutation to H_2O_2 according to reaction D, generate OH radicals in a genuine Fenton type process. (Scheme taken from Refs [20] and [23])

buffer complex proceeds through some mechanism analogous to "oxene" type oxygen insertion which has been extensively discussed in the literature[38,39,40,41] or one may assume that mixed oxidation states of iron (as contained in so-called "GIF-reagents"[42,43,44]) contribute to aromatic hydroxylations *via* intermediary epoxides.[45]

Evidently the decision where the oxygen atom in TA-OH comes from, cannot be solved without appropriate isotope experiments (analogous to those that have been conducted to show that the oxygen atom in the hydroxyl radicals derived from the "uncomplexed" Fenton reaction comes from H_2O_2 and not from O_2).[46] We are presently unable to explain the mechanism of TA hydroxylation in detail. The pathway outlined in Scheme 1 only seems to apply to free OH radicals and the question about the actual nature of the crypto-species and where the oxygen in TA-OH comes from has to await further investigation.

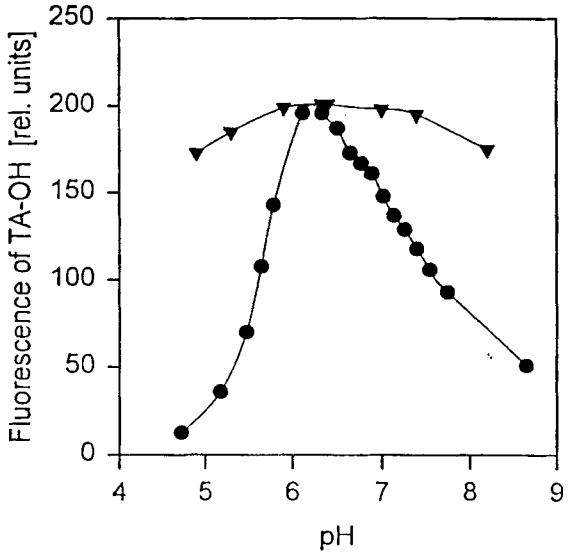


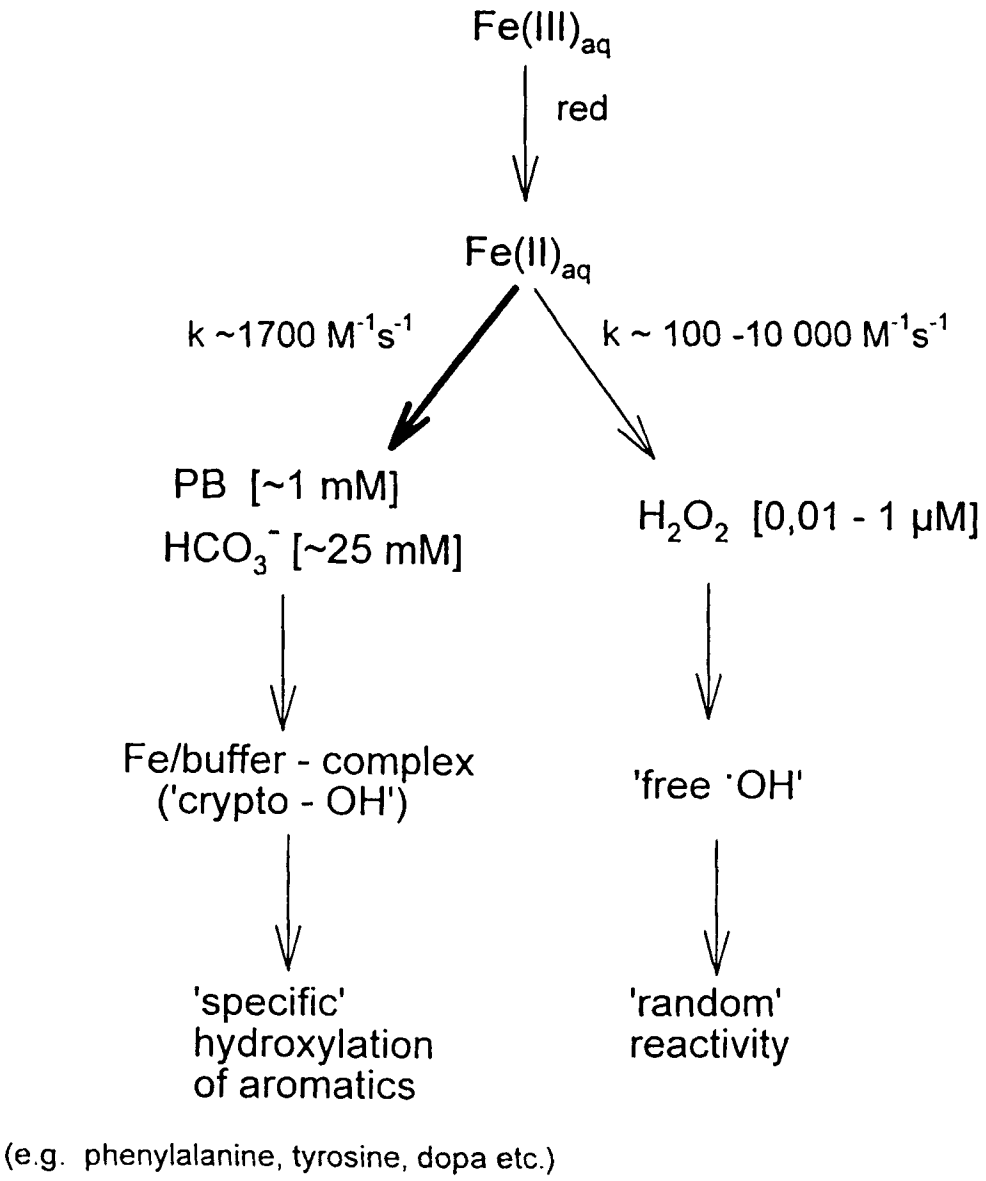
FIGURE 4 Hydroxylation of TA by "crypto-OH" (see text) strongly depends on pH whereas hydroxylation by "free" OH is almost independent of pH. The curve for free OH (triangles) was obtained by gamma-irradiation of TA-containing 50mM phosphate buffer; that for crypto-OH (circles) by injecting boli of 8 μM $FeSO_4$ into PB containing 460 μM TA; pH was adjusted by mixing Na_2HPO_4 and NaH_2PO_4

Biological consequences of iron complex formation

Reactive oxygen species (ROS) are increasingly discussed in the context of being involved in signalling processes, e.g. during apoptosis,[47] in the *inter*cellular induction of apoptosis,[48] the generation of signals by cells that have been treated with oxidants or UV-light,[49] the transmittance of signals between irradiated and unirradiated cells,[50,51,52,53] *extra*cellularly manifest responses to *intra*cellular ROS generation,[54,55] and *intra*cellularly manifest responses to *intra-* or *extra*cellularly generated ROS.[56,57] Furthermore it is increasingly appreciated that proliferation and differentiation of cells is connected to the oxidant state of cells[58,59,60] and the functioning of so called "iron switches" is thought to be correlated to hydrogen peroxide or other molecules involved in oxygen metabolism.[61,62,63]

In most of these cases mechanistic details about the interaction between ROS and the iron are not exactly known, some hypotheses favor the interaction of superoxide radicals with iron-sulfur aggregates, e.g. in the case of aconitase[64] acting as iron responsive element IRE. Other authors prefer the interaction of reduced iron with H_2O_2, i.e. Fenton-type reactions, to be the decisive determinant for the biological result.

We want to point out, however, that the idea of reduced iron interacting with an elevated level of H_2O_2 after it is released, e.g. from the ferritin store, cannot be an adequate explanation for understanding the interaction between both species. The assumption that iron remains "naked" for some period of time is certainly not justified even though it has been repeatedly shown that there is a "chelatable" iron pool[65] which is accessible by a variety of chelators. In fact, we are forced to consider that the chemistry occurring must be subject to the reaction probabilities that pertain to the local environment where iron appears on the scene, i.e. the actual composition of cellular and interstitial fluids. According to Figure 5, panel a, free hydroxyl radicals clearly



SCHEME 2 Fe(III), after being reduced to Fe(II) by a physiological reductant, may enter two pathways. The probability of each pathway depends on the abundance of the respective reaction partner and the respective reaction rate. Clearly the left reaction pathway leading to complexes which are able to hydroxylate aromatic compounds is dramatically favored over the reaction with hydrogen peroxide that generates "randomly" -and hence unspecifically- acting free hydroxyl radicals. (Note that besides TA, which was used as a test system, also physiological substrates such as aromatic amino acids are hydroxylated by crypto-OH (data not shown))

may be generated when Fe(II) encounters a hydrogen peroxide molecule, i.e. the Fenton reaction *may* occur. The chance for this to happen, however, is in competition with the crypto-OH-yielding reaction of Fe(II) with phosphate and/or bicarbonate which are present in

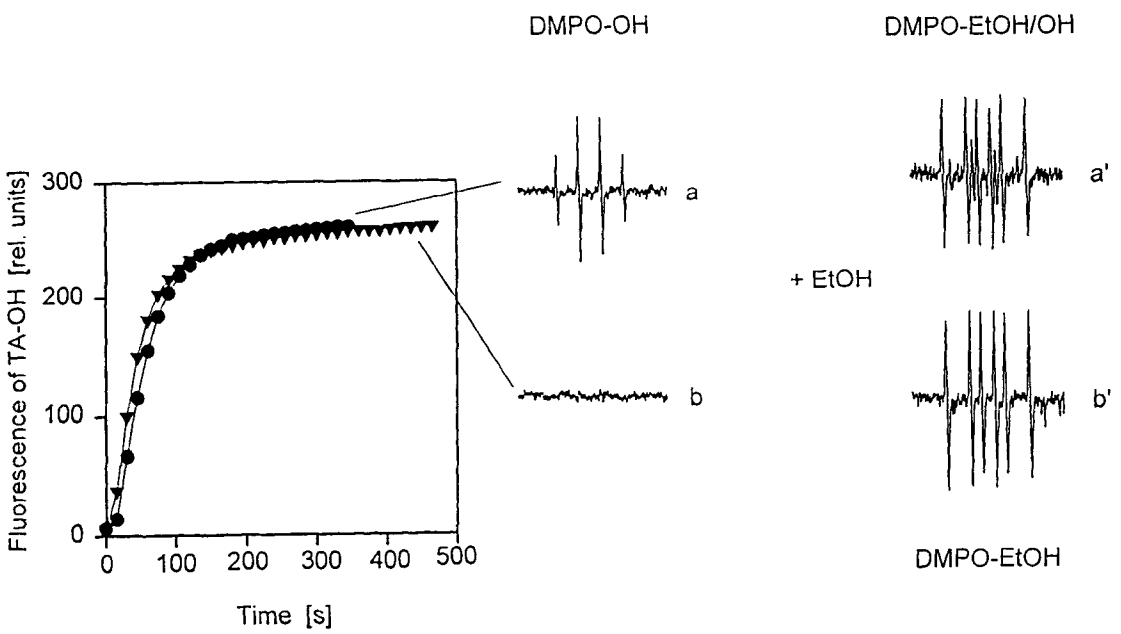


FIGURE 5 In contrast to "free" hydroxyl radicals "crypto-OH" does not produce DMPO-identifiable species. It only does so after "translation" of its oxidative capacity by ethanol. Left part: Boli of $FeSO_4$ (resulting in 20 μM end concentration) were injected in 50 mM PB, pH 7, containing 20 μM H_2O_2 (circles), or PB without any additive (triangles). Right part: Trace *a*: DMPO signal resulting from the Fe(II)/PB/H_2O_2 system; trace b shows the absence of any signal in the case of Fe(II)/PB. Traces a′ and b′ refer to the respective cases when 100 mM ethanol was additionally present. (Coupling constants of EPR signals were $a_N = a_H = 14.9$ G (trace a) and $a_N = 15.9$ G and $a_H = 23.1$ G (trace b′). (DMPO concentration in each case was 23 mM)

1 mM and 25 mM concentration, respectively. As a result, the two possible pathways outlined in Scheme 2 must be operative. From the time course displayed in Figure 3 follows that the overall process of TA-hydroxylation in physiological buffer proceeds to the half-maximal end concentration within about 30 seconds. The sequence consists of two steps, formation of the hydroxylating crypto-complex followed by the actual hydroxylation of TA. The second step, when depending on OH radicals generated by radiation, is known to proceed at a rate close to the diffusion-controlled limit,[20,23] and we assume that the analogous reaction by crypto-OH may also be rather fast. Hence formation of the Fe/buffer complex is the rate-determining step which sets the limits for the overall process. Estimating from the half-value in Figure 3 of about 30 seconds a reaction rate in the order of 1700 $M^{-1}s^{-1}$, we calculate a k*c product of 42.5 s^{-1} as a measure for the probability of Fe(II) to react with buffer molecules (k is the above rate constant of 1700 $M^{-1}s^{-1}$ and c the buffer concentration taken as 25×10^{-3} M, hence $k*c = 1700 \times 25 \times 10^{-3} = 42.5\ s^{-1}$). To arrive at an equal k*c-product, i.e. reaction probability, for the reaction of Fe(II) with H_2O_2, a hydrogen peroxide concentration of close to one molar(!) would have to be present to effectively compete with the buffer, when taking a rate constant close to 100 $M^{-1}s^{-1}$ [34] for the reaction of *uncomplexed* iron with H_2O_2. Allowing for the fact that reaction rates for Fenton reactions generally rise by several orders of magnitude when the catalytic metal is chelated, the concentration of H_2O_2 being necessary to compete with the buffer would still have to be in the millimolar range, i.e. at an unreasonably high steady state level which

would result in manifold "overkill" of any cell by this toxic agent.

CONCLUDING REMARKS

Admittedly there is a wealth of data implicating effects of hydrogen peroxide when cells become enriched in this reactive agent during oxidative stress. Furthermore a host of biological consequences of such conditions have been clearly shown to be related to metal catalysis. However, seeking an explanation for the generation of biological signals from H_2O_2/metal interactions by invoking iron-catalyzed generation of OH radicals by Fenton-type processes may be erroneous. The data presented suggest that any reduced iron in the physiological milieu will immediately be complexed by ubiquitous buffer anions and react in a *similar* way as does free OH, e.g. hydroxylate aromatic compounds, yet bypass H_2O_2 for genuine Fenton chemistry to occur. We are presently at a loss for a reasonable mechanistic explanation for the biological effects of hydrogen peroxide itself. We do suggest, however, that the molecular action mechanism of iron(II) must be independent of freely diffusible H_2O_2. Especially when considering that hydroxylated aromatic compound usually are effectors with high pharmacological activity (e.g. phenylalanine < tyrosine < dopa) buffer-promoted hydroxylations of biomolecules that are part of intra- or intercellular signalling pathways might be a better explanation for the bioactivity of iron at low concentration than invoking reactions of randomly acting OH radicals. Only at extremely high (local?) concentrations of H_2O_2 we may assume that an interaction of H_2O_2 with iron may be relevant for explaining "oxidative stress".

Interestingly, analogous conclusions concerning the minor importance of the Fenton reaction under *in vivo* conditions were recently derived by Qian and Buettner[66] who demonstrated that at concentration ratios $[O_2]/[H_2O_2] > 100$, which are to be assumed in almost all cellular compartments, pre-existing hydrogen peroxide is a negligible contributor to biological free radical oxidations and some hitherto not exactly identified "Fe-O" complex was suggested to outcompete hydrogen peroxide-dependent reaction pathways.

References

[1] C. Walling (1975) Fenton's reagent revisited. *Accounts of Chemical Research*, 8, 125–131.

[2] G. Cohen, P.M. Sinet (1980) Fenton's reagent – once more revisited. In: *Chemical and Biochemical Aspects of O_2^- and SOD*. (eds J.V. Bannister, H.A.O. Hill) Elsevier/North-Holland, New York, pp 27–37.

[3] S. Goldstein, D. Meyerstein, G. Czapski (1993) The Fenton Reagents. *Free Radical Biology and Medicine*, **15**, 435–445.

[4] W.H. Koppenol (1993) The Centennial of the Fenton Reaction. *Free Radical Biology and Medicine*, **15**, 645–651.

[5] C.C. Winterbourn (1995) Toxicity of iron and hydrogen peroxide: The Fenton reaction. *Toxicology Letters*, **82**, 969–974.

[6] P. Wardman, L.P. Candeias (1996) Fenton chemistry: An introduction. *Radiation Research*, **145**, 523–531.

[7] J.M.C. Gutteridge (1993) Free radicals in disease processes – A compilation of cause and consequence. *Free Radical Research Communications*, **19**, 141–158.

[8] R.J. Youngman, E.F. Elstner (1981) Oxygen species in paraquat toxicity: the crypto-OH radical. *FEBS-Letters*, **129**, 265–268.

[9] B.H.J. Bielski (1991) Studies on hypervalent iron. *Free Radical Research Communications*, **12–13**, 469–477.

[10] J.D. Melton, B.H.J. Bielski (1990) Studies of the kinetic, spectral and chemical properties of Fe(IV) pyrophosphate by pulse radiolysis. *Radiation Physics and Chemistry*, **36**, 725–733.

[11] B.H.J. Bielski (1992) Reactivity of hypervalent iron with biological compounds. *Annals of Neurology*, **32**, 28–32.

[12] B.H.J. Bielski (1990) Generation of iron(IV) and iron(V) complexes in aqueous solutions. *Methods in Enzymology*, **186**, 108–112.

[13] V. Dorovska-Taran, M.A. Posthumus, S. Boeren, M.G. Boersma, C.J. Teunis, I.M.C.M. Rietjens, G. Veeger (1998) Oxygen exchange with water in heme-oxo intermediates during H_2O_2-driven oxygen incorporation in aromatic hydrocarbons catalyzed by microperoxidase-8. *European Journal of Biochemistry*, **253**, 659–668.

[14] J.H. Dawson (1988) Probing structure function relations in heme-containing oxygenases and peroxidases. *Science*, **240**, 433–439.

[15] L.A. Reinke, J.M. Rau, P.B. McCay (1994) Characteristics of an oxidant formed during iron (II) autoxidation. *Free Radical Biology and Medicine*, **16**, 485–492.

[16] Y. Manevich, K.D. Held, J.E. Biaglow (1997) Coumarin-3-carboxylic acid as a detector for hydroxyl radicals generated chemically and by gamma radiation. *Radiation Research*, **148**, 580–591.

[17] A.V. Kachur, S.W. Tuttle, J.E. Biaglow (1998) Autoxidation of ferrous ion complexes: A method for the genera-

tion of hydroxyl radicals. *Radiation Research*, **150**, 475–482.

[18] M. Saran, G. Vetter, M. Erben-Russ, R. Winter, A. Kruse, C. Michel, W. Bors (1987) Pulse radiolysis equipment: a setup for simultaneous multiwavelength kinetic spectroscopy. *Reviews of Scientific Instruments*, **58**, 363–368.

[19] R.W. Matthews (1980) The radiation chemistry of the terephthalate dosimeter. *Radiation Research*, **83**, 27–41.

[20] X.W. Fang, G. Mark, C. von Sonntag (1996) OH radical formation by ultrasound in aqueous solutions. 1. The chemistry underlying the terephthalate dosimeter. *Ultrasonincs and Sonochemistry*, **3**, 57–63.

[21] J.C. Barreto, G.S. Smith, N.H.P. Strobel, P.A. McQuillin, T.A. Miller (1994) Terephthalic acid: A dosimeter for the detection of hydroxyl radicals in vitro. *Life Sciences*, **56**, PL89-PL96.

[22] T.J. Mason, J.P. Lorimer, D.M. Bates, Y. Zhao (1994) Dosimetry in sonochemistry: the use of aqueous terephthalate ion as a fluorescence monitor. *Ultrasonics and Sonochemistry*, **1**, S91-S95.

[23] M. Saran, K.-H. Summer (1999) Assaying for hydroxyl radicals: Hydroxylated terephthalate is a superior fluorescence marker than hydroxylated benzoate. *Free Radical Research*, **31**, 429–439.

[24] G.R. Buettner, L.W. Oberley (1978) Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5.5-dimethyl-1-pyrroline-1-oxide. *Biochemical Biophysical Research Communications*, **83**, 69–74.

[25] J.E. Biaglow, A.V. Kachur (1997) The generation of hydroxyl radicals in the reaction of molecular oxygen with polyphosphate complexes of ferrous ion. *Radiation Research*, **148**, 181–187.

[26] L.A. Reinke, D.R. Moore, J.M. Rau, P.B. McCay (1995) Inorganic phosphate promotes redox cycling of iron in liver microsomes: Effects on free radical reactions. *Archives of Biochemistry and Biophysics*, **316**, 758–764.

[27] B. Halliwell, J.M.C. Gutteridge (1992) Biologically relevant metal ion-dependent hydroxyl radical generation. An update. *FEBS-Letters*, **307**, 108–112.

[28] D.T. Sawyer, A. Sobkowiak, T. Matsushita (1996) Metal [ML(x); M=Fe, Cu, Co, Mn]/hydroperoxide-induced activation of dioxygen for the oxygenation of hydrocarbons: Oxygenated Fenton chemistry. *Accounts of Chemical Research*, **29**, 409–416.

[29] P.A. MacFaul, D.D.M. Wayner, K.U. Ingold (1998) A radical account of "oxygenated Fenton chemistry". *Accounts of Chemical Research*, **31**, 159–162.

[30] C. Walling (1998) Intermediates in the reactions of Fenton type reagents. *Accounts of Chemical Research*, **31**, 155–157.

[31] J.D. Rush, Z. Maskos, W.H. Koppenol (1990) Distinction between hydroxyl radical and ferryl species. *Methods in Enzymology*, **186**, 148–156.

[32] W.H. Koppenol, Z. Maskos, J.D. Rush (1991) Catalysis of oxyradical reactions by iron complexes. In: Oxidative Damage & Repair: Chemical, Biological and Medical Aspects. (ed K.J.A. Davies). Pergamon Press, Oxford, pp 814–819.

[33] E. Luzzatto, H. Cohen, C. Stockheim, K. Wieghardt, D. Meyerstein (1995) Reactions of low valent transition metal complexes with hydrogen peroxide. Are they "Fenton-like" or not? 4. The case of Fe(II)L, L=EDTA: HEDTA and TCMA. *Free Radical Research*, **23**, 453–463.

[34] H. Gallard, J. de Laat, B. Legube (1998) Influence du pH sur la vitesse d'oxydation de composés organiques par Fe^{II}/H_2O_2. Mécanismes réactionnels et modélisation. *New Journal of Chemistry*, 263–268.

[35] I.L. Yurkova, H.-P. Schuchmann, C. von Sonntag (1999) Production of OH radicals in the autoxidation of the Fe(II)-EDTA system. *Journal of the Chemical Society, Perkin Transactions* **2**, 2049–2052.

[36] S. Seibig, R. van Eldik (1997) Kinetics of [Fe(II)(edta)] oxidation by molecular oxygen revisited. New evidence for a multistep mechanism. *Inorganic Chemistry*, **36**, 4115–4120.

[37] S. Seibig, R. van Eldik (1999) Multistep oxidation kinetics of [Fe-II(Cdta)] [cdta = N,N', N'',N'''-(1,2-cyclohexanediamine)tetraacetate] with molecular oxygen. *European Journal of Inorganic Chemistry*, 447–454.

[38] R.E. White (1991) The involvement of free radicals in the mechanisms of monooxygenases. *Pharmacology and Therapy*, **49**, 21–42.

[39] G.A. Hamilton (1974) Chemical models and mechanisms for oxygenases. In: *Molecular Mechanisms of Oxygen Activation.* (ed. O. Hayaishi). Academic Press, New York, pp 405–451.

[40] S. Udenfriend, G. Cardinale, G. (1982) α-Ketoglutarate coupled dioxygenases. In: *Oxygenases and Oxygen Metabolism.* (eds. M. Nozaki, S. Yamamoto, Y. Ishimura, M.J. Coon, L. Ernster, R.W. Estabrook). Academic Press, New York, pp 99–110.

[41] E.F. Elstner (1990) *Der Sauerstoff. Biochemie. Biologie, Medizin.* Wissenschaftsverlag, Mannheim (and references therein).

[42] D.H.R. Barton, D.K. Taylor (1996) Models for non-heme oxidation enzymes. *Pure and Applied Chemistry*, **68**, 497–504.

[43] A.P. Sobolev, D.E. Babushkin, E.P. Talsi (1996) Formation of low-spin peroxoiron(III) complexes in Gif-type catalytic systems. *Mendeleev Communications*, **1**, 33–34.

[44] M.J. Perkins (1996) A radical reappraisal of GIF reactions. *Chemical Society Reviews*, 229–236.

[45] E.N. Jacobsen (1995) Transition metal-catalyzed oxidations: asymmetric epoxidation. *Comprehensive Organometallic Chemistry*, **12**, 1097–1135.

[46] R.V. Lloyd, P.M. Hanna, R.P. Mason (1997) The origin of the hydroxyl radical oxygen in the Fenton reaction. *Free Radical Biology and Medicine*, **22**, 885–888.

[47] S. Clutton (1997) The importance of oxidative stress in apoptosis. *British Medical Bulletin*, **53**, 662–668.

[48] G. Bauer (1996) Elimination of transformed cells by normal cells: A novel concept for the control of carcinogenesis. *Histology Histopathology*, **11**, 237–255.

[49] K. Bender, C. Blattner, A. Knebel, M. Lordanov, P. Herrlich, H.J. Rahmsdorf (1997) UV-induced signal transduction. *Journal of Photochemistry and Photobiology. B:Biol.*, **37**, 1–17.

[50] B.E. Lehnert, E.H. Goodwin (1997) Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. *Cancer Research*, **57**, 2164–2171.

[51] C. Mothersill, C. Seymour (1997) Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology*, **71**, 421–427.

[52] J.B. Little, C. Li, H. Nagasawa, T. Pfenning, H. Vetrovs (1996) Genomic instability and radiation mutagenesis. *Journal de Chimie Physique et de Physicochimie Biologique*, **93**, 157–164.

[53] P.K. Narayanan, E.H. Goodwin, B.E. Lehnert (1997) Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Research*, **57**, 3963–3971.

[54] P. Brenneisen, J. Wenk, L.O. Klotz, M. Wlaschek, K. Briviba, T. Krieg, H. Sies, K. Scharffetter-Kochanek (1998) Central role of ferrous/ferric iron in the ultraviolet B irradiation-mediated signaling pathway leading to increased interstitial collagenase (matrix-degrading metalloprotease (MMP)-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts. *Journal of Biological Chemistry*, **273**, 5279–5287.

[55] L.E. Crawford, E.E. Milliken, K. Irani, J.L. Zweier, L.C. Becker, T.M. Johnson, N.T. Eissa, R.G. Crystal, T. Finkel, P.J. Goldschmidt-Clermont (1996) Superoxide mediated actin response in post-hypoxic endothelial cells. *Journal of Biological Chemistry*, **271**, 26863–26867.

[56] M. Sundaresan, Z.X. Yu, V.J. Ferrans, D.J. Sulciner, J.S. Gutkind, K. Irani, P.J. Goldschmidt-Clermont, T. Finkel (1996) Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochemical Journal*, **318**, 379–382.

[57] L. Moldovan, K. Irani, N.I. Moldovan, T. Finkel, P.J. Goldschmidt-Clermont (1999) The actin cytoskeleton reorganization induced by Rac1 requires the production of superoxide. *Antioxidants and Redox Signaling*, **1**, 29–43.

[58] R.H. Burdon (1995) Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radicals in Biology and Medicine*, **18**, 775–794.

[59] M. Saran, C. Michel, W. Bors (1998) Radical functions in vivo: A critical review of current concepts and hypotheses. *Zeitschrift fuer Naturforschung C*, **53c**, 210–227.

[60] D.R. Crawford, G.P. Schools, K.J.A. Davies (1996) Oxidant-inducible adapt15 RNA is associated with growth arrest- and DNA damage-inducible gadd153 and gadd45. *Archives of Biochemistry and Biophysics*, **329**, 137–144.

[61] M. Sundaresan, Z.X. Yu, V.J. Ferrans, K. Irani, T. Finkel (1995) Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science*, **270**, 296–299.

[62] K. Irani, P.J. Goldschmidt-Clermont (1998) Ras, superoxide and signal transduction. *Biochemical Pharmacology*, **55**, 1339–1346.

[63] K. Pantopoulos, S. Mueller, A. Atzberger, W. Ansorge, W. Stremmel, M.W. Hentze (1997) Differences in the regulation of iron regulatory protein-1 (IRP-1) by extra- and intracellular oxidative stress. *Journal of Biological Chemistry*, **272**, 9802–9808.

[64] P.R. Gardner, I. Fridovich (1992) Inactivation-reactivation of aconitase in Escherichia coli. *Journal of Biological Chemistry*, **267**, 8757–8763.

[65] R.J. Rothman, A. Serroni, J.L. Farber (1992) Cellular pool of transient ferric iron, chelatable by deferoxamine and distinct from ferritin, that is involved in oxidative cell injury. *Molecular Pharmacology*, **42**, 703–710.

[66] S.Y. Qian, G.R. Buettner (1999) Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: an Electron Paramagnetic Resonance spin trapping study. *Free Radical Biology and Medicine*, **26**, 1447–1456.