THE CHEMISTRY OF PEROXYNITRITE, A BIOLOGICAL TOXIN*

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Oxyradicals play a role in several diseases. While for several decades the hydroxyl radical - produced *via* the Fenton reaction - has been considered the species that initiates oxyradical damage, new findings suggest that much of this damage can be ascribed to peroxynitrite, O=NOO$^-$, formed from the reaction of the superoxide anion with nitrogen monoxide near activated macrophages. The rate constant for the reaction of this reaction has been investigated by flash photolysis and was found to be significantly higher than previously described in the literature, 1.9 x 10^{10} $M^{-1}s^{-1}$. Studies of the isomerization to nitrate resulted in the discovery of a complex between peroxynitrite and its protonated form with a stability constant of 1 x 10^4 M^{-1}. Some of the harmful reaction of peroxynitrous acid have been ascribed to the hydroxyl radical as a product of homolysis of the O-O bond during the conversion to nitrate. Kinetics of the isomerization reaction as a function of pressure show that the activation volume is only +1.5±1.0 ml mol^{-1}, which is inconsistent with homolysis. Instead, an intermediate, possibly a distorted *trans*-isomer of O=NOOH could be responsible for the harmful reactions of peroxynitrite.

Keywords: peroxynitrite; nitrogen monoxide; superoxide; Fenton reaction; oxyradical damage; stopped-flow spectrophotometry; flash photolysis.

INTRODUCTION

Radicals are believed to play a role in many diseases. Some examples are: brain injury, reperfusion injury, iron overload, rheumatoid arthritis, cancer, asbestosis, and paraquat toxicity. One may ask the question: What are radicals? There is the official International Union of Pure and Applied Chemistry definition, namely: "Radicals are atoms or molecules with one or more unpaired electrons", and there is the definition found in the popular press, to wit: "Radicals are promiscuous molecules that react with anything that passes by". In the following I hope to show that this definition is not correct, that is, the word radical does not necessarily imply extreme reactivity.

Because of the discovery of superoxide dismutase in 1969 by McCord and Fridovich [1], it was argued that superoxide was a reactive species: if it was not toxic, we would not need an enzyme. Superoxide was already known for some time to radiation chemists, and they argued that superoxide is at best a mild reductant. A possible solution to this dilemma appeared to be two reactions proposed by Haber and Wilstätter [2] in a publication on radical chain mechanisms:

$$HO^\bullet + H_2O_2 = H_2O + O_2^{\bullet -} \quad (1)$$

$$O_2^{\bullet -} + H_2O_2 = O_2 + H_2O + HO^\bullet \quad (2)$$

In fact, the ability of superoxide to reduce hydrogen peroxide was considered a fundamental property of this radical [2]. These two reactions became known later as the Haber-Weiss cycle [3]. If these two reactions are important, then superoxide dismutase would protect by removing superoxide, so that hydroxyl radicals are not formed. George showed already in 1947 that the second reaction is too slow in comparison to the disproportionation of superoxide[4]. Nevertheless, a number of groups, unaware of George's work (see also[5-7], set out in the early seventies to determine the rate constant of Reaction 2, and all concluded, like George, that the rate constant was of the order of 1 $M^{-1}s^{-1}$, or smaller[8-14]. The recent suggestion[15] that Reaction 2 is significant and that the dioxygen is produced in the $^1\Delta_g$ singlet state must be rejected as erroneous on kinetic and thermodynamic grounds. In the seventies it was argued that if Reaction 2 is too slow, iron could catalyze it. The suggestion of iron as a catalyst was reasonable, as a human contains approximately four grams of iron, of which most is tied up in ferritin, haemoglobin, myoglobin, and various iron containing electron-transfer proteins, such as the iron-sulfur proteins and the cytochromes. The small amount of iron in transit between the various pools could catalyze Reaction 2, as follows[16]:

$$O_2^{\bullet -} + LFe^{3+} \rightarrow O_2 + LFe^{2+} \quad (3)$$

$$LFe^{2+} + H_2O_2 \rightarrow HO^\bullet + HO^- + LFe^{3+} \quad (4)$$

where L indicates a ligand. The sum of Reactions 3 and 4 is Reaction 2. Reaction 4, the one-electron reduction of hydrogen peroxide by iron(II) is known as the Fenton reaction.

THE FENTON REACTION

The reactions of iron(II) with hydrogen peroxide and with hypochlorite were described by Fenton in 1876[17], and the text of the original communication is reproduced here in part:

Chemical News **33**, 190 (1876)

On a New Reaction of Tartaric Acid

To the Editor of the Chemical News

I HAVE lately noticed the following reaction, which, besides presenting one or two rather interesting peculiarities, may as far as I can judge at present, be proposed as a test for tartaric acid. To a very dilute solution of ferrous sulphate or chloride,

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a small quantity of a solution of tartaric acid or a tartrate is added, followed by a few drops of chlorine water or hydrogen peroxide, and lastly, excess of caustic potash or soda, when a fine violet colour is obtained.

I have tried.....

H. J. H. FENTON

Fenton's discovery was a case of serendipity: he saw a student mix reagents at random[18]. Had there not been a violet colour, then, in all likelihood, there would not have been a Fenton reaction. It must be stressed that Fenton did not know the mechanism of the reaction that has been named after him. Hydroxyl radicals in context with the Fenton reaction were discussed in the fifties, more than twenty years after Fenton's death in 1929[18].

While Reactions 3 and 4 are now dogma for the origin of oxidative damage, there is little or no direct proof for the involvement of iron *in vivo*, except in the case of iron overload. Most importantly, no iron complex has been identified *in vivo* that participates in Reactions 3 and 4. If the nature and the amount of these iron complexes are unknown, then it is not known how fast they are reduced by superoxide and oxidized by hydrogen peroxide. This is important, because we have shown that the rate constants are influenced by the coordination of the iron: both reactions proceed by inner-sphere electron transfer[19]. Some examples are given in Table 1.

Table 1. Rate Constants for the Fenton Reaction [19]

Complex of Fe(II) with:	k, $M^{-1}s^{-1}$
diethylenetriamine-*N,N,N',N",N"*-pentaacetate	8.0×10^2
ethylenediamine-*N,N,N',N'*-tetraacetate	7.0×10^3
(*N*-hydroxyethyl)ethylenediamine-*N,N',N'*-tetraacetate	4.2×10^4
nitrilotriacetate	3.0×10^4
ethylenediamine-*N,N'*-diacetate	7.8×10^4
adenosinetriphosphate	6.6×10^3
adenosinediphosphate	1.1×10^4
citrate	4.9×10^3

For some complexes the rate constant for the reduction of iron(III) by superoxide, Reaction 3, is of the order of 10^6 $M^{-1}s^{-1}$ [20]. While this is fast, it is still three orders of magnitude slower than the reaction of superoxide with superoxide dismutase. As to concentrations, superoxide dismutase is intracellularly present at micromolar concentrations, and that of iron is similar, based on the bleomycin assay[21]. If the iron - hydrogen peroxide mechanism described above is responsible for oxidative damage, then the role of superoxide dismutase is difficult to understand, because the Fenton reaction is not inhibited by superoxide dismutase. This enzyme would inhibit the reduction of iron(III)complexes, but there are other cellular reductants available, such as ascorbate.

If the Fenton theory of oxyradical damage is unsatisfactory, then we must find a new one that still explains the footprints left behind by radicals, namely 8-hydroxydeoxyguanosine when DNA is damaged, lipid hydroperoxides in the case of lipids and increased carbonyl content of proteins. Furthermore, aromatic hydroxylation and spin adduct formation have been observed. As an example, the hydroxylation of salicylate is taken as evidence of hydroxyl radical formation[22]. However, one should be careful, because *in vivo* studies show that 2,5-dihydroxybenzoate is present in vast excess over 2,3-dihydroxy-benzoate, while *in vitro* studies on the reaction of the hydroxyl radical with salicylate yield 2,3-dihydroxybenzoate and 2,5-dihydroxybenzoate in a ratio of 5:1[23]. An explanation was provided by Ingelman-Sundberg *et al*. who showed that the reaction of microsomal cytochrome P450 with salicylate yields 2,5-dihydroxybenzoate[24]. Therefore, the presence of this metabolite should *not* be taken as evidence for the formation of the hydroxyl radical *in vivo*! That still leaves 2,3-dihydroxybenzoate as afootprint: if the Fenton reaction is not important, then a new oxidizing species must yield this product.

In summary, the use of iron in biology presents us with a paradox: on the one hand it is necessary for oxygen transport by haemoglobin and myoglobin and for electron transfer by the cytochromes and the iron-sulfur proteins. On the other hand, an excess of iron is clearly toxic. However, it would be wrong to ascribe all radical damage to the presence of catalytic amounts of iron. As shown above, this is for kinetic reasons most unlikely.

NITROGEN MONOXIDE

Recently an alternative to the the iron catalyzed formation of hydroxyl radicals has emerged. It was found that *in vivo* nitrogen monoxide - in the literature also known by the outdated name of nitric oxide[25] - is formed as a product of the oxidation of arginine by oxygen, catalyzed by the enzyme nitric oxide synthase. Nitrogen monoxide (i) acts as a messenger when it forms a nitrosyl complex with the haem of guanylate cyclase and initiates the formation of cyclic GMP, and (ii) functions in the immunological response by producing a potent oxidant, peroxynitrite. In many aspects this oxidant creates the same damage as the hydroxyl radical. Before discussing the properties of peroxynitrite, the side reactions of nitrogen monoxide should be mentioned:

$$Hb(II)O_2 + NO^{\bullet} \rightarrow Hb(III) + NO_3^- \quad (5)$$

$$O_2 + 2NO^{\bullet} \rightarrow 2NO_2^{\bullet} \quad (6)$$

$$RSH + NO^{\bullet} \rightarrow RSNO + H^+ + e^- \quad (7)$$

The last reaction requires an electron acceptor; in all likelyhood a nitrosation is carried out by an oxidation product of nitrogen monoxide, dinitrogen trioxide. Regarding Reaction 6, it can easily be shown that the reaction of nitrogen monoxide with oxygen proceeds very slowly under *in vivo* conditions. The reaction is second order in nitrogen monoxide, and first order in oxygen. Given a rate constant of 2×10^6 $M^{-2}s^{-1}$ [26] and cellular concentrations of 10 nanomolar nitrogen monoxide and 10 micromolar dioxygen, the half-life of nitrogen monoxide amounts to 10^6 s[27]! The reaction of nitrogen monoxide with haemoglobin, Reaction 5, accounts for its short biological half-life of 5 s. Our interest is in the formation and properties of peroxynitrite, Reaction 8:

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow O{=}NOO^- \quad (8)$$

which takes place near activated macrophages, and which may be the major oxidant formed. Peroxynitrite itself has not been detected *in vivo*. As will be discussed below, peroxynitrite nitrates phenolic compounds, and nitrotyrosine has been identified in inflamed tissues[28,29].

PEROXYNITRITE

Formation from superoxide and nitrogen monoxide: Superoxide reacts very fast with nitrogen monoxide to form the peroxynitrite anion, the rate is approximately 5×10^9 $M^{-1}s^{-1}$ [30-32]. We recently redetermined this rate and obtained a much higher rate constant by way of laser flash photolysis (R. Kissner, T. Nauser, P. Bugnon, P. G. Lye and W.H. Koppenol, in preparation). Solutions containing 0.1-1 mM peroxynitrite at

alkaline pH were irradiated at 266 and 355 nm with at a pulse width of 10 ns at room temperature. Kinetic analysis was initiated 1 μs after the pulse as the kinetics during the first microsecond may be influenced by cage effects and excited states. Irradiation resulted in bleaching of peroxynitrite and the appearance of an unstable product at 245 nm (Figure 1) with an extinction coefficient of approximately 2.4×10^3 $M^{-1}cm^{-1}$. After the flash, it decayed in a second-order process with a rate constant of 1.9×10^{10} $M^{-1}s^{-1}$, with simultaneous restoration of the peroxynitrite absorption. Given the similarity in spectrum (Figure 2) and extinction coefficient at 245 nm of the product to that of superoxide[33], we conclude that this product is indeed superoxide. Thus, photolysis of peroxynitrite leads to formation of superoxide an nitrogen monoxide.

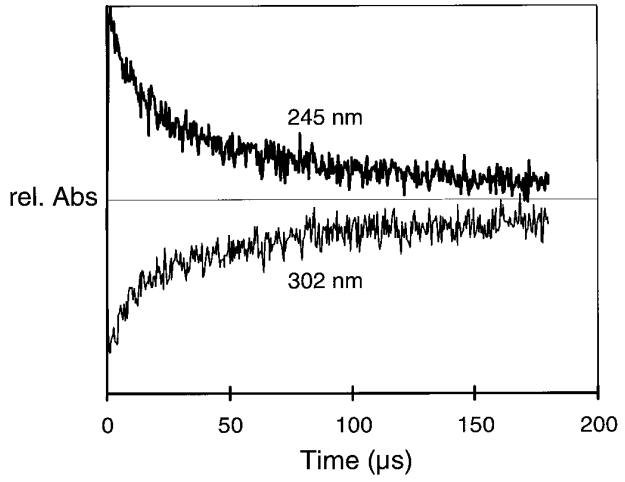

Figure 1. *Absorbance changes of a 100 μM peroxynitrite solution after irradiation with a 10 ns, 10 mJ laser pulse at 355 nm. At 300 nm (thin line) and at 245 nm (thick line) changes in the peroxynitrite and superoxide concentrations, respectively, are monitored. The traces show that the decay of superoxide and the appearance of peroxynitrite are correlated. From this it is inferred that the other photolysis product is nitrogen monoxide (Nauser, Kissner, Bugnon, Lye and Koppenol, in preparation).*

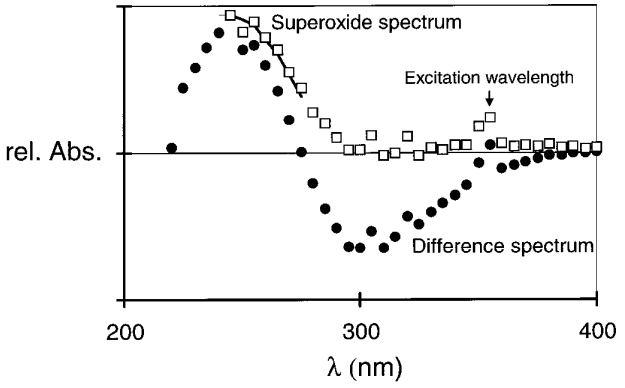

Figure 2. *Spectral changes upon photolysis of an alkaline solution of peroxynitrite. The spectrum of superoxide, characterized by a maximum at 245 nm is obtained. At the same time an equivalent amount of peroxynitrite has disappeared (Nauser, Kissner, Bugnon, Lye and Koppenol, in preparation).*

The rate constant of 1.9×10^{10} $M^{-1}s^{-1}$ is 3 to 4 times higher than those reported by other workers.[30-32] These rate constants reported in those studies were measured under experimental designs that involve reaction cascades to arrive at the reactants. The rapid one-step production of superoxide and nitrogen monoxide by flash photolysis has the advantage of allowing measurement of the recombination rate not complicated by other processes.

Thermodynamic properties: Before embarking on a discussion of the reactivity of this interesting molecule, it would be helpful to determine its thermodynamic properties. An enthalpy of formation of -10.8 kcal for $ONOO^-$ has been determined by Ray[34], a value that we recently confirmed[35]. Its absolute entropy is unknown. Taking the difference between the absolute entropies of the radical species $ONOO^{\Uparrow}$ and $NO_3^{\Uparrow}$ and applying that to the absolute entropy of nitrate, one estimates a value of 45 cal/(K·mol) for S·($ONOO^-$). A $\Delta_f G\cdot$ of +10 kcal/mol for the peroxynitrite anion can now be calculated[36]. Recenty, the estimate for the absolute entropy of the peroxynitrite anion has been criticised and an absolute entropy of 15 eu has been proposed (Merényi, 1997, personal communication). However, such a value implies an entropy of solvation for the peroxynitrite anion that seems unreasonably large, 55 e.u., rather than 25 e.u. implied here. On the basis of the 45 cal/(K·mol) for S·($ONOO^-$) mentioned above one calculates that peroxynitrite is an oxidizing species: E·($ONOO^-$, $2H^+$/ $NO_2^{\Uparrow}$, H_2O) is 1.4 V at pH 7, and that it is unstable with respect to disproportionation to nitrogen dioxide and the nitrosyldioxyl radical, $ONOO^{\Uparrow}$[36].

The ionization of peroxynitrous acid. The pKa of ONOOH has been reported as 6.8. It does not vary with temperature, from which it was concluded that the enthalpy of ionization is close to 0 kcal/mol[36]. Recently it has been discovered that the pKa is influenced by other solutes (R. Kissner, T. Nauser, P. Bugnon, P. G. Lye and W.H. Koppenol, in preparation). Extrapolated to zero ionic strength one finds that the pKa is 6.5. The value of 6.8 is valid in a buffer of 0.1 M phosphate buffer, but in a 0.5 M phosphate buffer one finds a value of over 7. Compound that form addducts with peroxynitrite, such as borate buffer move the pKa to values over 8, see Fig. 3.

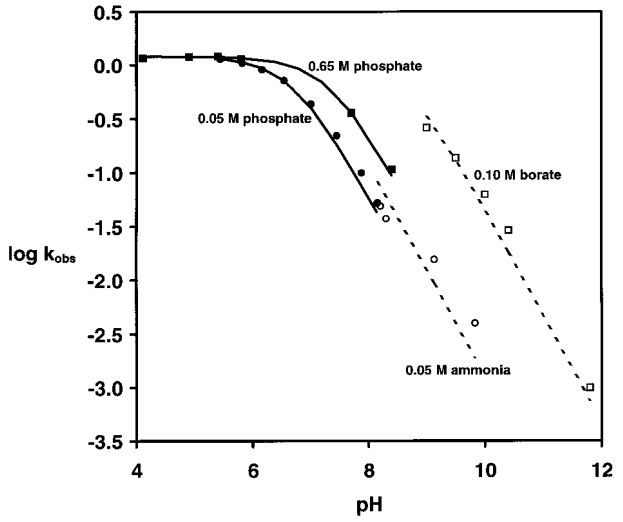

Figure 3. *Influence of solutes on the pKa of proxynitrous acid. Increasing concentrations of phosphate buffer shift the pKa to higher values. Ammonia and borate may form adducts with the peroxynitrite anion (Nauser, Kissner, Bugnon, Lye and Koppenol, in preparation).*

The isomerization of peroxtnitrous acid: An alkaline solution of peroxynitrite is yellow with a maximum absorption at 302 nm. It is stable for a couple of hours at room temperature. The rate of isomerization of peroxynitrous acid to nitric acid is 1.2 s^{-1} at 25 °C (R. Kissner, T. Nauser, P. Bugnon, P. G. Lye and W.H. Koppenol, in preparation), which is within the error of the measurement the same as the 1.3 s^{-1} reported before[36]. This isomerization reaction is strictly first-order in peroxynitrous acid, and has an activation enthalpy of 18 kcal mol^{-1}. The isomerization has been studied as a function of pressure and an activation volume of 1.5 cm^3 mol^{-1} was determined.

At pH values between 7 and 8.5 and at total peroxynitrite concentrations of more than 0.1 mM, the decay of peroxynitrite does not follow first-order kinetics, see Figure 4. Upon dilution first-order kinetics are observed again. Nitrite contributes somewhat to non-first-order behaviour, but, given the concentration required, is not the causal agent. The hypothesis that peroxynitrite forms an adduct with peroxynitrous fits the observations. A stability constant of 1 x 10^4 M has been determined (Nauser, Kissner, Bugnon, Lye and Koppenol, in preparation). It has recently been reported that the decay of peroxynitrite also yields nitrite and dioxygen[37]. The conditions under which these products have been observed match closely those where adduct formation is prevalent.

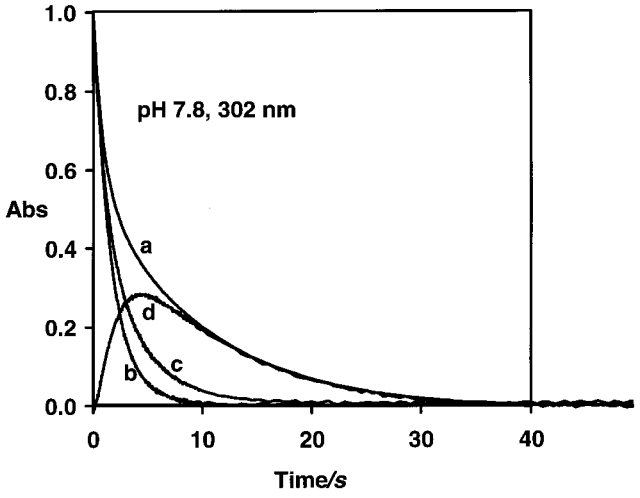

Figure 4. *Decay of peroxynitrite above pH = 7. Absorbance changes were recorded at 302 nm and at pH 7.9. a: c_{tot} = 0.48 mM; b: peroxynitrite absorbance x 10, c_{tot} = 0.048 mM; c: peroxynitrite absorbance x 10, c_{tot} = 0.048 mM, nitrite concentration 4.2 mM; d: Difference trace of a and b. At concentrations abouve -0.1 mM peroxynitrite and at pH above the pKa peroxynitrite may form and adduct with its protonated form that delays the decay (Nauser, Kissner, Bugnon, Lye and Koppenol, in preparation).*

Reactive Intermediate. During the isomerization a reactive intermediate is formed that can nitrate and hydroxylate phenolic compounds[38,39], such as tyrosine and salicylate; these reactions presumably proceed by an initial one-electron oxidation of the phenolic compound[40]. These reactions are zero-order in the phenolic compound, which shows that the formation of the reactive intermediate is rate-limiting. It has been suggested [41] that this reactive intermediate is the hydroxyl radical, formed by homolyis of the O-O bond in O=NOOH. This is not feasible for thermodynamic and kinetic reasons. Knowing that the homolysis reaction is endothermic by 21 kcal/mol, one can calculate the rate constant of the homolyis reaction, see Figure 5, because the rate constant for the reaction of the hydroxyl radical with nitrogen dioxide is known, 5 x 10^9 $M^{-1}s^{-1}$ [42]. The calculated 10^{-8} - 10^{-6} s^{-1} is much smaller than the experimentally observed rate constant of 1.2 s^{-1} for the isomerization to nitrate. Thus, peroxynitrous acid is not a source of hydroxyl radicals. However, if the value of 15 e.u. mentioned earlier is correct, then the revised Gibbs energy of formation of peroxynitrite **does** allow the formation of hydroxyl radicals. Experimentally, the activation volume of 1.5 cm^3 mol^{-1} is not compatible with a simple homolysis. Such reactions typically have activation volumes of 10 cm^3 mol^{-1} [43].

If no hydroxyl radicals are formed, then the question arises as to how peroxynitrous acid acts as a strongly oxidizing agent. Peroxynitrite is present in solution in the *cis*-form [44], see Fig. 6.

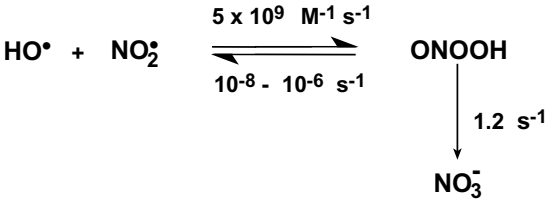

Figure 5. *Scheme to calculate the rate of homolysis of peroxynitrous acid. Based on the rate of peroxynitrous acid formation from nitrogen dioxide and the hydroxyl radical and the Gibbs energy change of formation of peroxynitrous acid from these reactants, the rate of homolysis is orders of magnitude lower than the rate of isomerization.*

Figure 6. *Isomers of peroxynitrite. Quantum mechanical calculations indicate that the N-peroxide-O bond has considerable double bond character.*

While only a single bond is drawn between nitrogen and the first peroxide oxygen, quantum mechanical calculations show that the bond has considerable double bond character. The energy barrier between the *cis* and *trans* forms amounts to approximately 25 kcal/mol for the anion, and approximately 12 kcal/mol for the protonated form. The *cis*-form of the anion is 3-4 kcal/mol more stable than the *trans*-form [45]. Since the nitration and hydroxylation of phenolic compounds take place with the same rate constant as the isomerization and do not depend on the concentration of the phenolic compound, it was concluded that the same intermediate is involved. It is this intermediate, presumed to be a twisted form of *trans*-peroxynitrite whereby the O-O bond is lengthened and the NOO angle is smaller than 90·, that is thought to be responsible for deleterious reactions such as the nitration of tyrosines [36].

Nitration by Peroxynitrite. The acid-base indicator phenolphtalein can be used to demonstrate that peroxynitrous acid nitrates pheolic compounds near neutral pH. When a few drops of a 1% phenolphtalein solution in ethanol are added to a yellow alkaline solution of peroxynitrite, the colour changes to that of pinkish red, the colour of the indicator in its deprotonated form. Upon addition of sodium hydrogencarbonate, the solution does not become colourless, as expected for protonated phenolphtalein, but yellow again. Disappearance of the pinkish red colour indicates that the pH is 8.1 or lower; under these conditions peroxynitrite decays within seconds. Thus, the yellow colour is not due to peroxynitrite. The fact that the yellow colour persists shows that the indicator has been nitrated.

The nitration reaction is catalyzed by some metal complexes, such as Fe(III)edta[40,46]. Surprisingly, it was found that Cu/Zn superoxide dismutase is capable of catalyzing the nitration of a phenolic compound[46]. This observation led to a hypothesis: In 25% of all cases of familial amyotrophic lateral sclerosis there is a mutation in the Cu/Zn superoxide dismutase[40]. Over 25 different mutations have been described sofar. It has been suggested that any of these mutations makes the copper of superoxide dismutase more accessible to peroxynitrite, so that it becomes a better nitration catalyst[47]. Beckman and coworkers have developed an antibody against nitrotyrosine, and with this antibody they showed that nitrotyrosines are formed near activated macrophages in arteriosclerotic tissues[48] and in human myocardial inflammation[49]. Certain kinases that have tyrosines

that become phosphorylated as part of the normal enzymatic cycle would be inactivated most efficiently by peroxynitrite.

Bimolecular Reactions. Peroxynitrite or peroxynitrous acid reacts with compounds that are thermodynamically easier to oxidize in a bimolecular fashion. A partial list of such compounds is shown in Table 2.

Table 2. Rate Constants for Peroxynitrite Reactions with small molecules.

Compound	k, $M^{-1}s^{-1}$	Ref.
cysteine	5.0×10^3	52
methionine	9.1×10^2	53
ascorbate	2.5×10^2	54
iodide	2.3×10^4	55
Nickel cyclam	3.2×10^4	55
tryptophan	1.1×10^2	56,57
ebselen	2.0×10^6	50
carbon dioxide	3×10^4	58

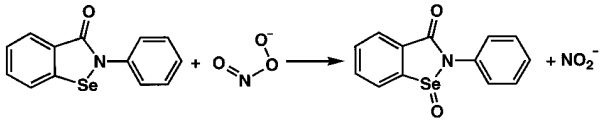

Figure 7. *The reaction with ebselen is the fastest reaction of the peroxynitrite anion sofar, 2.0 x 10^6 $M^{-1}s^{-1}$ [50].*

The highest rate constant, 2.0 x 10^6 $M^{-1}s^{-1}$ was found for the oxygen transfer from peroxynitrite to ebselen at 25· C^{50}:

Interesting is also that it is the anion that reacts, not peroxynitrous acid. It is hoped that this drug, or other compounds with an even higher reactivity, may be beneficial in diseases where peroxynitrite plays a role.

The role of superoxide dismutase. We can now return to the question: Why is superoxide dismutase necessary? Figure 8 may explain this.

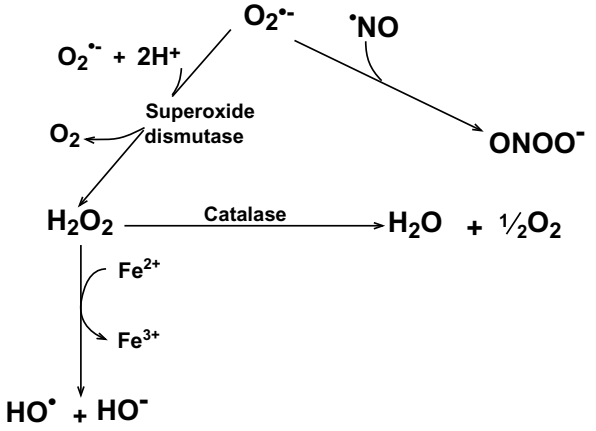

Figure 8. *Superoxide dismutase and nitrogen monoxide compete for superoxide.*

Under normal conditions the amount of superoxide dismutase is sufficient to channel all superoxide towards the dismutation products dioxygen and hydrogen peroxide. The product of the rate constant of superoxide with superoxide dismutase (2.4 x 10^9 M^{-1} s^{-1} [51]) with the superoxide dismutase concentration (approx. 10 μM) gives a rate of superoxide disappearance of 2 x 10^4 s^{-1}. Normal nitrogen monoxide concentrations are in the nanomolar range, say 10 nM. This concentration, multiplied with the rate constant of 5 x 10^9 M^{-1} s^{-1} for the reaction of nitrogen monoxide with superoxide, yields a rate of disappearance of 50 s^{-1}, much smaller than the 2 x 10^4 s^{-1} calculated above. Near activated macrophages the situation is different. With an estimated nitrogen monoxide concentration of 10 μM the rate of peroxynitrite formation is now 1 x 10^4 s^{-1}, which is half the rate of superoxide disappearance through superoxide dismutase. We must conclude that superoxide dismutase cannot prevent the formation of peroxynitrite near activated macrophages! Obviously, these crude approximations do not apply to the heterogeneous milieu inside or outside a cell, but they do indicate that under normal conditions the role of superoxide dismutase may be to inhibit the formation of peroxynitrite.

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REFERENCES

1. McCord, J. M.; Fridovich, I.; *J. Biol. Chem.* **1969**, *244*, 6049.
2. Haber, F.; Willstätter, R.; *Chem. Ber.* **1931**, *64*, 2844.
3. Haber, F.; Weiss, J.; *Proc.Roy.Soc.* **1934**, *147*, 332.
4. George, P.; *Discussions Faraday Soc.* **1947**, 196.
5. Barb, W. G.; Baxendale, J. H.; George, P.; Hargrave, K. R.; *Trans. Faraday Soc.* **1951**, *47*, 591.
6. Barb, W. G.; Baxendale, J. H.; George, P.; Hargrave, K. R.; *Trans. Faraday Soc.* **1951**, *47*, 462.
7. Barb, W. G.; Baxendale, J. H.; George, P.; Hargrave, K. R.; *Trans. Faraday Soc.* **1955**, *51*, 935.
8. Ferradini, C.; Seide, C.; *Int. J. Radiat. Phys. Chem.* **1969**, *1*, 219.
9. Melhuish, W. H.; Sutton, H. C.; *J. C. S. Chem. Commun.* **1978**, 970.
10. Koppenol, W. H.; Butler, J.; van Leeuwen, J. W.; *Photochem. Photobiol.* **1978**, *28*, 655.
11. Czapski, G.; Ilan, Y. A.; *Photochem.Photobiol.* **1978**, *28*, 651.
12. Rigo, A.; Stevanato, R.; Finazzi-Agro, A.; Rotilio, G.; *FEBS Lett.* **1977**, *80*, 130.
13. Weinstein, J.; Bielski, B. H. J.; *J. Am. Chem. Soc.* **1979**, *101*, 58-62.
14. Bors, W.; Michel, C.; Saran, M.; *FEBS Lett.* **1979**, *107*, 403.
15. Khan, A. U.; Kasha, M.; *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 12365.
16. McCord, J. M.; Day, E. D. Jr; *FEBS Lett.* **1978**, *86*, 139.
17. Fenton, H. J. H.; *Chem. News.* **1876**, *33*, 190
18. Koppenol, W. H.; *Free Radical Biol.Med.* **1993**, *15*, 645.
19. Koppenol, W. H.; *Chemistry of iron and copper in radical reactions*. In: *Free Radical Damage and its Control*; Rice-Evans, C. A.; Burdon, R. H. Eds., Elsevier Science B.V. Amsterdam, 1994; pp 3-24.
20. Butler, J.; Halliwell, B.; *Arch.Biochem.Biophys.* **1982**, *218*, 174.
21. Gutteridge, J. M. C.; Rowley, D. A.; Halliwell, B.; *Biochem. J.* **1981**, *199*, 263.
22. Grootveld, M.; Halliwell, B.; *Biochem. J.* **1986**, *237*, 499.
23. Maskos, Z.; Rush, J. D.; Koppenol, W. H.; *Free Radical Biol.Med.* **1990**, *8*, 153.
24. Ingelman-Sundberg, M.; Kaur, H.; Terelius, Y.; Persson, J.-O.; Halliwell, B.; *Biochem. J.* **1991**, *276*, 753.

25. Koppenol, W. H.; *Nature* **1994**, *367*, 28
26. Ford, P. C.; Wink, D. A.; Stanbury, D. M.; *FEBS Lett.* **1993**, *326*, 1-3.
27. Koppenol, W. H.; *The chemistry and biochemistry of oxidants derived from nitric oxide*. In: *Nitric Oxide and Radicals in the Pulmonary Vasculature*; Weir, E. K.; Archer, S. L.; Reeves, J. T. Eds., Futura Publishing Co., Inc. Armonk, NY, 1996; pp 355.
28. Beckman, J. S.; Ye, Y. Z.; Anderson, P. G.; Chen, J.; Accavitti, M. A.; Tarpey, M. M.; White, C. R.; *Biol. Chem. Hoppe-Seyler* **1994**, *375*, 81.
29. Ye, Y. Z.; Strong, M.; Huang, Z. Q.; Beckman, J. S.; *Meth.Enzymol.* **1996**, *269*, 201
30. Huie, R. E.; Padmaja, S.; *Free Radical Res. Commun.* **1993**, *18*, 195.
31. Goldstein, S.; Czapski, G.; *Free Radic. Biol. Med.* **1995**, *19*, 505.
32. Kobayashi, K.; Miki, M.; Tagawa, S.; *J. Chem. Soc. Dalton Trans.* **1995**, 2885.
33. Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; *J.Phys.Chem.Ref.Data* **1985**, *14*, 1041.
34. Ray, J. D.; *J. Inorg. Nucl. Chem.* **1962**, *24*, 1159.
35. Manuszak, M.; Koppenol, W. H.; *Thermochim. Acta* **1996**, *273*, 11.
36. Koppenol, W. H.; Moreno, J. J.; Pryor, W. A.; Ischiropoulos, H.; Beckman, J. S.; *Chem. Res. Toxicol.* **1992**, *5*, 834.
37. Pfeiffer, S.; Gorren, A. C. F.; Schmidt, K.; Werner, E. R.; Hansert, B.; Bohle, D. S.; Mayer, B.; *J. Biol. Chem.* **1997**, *272*, 3465.
38. Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S.; *Arch. Biochem. Biophys.* **1992**, *298*, 431.
39. Van der Vliet, A.; O'Neill, C. A.; Halliwell, B.; Cross, C. E.; Kaur, H.; *FEBS Lett.* **1994**, *339*, 89.
40. Ramezanian, M. S.; Padmaja, S.; Koppenol, W. H.; *Chem. Res. Toxicol.* **1996**, *9*, 232.
41. Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A.; *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1620.
42. Logager, T.; Sehested, K.; *J. Phys. Chem.* **1993**, *97*, 6664.
43. Asano, T.; le Noble, W. J.; *Chem. Rev.* **1978**, *78*, 407.
44. Tsai, J. -H. M.; Harrison, J. G.; Martin, J. C.; Hamilton, T. P.; van der Woerd, M.; Jablonsky, M. J.; Beckman, J. S.; *J. Am. Chem. Soc.* **1994**, *116*, 4115.
45. Tsai, H. H.; Hamilton, T. P.; Tsai, J. H. M.; van der Woerd, M.; Harrison, J. G.; Jablonsky, M. J.; Beckman, J. S.; Koppenol, W. H.; *J. Phys. Chem.* **1996**, *100*, 15087.
46. Beckman, J. S.; Ischiropoulos, H.; Zhu, L.; van der Woerd, M.; Smith, C. D.; Chen, J.; Harrison, J.; Martin, J. C.; Tsai, M.; *Arch.Biochem.Biophys.* **1992**, *298*, 438.
47. Beckman, J. S.; Carson, M.; Smith, C. D.; Koppenol, W. H.; *Nature* **1993**, *364*, 584
48. Beckman, J. S.; Ye, Y. Z.; Anderson, P. G.; Chen, J.; Accavitti, M. A.; Tarpey, M. M.; White, C. R.; *Biol. Chem. Hoppe Seyler* **1994**, *375*, 81.
49. Kooy, N. W.; Lewis, S. J.; Royall, J. A.; Ye, Y. Z.; Kelly, D. R.; Beckman, J. S.; *Crit. Care Med.* **1997**, *25*, 812.
50. Masumoto, K.; Kissner, R.; Koppenol, W. H.; Sies, H.; *FEBS Lett.* **1996**, *398*, 179.
51. Fielden, E. M.; Roberts, P. B.; Bray, R. C.; Lowe, D. J.; Mautner, G. N.; Rotilio, G.; Calabrese, L.; *Biochem. J.* **1974**, *139*, 49.
52. Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A.; *J. Biol. Chem.* **1991**, *266*, 4244.
53. Pryor, W. A.; Jin, X.; Squadrito, G. L.; *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11173.
54. Bartlett, D.; Church, D. F.; Bounds, P. L.; Koppenol, W. H.; *Free Radical Biol.Med.* **1995**, *18*, 85.
55. Goldstein, S.; Czapski, G.; *Inorg. Chem.* **1995**, *34*, 4041-4048.
56. Padmaja, S.; Ramezanian, M. S.; Bounds, P. L.; Koppenol, W. H.; *Redox Reports* **1996**, *2*, 173.
57. Alvarez, B.; Rubbo, H.; Kirk, M.; Barnes, S.; Freeman, B. A.; Radi, R.; *Chem. Res. Toxicol.* **1996**, *9*, 390.
58. Lymar, S. V.; Hurst, J. K.; *J. Am. Chem. Soc.* **1995**, *117*, 8867.