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REVIEW

Peroxynitrite: *In Vivo* and *In Vitro* Synthesis and Oxidant Degradative Action on Biological Systems Regarding Biomolecular Injury and Inflammatory Processes

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This review summarizes all significant data regarding peroxynitrite chemistry, the ways of its synthetic preparation as well as the degradative action of this species on biomolecules, in particular glycosaminoglycans, among which the hyaluronan degradation by peroxynitrite has recently been the subject of greater interest than ever before. The complex chemical behavior of a peroxynitrite molecule is strongly influenced by a few factors; conformational structural forms, active intermediates release, presence of CO_2 and trace transition metals, different reaction conditions, as well as the rules of kinetics. Special attention was focused on monitoring of the kinetics of the degradative action of peroxynitrite in or without the presence of residual hydrogen peroxide on high-molar-mass hyaluronan.

Keywords: peroxynitrite, biosynthesis, synthesis, pathophysiological aspects, polymer degradation

INTRODUCTION

Over the last decade, several review papers have been published on peroxynitrite, the reaction product of nitric oxide radical ($^{\bullet}NO$) and superoxide anion radical ($O_2^{\bullet -}$), a mediator of cellular and tissue injury in various pathological situations by its oxidative and nitrative pathways. The review paper by *Murphy et al.* [1] offers comments on peroxynitrite properties and *in vivo* formation in biological systems. *Pacher et al.* [2] demonstrate peroxynitrite generation *in vivo* and further interaction with lipids, DNA, and proteins *via* direct oxidative reactions or indirect radical-mediated mechanisms which result in pathophysiological events (stroke, myocardial infarction, diabetes, chronic heart failure, circulatory shock, chronic inflammatory diseases, cancer).

Ducrocq et al. [3] gathered, over a few years, all important facts regarding peroxynitrite biosynthesis, synthesis, its oxidation and nitration reactions as well as reactions with various antioxidants. The paper by *Evgenov* and *Liaudet* [4] demonstrates peroxynitrite as one of the reactive oxygen and nitrogen species potentially inducing tissue damage during systemic inflammatory response and circulatory shock. Also the review paper by *Kamat* [5] deals with biological significance of peroxynitrite, its precursors, reactions, as well as with cytotoxic aspects such as formation of 3-nitrotyrosine, "biomarker" of the peroxynitrite reaction with a given protein, which was identified in various pathologies such as Lou Gehrig's and Parkinson's diseases, cancer, atherosclerosis, and in biological aging. The role of peroxynitrite in neurodegenerative inflammatory disorders leading to the development of the Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, and the Parkinsonism dementia complex of Guam is reflected on in the review paper by *Torreilles et al.* [6]. An extensive study

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was carried out to elucidate a possible role of peroxynitrite in the course of excitotoxic events evoked by quinolinic acid in the brain [7]. *Brookes et al.* [8] described in detail the role of peroxynitrite in brain mitochondria. Extra- or intramitochondrial formation of peroxynitrite, its diffusion through mitochondrial compartments causing alteration in mitochondrial energy and calcium homeostasis; its fast, direct, and free radical-dependent target molecule reactions resulting in oxidation, nitration, and nitrosation of inner and outer membrane and intermembrane space; its fate, as well as signaling of cell death were reported by *Radi et al.* [9].

The preferential protein targets of peroxynitrite and the role of proteins in peroxynitrite detoxifying pathways are discussed in the review paper by *Alvarez* and *Radi* [10]. Possible mechanisms of peroxynitrite reaction pathways conferring to the proteins (hemoglobin, myoglobin, cytochrome *c*) and, especially, its scavenging role were discussed by *Pietraforte et al.* [11]. Peroxynitrite as a potent cytotoxin plays a key role in the pathogenesis of cardiovascular dysfunction leading to progression of diabetic retinopathy, neuropathy, and nephropathy by attacking various biomolecules in vascular endothelium, vascular smooth muscle, and myocardium [12]. Peroxynitrite impairment of cardiovascular functions by activation of matrix metalloproteinases and nuclear enzyme poly(ADP-ribose) polymerase (PARP) is discussed offering novel emerging therapeutic strategies [13], taking into account that its toxicity is given by the ability to trigger activation of PARP-1 [4]. Peroxynitrite plays a key role in the pathogenesis of doxorubicin (DOX)-induced cardiac failure. A new cardioprotective strategy may be represented after the DOX exposure targeting the peroxynitrite formation because peroxynitrite is a major mediator of myocardial injury [14].

Reduction of the oxygen molecule is one of the main reactions by which animal cells, including the human ones, produce metabolic energy

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \qquad (A)$$

The substrate (O_2) is, by a cascade of enzymatically driven reactions, reduced within subcellular organelles, mitochondria, to a completely harmless substance, water. Along with this four-electron reaction, several specialized cells, more precisely their specific (sub)cellular structures, reduce O_2 molecules, producing the superoxide anion radical ($O_2^{\bullet -}$)

$$O_2 + 1e^- \rightarrow O_2^{\bullet -} \qquad (B)$$

which in aqueous (acidic) milieu can form the perhydroxyl radical ($^{\bullet}O_2H$)

$$O_2^{\bullet -} + H^+ \rightarrow {}^{\bullet}O_2H \qquad (C)$$

Since the reverse reaction represents the dissociation of a weak acid of the perhydroxyl radical, its pK_a value of 4.8 and the pH value of the aqueous milieu govern the actual mole ratio between the two forms, *i.e.* between $O_2^{\bullet -}$ and $^{\bullet}O_2H$. Under slight acidosis accompanying the inflammation processes, *e.g.* at pH 6.8, the mole ratio of $n(O_2^{\bullet -})/n(^{\bullet}O_2H)$ equals 99.

Nitrogen monoxide ($^{\bullet}NO$), a (bioactive) free radical, is produced in various cells/tissues by the enzyme NO synthase. The level of $^{\bullet}NO$ increases markedly during inflammation, it is a process accompanied by abundant production of the superoxide anion radical [15].

The nitric oxide synthases (NOS) are a group of enzymes (EC 1.14.13.39) first identified by Furchgott in 1980. They are responsible for the synthesis of nitric oxide from the terminal nitrogen atom of L-arginine in the presence of O_2 and the cofactors nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, tetrahydrobiopterin (BH_4). Different forms of NO synthase have been classified as follows: neuronal NOS (nNOS or NOS1: $^{\bullet}NO$ production in neuronal tissue in both the central and the peripheral nervous system), inducible NOS (iNOS or NOS2: found in both the immune and the cardiovascular system), endothelial NOS (eNOS or NOS3), and constitutive NOS (cNOS: $^{\bullet}NO$ generation in blood vessels, also involved in regulating vascular function) [16].

The two radical intermediates, $O_2^{\bullet -}$ and $^{\bullet}NO$, serve as precursors of various reactive oxygen species (ROS), including hydrogen peroxide, peroxynitrite, hypochlorous acid, *etc.* On respiring air, approximately 1—3 % of ingested oxygen is assigned to the generation of ROS that defends the organism against viral/bacterial invaders. In some cases, however, the intermediate and/or the "final" reactive oxidative species may also damage cells/tissues of the human host. Imbalance between the extent of damage and self-repair of functionally essential structures may result in a broader host tissue injury, eventually leading to a specific disease.

There are numerous diseases, the pathology of which involves reactive oxidative/oxygen-derived species at the onset and/or at later stages of the disease. One of the classes of such diseases includes arthritic conditions, *e.g.* inflammatory diseases of joints. Substantial evidence exists of increased generation of oxidants in patients suffering from acute and chronic inflammatory joint diseases [15]. The oxidants also contribute to the pathophysiology of myocardial ischemia reperfusion, heart failure, circulatory shock, stroke, atherosclerosis, neurodegenerative disorders, diabetes, and diabetic complications [2, 4, 9, 12—14].

Protein oxidation and nitration are important features of peroxynitrite-induced biomolecular injury as well as the reactions of peroxynitrite with aliphatic

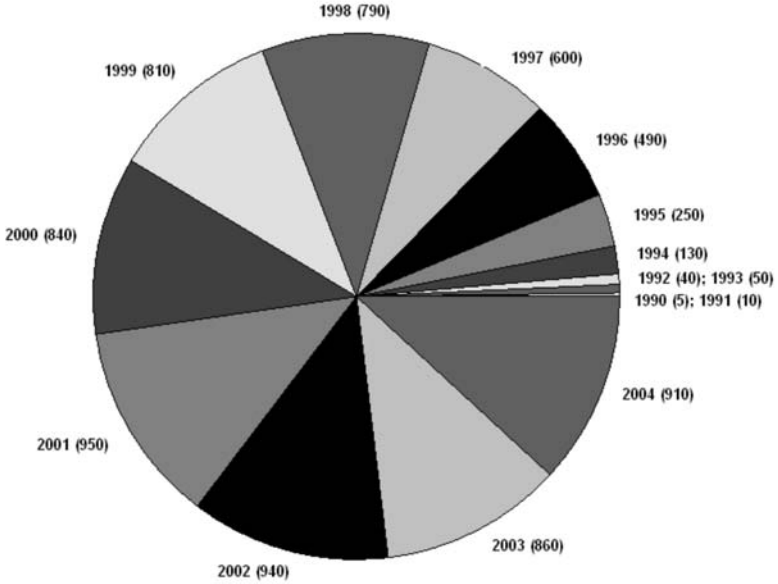


Fig. 1. Overview of the number of papers (in the parentheses) published with the research topic "peroxynitrite" per year.

aldehydes and ketones are important in modulation of biomolecular damage.

PEROXYNITRITE: HISTORICAL ASPECTS AND PROPERTIES

Toxicology was greatly revolutionized by the hypothesis of *in vivo* peroxynitrite formation resulting in an intense investigation by biochemists, radical, and inorganic chemists. This process is reflected in a number of publications. According to "SciFinder", starting from 1990 the number of publications significantly increased and in 2001—2004 their number reached the level of *ca.* 900 manuscripts per year [17] (Fig. 1). Based on the data available in "PubMed", in 2006 over 3000 peroxynitrite-related papers were published [18].

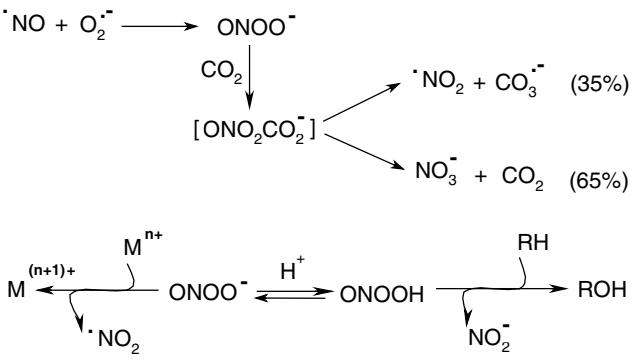
Peroxynitrite (peroxonitrite, less commonly named hydroperoxide), $ONOO^-$, is a reactive species, a potent harmful oxidant known since 1904 [19] and a major cytotoxic agent, the first known peroxyacid [20]. Abel's formula for "pernitrous acid" was acid nitrosyl peroxide ($NO^+HO_2^-$) [21]. *Petriconi* and *Papee* [22] suggested a simple peroxy formula of the type H—O—O—N═O. The recommended IUPAC nomenclature for peroxynitrite is oxoperoxonitrate and for peroxynitrous acid it is hydrogen oxoperoxonitrate. The term oxoperoxonitrate/oxoperoxynitrate (1-) [23—27] was first suggested by *Beckman et al.* [28]. Peroxynitrite formation is characterized by a second order rate constant, the value of which (3.7×10^7 M^{-1} s^{-1}, 4.3×10^7 M^{-1} s^{-1}, and 6.7×10^7 M^{-1} s^{-1} [23, 24, 29], respectively) depends on the preparation method used. Professor Pryor is a pioneer chemist in investigating the role of free radicals and other oxidants, including peroxynitrite, in health and disease [30].

Peroxynitrite is relatively stable in alkaline solution (pH > 12, half-life time $t_{1/2} \geq 10^5$ s), whereas at physiological pH, peroxynitrous acid ($pK_a = 6.8$) rapidly isomerizes/decomposes to nitrate (NO_3^-) with $t_{1/2} = 10$ s at 1 °C [19, 25, 31, 32] or 0.53 s at 25 °C [33]. The decomposition rate constant of 1.3 s^{-1} at 25 °C was reported in [23], while the value of 0.6 s^{-1} was calculated for the reaction occuring in a phosphate buffer solution at 37 °C [31]. *Pryor* and *Squadrito* [34] reported $t_{1/2} = 1$ s at pH 7 and 37 °C. Peroxynitrite anion can be preserved in a strongly basic media at −18 °C for weeks [35]. Peroxynitrite decays spontaneously with $t_{1/2}$ of approximately 1 s at 37 °C and a pseudo-first-order rate constant of 38 min^{-1} [36], or 1.9 s^{-1} at pH 7.4 [37]. At pH 7.4, 80 % of dissolved peroxynitrite could be found in its anionic form [27]. Peroxynitrite is a mild oxidant with the reduction potential of 0.44 V, whereas that of $^{\bullet}NO$ is 0.39 V [28, 38]. The redox potential at pH 7.0 of the nitrosodioxyl radical ($ONOO^{\bullet}$) and the peroxynitrite anion ($ONOO^-$) is 0.43 V [39]. *Goldstein et al.* [33] reported $E^0(ONOO^{\bullet}/ONOO^-) = 0.80$ V. Peroxynitrite is a relatively "long-living" toxic agent under physiological conditions [39] capable to diffuse away from the site of its formation and reach the critical targets [23, 28, 38]. The peroxynitrite anion is relatively non-reactive, however, its complexation with metal ions leads to formation of reactive species responsible for either oxidation or rapid degradation processes rearranging it to NO_3^- anion [40].

Biosynthesis of Peroxynitrite Anion

The Role of Nitric Oxide

Peroxynitrite is generated *in vivo* by a fast radical



Scheme 1

—radical recombination reaction of a superoxide anion radical ($O_2^{\bullet -}$) and a free nitric oxide radical ($^{\bullet}NO$)

$$^{\bullet}N{=}O + {}^{\bullet}O{-}O^{-} \rightarrow O{=}N{-}O{-}O^{-} \qquad (D)$$

In vivo nitric oxide diffuses within seconds to a distance of over 100 μm through tissues to enter red blood cells and react with oxohemoglobin [41]. *Al-Ajlouni* and *Gould* [35] reported the rate constant $k = 6 \times 10^9$ M^{-1} s^{-1} of rapid coupling/binding of the two odd-electron diatomic species of $^{\bullet}NO$ and $O_2^{\bullet -}$. It is a near-diffusion-controlled/limited process [32, 42, 43].

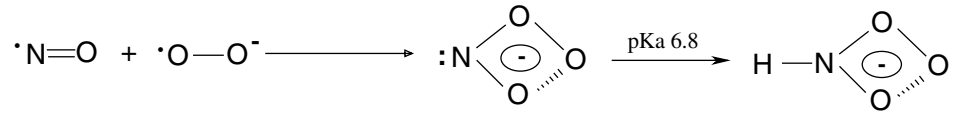
The nitric oxide radical, a peroxynitrite precursor, is a biologically ubiquitous molecule involved in many responses of chondrocytes which can generate it along with the superoxide anion radical [44]. $^{\bullet}NO$ is synthesized by a wide variety of cell types, including macrophages, vascular endothelial cells, neutrophils, hepatocytes, phagocytes, and neurons and it is a major participant in several physiological functions such as blood pressure regulation [37, 38]. Neutrophils and macrophages generate $^{\bullet}NO$ radical *via* an L-arginine-dependent pathway [29]. Polymorphonuclear leukocytes, which contain myeloperoxidase, generate both nitric oxide and superoxide anion radicals forming peroxynitrite by their recombination [45]. As demonstrated by *Mazzetti et al.* [46], the osteoarthritis-affected chondrocytes are a probably more important source of $^{\bullet}NO$ than the rheumatoid arthritis-affected cartilage. $^{\bullet}NO$ is known as a regulator of cell survival and death [47].

Large quantities of $^{\bullet}NO$ are produced by the inducible form of nitric oxide synthase enzyme in neutrophils and monocytes upon inflammatory stimulation [48—51]. Measurements of nitrate and nitrite concentration in human synovial fluid certify the presence of $^{\bullet}NO$ synthesized in arthritic joints. Sodium nitroprusside, which liberates $^{\bullet}NO$ in solution, reduces the viscosity of human synovial fluid [52]. Interaction between $^{\bullet}NO$ and $O_2^{\bullet -}$ may dictate the type of mutagenic reactions taking place at sites where both these free radicals are produced [29]. Nitric oxide, a small lipophilic molecule [52], is a powerful vascular and neural regulator [53] and, unlike peroxynitrite, is neither a strong oxidant nor a nitrating agent [27]. $^{\bullet}NO$ was shown to suppress proteoglycan synthesis [44].

Biomolecular Pathways of Peroxynitrite Anion and the Role of Carbon Dioxide

Peroxynitrite is a strong oxidizing species which becomes protonated at physiological pH to form peroxynitrous acid (ONOOH) [30, 54], a precursor of the toxic hydroxyl radical ($^{\bullet}OH$), however, its $^{\bullet}NO$ group exhibits a strong electron-withdrawing effect [19]. It exists in the protonation equilibrium with a conjugate acid ONOOH/ONOOH* (pK_a of peroxynitrous acid 6.6—6.8) which is unstable and undergoes, at physiological pH, homolysis to nitrogen dioxide radical ($ONO^{\bullet}$) and a highly reactive and dangerous hydroxyl radical, both radicals mediate oxidation reactions [19, 27, 29, 32, 52]. $^{\bullet}OH$ may be formed by spontaneous homolysis of the ONO—OH bond [35]. *Radi et al.* [27] proposed possible biomolecular pathways of peroxynitrite anion, which are demonstrated in Scheme 1.

However, *Koppenol et al.* [23] claim that peroxynitrous acid is unlikely to dissociate into a hydroxyl radical which is not involved in oxidation reactions of peroxynitrite and nitrogen dioxide. Formation of free hydroxyl radicals is thermodynamically not feasible [23, 25]. Various organo—transition metal complexes have been used as peroxynitrite decomposition catalysts protecting against the effects of the exo/endogenous peroxynitrite [27]. These compounds, *e.g.* highly active water-soluble iron(III) and manganese porphyrins, catalyze the isomerization of peroxynitrite to nitrate [55]. Synthetic metalloporphyrins act as peroxynitrite decomposition catalysts improving cardiac function [14]. *Szabó et al.* [56] examined a potent novel porphyrinic catalyst FP15 inhibiting tyrosine nitration and peroxynitrite-induced cytotoxicity *in vitro* and *in vivo* in both the pancreatic islets and the cardiovascular system of diabetics and also preventing from developing of vascular dysfunction. According to *Mabley et al.* [57], the treatment with FP15 significantly reduced the inflammation and oxidative stress in arthri-



Nitric oxide radical Superoxide anion radical Peroxynitrite Peroxynitrous acid

Scheme 2

tis and colitis, thus confirming that peroxynitrite plays a significant role in such inflammatory diseases.

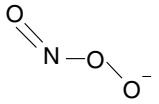
The rate of decomposition of peroxynitrite to free radicals, as reported by *Pryor et al.* [20], is insensitive to changes in medium viscosity. They suggest that free radicals are not produced during the spontaneous self-decomposition of peroxynitrite. The geminate $^{\bullet}OH/ONO^{\bullet}$ radical pair [30, 34] may be, due to the hydrogen bonding polar effect of water molecules, transformed into a geminate hydroxide anion/nitronium cation (HO^-/NO_2^+) pair which is more stable in water than the radical pair as shown by the difference of their respective Gibbs energies of about 37.8 kJ mol^{-1} [30]. Determination of nitrite (NO_2^-) and nitrate (NO_3^-) ions in solutions of decomposed peroxynitrite showed that the relative amount of NO_2^- increased with increasing pH, *i.e.* peroxynitrite decomposition rates to NO_2^- and oxygen (O_2) at physiological pH are significant [58].

Peroxynitrite-dependent oxidation involves directly ground-state peroxynitrous acid (one/two electron oxidations) or indirectly an activated intermediate ($ONOOH^*$) as a one-electron oxidant [32, 34, 59]. $ONOOH^*$ is a high-energy, metastable form of ONOOH that is present in steady-state together with ground-state peroxynitrous acid [1, 30]. $ONOO^-$ and ONOOH can cross biological membranes *via* anion channels and passive diffusion [27]. It was found that some of the reactions of peroxynitrite with various substrates are of first order, second order (direct oxidation pathway by peroxynitrite; 100 % oxidation yields), and of mixed order. It was found that about 40—50 % yield is obtained for the indirect oxidation pathway by peroxynitrite [32]. More than 95 % of all peroxynitrite formed is *in vivo* consumed by direct reactions [27]. Nitroxyl anion (NO^-) reacts with molecular oxygen to give peroxynitrite [39]. As indicated by *Khan et al.* [60], peroxynitrite, upon protonation, decomposes into singlet oxygen ($^1O_2$) and nitroxyl anion (NO^-). On the other hand, peroxynitrous acid is generated by the protonation of peroxynitrite formed from nitric oxide and a superoxide anion radical as illustrated in Scheme 2.

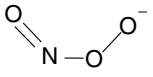
Approximately 30—33 % (30—35 % according to *Goldstein et al.* [33]) of the nitrosoperoxycarboxylate anion ($ONOOCO_2^-$), an adduct of peroxynitrite and carbon dioxide, decomposes into $ONO^{\bullet}$ and carbonium anion radical ($CO_3^{\bullet -}$) [61]. The rate constant of this rapid reaction is 3×10^4 M^{-1} s^{-1} [26, 61] that means the predominant pathway of peroxynitrite disappearance in normal physiological fluids. Both, $ONO^{\bullet}$ and $CO_3^{\bullet -}$, radicals can also oxidize $ONOO^-$ [33]. Homolytic cleavage of the weak O—O bond of $ONO—OCO_2^-$ produces toxic bicarbonate radical ($HCO_3^{\bullet}$) [26, 62], while the heterolytic one yields the nitrogen dioxide cation (ONO^+), a highly reactive nitrating agent [26]. This very short-living nitronium cation is also formed by dissociation of nitryl chloride ($ClNO_2$) in water [63]. ONO^+ is a strong oxidant ($E^0 = 1.6$ V) [39]. The O—NO bond may be broken heterolytically to yield the nitrosyl cation (NO^+), a powerful nitrosating agent [35]. As shown by *Goldstein* and *Czapski* [61], 30—33 % of peroxynitrite ($ONOOH/ONOO^-$) added reacts with the excess of CO_2 to form peroxynitrate (O_2NOOH/O_2NOO^-) in the presence of the bicarbonate anion (HCO_2^-), hydrogen peroxide (H_2O_2), methanol, 2-propanol, diethylenetriamine-*N*, *N′*, *N‴*-pentaacetate (DTPA). The activated form of peroxynitrite ($ONOOH^*$) as well as peroxynitrite itself, is a much more selective and reactive oxidant than the hydroxyl radical [27, 41, 64]. On the other hand, the hydroxyl radical is a more powerful oxidant than $CO_3^{\bullet -}$ and $ONO^{\bullet}$ [27].

Peroxynitrite Molecule Geometry

Peroxynitrite is not a free radical as the unpaired electrons of the superoxide anion radical and nitric oxide recombine to form a new bond ($ON—OO^-$) [31]. Peroxynitrite and its conjugated acid can potentially exist in two planar geometries (Chart 1), the *cis* and *trans* conformational isomers, of which the *cis* isomer is by *ca.* 12.6 kJ mol^{-1} more stable than the *trans* isomer as revealed by the measurement of vibrational spectra according to the differences found at the laser excitation wavelengths range of 488—514.5 nm [65, 66]. These isomers are also called rotamers [62]. Owing to the $ON—OO^-$ partial double bond [1], the energy of peroxynitrite is by 151.2 kJ mol^{-1} higher than that of nitrate formed by a direct attack of the terminal peroxide oxygen atom on the nitrogen atom of *trans*-peroxynitrite [66]. Formation of the tentative structure of a peroxynitrite isomer, a cyclic (three-membered ring) intermediate (Scheme 3) was reported in [35, 67, 68]. The weakest bond of this structure is the $ONO—O^-$ single bond [19]. As demonstrated



Trans-peroxynitrite



Cis-peroxynitrite

Chart 1

Scheme 3

by both, the ^{15}N NMR and the laser Raman, spectroscopy methods, the band at 642 cm^{-1} is attributed to the $ONOO^-$ torsional motion of its *cis* form due to the heterogeneous interactions with water molecules, thus confirming the *cis* geometry of peroxynitrite in alkaline aqueous solutions as the most stable and dominant isomeric form [60, 65]. X-ray crystallography confirmed peroxynitrite crystallization in the *cis* form. It can be concluded that the negative charge is delocalized over the entire planar *cis* $ONOO^-$ molecule and a weak hydrogen bond-like interaction exists between the terminal oxygen atoms causing its cyclic structure [68] as shown in Scheme 3.

Peroxynitrite Scavengers Acting as Antioxidants

Regoli and *Winston* [59] used the total oxidant scavenging capacity (TOSC) assay for a quantitative comparison of the scavenging efficiency of antioxidants, namely reduced glutathione (GSH), uric acid, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) toward various oxidants, *e.g.* peroxynitrite generated from 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1), hydroxyl, and peroxyl radicals. With the exception of GSH, the scavenging capacity of antioxidants was higher regarding peroxyl radicals and peroxynitrite compared to hydroxyl radicals. Trolox is known to have a protective effect on apoptosis triggered by peroxynitrite and other oxidants [64]. Peroxynitrite triggers apoptosis in cardiomyocytes *in vitro* and in the reperfused myocardium *in vivo* [69]. Treatment with a natural peroxynitrite scavenger, uric acid, was shown to reduce pathophysiological changes [70]. Uric acid is a powerful inhibitor of tyrosine nitration induced by peroxynitrite, but it fails to prevent α_1-antiproteinase inactivation induced by this species. Physiological concentrations of bicarbonate modify the ability of uric acid to prevent peroxynitrite-mediated reactions [71]. It was found that normal physiological concentrations of ascorbic acid, an important antioxidant, are insufficient for scavenging peroxynitrite, thus, ascorbate cannot play a direct role in the defense against this species [25]. Stable cyclic nitroxide radicals are known as effective antioxidants employing for this purpose a cyclic mechanism of electron transfer among three oxidation states; the oxoammonium cation, nitroxide, and hydroxylamine. These species protect hyaluronan from hydroxyl radicals generated enzymatically or radiolytically not due to their scavenging, but due to their reaction with secondary carbohydrate (peroxyl) radicals. Nitroxides, however, can also oxidize polysaccharides and facilitate degradation of hyaluronan induced by oxidants such as hypochlorite [72]. Peroxynitrite oxidizes cysteine to cystine and it may also exert cytotoxic effects in part by direct oxidation of tissue thiols at an apparent pK_a 6.8 [66, 73]. The oxidation of cysteine, possible peroxynitrite scavenger, to cystine is characterized by a rate constant of 5900 M^{-1} s^{-1} [31]. Vitamin E, acting as a major lipophilic antioxidant consisting of α-, β-, and γ-tocopherols, plays a very important role in protecting biological membranes against peroxynitrite-induced oxidative damage [3, 36]. CO_2/bicarbonate is an efficient antioxidant against peroxynitrite damage in extracellular fluids, similarly to bilirubin, β-carotene, and melatonin [3]. Nucleophilic addition of peroxynitrite to the keto group of pyruvate acting as an antioxidant results in the pyruvate decarboxylation [74]. Peroxynitrite scavenger Mn(III)-tetrakis(4-benzoic acid)porphyrin (MnTBAP) causes considerable reduction of myocardial injury [69]. Tyrosine-containing peptides may offer a novel strategy to neutralize the toxic effects of peroxynitrite by scavenging peroxynitrite-derived radicals [75].

The Role of Transition Metals on Peroxynitrite Reactivity

The reactivity of peroxynitrite is highly pH dependent and is influenced by the presence of metals, thiols, and bicarbonate [31]. Reactions of peroxynitrite with thiols, transition metal (Fe, Cu, Mn) complexes, carbon dioxide, and tyrosine residues represent the major pathways accounting for biological effects of this species [27, 39, 76]. Fe(III) and Cu(II) catalyze heterolysis and react with peroxynitrite to form a potent nitrating agent with reactivity comparable to that of a nitryl cation, even taking into account that the metals are bound to proteins [23]. Trace metals, particularly copper, in alkaline solutions catalytically increase the rate of peroxynitrite decomposition to nitrite and dioxygen by at least 75 %. Diaminoethanetetraacetic acid (EDTA) addition inactivates the metal catalytic activity by forming the respective metal chelates [19]. As reported by *Ducrocq et al.* [3], transition metals including Fe(III)—EDTA and Cu/Zn—superoxide dismutase (SOD) can catalyze peroxynitrite-mediated nitration of phenolic compounds

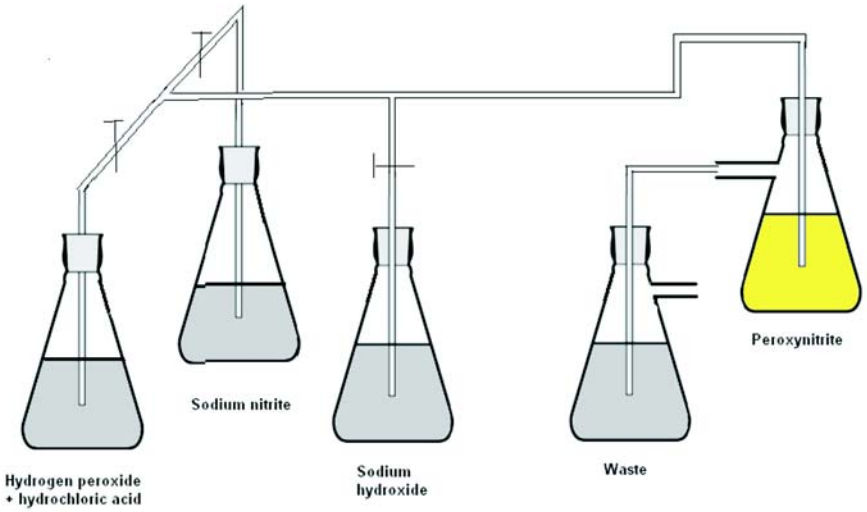


Fig. 2. The apparatus for peroxynitrite synthesis.

$$ONOO^- + Fe(III)—EDTA \rightarrow \rightarrow NO_2^+—O—Fe(III)—EDTA \qquad (E)$$

PEROXYNITRITE SYNTHESIS

Synthesis from acidic hydrogen peroxide and sodium nitrite, as described by *Saha et al.* [77], seems to be the most convenient, simplest, and possibly cleanest way of synthesizing peroxynitrite. The authors report that peroxynitrite can be synthesized using an efficient quenched-flow reactor at optimum flow rate of 162 mL min^{-1} at 20—25 °C obtaining 178 mmol L^{-1} peroxynitrite with a yield of approximately 85—95 %, with residual nitrite of about 0.001 %. On the other hand, *Edwards* and *Plumb* [19] claim that in order to obtain satisfactory yields of peroxynitrite, all reactants must be kept at 0 °C, and a strong base must be added immediately after the admission of the strong acid into the solutions of $NaNO_2$ and H_2O_2. Under basic conditions, peroxynitrite may either partially isomerize to nitrate or decompose to nitrite

$$H_2O_2 + HNO_2 \rightarrow ONOOH + H_2O \qquad (F)$$

$$ONOOH + OH^- \rightarrow ONOO^- + H_2O \qquad (G)$$

Pfeiffer et al. [58] report preparation of alkaline solutions of peroxynitrite (80—100 mmol L^{-1}) according to the Baeyer—Villiger reaction. Peroxynitrite can be prepared by the method of *Hughes* and *Nicklin* [78], as described by *Beckman et al.* [79]. A solution containing 0.5 mol L^{-1} $NaNO_2$ and 0.5 mol L^{-1} H_2O_2 was freshly prepared and cooled on ice. The solution was stirred rapidly using a magnetic stirrer, 1 mol L^{-1} of precooled HCl followed within approximately 0.5 s by precooled 1.5 mol L^{-1} NaOH were added into the solution. *Yi* [80] also prepared peroxynitrite *in vacuo* according to *Beckman et al.* [79] employing a laboratory apparatus illustrated in Fig. 2. Optimization showed that the flow rate of 10 mL min^{-1} allowed to obtain the maximum peroxynitrite concentration in this experimental setup.

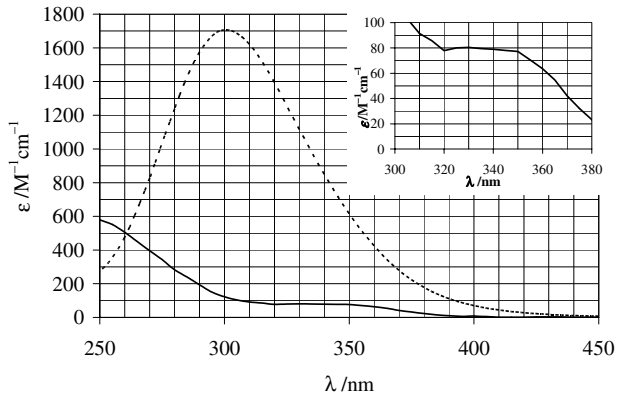


Fig. 3. Normalized spectrum of 500 μmol L^{-1} peroxynitrous acid recorded in 0.1 mol L^{-1} phosphoric acid/phosphate buffer at pH 3 (solid line), and of 500 μmol L^{-1} peroxynitrite at pH 13 (dotted line) (The figure inserted with permission of Professor Koppenol, ETH Zürich, Switzerland) [84].

Yeh et al. [81] modified the method described by *Beckman et al.* [79] using different concentrations of the reactants, namely 0.6 mol L^{-1} $NaNO_2$ and H_2O_2, 0.7 mol L^{-1} HCl, and 1.2 mol L^{-1} NaOH. The product was stored in 1.2 mol L^{-1} NaOH at −20 °C and the presence of peroxynitrite was indicated by an intense yellow color. The peroxynitrite concentration was assayed by absorption spectrophotometry using ε_{302} = 1670 M^{-1} cm^{-1}.

According to *Sandoval-Chacón et al.* [82], a fresh peroxynitrite solution (5 mmol L^{-1}) was prepared in 5

mmol L^{-1} KOH and filtered through an 0.2 μm membrane filter. *Grune et al.* [83] and *Kurz* [84] report molar absorptivity coefficient of peroxynitrite ε_{302} = 1705 M^{-1} cm^{-1}. The absorption maxima with the respective values of molar absorptivity coefficients of both peroxynitrite and peroxynitrous acid are shown in Fig. 3 [84].

Corsaro et al. [85] prepared peroxynitrite as reported by several authors [19, 23, 28, 35, 39, 40, 43, 73, 83, 84, 86—90] using a quenched flow-mixed reactor/stopped flow apparatus as shown in Fig. 4 [77]. It was rinsed with an EDTA solution prior to the reaction to remove trace metals which may cause rapid decomposition of the product [45]. In such an experimental setup *Radi et al.* [73] obtained a final peroxynitrite concentration of 170—220 mmol L^{-1} at the flow rate of 26 mL min^{-1}. The product was contaminated with NO_2^-, NO_3^-, and H_2O_2. The excess of H_2O_2 was removed by the addition of MnO_2. *Koppenol et al.* [23] investigated the influence of the MnO_2 addition on the removal of residual H_2O_2. The treatment of peroxynitrite solution with MnO_2, however, led to a 10—15 % loss of peroxynitrite due to the contamination with metal ions. *Scorza* and *Minetti* [91] treated peroxynitrite with MnO_2 (1 mg mL^{-1}, 30 min at 4 °C) which was subsequently removed by centrifugation (for 5 min at 4 °C) and ultrafiltration (0.45 μm). The product contained 200—500 mmol L^{-1} of peroxynitrite and it was found that the solution stored at –20 °C for 1—2 weeks showed negligible changes in its concentration. *Li et al.* [54] obtained the solution with peroxynitrite concentration ranging between 0.07 mol L^{-1} and 0.08 mol L^{-1}, whereas *Di Mascio et al.* [92] obtained a product containing 200—400 mmol L^{-1} of peroxynitrite.

A control reaction was carried out using the same procedure, except that the delay between the HCl and NaOH additions was increased to 10 s. As a result, the decomposition of peroxynitrous acid before neutralization caused discoloration of the product. Peroxynitrite prepared *via* this procedure may contain residual hydrogen peroxide. Application of peroxynitrite solution containg H_2O_2 in the study of hyaluronan (HA) degradation could lead to the formation of $^{\bullet}OH$ by the Fenton reaction due to the contamination of HA solution with iron. *Coddington et al.* [40] reported the storage of product containing *ca.* 140 mmol L^{-1} peroxynitrite and 21 mmol L^{-1} of residual nitrite at −80 °C. When stored frozen at −20 °C, the peroxynitrite decomposition rate was about 1.7 % per day and the product had to be used within 2—4 weeks [93]. *Goldstein* and *Czapski* [61] accomplished the reaction at room temperature applying 0.606 mol L^{-1} nitrite, 0.60 mol L^{-1} H_2O_2 in 0.7 mol L^{-1} $HClO_4$, and 3.60 mol L^{-1} NaOH at the flow rate of 45 mL min^{-1}. The stock solution contained 0.20 mol L^{-1} peroxynitrite (85 % yield), 2.5 % nitrite, and 1.5 % H_2O_2. Final peroxynitrite concentration of 0.120—0.180 mol L^{-1} was reported in [33]. *Kurz* [84] obtained a final yield of peroxynitrite of about 90 %. *Lymar et al.* [62] prepared peroxynitrite as described by *Keith* and *Powell* [94]. At room temperature as-

Table 1. Representative Comparative Study of Peroxynitrite Synthesis from Sodium Nitrite and Hydrogen Peroxide

Initial concentration/(mol L^{-1})				MnO_2 addition	Product composition/(mmol L^{-1})					Ref.
$NaNO_2$	H_2O_2	HCl	NaOH	g L^{-1}	$ONOO^-$	H_2O_2	NO_2^-	NO_3^-	Mn	
0.60	0.70	0.60	1.50	0.80	170—220	–	–	–	–	[28]
0.63	0.60	0.70[b]	3.60	N	180 (188[e])	0.32—0.49 (0.29[e])	12.30[e]	52[e]	–	[33]
0.60	0.60	0.70[b]	3.60	N	120 (122[e])	0.94—1.10 (1.19[e])	1.19[e]	117[e]	–	[33]
0.45	8.82	0.30[c]	1.25	A	–	–	–	–	–	[45]
0.243[a]	0.243	1.00	1.20	1.00	–	–	–	–	–	[62]
0.60[a]	0.70	0.60	3.00	N	44—82[f]	–	–	–	–	[86]
0.233	0.233	1.00	1.00	A	–	–	–	–	–	[36]
0.50	0.50	1.00	1.50	N	70—80	–	–	–	–	[54]
0.60	0.60	0.70	1.20	N	70—100	–	–	–	–	[81]
0.606	0.60	0.70[b]	3.60	N	200 (85[f])	1.50[g]	2.50[g]	–	–	[61]
0.50	0.50	1.00	1.50	0.80	13—17	–	–	–	–	[134]
1.00	0.20	1.00	1.50	N	1.00[g]	–	–	–	–	[42]
0.70	0.70	0.60	1.50[d]	0.80	35—50	–	–	–	–	[82]
0.60[a]	0.70	0.60	1.20	A	200—250	–	–	–	–	[71]
0.50	0.50	0.50	1.00	A	–	< 1.00[g]	–	–	–	[88]
0.31	0.30	0.35[b]	1.20	N	178	0.32	0.001—1[g]	52[e]	–	[77]

Steady-flow quenched-mixed reactor/apparatus, temperature (0—1 ± 1) °C (20—25 °C referred by [33, 61, 77]), reaction time in seconds, pH approximately 12. Yield of peroxynitrite dependent on the rate of reaction quenching and the flow rate. Direct spectrophotometric (thermostated equipment) determination of peroxynitrite concentration in a 1 mol L^{-1} NaOH solution: ε_{320} = (1670 ± 50) M^{-1} cm^{-1}.

a) KNO_2. *b*) $HClO_4$. *c*) H_2SO_4. *d*) KOH. *e*) Simulated. *f*) Yield in %. *g*) Content in %.

N – not applied, A – applied.

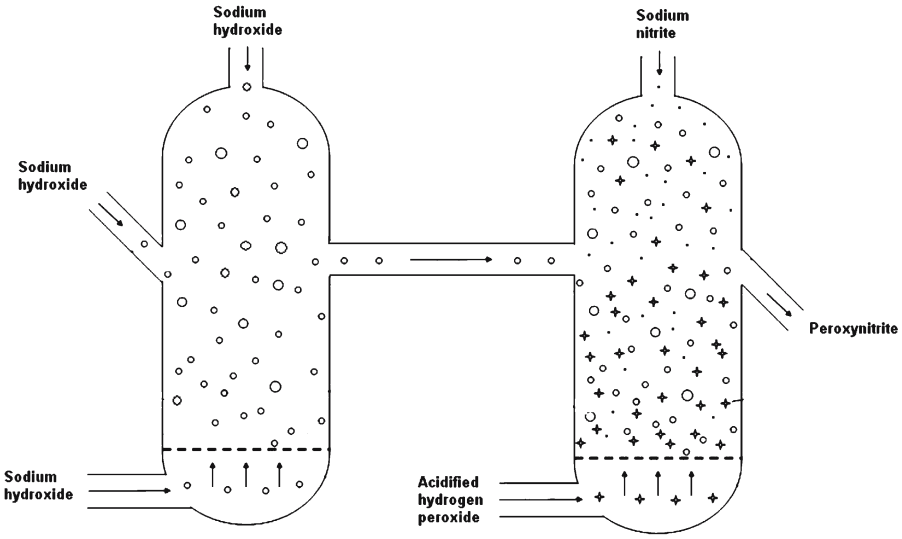
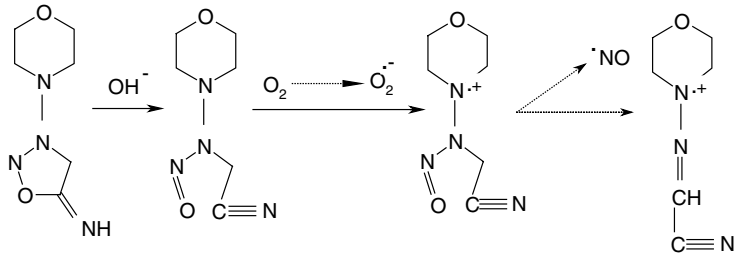


Fig. 4. Schematic design of a quenched flow-mixed reactor for peroxynitrite synthesis.



Scheme 4

suming the initial concentration of 10 mmol L^{-1} in Krebs' buffer, the rate of peroxynitrite decomposition reached 50 %, 92 %, and 100 % after 15 min, 30 min, and 60 min, respectively [89]. *Robinson* and *Beckman* [95] obtained within 1 h a solution containing 180 mmol L^{-1} of peroxynitrite, less than 2 % of nitrite, 0.28 mol L^{-1} sodium chloride, and 0.1 mol L^{-1} sodium hydroxide. *Fontana et al.* [96] prepared peroxynitrite (600—700 mmol L^{-1}) from potassium nitrite and hydrogen peroxide. For more details see Table 1.

Peroxynitrite can be generated *in situ* by the decomposition of SIN-1 in the presence of oxygen, releasing the superoxide anion radical and nitric oxide [97] spontaneously forming $ONOO^-$ in the presence of α-keto-γ-methiolbutyric acid (KMBA), potassium phosphate buffer, and diethylenetriamine-*N*,*N*′,*N*‴-pentaacetate (DTPA) at 35 ℃ [59, 98] or in the presence of phosphate-buffered saline-containing DTPA, pH 7.2, at 37 ℃ [37]. As shown by *Kurz* [84], in the first step, SIN-1 is oxidized to SIN-$1^{\bullet+}$ (SIN-1B) generating a superoxide anion radical from O_2 (Scheme 4). In the second step, SIN-$1^{\bullet+}$ releases a nitrogen monooxide molecule and becomes inactive (SIN-1C). $^{\bullet}NO$ recombines in equimolar ratio with $O_2^{\bullet-}$ to form peroxynitrite as reported by *Espey et al.* [88].

Peroxynitrite was synthesized by ozonation (5 % ozone in oxygen) of an aqueous solution of 0.1 mol L^{-1} or 0.02—0.2 mol L^{-1} sodium azide at pH 12, 0—4 ℃ for 1.5 h. The preparation contained 32—34 × 10^{-3} mol L^{-1} or 80 × 10^{-3} mol L^{-1} peroxynitrite [84, 99, 100].

$$N_3^- + 2O_3 \rightarrow ONOO^- + N_2O + O_2 \qquad (H)$$

The same method was employed also by other researchers [20, 64, 99, 101] using an ozonator (5 % ozone in oxygen). The method is convenient as the product is not contaminated with any residual hydrogen peroxide, traces of azide, or trace metals [64, 99]. *Pryor et al.* [99] report that peroxynitrite preparations frozen at −20 ℃ after about 3 weeks of storage showed negligible decomposition. *Richeson et al.* [30] obtained a solution containing *ca.* 0.075 mol L^{-1} of peroxynitrite which was stored at −80 ℃ excluding carbon dioxide to avoid contamination. *Koppal et al.* [102] reported the decrease of peroxynitrite concentration from 40 mmol L^{-1} to 30 mmol L^{-1} after 4 months of storage at −80 ℃.

Peroxynitrite could be also prepared by autoxidation of hydroxylamine in 0.5 mol L^{-1} NaOH solution containing 100 μmol L^{-1} DTPA. The concentration of peroxynitrite in the product was assayed by absorption spectrophotometry using $\varepsilon_{302} = 1670$ M^{-1} cm^{-1} [25, 101, 103]. *Yagil* and *Anbar* [104] reported enhanced formation of relatively stable peroxynitrite solutions when introducing hypochlorite to aqueous alkaline solution of chloramine (NH_2Cl), hydroxylamine (NH_2OH), and sodium nitrohydroxamate ($Na_2N_2O_3$) in the presence of oxygen. The authors estimated the product concentration using ε_{302} = 1300 M^{-1} cm^{-1}. *Kurz* [84] report about 25 % yield of peroxynitrite.

$$NH_2OH + O_2 + OH^- \rightarrow H_2O_2 + NO^- + H_2O \quad (I)$$

$$NO^- + O_2 \rightarrow ONOO^- \quad (J)$$

Petriconi and *Papee* [22] reported gaseous oxygen and nitrogen oxides evolution *via* irradiation of solutions of concentrated alkali metal nitrates with ultraviolet light at acid/alkaline pH values. Peroxynitrite can be prepared by UV irradiation/photolysis or γ radiolysis of sodium nitrate aqueous solutions with photons of wavelength 254—280 nm [19].

Preparation of peroxynitrite from hydrogen peroxide and isoamyl nitrite involves a two-phase system using isoamyl nitrite ($(CH_3)_2CH(CH_2)_2ONO$) and hydrogen peroxide. Product of this reaction, about 0.45 mol L^{-1} peroxynitrite solution, is usually contaminated with nitrite, alkali, and residual hydrogen peroxide. The use of fresh isoamyl nitrite minimizes the contamination by NO_2^- and the washing of isoamyl nitrite with water reduces the isoamyl alcohol contamination.

$$H_2O_2 + (CH_3)_2CH(CH_2)_2ONO + OH^- \rightarrow \\ \rightarrow ONOO^- + (CH_3)_2CH(CH_2)_2OH + H_2O \quad (K)$$

Uppu and *Pryor* [93] carried out this reaction at pH 12.5—13, and 0—25 °C during 1—15 h obtaining a solution containing 1 mol L^{-1} of peroxynitrite. Similar results were reported by *Kurz* [84].

According to *Khan et al.* [60], peroxynitrite could be generated by the reaction of potassium superoxide with nitric oxide. For this purpose, $^{\bullet}NO$ was bubbled into a deoxygenated 1 mol L^{-1} KOH solution at 0 °C and pH 13 adding 5 mg of KO_2. The concentration of peroxynitrite prepared was in the range of 70—110 mmol L^{-1}. A solid-phase synthesis of peroxynitrite by this reaction was described by *Kurz* [84]. Solid potassium superoxide was diluted by mixing it with quartz sand and after flushing the reactor vessel with argon, nitrogen monooxide was added. The obtained solid peroxynitrite was mixed with a small amount of MnO_2 to eliminate H_2O_2 produced by the remnants of KO_2 upon dissolution in cooled NaOH. The advantage of this method is much lower contamination of the product with nitrite and nitrate ions.

$$KO_2 + {}^{\bullet}NO + 2OH^- \rightarrow ONOO^- + H_2O_2 + K^+ \quad (L)$$

Sodium nitrite (99.99 %), ferrous sulfate heptahydrate, sulfuric acid, tetramethylammonium superoxide, dry liquid ammonia, sodium hydroxide, phosphorus pentoxide, and potassium hydroxide are the reagents used in the synthesis of tetramethylammo-

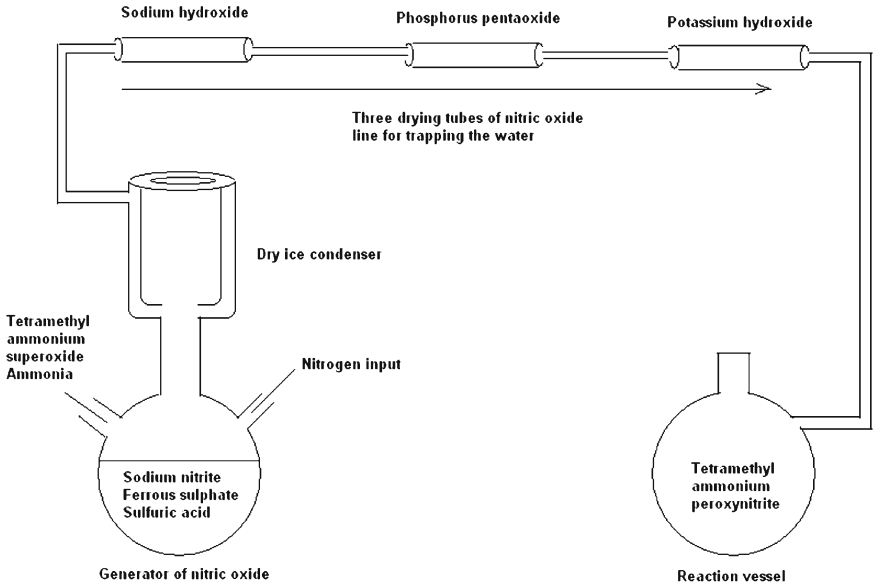


Fig. 5. Experimental setup for $[N(CH_3)_4]^+[ONOO]^-$ synthesis.

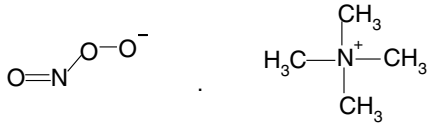


Chart 2

nium peroxynitrite, of the chemical formula given in Chart 2. The reaction was carried out in an experimental setup consisting of an $^{\bullet}NO$ generator, dry-ice condenser, three drying tubes and a reaction vessel, as shown in Fig. 5. After lyophilization, the fluffy orange solid, tetramethylammonium peroxynitrite, was obtained in a yield of 97 % [105].

$$2NaNO_2 + FeSO_4 + 2H_2O \rightarrow \\ \rightarrow 2^{\bullet}NO + Fe^{3+} + SO_4^{2-} + 2Na^+ + 4OH^- \quad (M)$$

$$[N(CH_3)_4]^+[O_2^{\bullet}]^- + {}^{\bullet}NO(g) + NH_3(l) \rightarrow \\ \rightarrow [N(CH_3)_4]^+[ONOO]^- \quad (N)$$

Another method of tetramethylammonium peroxynitrite preparation in liquid ammonia was described by *Kurz* [84]. In an inert and dry atmosphere, tetramethylammonium hydroxide was mixed with potassium superoxide and shaken under vacuum for three days. Then, dry ammonia was added and the dissolved tetramethylammonium superoxide was purified and exposed to nitrogen monooxide. Ammonia was slowly removed within two days *in vacuo*. The obtained solid tetramethylammonium peroxynitrite was of high purity and could be stored at −70 °C for months. *Latal et al.* [106] prepared tetramethylammonium peroxynitrite from superoxide and nitrogen monooxide in liquid ammonia observing low levels of nitrite as a contaminant when the frozen peroxynitrite solution was first kept at +1 °C and then at room temperature. The product underwent only a 2—3 % decomposition during an hour. The tetramethylammonium salt of peroxynitrite ($[N(CH_3)_4]^+[ONOO]^-$) was also synthesized by *Pfeiffer et al.* [58] from $[N(CH_3)_4]^+[O_2^{\bullet}]^-$ and $^{\bullet}NO$ showing that no detectable amount of paramagnetic ($O_2^{\bullet-}$) impurity was present in the stock solution kept at −70 °C.

As stated by *Leis et al.* [107] the routine method of peroxynitrite preparation using acidic hydrogen peroxide and sodium nitrite yields at best only about 45—50 % of the product. The authors summarized the shortcomings of this method, the unavoidable presence of nitrite and nitrate ions in the final solutions as well as the contamination with residual hydrogen peroxide usually decomposed by the addition of MnO_2, which resulted in a further loss of peroxynitrite. However, a few years later, *Saha et al.* [77] synthesized free peroxynitrite from nitrite using slightly higher concentration of hydrogen peroxide than that of nitrite and *vice versa* (see Table 1). On studying the reaction of highly nucleophilic HOO^- with the nitroso group of alkyl nitrites using equimolar amounts of both reactants, *Leis et al.* [107] introduced a new, simple, clean, and fast method yielding stable solutions of peroxynitrite.

$$CH_3CH_2O(CH_2)_2ONO + HOO^- \rightarrow \\ \rightarrow CH_3CH_2O(CH_2)_2OH + ONOO^- \quad (O)$$

Peroxynitrite is quantitatively (over 97 % yield) formed in basic media (0.2—1 mol L^{-1} NaOH) without any residual hydrogen peroxide as well as without any interference of the competitive process of alkaline hydrolysis of alkyl nitrite.

Petriconi and *Papee* [22] reported a method of peroxynitrite preparation by the reaction of aqueous solution of alkaline hydrogen peroxide and freshly prepared nitric oxide. $^{\bullet}NO$ was obtained by the reaction of diluted nitric acid and metallic copper and subsequently dissolved in concentrated sulfuric acid under stirring and in the presence of 99.9 % pure oxygen forming thus nitrosyl-sulfuric acid. The introduction of the acid into solutions of about 1 % hydrogen peroxide at pH 12.5—13.5 and 0 °C resulted in the formation of peroxynitrite (0.006 mol L^{-1})

$$2HNOSO_4 + H_2O \rightarrow 2H_2SO_4 + {}^{\bullet}NO + NO_2 \quad (P)$$

$$2HOO^- + 2OH^- + {}^{\bullet}NO \rightarrow ONOO^- + 2H_2O + O_2 (Q)$$

Increased peroxynitrite formation was observed until the mole ratio of reactants increased to $n(H_2O_2)/n(^{\bullet}NO) = 10$, when the peroxynitrite concentration of 0.5 mmol L^{-1} was achieved. For more details see Table 2.

Methods of Peroxynitrite Determination

As reported by *Ischiropoulos et al.* [31], the detection of peroxynitrite *in vivo* is complicated by the multiple pathways of its reactivity with biological molecules. *Radi et al.* [27] examined the formation of cell- and tissue-derived oxidants ($^{\bullet}OH$, oxo-iron complexes, and peroxynitrite mediators) by oxidation of fluorescent probes, dichlorofluorescin (DCFH) and dihydrorhodamine (DHR) at pH 7.4 and 37 °C resulting in approximately 35—42 % oxidation yields. Laser Raman spectra of peroxynitrite-treated SOD (pH 10, 438 nm absorption band) confirmed the 3-nitrotyrosine formation [39]. *Ischiropoulos et al.* [31] used HPLC to detect 3-nitro-*p*-hydroxyphenylacetic acid produced by the nitration of *p*-hydroxyphenylacetic acid with peroxynitrite catalyzed by SOD. The author found that only 8 % of peroxynitrite was consumed by this reaction. Indirect peroxynitrite determination by nitrotyrosine detection [31] was based on UV-visible spectropho-

Table 2. Representative Pattern of Various Methods of Peroxynitrite Synthesis and Determination Methods Applied

Precursor/method	Reaction pathway	Reaction conditions	$ONOO^-$ concentration / mol L^{-1}	Determination method	Ref.
SIN-1	Simultaneous decomposition of SIN-1	35—37 °C, pH 7.2, aerobic conditions	–	Fluorescence[d]	[37, 59, 98]
		KMBA, DTPA	–	–	[84]
O_3 and NaN_3	Ozonation (free of H_2O_2)	0.1 mol L^{-1} NaN_3, pH 12, 0—4 °C, 1.5 h	0.032—0.034	Spectrophotometry[e]	[84, 100]
		5 % O_3 in O_2	–	–	[20, 64]
		–	0.075	Spectrophotometry[e]	[30]
		0.1 mol L^{-1} NaN_3, pH 12	0.042	Spectrophotometry[e]	[102]
		5 % O_3, 0.02—0.20 mol L^{-1} NaN_3	0.08	–	[99]
$NH_2OH/NH_2Cl/Na_2N_2O_3$	Autoxidation	pH 12—13, 25 °C, 0.01 mol L^{-1} NH_2OH, 1 mmol L^{-1} EDTA, O_2	0.0033	Spectrophotometry[e]	[103]
		0.5 mol L^{-1} NaOH; 100 μmol L^{-1} DTPA	–	Spectrophotometry[e]	[25]
		O_2/NaOCl	25[a]	–	[84]
UV irradiation/photolysis/ γ-radiolysis	Photons (λ = 254—280 nm)	Aqueous Na^+/K^+ NO_3^- solution	–	Potentiometry[d] (permanganate in basic solution), diffuse reflectance FTIR[e]	[19]
H_2O_2 and isoamyl nitrite	Two-phase synthesis/ nitrosation	0.20—2.00 mol L^{-1} H_2O_2; pH 12.5—13, 0.04 mol L^{-1} DTPA in 0.05 mol L^{-1} NaOH, 0.02—0.20 mol of isoamyl nitrite, 1—15 h; 0—25 °C, MnO_2	1.0	Spectrophotometry[e]	[84, 93]
KO_2 and $^{\bullet}NO$	Synthesis in aqueous medium	$^{\bullet}NO$, 1mol L^{-1} KOH, 0 °C, pH 13, 5 mg KO_2	0.07—0.11	Spectrophotometry[e]	[60]
	Solid-phase synthesis	KO_2; quartz sand; $^{\bullet}NO$	–	–	[84]
$[N(CH_3)_4]^+[O_2^{\bullet}]^-$ and $^{\bullet}NO$	Synthesis in liquid ammonia	3 mmol L^{-1} $NaNO_2$, 18 mmol L^{-1} $FeSO_4$, H_2SO_4, NH_3(l), 1.13 mmol L^{-1} $[N(CH_3)_4]^+[O_2^{\bullet}]^-$	0.162[b] (97[c])	–	[105]
		$[N(CH_3)_4]^+[OH]^-$, KO_2, NH_3(l), $^{\bullet}NO$	–	–	[84]
H_2O_2 and 2-ethoxyethyl nitrite	Reaction of nucleophiles with alkyl nitrites	0.109 mol L^{-1} H_2O_2, 2 mol L^{-1} NaOH, H_2O, 0.195 g $C_2H_5O(CH_2)_2NO_2$, 5 min, 25 °C	0.015—0.016	Spectrophotometry[e]	[107]
$^{\bullet}NO$ and alkaline H_2O_2	Alkaline nitrosation of hydrogen peroxide	0.096 mol L^{-1} H_2O_2; concentrated H_2SO_4 with 0.032 mol of $^{\bullet}NO$, 50 % NaOH, pH 12.5—13.5, 0 °C	0.0005—0.006	Colorimetry[d]	[22]

a) Content in %. *b*) Mass in g. *c*) Yield in %. *d*) Indirect determination. *e*) Direct determination.

tometry of alkaline solution with absorbance maximum at 420—440 nm and ε_{438} = 4200 M^{-1} cm^{-1} or acidic medium at 350—360 nm with ε_{360} = 3400 M^{-1} cm^{-1}, gas-chromatography with thermal analyzer, coupled gas chromatography mass spectrophotometry (detection of 3-nitro-*p*-hydroxyphenylacetic acid, the major metabolite of nitrotyrosine in human urine), amino acid analysis (dithionite reduction of 3-nitrotyrosine to 3-aminotyrosine, 74—76 % yield), HPLC analysis (based on the intrinsic absorbance of 3-nitrotyrosine and 3-aminotyrosine at 365 nm, tyrosine at 280 nm), or detection of polyclonal and

Table 3. Overview of Peroxynitrite "Large-Scale" Producers [108]

Product	Code	Producer	Distributor
Peroxynitrite	CAY-81565 20-107/5 mL 20-247/500 μL	Alexis Corporation, Lausen, Switzerland UPSTATE, MILLIPORE, Billerica, MA 01821, USA	LAMBDA LIFE, Bratislava, Slovak Republic SCINTILA, Jihlava, Czech Republic
Tetramethyl-ammonium peroxynitrite	ALX-400-036-5001/5 × 1 mL	AXXORA PLATFORM: Alexis Corporation, Lausen, Switzerland AXXORA (UK) Ltd., Nottingham, United Kingdom AXXORA, LLC, San Diego USA AXXORA Deutschland GmbH, Lörrach, Germany	

monoclonal antibodies specific for nitrotyrosine. Direct methods of peroxynitrite determination [31] are represented by fluorescence assays [oxidation of non-fluorescent dihydrorhodamine 123 (DHR 123) to fluorescent rhodamine 123 (RH 123) used to monitor the generation of peroxynitrite from SIN-1, or oxidation of 2′,7′-dichlorofluorescin], chemiluminescence assays (peroxynitrite induces luminol chemiluminescence with a quantum yield of approximately 10^{-3} in bicarbonate buffer and also lucigenin chemiluminescence equivalent to 25 % of the luminol chemiluminiscence intensity), spectrophotometric assays (*e.g.* oxidation of 1 mmol L^{-1} *o*-phenylenediamine at pH 7.4 and $\varepsilon_{420} = 5300\ M^{-1}\ cm^{-1}$). *Edwards* and *Plumb* [19] reported indirect quantitative potentiometric oxidative determination of peroxynitrite anion with permanganate in basic solution and the measurement of IR spectrum of $ONOO^-$ in solid nitrates by diffuse reflectance FTIR. The rate constants for the oxidation of peroxynitrite by inorganic radicals ($CO_3^{\bullet-}$, $^\bullet N_3$, and $ClO_2^\bullet$) were determined from their decay kinetics using a pulse radiolysis technique [33].

Table 3 lists major worldwide producers of peroxynitrite and distributors in the Slovak and Czech Republic.

PATHOPHYSIOLOGICAL ASPECTS OF PEROXYNITRITE IN TISSUE INJURY AND INFLAMMATION

Peroxynitrite was shown to be involved in pathogenesis of many diseases including acute and chronic inflammatory processes, atherosclerosis, rheumatoid arthritis, inflammatory bowel disease, adult respiratory distress syndrome, sepsis, ischemia-reperfusion, vascular injuries, or neurodegenerative disorders [27, 35, 76]. Peroxynitrite and other reactive oxygen species are linked to various cellular injuries including membrane lipid peroxidation, DNA alteration, damage of proteins and enzyme inactivation [39, 59]. Peroxynitrite is a potent degradative agent capable of reacting with nucleic acids, proteins, lipids, and glycosaminoglycans (GAGs) [51, 54, 79, 109]. A summary of peroxynitrite reactions with biomolecules is given in Table 4.

Peroxynitrite Reactions with Nucleic Acids (DNA, RNA)

Peroxynitrite can cause chain scission in DNA [27, 64, 110] promoting oxidation and nitration of isolated DNA resulting in DNA strand breaks by nitration of guanine to form 8-nitroguanine [29, 111, 112]. It can also initiate the apoptotic process responsible for genomic degradation of nuclear DNA in rat thymocytes, apoptosis in normal cells of primary structure [25, 30], and it is responsible for DNA nicks [64]. Studies using HPLC analysis with electrochemical detection suggest that $ONOO^-$ nitrates xanthine and adenine nucleosides [81, 113]. 8-nitroxanthine is generated by $ONOO^-$ at physiological pH and plasma concentrations of $NaHCO_3$. It is a potential biomarker of oxidation caused by reactive nitrogen species [81]. HIV-1 infection of brain tissue macrophages of AIDS patients with and without the AIDS dementia complex resulted in both increased superoxide anion radical production and elevated SOD mRNA levels, *i.e.* peroxynitrite may contribute to the neuropathogenesis of HIV-1 infection [114]. Peroxynitrite can cause nitrosative deamination of DNA bases (guanine, cytosine) as well as DNA base and sugar oxidative modifications [115].

Peroxynitrite Reactions with Proteins and Enzymes

Nitration of aromatic amino acid residues by peroxynitrite (by direct $ONOO^-$ pathway or by NO_2^+ and $^\bullet OH$), the presence of which (3-nitrotyrosine) may be a marker of peroxynitrite-mediated (*i.e.* $^\bullet NO$-dependent) damage *in vivo*. It was found that the patients with rheumatoid arthritis have elevated levels of this marker [39, 79, 116]. As demonstrated by *Kastenbauer et al.* [70], cerebral blood vessels of a brain suffering from bacterial meningitis are affected by increased tyrosine nitration and lipid peroxidation due to the peroxynitrite contribution to the devel-

Table 4. Representative Studies of the Influence of Peroxynitrite on Physiological and Pathological Processes

Level of affection	Substrate	Product	$ONOO^-$ effect	Commentary	Determination methods	Ref.
(Macro)molecular	α-Tocopherol	Tocopherolquinone	*In vitro* rapid and selective $ONOO^-$-induced oxidation.	Mitochondrial α-tocopherol more susceptible to oxidation than synaptosomal. Without cholesterol oxidation.	Liquid chromatography	[36]
	Tyrosine	3-Nitrotyrosine (footprint of $ONOO^-$)	Direct $ONOO^-$ pathway (NO_2^+ and $^{\bullet}OH$). $ONOO^-$-mediated nitration of tyrosine.	3-Nitrotyrosine (elevated levels), marker of peroxynitrite-mediated (*i.e.* $^{\bullet}NO$-dependent) damage *in vivo*.	Raman spectrometry	[39, 79]
Subcellular/ supramolecular	High-molar-mass hyaluronan (HA)	Degradation products of HA of low-molar-mass, formation of C-centered radicals on HA	*In vitro* rapid degradation of HA *via* nucleophilic attack of *trans*-$ONOO^-$ on a HA-glycosidic bond.	*In vivo* production of $ONOO^-$ under inflammatory conditions. Reduced limiting viscosity number of HA and progression of rheumatoid arthritis.	Agarose gel electrophoresis, capillary viscometry, EPR, NMR	[54, 85]
Cellular	Rat brain mitochondria	Lipid peroxidation (high polyunsaturate content of brain mitochondrial phospholipids), mitochondrial proton leak	Irreversible inhibition of mitochondrial respiration.	Lipid peroxidation (proposed mechanism of $ONOO^-$ cytotoxicity), increased membrane bilayer proton permeability, decreased mitochondrial membrane potential.	NMR	[8]
	Brain neocortical synaptosomal membrane proteins, oxidation-sensitive enzyme GS	Inactivation of GS, protein conformational changes, protein carbonyls	Neuronal cell death. Oxidation of membranous and cytosolic proteins.	Potential involvement of $ONOO^-$ in AD neurodegeneration. Protection by a thiol-containing antioxidant tripeptide glutathione.	EPR, protein-specific spin label	[102]
Tissue	Pressure-induced myogenic activity, VSM actin of isolated PCAs	Depolymerized F-actin in VSM	Low concentration of $ONOO^-$ ($\leq 10^{-6}$ mol L^{-1}) to isolated PCAs, constriction from (129 ± 16) μm to (115 ± 15) μm, ($> 10^{-6}$ mol L^{-1}), dilatation of spontaneous tone.	Loss of myogenic activity in 50—125 mm Hg ($> 10^{-6}$ mol L^{-1} of $ONOO^-$), 4.5-fold decrease in F-actin content of VSM, 27 % increase in G-actin content.	Confocal microscopy	[117]
	Brain tissue of AIDS patients with/without AIDS dementia complex and HIV-seronegative controls	RNA expression, inducible NO synthase and SOD, higher in DP compared with NP	Higher content and more frequent presence of nitrotyrosine in brain sections of DP compared with NP. Possible $ONOO^-$ contribution to the neuropathogenesis of HIV-1 infection.	HIV-1 infection of macrophages, increased SAR production and elevated SOD mRNA levels compared to uninfected macrophages.	Immunohistochemical analysis	[114]

opment of cerebrovascular complications and blood-brain barrier disruption. Nitration of structural proteins, including neurofilaments and actin, can disrupt the filament assembly with major pathological consequences [41]. The study by *Maneen et al.* [117] exhibits peroxynitrite effect on the myogenic activity of

cerebral arteries causing filamentous actin (F-actin) depolymerization in VSM (vascular smooth muscle) possibly promoting vascular damage and brain injury. *Obrosova* [118] reports toxic effects of peroxynitrite in heart including the inhibition of key myocardial enzymes such as reticulum sarcoplasmic Ca^{2+} ATPase and creatine kinase and the activation of metalloproteinases. Apoptosis represents a crucial mechanism of the cardiomyocyte loss in a great number of cardiac pathologies. As shown by *Estevéz et al.* [119], acidic and basic fibroplast growth factors greatly increased the peroxynitrite-initiated apoptosis up to 63 % and 70 %, respectively. Specific neurotrophic factors demonstrate diferential regulation of peroxynitrite-induced apoptosis *in vitro*.

Pryor et al. [99] report reaction of peroxynitrite at pH 7.0 with L-tyrosine giving a 7.3 mol % yield of nitrotyrosine(s). As found by *Espey et al.* [88], exposure of the purified green fluorescent protein (GFP) to peroxynitrite resulted in an increased 3-nitrotyrosyl immunoreactivity concomitant with the disappearance of intrinsic fluorescence.

Peroxynitrite oxidizes mitochondrial (more susceptible) and synaptosomal α-tocopherol at initial concentration of 0.21—5 μmol L^{-1} yielding tocopherolquinone. It was observed that oxidation of this species induced by ferrous ion and ascorbate was much slower compared to that caused by peroxynitrite [36]. Peroxynitrite blocks prostacyclin synthase at very low concentrations [43]. Peroxynitrite serves as an efficient peroxidase substrate and cyclooxygenase activator [44]. *Trans*-peroxynitrite nitrosylates/nitrates tyrosine residues on Fe, Mn, and bovine Cu—Zn superoxide dismutases forming a stable 3-nitrotyrosine [31, 39, 52]. Both nitration and dimerization of tyrosine are two-electron processes [3, 41]. The SOD and Fe(III)-EDTA catalyze phenolic nitration of tyrosine in proteins by peroxynitrite [31, 65]. The reaction of SOD with peroxynitrite is rate-limited by the isomerization of the *cis* peroxynitrite conformer to the *trans* one and, therefore, could be pathologically relevant in biological systems. However, Fe(III)-EDTA reacts directly with the *cis* peroxynitrite anion [65]. Peroxynitrite can increase protein susceptibility towards proteasomal proteolysis, as evidenced by the 40 % loss of proteasomal activity after the treatment of cells with peroxynitrite [83]. Some oxidative reactions of biological targets, however, are not induced by peroxynitrite itself. $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ radicals are probably responsible for these reactions. These radicals are formed by a rapid *in vivo* reaction of nucleophilic terminal oxygen atom of peroxynitrite with an electron-deficient central carbon atom of CO_2 (Lewis acid) [26, 40, 85, 120—122] through an adduct, a nitrosoperoxycarboxylate anion ($ONO_2CO_2^-$) [27, 81] with the lifetime < 3 ms and the redox potential of approximately 1 V capable to form 3-nitrotyrosine, 3,3′-dityrosine, and 3,5-dinitrotyrosine by nitration and dimerization of tyrosine (two-electron oxidation) [62].

Peroxynitrite can oxidize (seleno)methionine, protein and nonprotein thiols [32, 73] as well as cellular thiols [25, 30]. The formation of methionine sulfoxide by peroxynitrite is a one- and two-electron process [3, 101]. The 3-nitrotyrosine formation by peroxynitrite and its detection in injured tissues were reported [27, 64, 83, 123], as well as the treatment of *Saccharomyces cerevisiae* with this species resulting in nitration of glyceraldehyde-3-phosphate dehydrogenase [83]. It was shown that peroxynitrite is highly bactericidal to *Escherichia coli* [23, 62]. However, already low concentrations of bicarbonate protect *Escherichia coli* from the toxic effect of peroxynitrite [61]. Reactive nitrating species from peroxynitrite decomposition may give rise to nitro forms of tryptophane [43]. As shown by rapid-scan spectra of the myeloperoxidase reaction with peroxynitrite in a 1.2 mol L^{-1} chloride solution at pH 7.0 [45], chloride anion inhibits the reaction by reacting with a protonated form of peroxynitrite. A potential involvement of peroxynitrite in Alzheimer's disease (AD), neurodegeneration by oxidative modification of membranous and cytosolic proteins, is discussed in the paper by *Koppal et al.* [102]. As shown by *Kinobe et al.* [90], peroxynitrite inhibits the *in vitro* catalytic activity of rat spleen (HO-1) and brain (HO-2) microsomal heme oxygenases. Addition of SIN-1 or peroxynitrite, involving $CO_3^{\bullet -}/ONO^{\bullet}$ radicals, to mixtures containing α-synuclein (a neuronal presynaptic protein) and HCO_3^- markedly enhanced both nitration and aggregation of α-synuclein through the dityrosine formation [97]. *Fontana et al.* [96] report that direct oxidation of sulfinates (hypotaurine, cysteine) to sulfonates, mediated by both radicals, $CO_3^{\bullet -}/ONO^{\bullet}$, generated by the decomposition of the peroxynitrite-CO_2 adduct, is inhibited in the presence of bicarbonate.

Peroxynitrite Reactions with Polysaccharides

Peroxynitrite Reactions with Glycosaminoglycans (GAGs)

Heparin and hyaluronan are glycosaminoglycans widely distributed in the extracellular matrix (ECM) of tissues. The cartilage matrix, a relatively anaerobic environment [52], contains two major protein groups, collagens and proteoglycans. Regulation of breakdown *vs.* synthesis of these complex macromolecules containing a core protein with one or more covalently bound glycosaminoglycan chains determines the cartilage matrix integrity [44]. GAGs are complex polysaccharides including heparan sulfate, keratan sulfate, and chondroitin sulfates. They bind large quantities of water which expands their three-dimensional space [51, 124]. The major proteoglycan of the endothelial cell extracellular matrix is heparan sulfate which binds growth factors, chemokines, and enzymes such

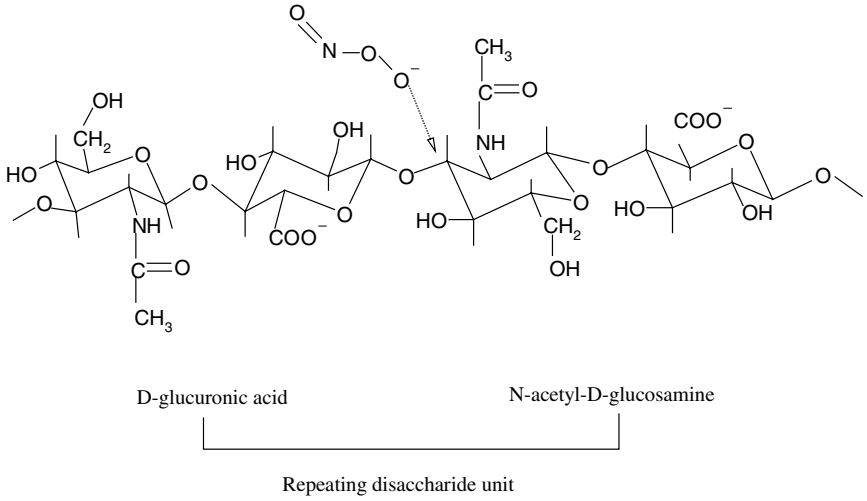
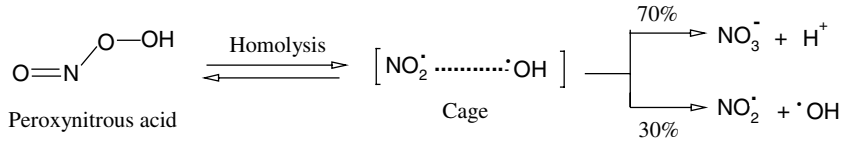


Fig. 6. Proposed attack of the more reactive *trans*-peroxynitrite on the glycosidic bond of high-molar-mass HA.



Scheme 5

as extracellular superoxide dismutase [50]. This enzyme catalyzes dismutation of the superoxide anion radical to hydrogen peroxide [46].

Chondroitin-4-sulfate (A), dermatan sulfate (B), and chondroitin-6-sulfate (C) are constituents of the basement membranes of many tissues, including the intestine. Glycosaminoglycans differ in their sugar composition and degree of sulfation. They are typically heterogeneous in chain length and are negatively charged. They differ in the position of the sulfate group and in the type of uronic acid, *e.g.* glucuronic or iduronic acid [51]. Rheumatoid arthritis and osteoarthritis due to the degradation of glycosaminoglycans by ROS (hydroxyl radical) have been reported in [49, 51, 125]. It was found that the $^{\bullet}$NO-mediated degradation of GAG has two pathways. The first one is initiated by the conversion of nitric oxide to nitrous acid, while the second one includes peroxynitrite. Heparin and heparan sulfate are susceptible to degradation by nitrous acid, hyaluronan by peroxynitrite, and chondroitin sulfates (A, C) partially by both reagents [51]. Decomposition of heparan sulfate and other GAGs of the extracellular matrix by $^{\bullet}$NO may be important in physiological or pathological states (bone development, apoptosis, atherosclerotic plaque release, metastatic, inflammatory conditions) [53]. Endothelial-cell-derived $^{\bullet}$NO is capable of degrading heparin and heparan sulfate *via* HNO_2 rather than peroxynitrite. Along with the cleavage of the glycosidic bond, the amino and sulfate groups are both eliminated. Inflammatory processes may release excess $^{\bullet}$NO and superoxide anion radicals forming peroxynitrite capable to degrade hyaluronan but not heparan sulfate. The balance between $^{\bullet}$NO and superoxide anion radicals determines which glycosaminoglycan component of the extracellular matrix will be destroyed and it may be important in regulating the disease processes [50, 126].

Peroxynitrite Reactions with High-Molar-Mass Hyaluronan

As shown in Fig. 6, hyaluronan is a linear non-sulfated glycosaminoglycan, composed of a repeating disaccharide unit (D-glucuronic acid and *N*-acetyl-D-glucosamine), a naturally occurring biopolymer widely distributed in vertebrate tissues. At higher concentrations, HA solutions exhibit a pseudo-plastic behavior. In aqueous solutions, HA, at the pK_a 3.2, is represented by negatively charged macromolecules with extended conformations, which impart high viscosity/viscoelasticity, accompanied also by low compressibility of the synovial fluid [15]. Peroxynitrite (the more reactive *trans*-from) degrades hyaluronan at the neutral pH [50, 51, 54]. Also other ROS (hydroxyl radical, hypochlorite) degrade hyaluronan, a joint-lubricating agent, resulting in the loss of solution viscosity [72]. Bubbling $^{\bullet}$NO gas into hyaluronan solutions lowers their viscosity only under aerobic conditions [52]. The chain breaks are caused by hydroxyl radicals, produced by the catalytic action of the transition metal ions, iron(II/III) and copper(I/II), which

escape a cage containing the $^{\bullet}OH/ONO^{\bullet}$ radical pair [127] as shown in Scheme 5 and, also by ONOOH and trioxocarbonate anion radical $CO_3^{\bullet -}$.

The concentration of HA fragments increases almost linearly with peroxynitrite concentration up to 0.15 mmol L^{-1} before reaching the steady level of chain breaks of about 4 μmol L^{-1} at high peroxynitrite concentration. Each peroxynitrite molecule produces 1.12×10^{-2} chain breaks. The SEC/MALS techniques are used in detection of HA chain breaks [100]. These breaks result in altered mobility of HA fragments on the agarose gel electrophoresis as well as in reduced limiting viscosity number. Protective agents/target molecules in the HA attack by peroxynitrite are thiourea (extremely effective), dimethyl sulfoxide (moderately effective), sodium benzoate, and mannitol (slightly effective). Peroxynitrite is said to exhibit hydroxyl radical-like reactivity derived from the vibrationally exciting state of *trans*-peroxynitrous acid [66], which also appears in the degradation of HA [54]. In the normal knee joint synovial fluid, the average molar mass of HA is approximately $(6—8) \times 10^6$ Da and its concentration is 2.5 mg mL^{-1} [54, 128, 129, 130]. According to *Al-Assaf et al.* [100] the average HA molar mass is 7 MDa. Hyaluronan is not susceptible to degradation by $^{\bullet}NO$ and HNO_2 because of its *N*-acetyl groups [50, 131, 132, 133]. Spin-trapping EPR experiments revealed that at acidic pH peroxynitrite-dependent C-centered carbon radicals are formed in monomers, in the tetra-saccharide as well as in the hyaluronan polymer. This fact supports the hypothesis of the oxidative pathway involved in the degradation of hyaluronan playing a key role in the development and progression of rheumatoid arthritis [85].

According to *Stankovská et al.* [134], monitoring of the kinetics of hyaluronan degradation by peroxynitrite containing H_2O_2 or H_2O_2-free peroxynitrite using a Brookfield rotational viscometer revealed that although MnO_2 decomposed the residual H_2O_2, trace amounts of manganese ions dissolve in the alkaline solution containing pure peroxynitrite. Since manganese is a transition metal, its presence in the solution could induce further side reactions resulting in a more rapid time-dependent degradation of the biopolymer.

Peroxynitrite Reactions with Lipids

Peroxynitrite and its conjugated acid (peroxynitrous acid) are potent and versatile oxidants [34] that can initiate/mediate lipid peroxidation [32, 73]. Vitamin E plays an important role in lipid peroxidation which increases the membrane bilayer proton permeability. The high polyunsaturated content of brain mitochondrial phospholipids may predispose them to peroxidation. Peroxynitrite inhibits mitochondrial respiration irreversibly [8]. *Sandoval-Chacón et al.* [82] report effective anti-inflammatory properties of *Uncaria tomentosa*, a vine known as cat's claw used in traditional Peruvian medicine for the treatment of arthritis and other diseases. The active components of *Uncaria tomentosa* inhibit the lipopolysaccharide-induced iNOS gene expression, nitrite formation, and cell death, protecting the affected biomolecules against oxidative stress caused by peroxynitrite, as well as the activation of NF-κB.

Peroxynitrite Reactions with Other Low-Molar-Mass Compounds

Al-Ajlouni and *Gould* [35] report the reduction of peroxynitrite with sulfite at pH 12—14 catalyzed by Cu(II) forming sulfate and nitrite [32, 59]. As reported by *Alvarez et al.* [135], *Coddington et al.* [40], *Ischiropoulos et al.* [31], and *Edwards* and *Plumb* [19], peroxynitrite indirectly oxidizes hydrogen peroxide forming nitrite and oxygen, the process is catalyzed by cupric ions. Peroxynitrite can also oxidize benzene, hydrazine, formaldehyde, and arsenite, and it is reduced by cyanide and thiocyanate [19]. Peroxynitrite is capable of inducing luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) chemiluminescence in the presence of bicarbonate/carbon dioxide [27]. It is also able to oxidize and nitrate aromatic amino acids [116], it oxidizes dimethylsulfoxide to formaldehyde [31]. *Pryor et al.* [99] report an 8.2 mole % yield of formaldehyde for the dimethylsulfoxide oxidation by peroxynitrite. Peroxynitrite also undergoes direct bimolecular reactions with ascorbate and bicarbonate [25, 30]. Peroxynitrite promotes hydroxylation and nitration of aromatic compounds [3, 27, 28, 31, 38, 65] and can rapidly oxidize thiols and thioethers forming a strongly nitrating species in the presence of metal ions or complexes [23]. A two-electron oxidation of peroxynitrite with thiols leads to the formation of disulfides [3]. *Scorza* and *Minetti* [91] presented the evidence that in human blood plasma peroxynitrite induces the formation of a disulfide cross-linked protein, a dimer of serum albumin which is promoted by bicarbonate and ascorbate. The selenoorganic compound ebselen, [2-phenyl-1,2-benzisoselenazol-3(2*H*)-one] [3], is known to reduce peroxynitrite [83, 136] yielding selenoxide [3]. Peroxynitrite reacts with glutathione giving a strongly reducing $GSSG^{\bullet -}$ anion radical, which reacts with oxygen to form the superoxide anion radical [25]. According to *Kirsch et al.* [122], glutathione, at low concentrations, strongly inhibits the $ONO^{\bullet}/CO_3^{\bullet -}$ mediated formation of 3-nitrotyrosine. Thus, glutathione could be a promissing agent protecting tyrosine against peroxynitrite attack in the presence of carbon dioxide.

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SYMBOLS

$ClNO_2$	Nitryl chloride
$ClO_2^{\bullet}$	Chlorite radical
$CO_3^{\bullet-}$	Carbonium anion radical (trioxocarbonate radical anion)
HCO_2^-	Bicarbonate anion
$HCO_3^{\bullet}$	Bicarbonate radical
HO^-	Hydroxide anion
HOO^-	Hydrogen peroxyl anion
H_2O_2	Hydrogen peroxide
$^{\bullet}N_3$	Azide radical
$NaNO_2$	Sodium nitrite
$Na_2N_2O_3$	Sodium nitrohydroxamate
$[N(CH_3)_4]^+[ONOO]^-$	Tetramethylammonium peroxynitrite
$[N(CH_3)_4]^+[O_2^{\bullet}]^-$	Tetramethylammonium superoxide
NH_2Cl	Chloramine
NH_2OH	Hydroxylamine
$^{\bullet}NO$	Nitric oxide radical
NO^+	Nitroxyl cation
NO^-	Nitroxyl anion
$NO_2^+ (ONO^+)$	Nitronium cation (nitrogen dioxide cation)
NO_2^-	Nitrite anion
NO_3^-	Nitrate anion
$NO^+HO_2^-$	Nitrosyl peroxide
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide anion radical
$^{\bullet}OH$	Hydroxyl radical
$^{\bullet}O_2H$	Perhydroxyl radical
$ONO^{\bullet}$	Dioxide radical
$ONOO^{\bullet}$	Nitrosodioxyl radical
$ONOO^-$	Peroxynitrite anion
$ONOOCO_2^-$	Nitrosoperoxycarboxylate anion
ONOOH	Peroxynitrous acid
O_2NOOH/O_2NOO^-	Peroxynitrate
$t_{1/2}$	Half-life time

ABBREVIATIONS

AD	Alzheimer's disease
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
ATPase	Adenosinetriphosphatase
B	Blood
BH_4	Tetrahydrobiopterin
CB	Crystalloid buffer
DCFH	Dichlorofluorescein
DHR	Dihydrorhodamine
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DP	Demented patients
DTPA	Diethylenetriamine-N,N',N'''-pentaacetate
ECM	Extracellular matrix
EDTA	Diaminoethanetetraacetic acid
F-actin	Filamentous actin
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FP15	Porphyrinic catalyst
G-actin	Globular actin
GAGs	Glycosaminoglycans
GFP	Green fluorescent protein
GS	Glutamine synthetase
GSH	Glutathione
$GSSG^{\bullet-}$	Glutadithionyl anion radical
HA	Hyaluronan
HIV	Human immunodeficiency virus
iNOS	Inducible nitric oxide synthase
KMBA	α-Keto-γ-methiolbutyric acid
MALS	Multi-angle laser light scattering
MnTBAP	Mn(III)-tetrakis(4-benzoic acid)-porphyrin
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa B (Transcription factor)
NP	Nondemented patients
PARP	Poly(ADP-ribose) polymerase
PCAs	Posterior cerebral arteries
RH	Rhodamine
ROS	Reactive oxygen species
SAR	Superoxide anion radical
SEC	Size-exclusion chromatography
SIN-1	3-Morpholinosydnonimine N-ethylcarbamide
SOD	Cu/Zn-superoxide dismutase
TOSC	Total oxidant scavenging capacity
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Vitamin E	α-Tocopherol
VSM	Vascular smooth muscle

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