Oxygen 2002

Sunrise Free Radical School

November 21-24, 2002
San Antonio, TX

What do $^{\cdot}NO$, $O{=}NOO^{-}$, NO_2, N_2O_3, *etc.* do, and how do they do it: Basics

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Lecture Notes--

Biologically Relevant Chemistry of ONOOH and Its Reaction Products

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- ***Goal:*** To provide the chemical background required to productively contemplate origins and consequences of peroxynitrite formation in biological environments

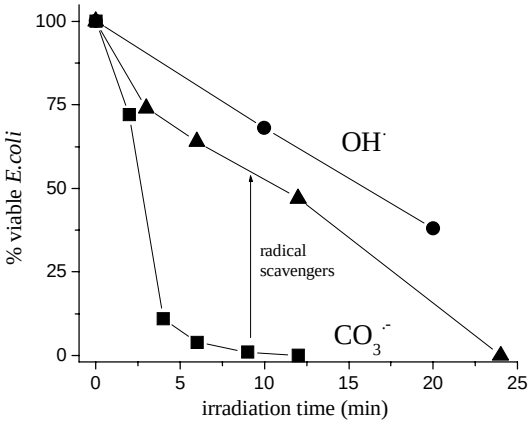
- ***Challenges:*** Critical errors exist in the chemical literature on reactive nitrogen species (RNS) and these have been inadvertently incorporated into discussions of biological reaction mechanisms.

  "An expert seldom gives an objective view; he gives his own view"--
  Morarji Desai

- ***Caveats:*** The pathophysiology of oxidative stress may bear no simple relationship to the major reaction pathways of RNS and reactive oxygen species (ROS).* This seems particularly likely for c hronic diseases whose progression involves long periods of insult, but could also be an issue under conditions of acute exposure to oxidants.

  *e.g., toxicity of $OH^{\bullet}$ toward bacteria:

  Premise 1: $OH^{\bullet}$ is not very toxic in biological environments because it is too reactive to reach vulnerable targets.



$H_2O \longrightarrow OH^{\bullet} + H^+ + e_s^-$

$e_s^- + N_2O + H^+ \longrightarrow N_2 + OH^{\bullet}$

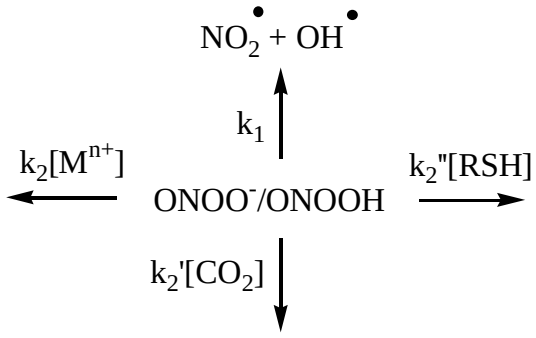
Net: $H_2O + N_2O \longrightarrow N_2 + 2OH^{\bullet}$

Toxin (*E. coli*)	LD_{90} (molecules/bacterium)	rel. toxicity
HOCl	$\sim 10^8$	1.0
H_2O_2	$\approx 3\times10^{11}$	$\sim$0.003
$CO_3^{\bullet-}$	$\sim 10^{10}$	$\sim$0.01
$OH^{\bullet}$	$\sim 2\times10^{11}$	$\sim$0.005
(however, killing by $OH^{\bullet}$ is intracellular!)		
$OH^{\bullet}$(internal)*	$\sim 10^5$	$\sim$1000

*internal volume of 10^6 cells/mL $\simeq 5\times10^{-7}$ cm^3

Conclusion: intracellularly generated $OH^{\bullet}$ is highly toxic!

Premise 2: $OH^{\bullet}$ formed by ONOOH bond homolysis cannot be a significant component of the oxidative load because bimolecular reactions of $ONOO^-$/ONOOH (e.g., with CO_2, thiols (RSH), metalloproteins) will predominate in biological environments.



At pH 7.4,

$k_1 \simeq 0.34\ s^{-1}$

$k_2[M^{n+}] \lesssim 300\ s^{-1}$

$k_2'[CO_2] \simeq 20\ s^{-1}$

$k_2''[RSH] \lesssim 3\ s^{-1}$

Simple competition kinetics predicts:

$$[OH^{\bullet}]/[ONOO^-/ONOOH] = k_1/\Sigma(k_i[X_i]) \lesssim 0.015$$

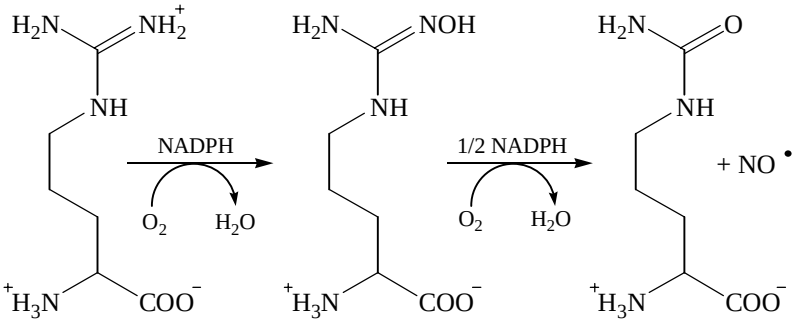
i.e., less than 2% of the peroxynitrite formed will generate $OH^{\bullet}$. Nonetheless, if intracellular $OH^{\bullet}$ is 10^3 times more toxic than other oxidants, it could still be a significant component of oxidative stress.

Possible Biological Origins of $ONOO^-$/ONOOH

A. **$NO^\bullet + O_2^{\bullet-} \rightarrow ONOO^-$** (radical coupling, $k \approx 10^{10}\ M^{-1}s^{-1}$)

Primary sources of $NO^\bullet$ and $O_2^{\bullet-}$ in respiring tissues:

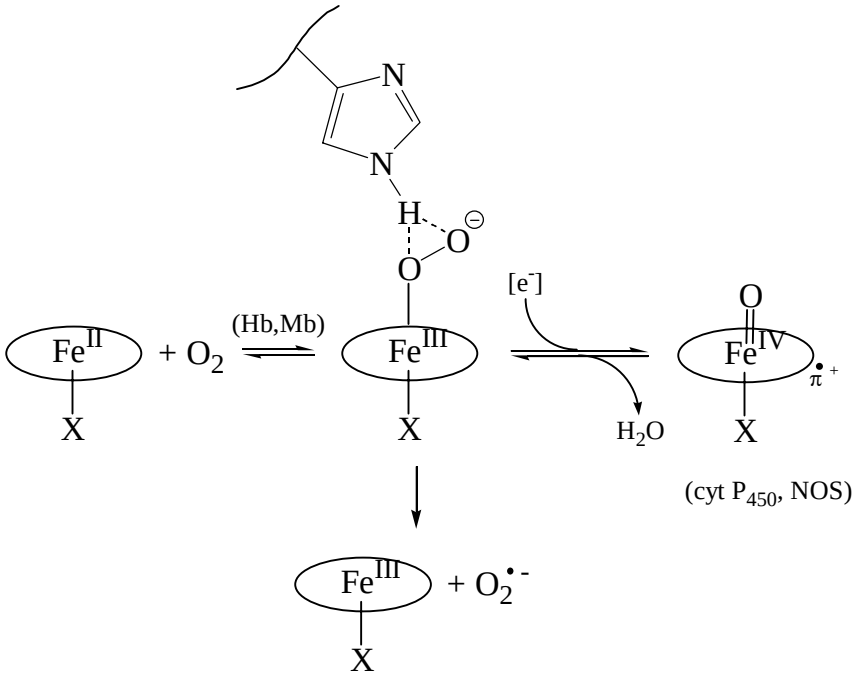
$NO^\bullet$:



catalyzed by NOS isozymes (nNOS(I), iNOS(II), eNOS(III))

$O_2^{\bullet-}$:

i) O_2-binding heme proteins--



e.g., hemoglobin (Hb); myoglobin (Mb);
cytochrome $P_{450;}$ L-arg or tetrahydrobiopterin (H_4B)-depleted NOS.

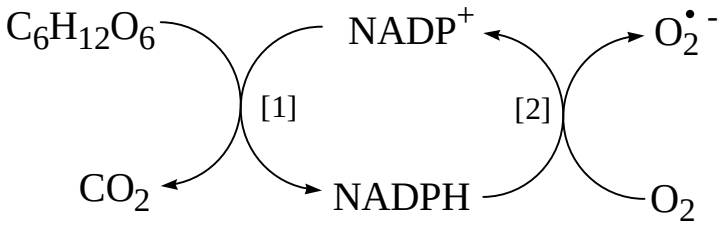
ν(O-O) = 1105 cm^{-1} (Hb-O_2, Mb-O_2)
1140 cm^{-1} (P_{450}-O_2)

cf. 1556 cm^{-1} (O=O)
1145 cm^{-1} (KO_2)
836 cm^{-1} (NH_4O_2H)

ii) endogenous semiquinones (e.g., mitochondrial bc complex (III))--

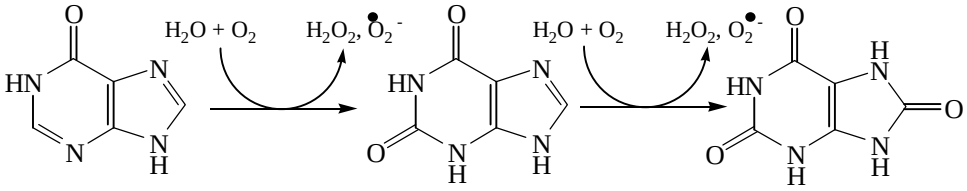
$$Q^{\bullet -}/QH + O_2 \rightarrow Q(+ H^+) + O_2^{\bullet -}$$

iii) stimulated phagocytes (e.g., neutrophil)--



[1] pentose phosphate pathway
[2] NADPH oxidase

iv) molybdoflavoenzymes (e.g., xanthine oxidase)

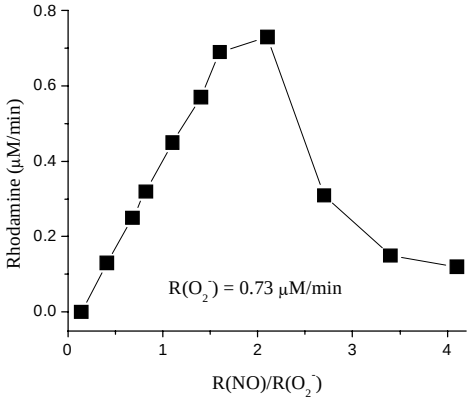


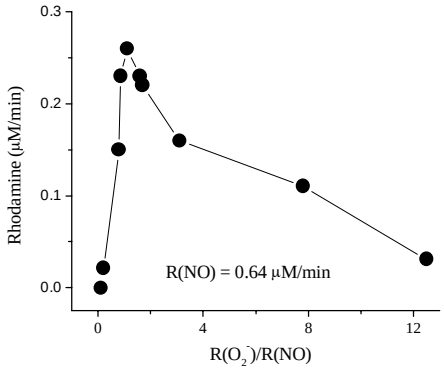
hypoxanthine xanthine uric acid

- A conceptual problem (?)--

  optimal formation of $ONOO^-/ONOOH$ requires approximately equal fluxes of $NO^\bullet$ and $O_2^{\bullet -}$

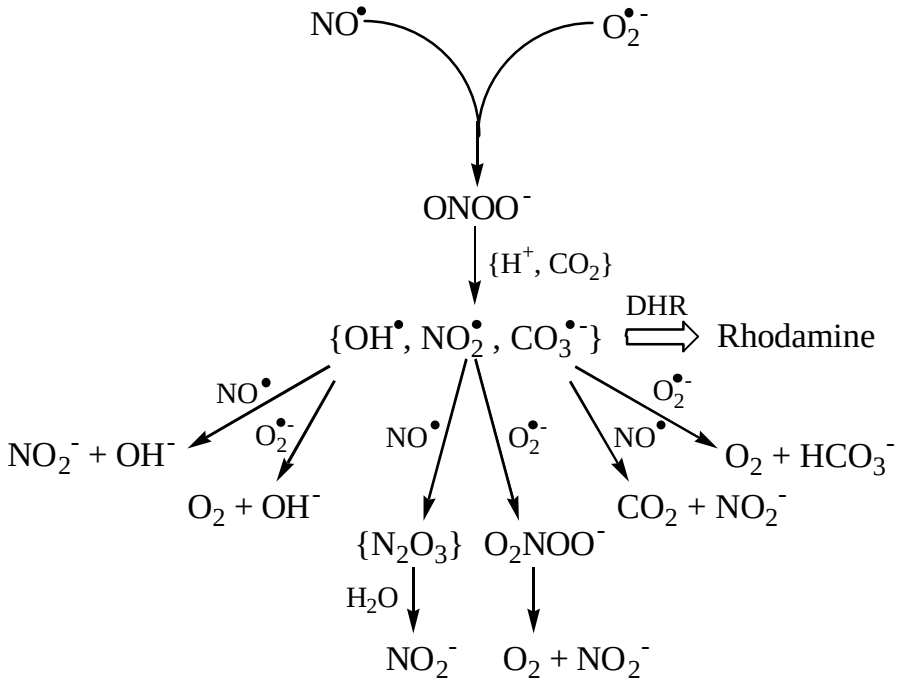
  e.g., $ONOOH \xrightarrow{k_1(\text{slow})} \{int\} \xrightarrow{DHR} Rh\ (hv) + H_2O + NO_2^-$



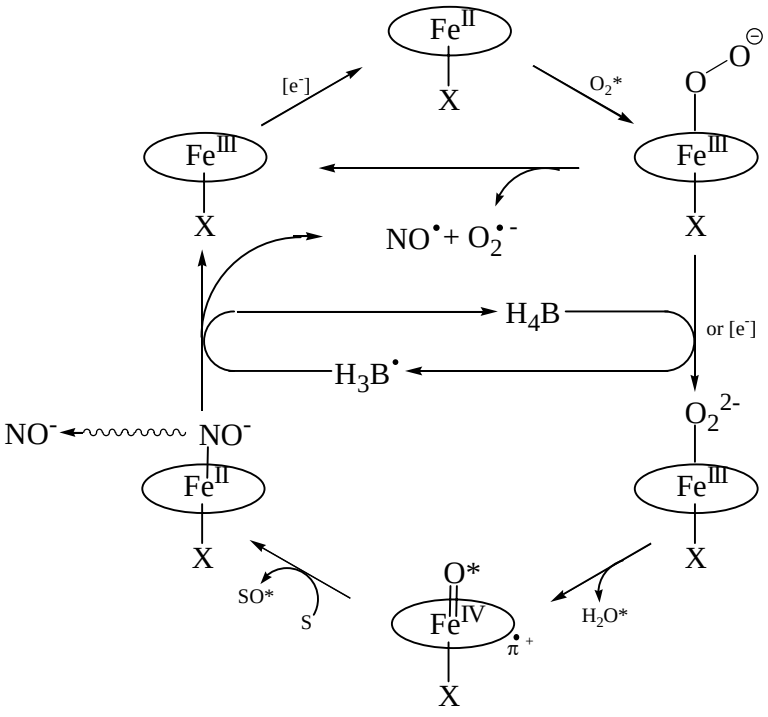


data from: [Jourd"heuil, et al. *J.Biol Chem*. **2001**, *276*, 28799-28805]

Interpretation:

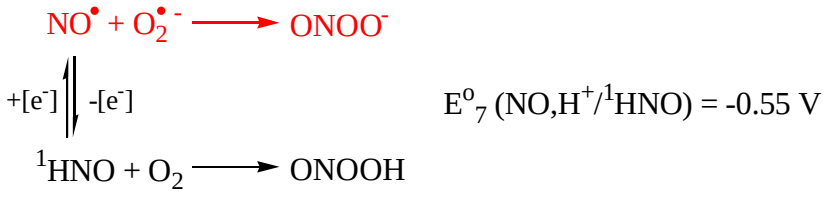


- Is NOS capable of simultaneous generation of $NO^{\bullet}$ and $O_2^{\bullet -}$?



[Stuehr et al. *J. Biol. Chem.* **2001**, 276, 14533-14536]

B. "Nitroxyl" reactions--

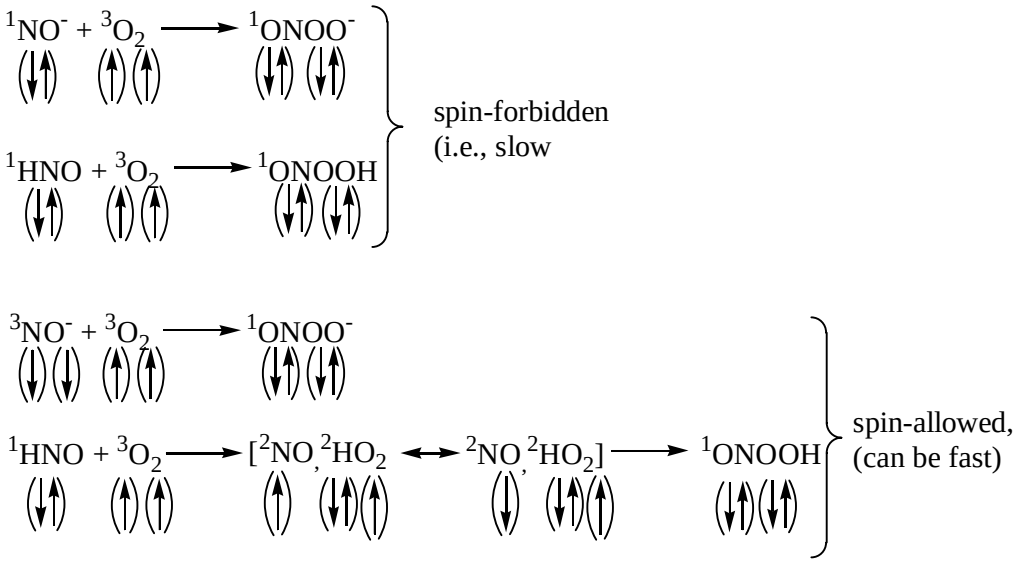


Potential biological sources of HNO--

- NOS (H_4B-depleted environments)
- RS-NO, e.g.: $RSH + N_2O_3 \longrightarrow RS\text{-}NO + NO_2^- + H^+$ ($\{NO^+\text{-}NO_2^-\}$)

  $RSH + RS\text{-}NO \longrightarrow RSSR + HNO$

- Spin restrictions and kinetics—



$ONOO^-$/ONOOH formation from "nitroxyl" depends upon its physical properties (e.g.,spin state, acidity, redox potential).

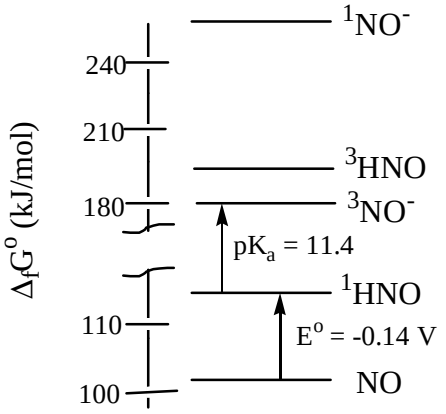
Previous description--

- $HNO \rightleftharpoons H^+ + {}^1NO^-$, $pK_a \simeq 4.7$
  (weak acid, present as singlet anion in physiological milieu)
- reaction with O_2 is spin-forbidden

Reassessment--

"There is only as much truth in any natural science as there is mathematics in it"--
as translated by Sergei Lymar from obscure Russian origins

[Shafirovich & Lymar, *Proc. Natl. Acad. Sci, USA* **2002**, *99*, 7340-7345]



$\Delta G^o = \Sigma\ \Delta_fG^o_{products} - \Sigma\ \Delta_fG^o_{reactants}$

acidity of HNO--

$^1HNO \rightleftharpoons H^+ + {}^3NO^-$

Δ_fG^o: 115 0 180

$\Delta G^o = -RT\ lnK_a = 65$ kJ/mol; $pK_a = 11.4$

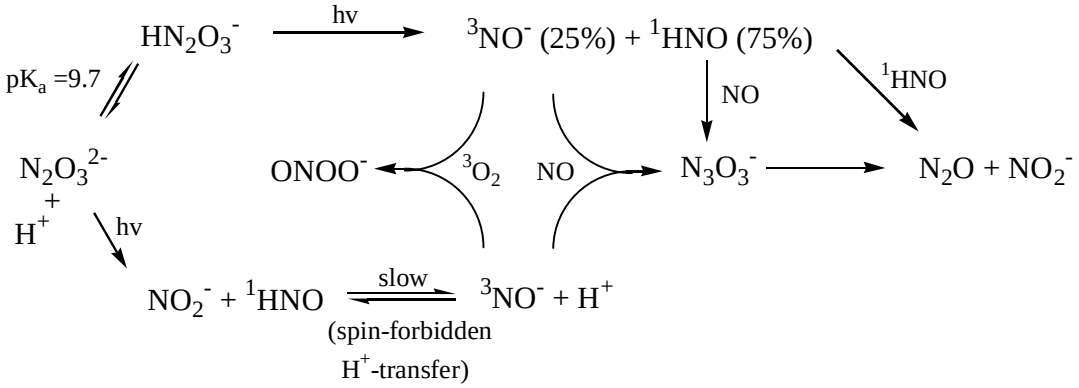
NO reduction potential--

$H^+ + NO + e^- \rightarrow {}^1HNO$

Δ_fG^o: 0 102 0 115

$\Delta G^o = -nFE^o = 13$ kJ/mol; E^o = -0.14V (pH 0)
-0.55V (pH 7)

Photoreaction Dynamics (reactions with O_2 and $NO^{\bullet}$):



Biological implications--

- "nitroxyl" in physiological environments is ^{1}HNO (the acid) and is ***strongly reducing*** ($E^o_7 \simeq -0.55$V)

- ^{1}HNO and NO$^\bullet$ are nitroxyl "sinks"--
  can $ONOO^-$/ONOOH be formed from ^{1}HNO in physiological environments?

  $$PN/[^1HNO] \approx (10^3 \times [O_2] + 10^7 \times [OH^-])/\{10^7 \times ([NO^\bullet] + [^1HNO])\}$$
  where $PN = [ONOO^-] + [ONOOH]$

  If $[O_2] > [NO^\bullet]$, $[O_2] = 10\ \mu M$, $[NO^\bullet] + [^1HNO] = 10$ nM, then at pH 7.4,
  $PN/[^1HNO] \approx 0.3$ (or 30% of the ^{1}HNO decays to PN)
  However, if either $[NO^\bullet]$ or $[^1HNO] >> 10$ nM, the PN yield is negligible.

  (Experimentally--$HN_2O_3^-$ decomposition causes O_2 to decrease, implying reaction with ^{1}HNO)

- Low reduction potential of NO$^\bullet$ accounts for ability of "nitroxyl" to reduce redox-active metalloproteins (e.g., (Cu-Zn)SOD, (Fe^{3+})cyt c, (Fe^{3+})Hb). What accounts for its ability to oxidize RSH and Hb-O_2? ^{1}HNO is also a ***moderately strong oxidant***, i.e.,

  $$^1HNO + 2H^+ + 2e^- \rightarrow NH_2OH \quad (E^o_7 \simeq 0.3\ V)$$

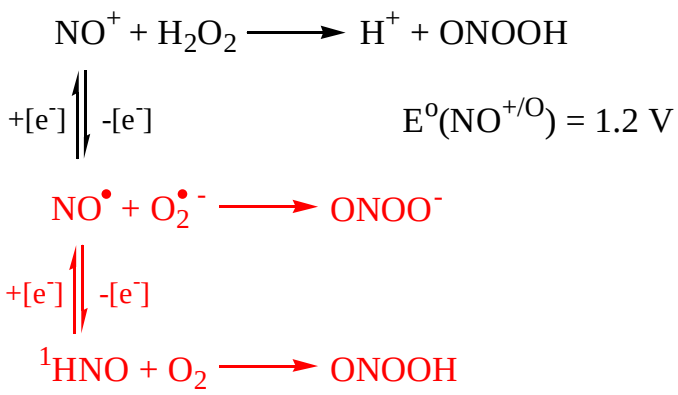
  e.g., $$RSH + {}^1HNO \longrightarrow RS\text{-}NHOH \longrightarrow RSSR + NH_2OH$$

  $$\left.\begin{array}{l} H^+ + \underset{(Fe^{3+}\text{-}O_2^-)}{Hb\text{-}O_2} + {}^1HNO \longrightarrow (Fe^{3+})Hb + NO + H_2O_2 \\ Hb\text{-}O_2 + NO \longrightarrow (Fe^{3+})Hb + NO_3^- \end{array}\right\}$$

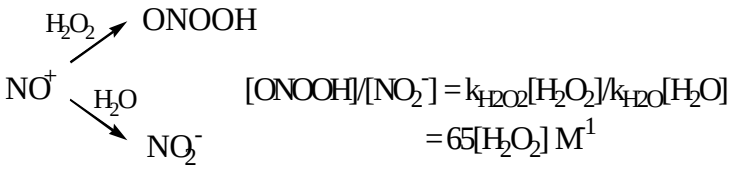
- Since ^{1}HNO is both strongly oxidizing and reducing, it is unstable to disproportionation:
  $$3\ {}^1HNO \rightarrow 2\ NO^\bullet + NH_2OH, \quad \Delta E^o_7 \simeq 0.85\ V$$

  Thus, many redox-active metalloproteins are potentially capable of catalyzing ^{1}HNO decay.

C. "Nitrosyl" reactions--



- $NO^+ + H_2O \rightleftharpoons NO_2^- + 2H^+$; $K = [NO_2^-][H^+]^2/[NO^+] = 10^3\ M^2$
  at pH 7.4, $[NO^+]/[NO_2^-] \approx 10^{-18}$!

- 

  at $[H_2O_2] \leq 100\ \mu M$, $[ONOOH]/[NO_2^-] \leq 0.007$, i.e.,
  less than 1% of the generated NO^+ will form ONOOH.

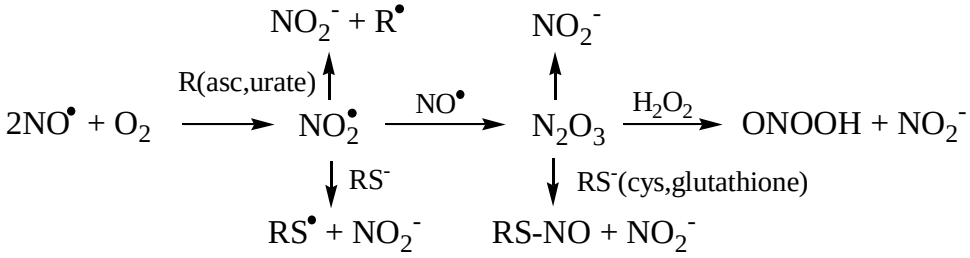
Conclusion--

free NO^+ is not involved in biological $ONOO^-$/ONOOH formation

— too difficult to oxidize $NO^\bullet$

— at physiological [ONOOH], reaction cannot compete with hydration by H_2O.

"Stabilized" NO^+--i.e., N_2O_3 *(or NO^+-NO_2^-)*--as a nitrosating agent?



N_2O_3 reactions--

at pH 7.4: $k_{RSH} \simeq 10^6\ M^{-1}s^{-1}$; $k_{H2O2} \leq 10^5\ M^{-1}s^{-1}$; $k_{H2O}[H_2O] \simeq 10^3\ s^{-1}$

if $[H_2O_2]$ = 0.1 mM; [RSH] = 5 mM,

$$[ONOOH]/[N_2O_3] \leq k_{H2O2}[H_2O_2]/\Sigma (k_i[X_i]) = 0.007\ (0.7\%)$$

NO_2 reactions--

at pH 7.4: $k_R \simeq k_{RSH} \simeq 3\times10^7\ M^{-1}s^{-1}$; $k_{NO} = 10^9\ M^{-1}s^{-1}$

if $[NO^{\bullet}] \simeq 1\ \mu M$; [urate] ≃ 0.3 mM (plasma); [asc] ≃ 0.5 mM (cytosol); [RSH] ≃ 5 mM (cytosol) or 0.1 mM (plasma),

$$[N_2O_3]/[NO_2^{\bullet}] = k_{N2O3}[N_2O_3]/\Sigma (k_i[X_i]) \simeq 0.006\ \text{(cytosol)}\ 0.08\ \text{(plasma)}$$

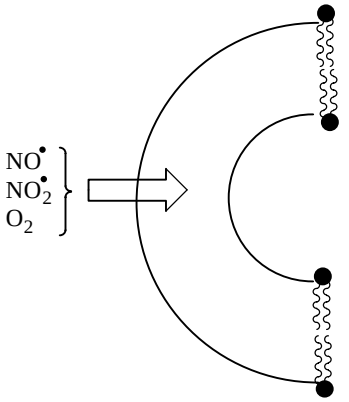
Overall--

$$[ONOO]/[NO_2^{\bullet}] = [N_2O_3]/[NO_2^{\bullet}]\times[ONOOH]/[N_2O_3] \leq 0.08\times0.007 = 6\times10^{-4}\ (\mathbf{< 0.1\%})$$

Primary pathway for $ONOO^-$/ONOOH formation under physiological conditions is yet to be identified!

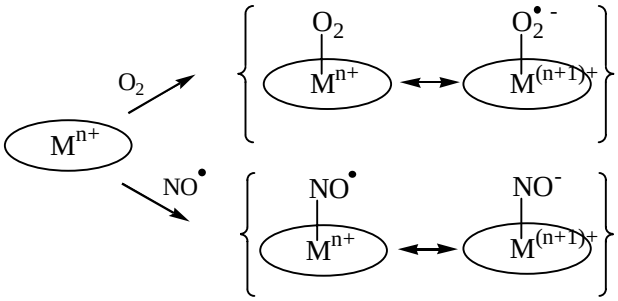
What might change this viewpoint?

- Predictions are based upon rate ratios measured for aqueous solution. However,



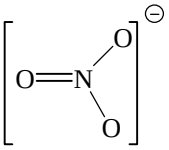
- Gases are more soluble in hydrocarbons than H_2O;
- In lipidic regions of tissues, reaction dynamics will be very different--s.p.,
  — reactions forming neutral products will be favored;
  — intermediates susceptible to hydrolytic disproportionation by H_2O will be protected ($t_{1/2}$ will increase).

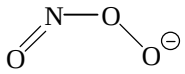
- Binding to metalloproteins can activate and stabilize ONOOH precursors:



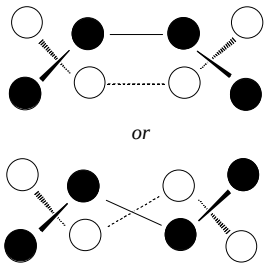
- E^o of bound O_2, $NO^{\bullet}$ increased
- $t_{1/2}$ of reactive intermediates increased
- spin restrictions removed

What is peroxynitrite? (Biologically relevant chemical properties)

a structural isomer of nitrate 

that contains a peroxo bond: 

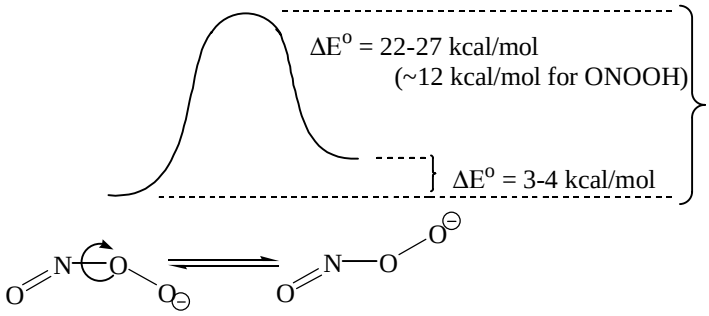
X-ray structure $\{(CH_3)_4N^+/OONO^-\}$:
[Worle et al., *Chem. Res. Toxicol*. **1999**, *12*, 305-307]



N-O(peroxo) bond length = 1.35 Å
N-O single bond length = 1.41 Å
N=O double bond length = 1.20 Å

significant multiple bond character in the N-O peroxo bond indicated by bond length; delocalization of N lone pair electrons over nuclear framework stabilizes *cis* and *trans* planar geometries.

from *ab initio* calculations:



a weak acid: $ONOOH \rightleftharpoons ONOO^- + H^+$ $pK_a = 6.8$

cf, $HOOH \rightleftharpoons HOO^- + H^+$ $pK_a = 11.6$

O-O bond dissociation energies:

$ONO\text{-}OH \longrightarrow NO_2^{\bullet} + OH^{\bullet}$ BDE = 20 kcal/mol

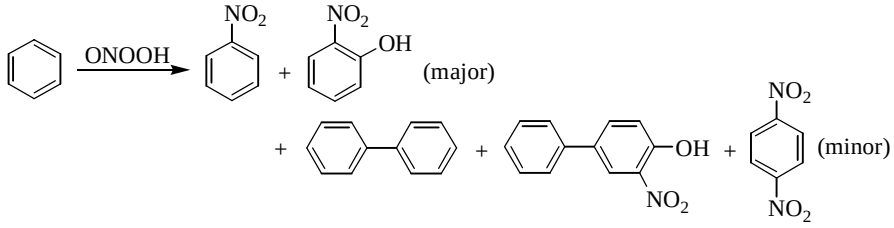
$HO\text{-}OH \longrightarrow OH^{\bullet} + OH^{\bullet}$ BDE = 52 kcal/mol

A little peroxynitrite history:

- First prepared at the turm of the century [Baeyer & Villiger, *Berichte***1901**, *34*, 755], but not characterized until much later (1930's).

- Regarded as little more than a laboratory curiosity--e.g., the classic 1st text on mechanistic inorganic chemistry by Yost & Russell ("Systematic Inorganic Chemistry", 1946) gives it just one line.

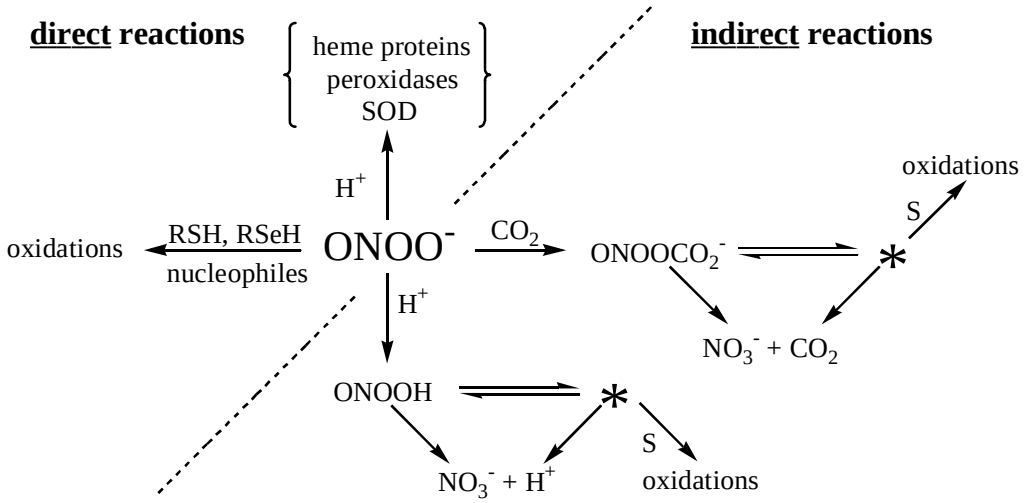
- Some interesting research on aromatic hydroxylations and nitrations appeared in the 1950's [e.g., Halfpenny, E., Robinson, P. L. *J. Chem. Soc*. **1952**, 928-936, ibid. 939-946.]:



but was subsequently largely forgotten. Reactivity was attributed to formation of radical intermediates because diaryl coupling was observed among the minor products.

- An early mechanistic study providing evidence for formation of $OH^{\bullet}$ and $NO_2^{\bullet}$ during ONOOH decomposition was published [Mahoney, L. R. *J. Am. Chem. Soc*. **1970**, *92*,1562-1563], but also apparently overlooked by later researchers.

Chemical reactivity:



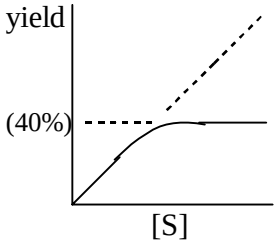
direct reactions:

— $1e^-$ and $2e^-$ oxidations
— stoichiometric (%100%) yields
— R = k[ONOOH][S];
(i.e., bimolecular)

indirect reactions:

— $1e^-$ oxidations
— substoichiometric ($\leq$ 40%) yields†
— R = k(ONOOH) or k[$ONOO^-$][CO_2]
(i.e., independent of S!)

†:

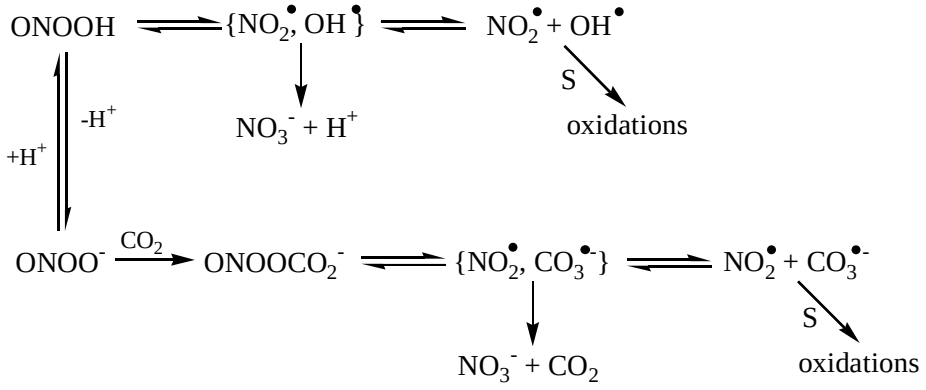


What is "✻"? (i.e., the reactive intermediate):

"One of the symptoms of an approaching nervous breakdown is the belief that one's work is terribly important."--Bertrand Russell

Two alternative viewpoints:

(1) Unreactive forms are geminate radical pairs; "✻" represents radicals that escape the cage:

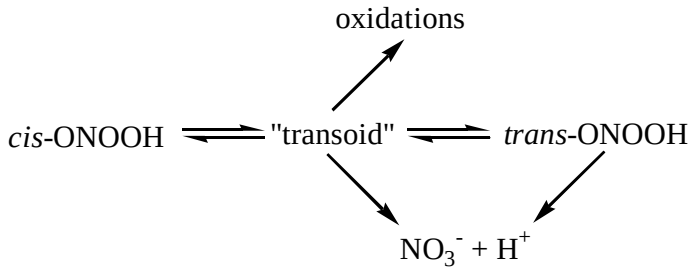


support:

ONO-OH	BDE(calc.)	~20 kcal/mol
$ONO-OCO_2^-$	BDE(calc.)	~9 kcal/mol
cf. HO-OH	BDE (exptl.)	~52 kcal/mol
H_3C-CH_3	BDE (exptl.)	88 kcal/mol

peroxo O-O bonds are very weak!

(2) Unreactive and reactive forms are geometrical isomers:



support:

thermodynamic calculations show rate of O-O bond homolysis in ONOOH is too slow to account for observed reaction rates (?)

Thermodynamic estimates of ONOOH homolysis rates:

$$ONOOH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NO_2^{\bullet} + OH^{\bullet}$$

[Koppenol et al., *Chem. Res. Toxicol*. **1992**, *5*, 834-842]: **k_1(calc.) = 10^{-4}-10^{-6} s^{-1}**

conclusion: since the experimentally determined 1st order rate constant for ONOOH decay is **k_{decay} = 0.8 s^{-1}** (4-6 orders of magnitude larger than the estimated k_1, for bond homolysis), the reaction cannot occur by this pathway!

[Mereyni &Lind, *Chem. Res. Toxicol*. **1997**, *10*, 1216-1220]: **k_1(calc.) = 2.5 s^{-1}**

conclusion: the estimated O-O homolysis rate constant (k_1) is totally consistent with the measured k_{decay}.

[Koppenol & Kissner, *Chem. Res. Toxicol*. **1998**, *11*, 1216-1220]: **k_1(calc.) = 0.01 s^{-1}**

conclusion: perhaps a few % decay by this pathway, but not much.

What's the problem? Why can't our experts agree?

(1) $K = [NO_2^{\bullet}][OH^{\bullet}]/[ONOOH] = k_1/k_{-1}$ ($k_{-1} \simeq 5\times10^9\ M^{-1}s^{-1}$)

(2) $-RTlnK = \Delta G^o = \Delta_f G^o(NO_2^{\bullet})_{aq} + \Delta_f G^o(OH^{\bullet})_{aq} - \Delta_f G^o(ONOOH)_{aq}$

(3) $\Delta_f G^o = \Delta_f H^o - T\Delta_f S^o$

Enthalpies:

$\Delta_f H^o(ONOO^-)_{aq} = \Delta_f H^o(NO_3^-)_{aq} - \Delta H_1$ (ΔH_1 = measured heat of isomerization, i.e., $ONOO^- \rightarrow NO_3^-$)

$\Delta_f H^o(ONOOH)_{aq} = \Delta_f H^o(ONOO^-)_{aq} - \Delta H_2$ (ΔH_2 = measured heat of ionization, i.e., $ONOOH \rightleftharpoons ONOO^- + H^+$)

Entropies:

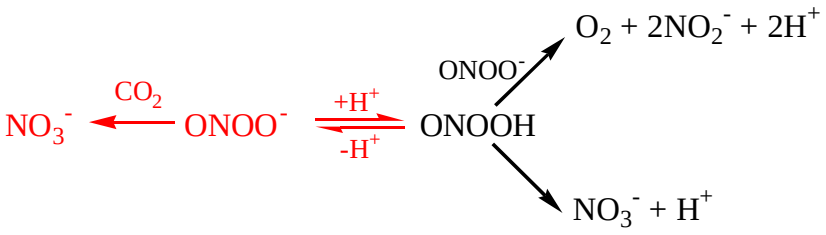
$ONOOH(g) \rightarrow ONOOH(aq)$

or for which $S^o(aq) = S^o(g) + \Delta S^o$, where ΔS^o = solvation entropy

$ONOO^-(g) \rightarrow ONOO^-(aq)$

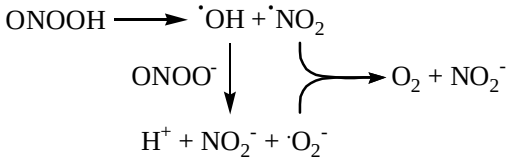
ΔS^o is very difficult to estimate!

A kinetic approach:



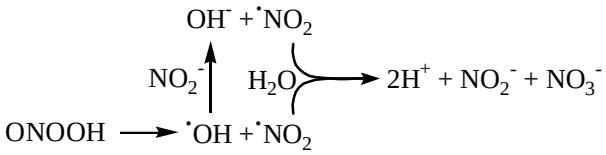
radical pathways for ONOOH decay *(note: these exist!--the issue is whether or not they can account for the observed product yields*):

i) *direct decomposition:*



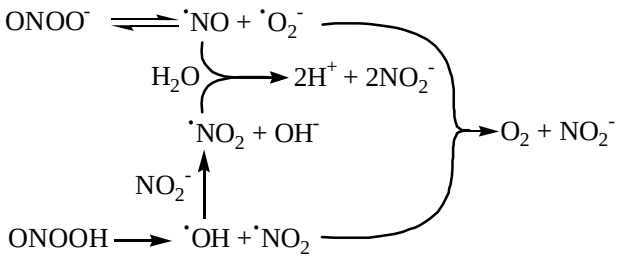
Net: **ONOOH + ONOO⁻ ⟶ O_2 + 2NO_2^- + H^+**

ii) *NO_2^--mediated isomerization:*



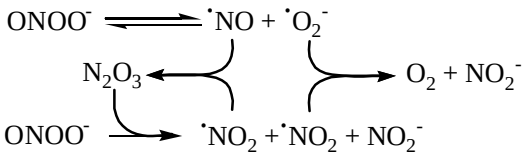
Net: **ONOOH ⟶ H^+ + NO_3^-**

iii) *NO_2^--mediated decomposition:*

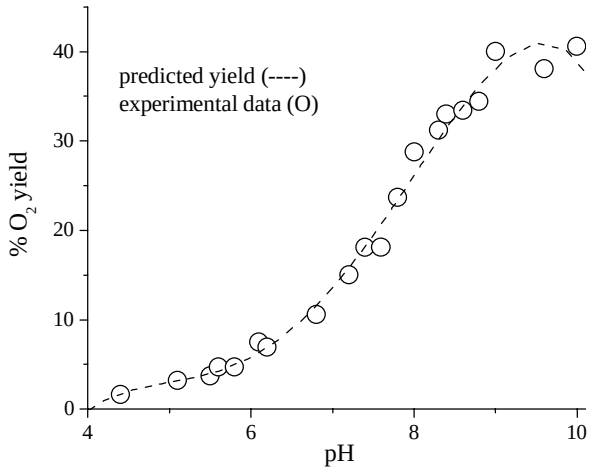


Net: **ONOOH + ONOO⁻ ⟶ O_2 + 2NO_2^- + H^+**

iv) *N_2O_3-catalyzed decomposition:*



Net: **2ONOO⁻ ⟶ O_2 + 2NO_2^-**

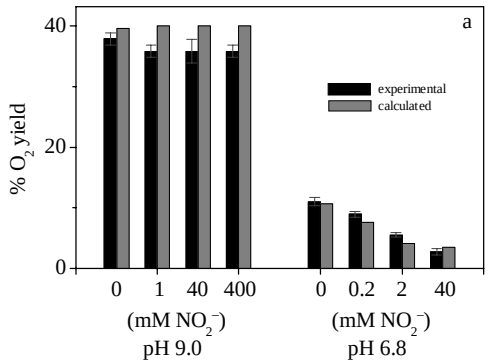


Conclusion: the radical pathway accurately predicts decomposition yields

More predictions of the radical model:

(i) NO_2^- inhibits O_2 formation in acidic media; not in alkaline;
(ii) inhibition by organic radical scavengers will be reversed by NO_2^- in alkaline media;
(iii) inhibition by $Fe(CN)_6^{4-}$ will not be reversed by NO_2^-.

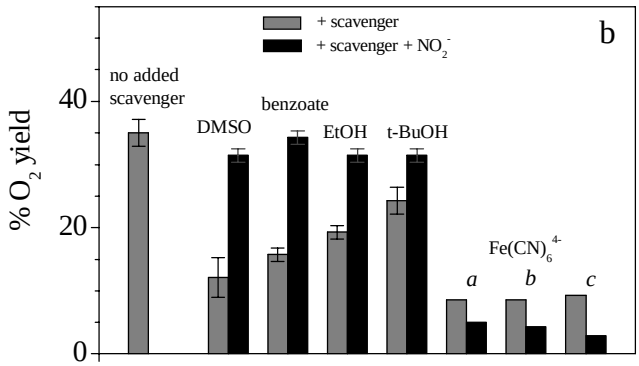
Prediction (i):



In alkaline media, $NO_2^{\bullet}$ formed from reaction of NO_2^- with $OH^{\bullet}$ with $NO^{\bullet}$ formed by $ONOO^-$ dissociation to give same products as direct decomposition; in acid $NO_2^{\bullet}$ reacts with another $NO_2^{\bullet}$ to give net isomerization.

Predictions (ii) and (iii):

Organic radicals formed in reactions with $OH^{\bullet}$ decay by pathways that do not form O_2; the more reactive NO_2^- effectively scavenges $OH^{\bullet}$ in the presence of these compounds, generating O_2 by pathway iii. $Fe(CN)_6^{4-}$ reacts rapidly with both $NO_2^{\bullet}$ and $OH^{\bullet}$, effectively quenching any further reaction, e.g.,



v) *$Fe(CN)_6^{4-}$ inhibition of O_2 formation:*

(NO_2^- absent):

$$ONOOH \longrightarrow {}^{\bullet}OH + {}^{\bullet}NO_2 \xrightarrow{2\ Fe(CN)_6^{4-}} OH^- + NO_2^- + 2\ Fe(CN)_6^{3-}$$

(NO_2^--mediated):

$$OH^- + {}^{\bullet}NO_2 \xrightarrow{Fe(CN)_6^{4-}} NO_2^- + Fe(CN)_6^{3-}$$

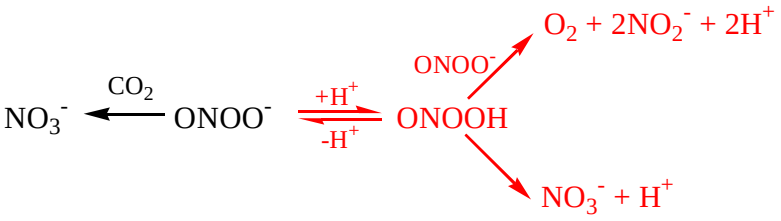
$$ONOOH \xrightarrow{NO_2^-} {}^{\bullet}OH + {}^{\bullet}NO_2 \xrightarrow{Fe(CN)_6^{4-}} NO_2^- + Fe(CN)_6^{3-}$$

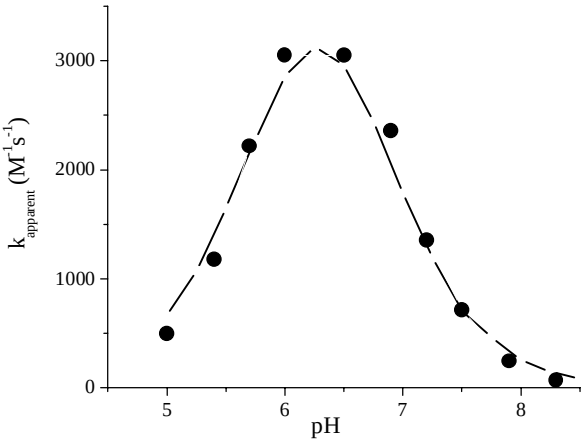
Net: $\mathbf{ONOOH + 2\ Fe(CN)_6^{4-} \longrightarrow NO_2^- + OH^- + 2\ Fe(CN)_6^{3-}}$

***Conclusion*: $OH^{\bullet}$ and $NO_2^{\bullet}$ radical formation during ONOOH decay is extensive.** At present no undisputed evidence exists that is inconsistent with this reaction model--[Coddington, J.W. et al. *J. Am. Chem. Soc*. **1999**, *121*, 2438-2443].

Reactive intermediates in bicarbonate media:

How do we know that $ONOO^-$ and CO_2 are the reactants?





$$-d[PN]/dt = k_{apparent}[PN][HCO_3]_T$$

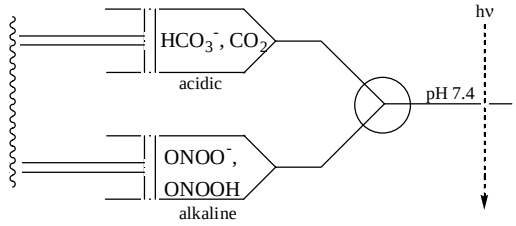
$$[PN] = [ONOO^-] + [ONOOH]$$
$$[HCO_3]_T = [CO_2] + [HCO_3^-]$$

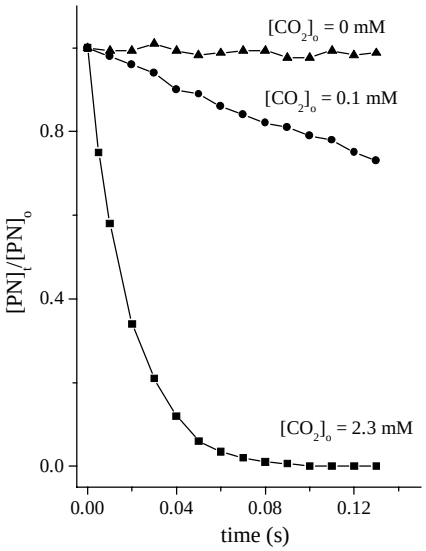
$$k_{apparent} = k_2/(1 + [H^+]/K_a)(1 + K_a'/[H^+])$$
or
$$k_2'/(1+ K_a/[H^+])(1 + [H^+]/K_a')$$

pH-rate profile indicates that reactant pairs are ***either (ONOOH, HCO_3^-) or ($ONOO^-$, CO_2).*** These can be distinguished by a stopped-flow pH-jump experiment because the equilibrium:

$$HCO_3^- + H^+ \rightleftharpoons \{H_2CO_3\} \rightarrow H_2O + CO_2$$

is slow in neutral solutions:

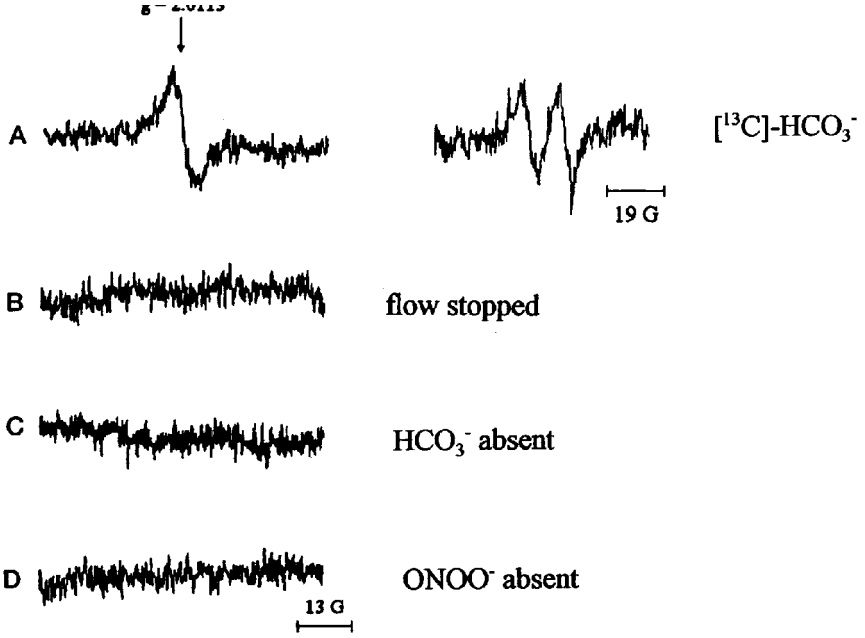


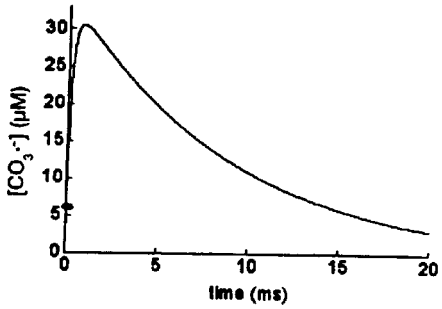


Since the reaction rate depends upon $[CO_2]$, the isomerization reaction involves $ONOO^-$ and CO_2 ($k_2 = 3\times10^4\ M^{-1}s^{-1}$ @ 23 °C)

What is "✻" (the reactive intermediate) formed in the indirect reactions of $ONOOCO_2^-$?

direct flow-EPR detection of $CO_3^{\bullet-}$ from reaction of $ONOO^-$ with CO_2:
[Bonini et al. *J. Biol. Chem.* **1999**, *274*, 10802-10806]





Conclusion: "Indirect" reactions occur by O-O bond homolysis in peroxynitrite adducts (ONOOH, $ONOOCO_2^-$) to generate reactive radicals ($OH^{\bullet}$, $CO_3^{\bullet-}$, $NO_2^{\bullet}$) as secondary oxidants