POLITECNICO DI MILANO

Department CMIC

Lecture 16 – FR16





Free-Radicals: Chemistry and Biology

Prof. Attilio Citterio

Dipartimento CMIC “Giulio Natta”

http://iscamap.chem.polimi.it/citterio/education/free-radical-chemistry/

Content

1. **Introduction**
   - Current Status of Radicals Chemistry
   - What is a Radical
   - Free Radicals and Life
2. **Historical Aspects**
3. **Electronic Structure and Bonding**
4. **Active Oxygen Specie,**
   - O_2, $O_2^{\bullet-}$, $HO_2^{\bullet}$, $^1O_2$, H_2O_2, $HO^{\bullet}$
   - Chemistry
   - H_2O_2 and peroxides
5. **Radical Reactions**
   - Atom transfer
   - Addition to multiple bonds
   - Homolytic Aromatic Substitution
   - Electron Transfer (oxidation-reduction)
6. **Thermodynamics**
7. **Free Radical Kinetics**
   - First-order Reaction
   - Second-order Reaction
   - Steady-State
   - Chain-reactions
   - Redox chain reactions
   - Inhibition
8. **Radiation Chemistry**
   - Tools
   - Specie: e^-(aq), $H^{\bullet}$, $HO^{\bullet}$, H_2O_2, H_2, $O_2^{\bullet-}$
   - Pulse Radiolysis/Flash Photolysis
9. **Lipid Peroxidation**
   - Chemistry
   - Measurement
   - Effects
10. **Antioxidants**
    - Preventive
    - Chain-breaking
    - Small molecule (Vit. C/E, CoQ, Urate).
    - Enzymes
    - Chelates
11. **Iron and Free Radical Chemistry**
    - Reactions
    - Complexes and redox chemistry
12. **DNA and Protein** (As radical targets)
13. **Photo reactions**
    - Photochemistry
    - Photosensitization
14. **Detection of Radicals**
    - TBARS
    - Fluorescence
    - Cyt. C /NBT
    - Chemiluminescence
15. **EPR Detection of Radicals**
    - Direct Detection
    - Spin Trapping
    - Transition metal
16. **Nitric Oxide/NOS**
17. **Oxygen radicals/ROS**

POLITECNICO DI MILANO





Reactive Nitrogen Species (RNS) Nitric Oxide / NOS

Prof. Attilio Citterio
Dipartimento CMIC “Giulio Natta”

Reactive Nitrogen Species (RNS)

Radicals:

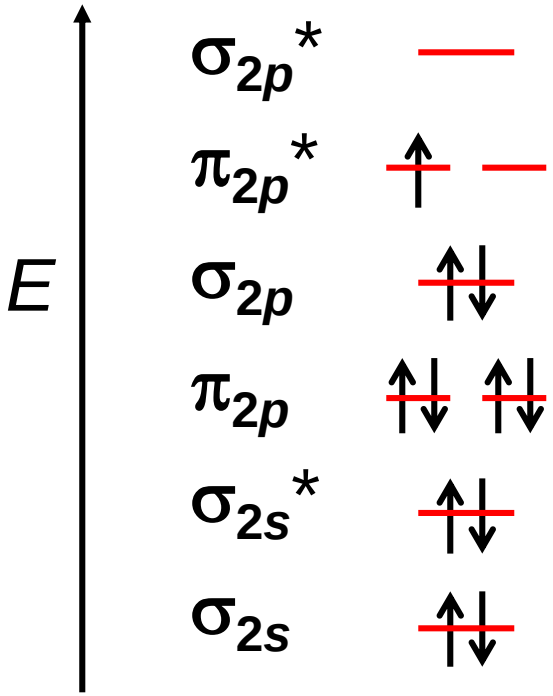
$NO^{\bullet}$	Nitric Oxide
$NO_2^{\bullet}$	Nitrogen dioxide

Non-Radicals:

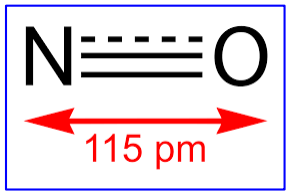
$ONOO^-$	Peroxynitrite
$ROONO$	Alkyl peroxynitrites
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetroxide
HNO_2	Nitrous acid
NO_2^+	Nitronium cation
NO^-	Nitroxyl anion
NO^+	Nitrosyl cation
NO_2Cl	Nitryl chloride

Nitric Oxide

Persistent radical, until 1989 considered only a pollutant. Then, identified as a fundamental signaling agent in cells biochemistry with relevant physiological roles.



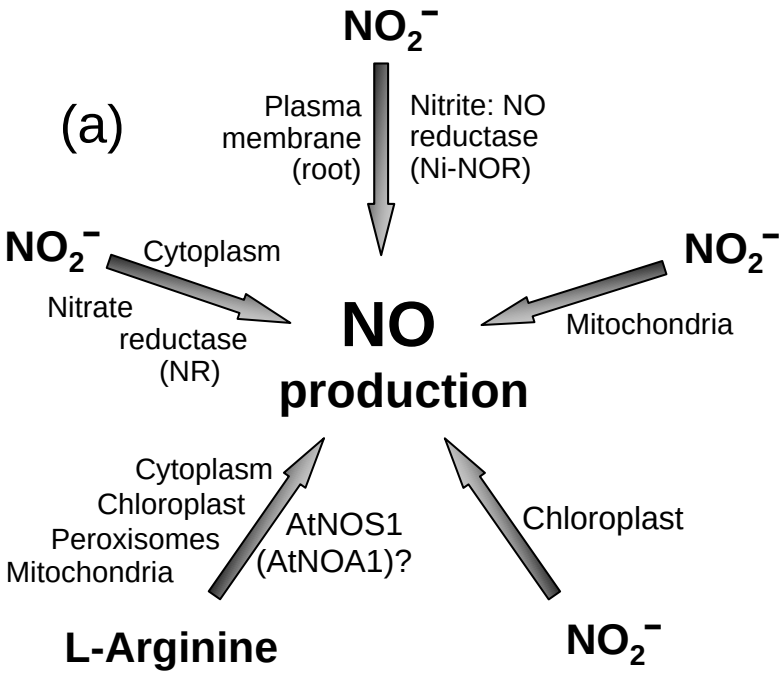
Bond Order
= (1/2) [8– 3]
= 2.5

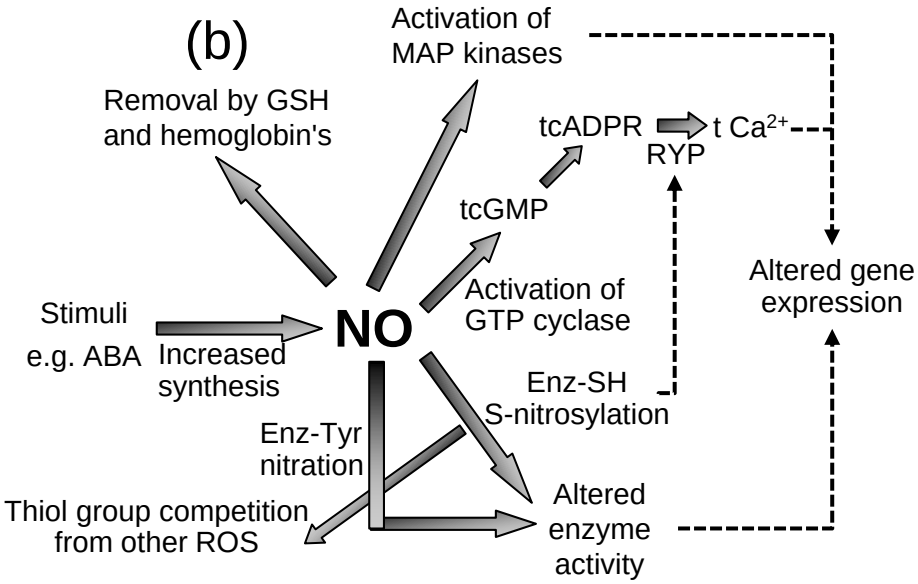


1 σ bond
1 π bond
1 “3 electrons” bond

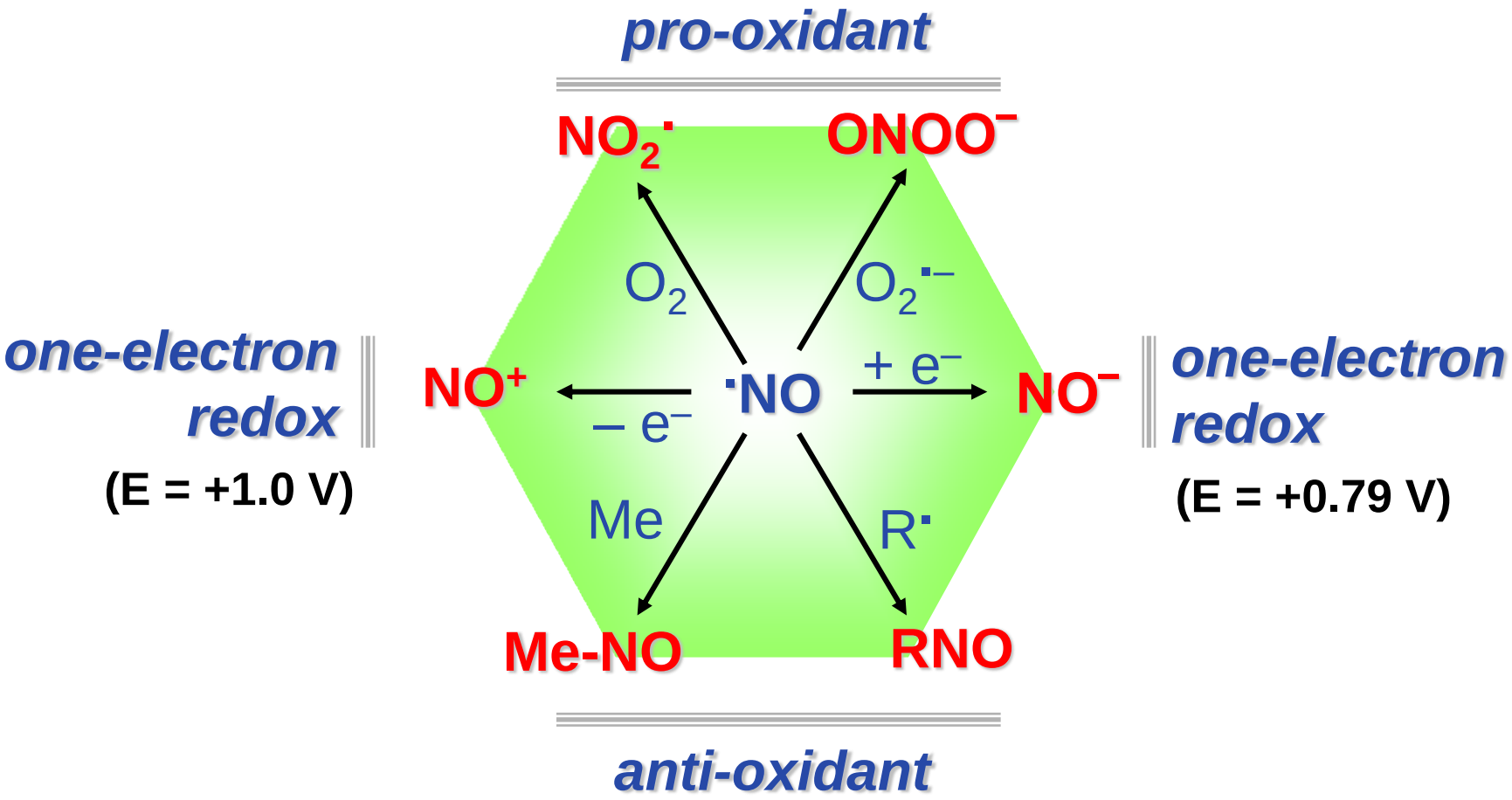
Nitric oxide: a molecule of millennium! *Circ Res* **2000**, *87*, 85-7.

Synthesis and Fate of Nitric Oxide in Living Systems



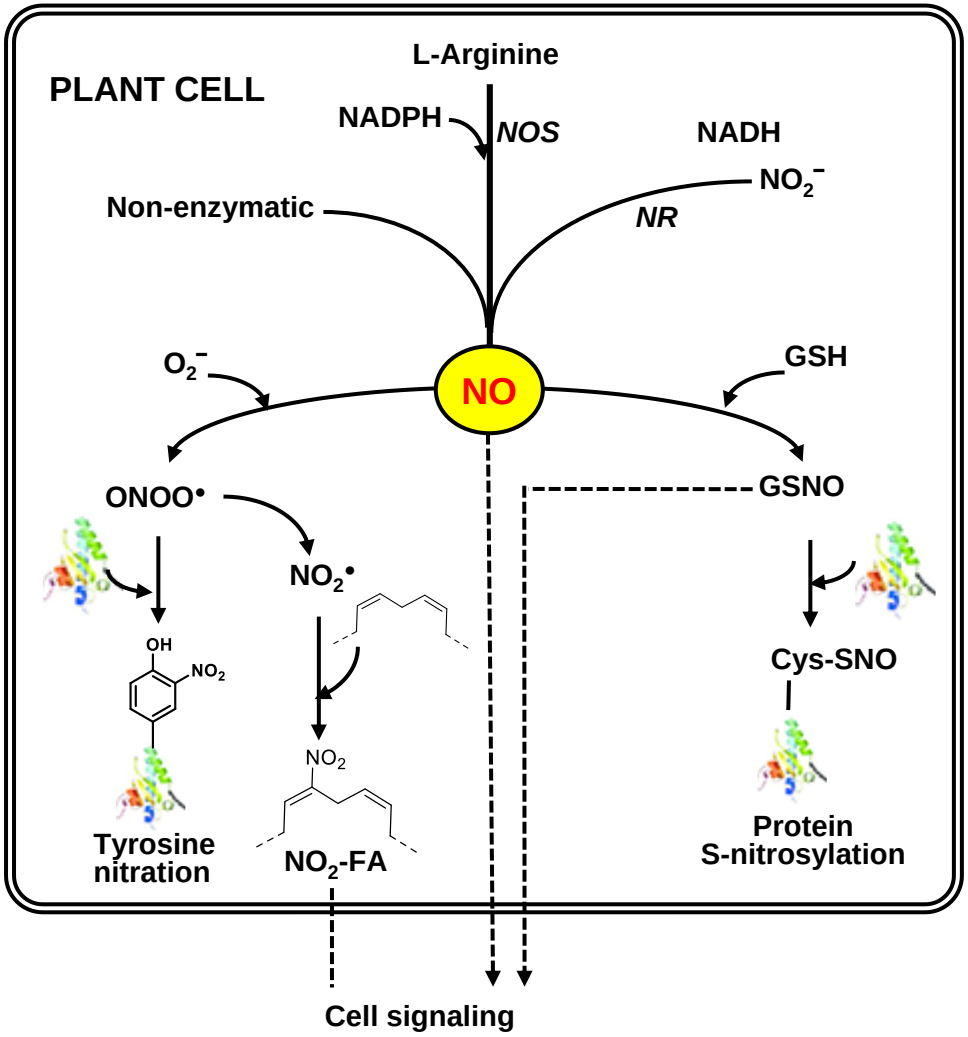


Brief Summary of Nitric Oxide Chemistry



H. Rubo & R. Radi In Handbook of Antioxidants Cadenas & Packer, Eds., pp. 689-707 (2001)

NO in Plant



Journal of Experimental Botany 66(10):2827-2837 · April 2015

Reactions of •NO with Other Free Radicals

Oxidation of •NO to $•NO_2$ and nitrite:

$$2\ •NO + O{:}{:}O \rightarrow 2\ •NO_2$$

$$4\ •NO + O{:}{:}O + 2H_2O \rightarrow 4HNO_2$$

Nitric oxide reaction with organic alkyl and peroxy radicals:

$$•NO + R• \rightarrow RNO \xrightarrow{R•} R_2N\text{-}O•$$

$$•NO + ROO• \rightarrow ROONO \rightarrow ??$$

Reaction with superoxide ion and $•NO_2$:

$$O_2•^- + •NO \rightarrow {}^-OON{=}O$$

$$ONOONO \rightarrow 2NO_2•$$

$$NO_2• + •NO \rightarrow N_2O_3$$

Nitric Oxide Biological Functions

- Cell signaling/effector agent
- increases intracellular levels of cGMP in smooth muscle tissue
- vase-relaxant
- inhibits cell (platelet) adhesion and aggregation
- also made in brain and mitochondria

- Drugs sources of •NO: nitro-vasodilator (amyl-nitrite, nitroglycerine)
- Modification of biological molecules by •NO via reaction with R-SH
  → regulator of metal metabolism

$$RS^{\bullet} + {}^{\bullet}NO \rightarrow RS\text{-}NO$$

Main Biological Chemistry of •NO

- ***Binding of NO to ferrous heme iron of guanylate cyclase***
  - The first major reaction is binding of NO to ferrous heme iron of guanylate cyclase or other proteins. This is important for the activation of signal transduction pathways

$$\text{Heme—Fe}^{2+} + {}^{\bullet}\text{NO} \rightarrow \text{Heme—Fe}^{2+}\text{—NO}$$

- ***Reaction with oxyhemoglobin***
  - The second reaction and certainly the major route for the destruction of NO in vivo is the fast and irreversible reaction with oxyhemoglobin to nitrate:

$$\text{Hb—Fe}^{2+}\text{—O}_2 + {}^{\bullet}\text{NO} \rightarrow \text{Hb—Fe}^{3+} + \text{NO}_3^-$$

- ***Reaction with superoxide anion***
  - The third reaction is the fast (diffusion-controlled) and irreversible reaction of nitric oxide with superoxide anion to yield peroxynitrite ($ONOO^-$):
- ***Reaction with oxygen***

Reaction with Superoxide Anion

$$O_2^{\bullet -} + {}^{\bullet}NO \rightarrow ONOO^-$$

$$k = 1.9 \times 10^{10}\ M^{-1} \cdot s^{-1}$$

Peroxynitrite: $E^{\circ\prime} = +1.4$ V

- Peroxynitrite is in rapid protonation equilibrium with peroxynitrous acid

$$ONOO^- \rightleftarrows ONOOH \quad pK_a = 6.8$$

- Peroxynitrous acid may decompose to $HO^{\bullet}$ and $NO_2^{\bullet}$

$$ONOOH \rightleftarrows [HO^{\bullet} \cdots NO_2^{\bullet}]$$

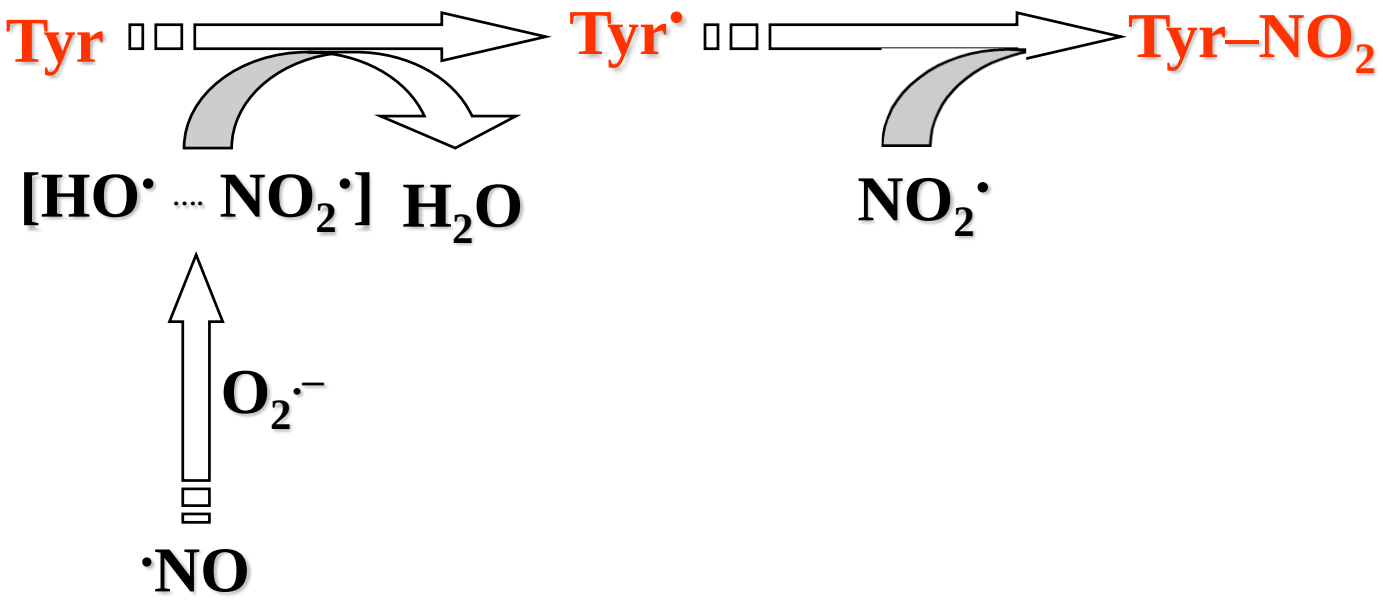
- Chemical reactivity includes oxidations and nitrations

$$[HO^{\bullet} \cdots NO_2^{\bullet}] + RH \rightarrow R^{\bullet} + H_2O + NO_2^{\bullet}$$

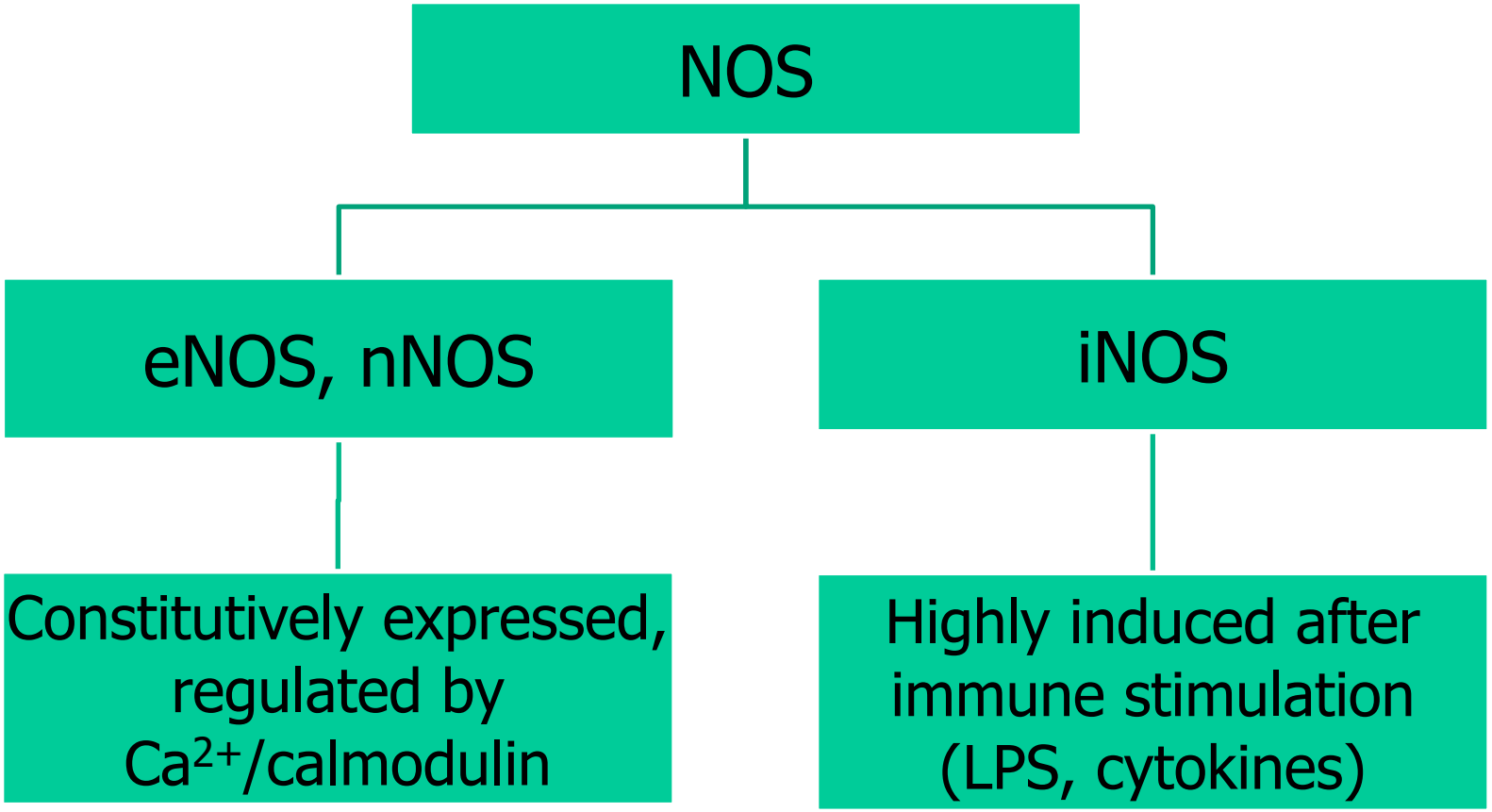
$$R^{\bullet} + NO_2^{\bullet} \rightarrow RNO_2$$

Pro-oxidant Properties of Nitric Oxide

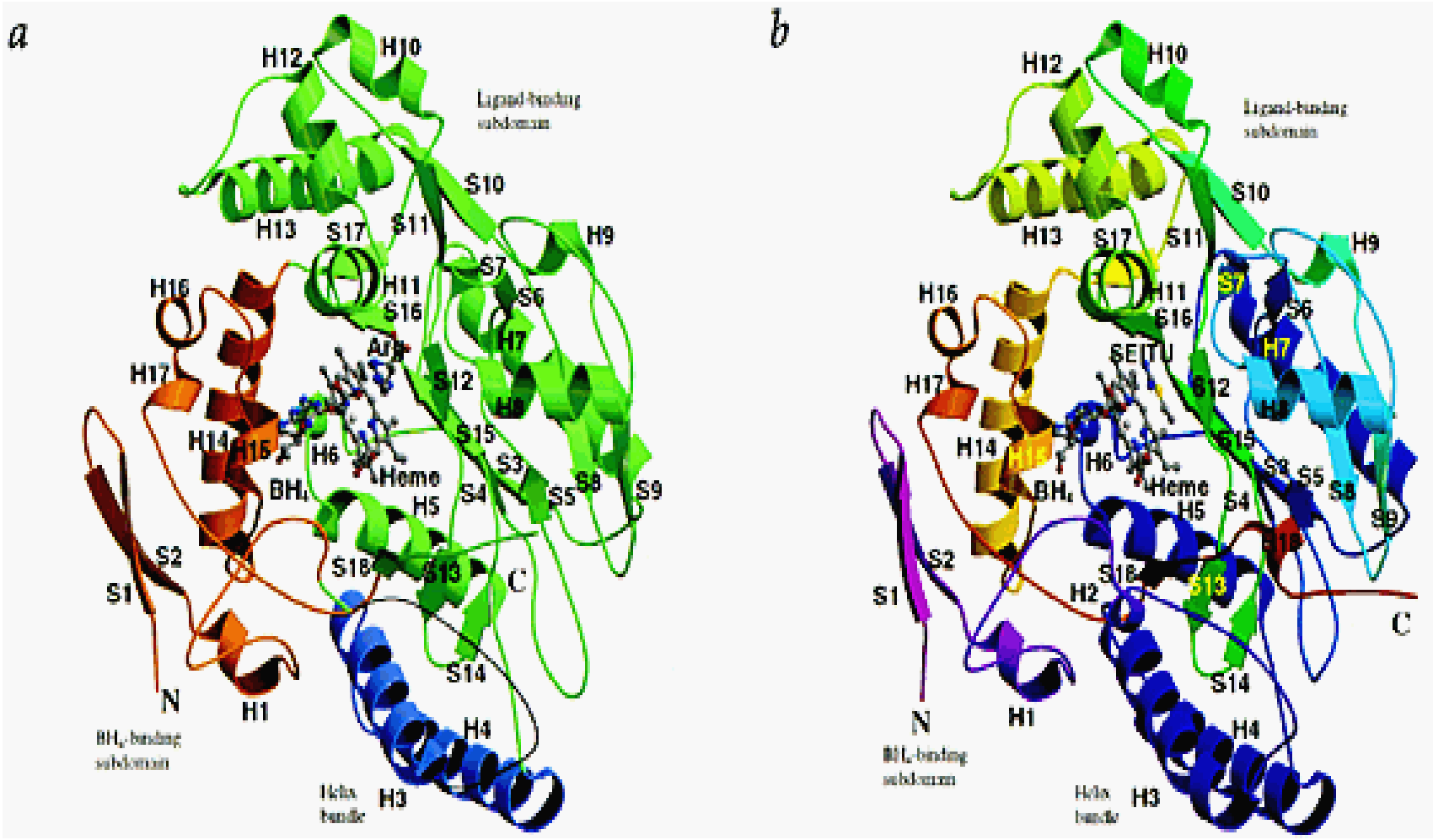
- 3-Nitrotyrosine, a fingerprint of peroxynitrite reactivity



Nitric Oxide Synthases (NOS-family)



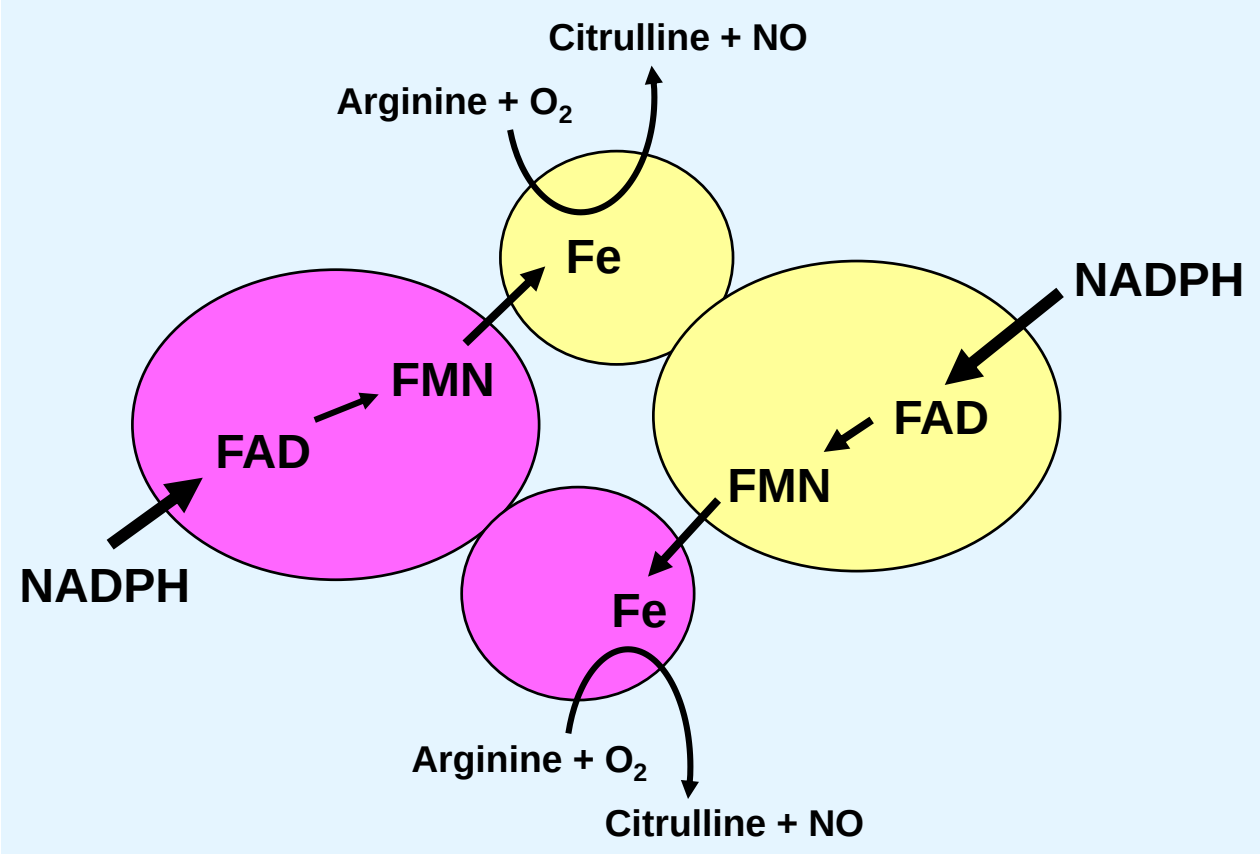
Nitric Oxide Synthase

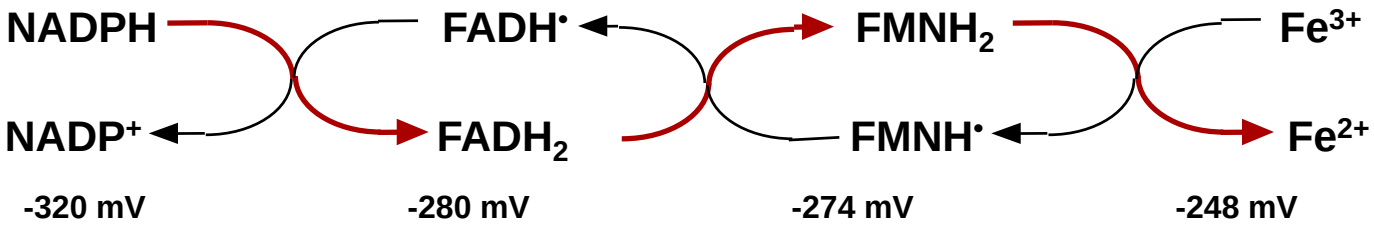


Biochemistry, **2002**, *41*, 11073

Nature Structural Biology **1999**, *6*, 233 – 242.

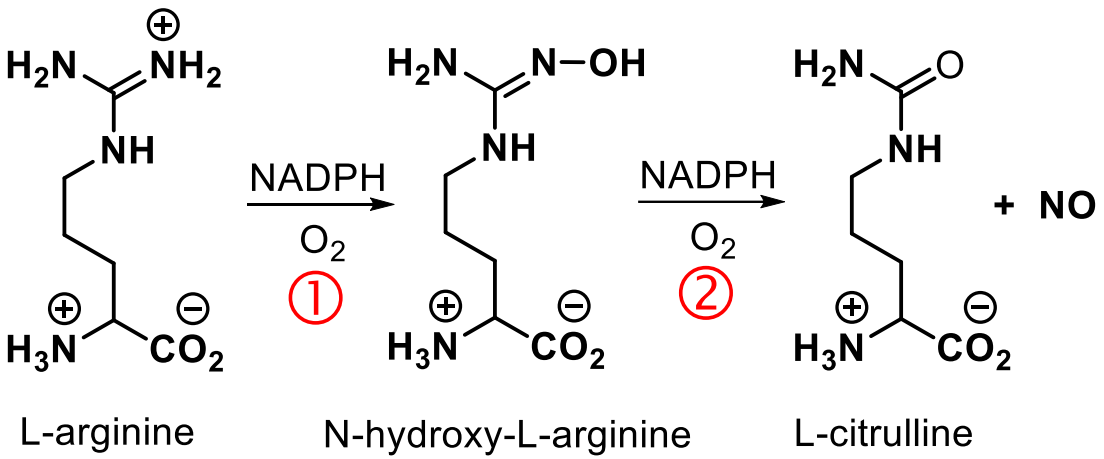
Redox Cascade to Fe^{2+}/Fe^{3+}

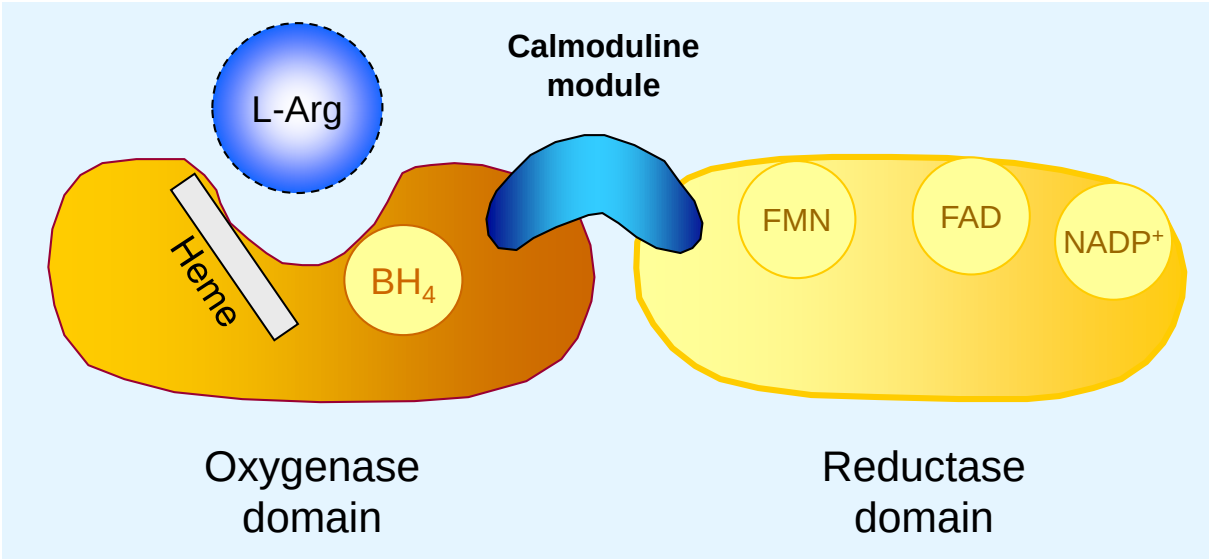




In vivo Generation of •NO: Nitric Oxide Synthase (NOS)

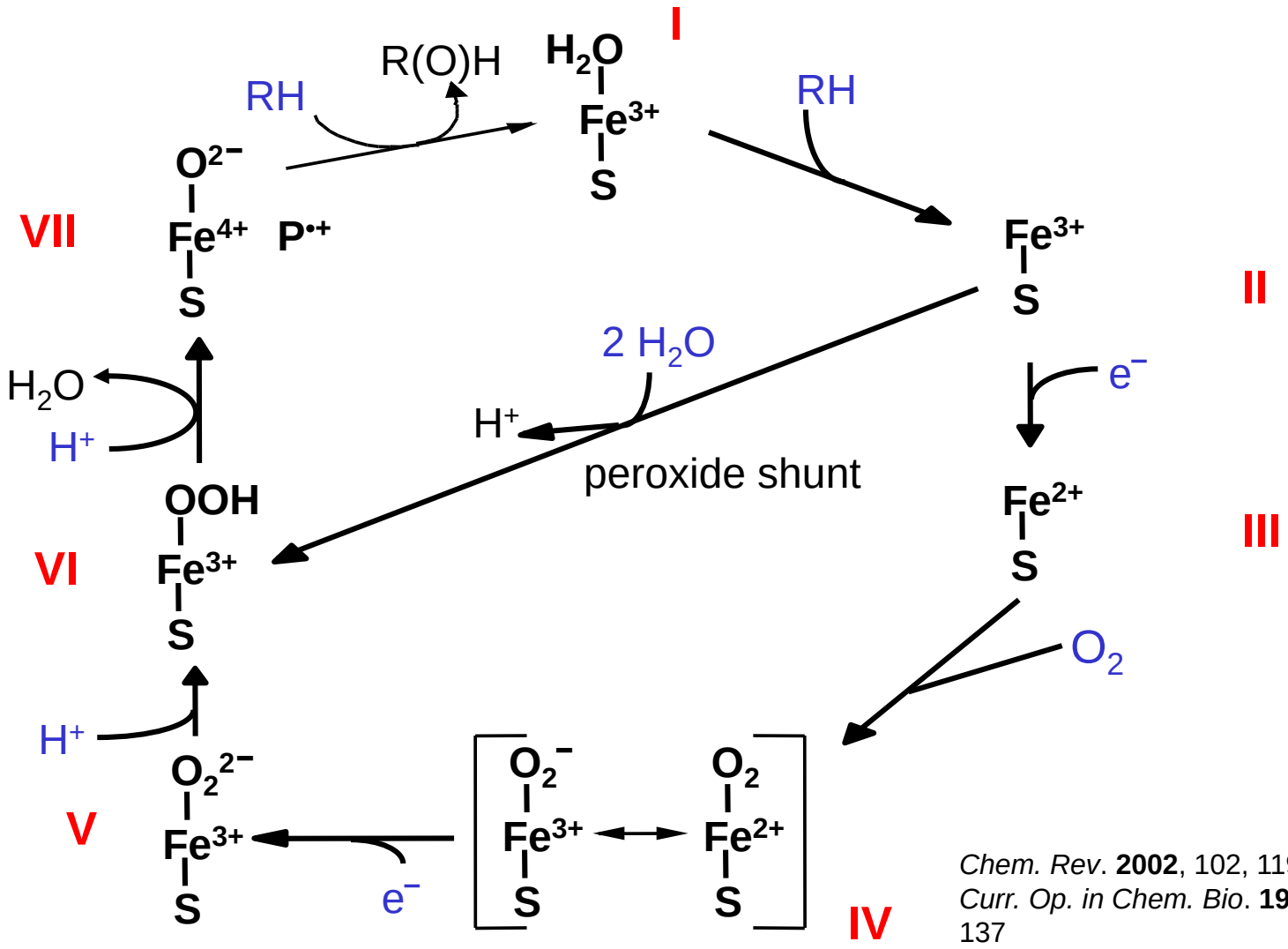
Two cycles





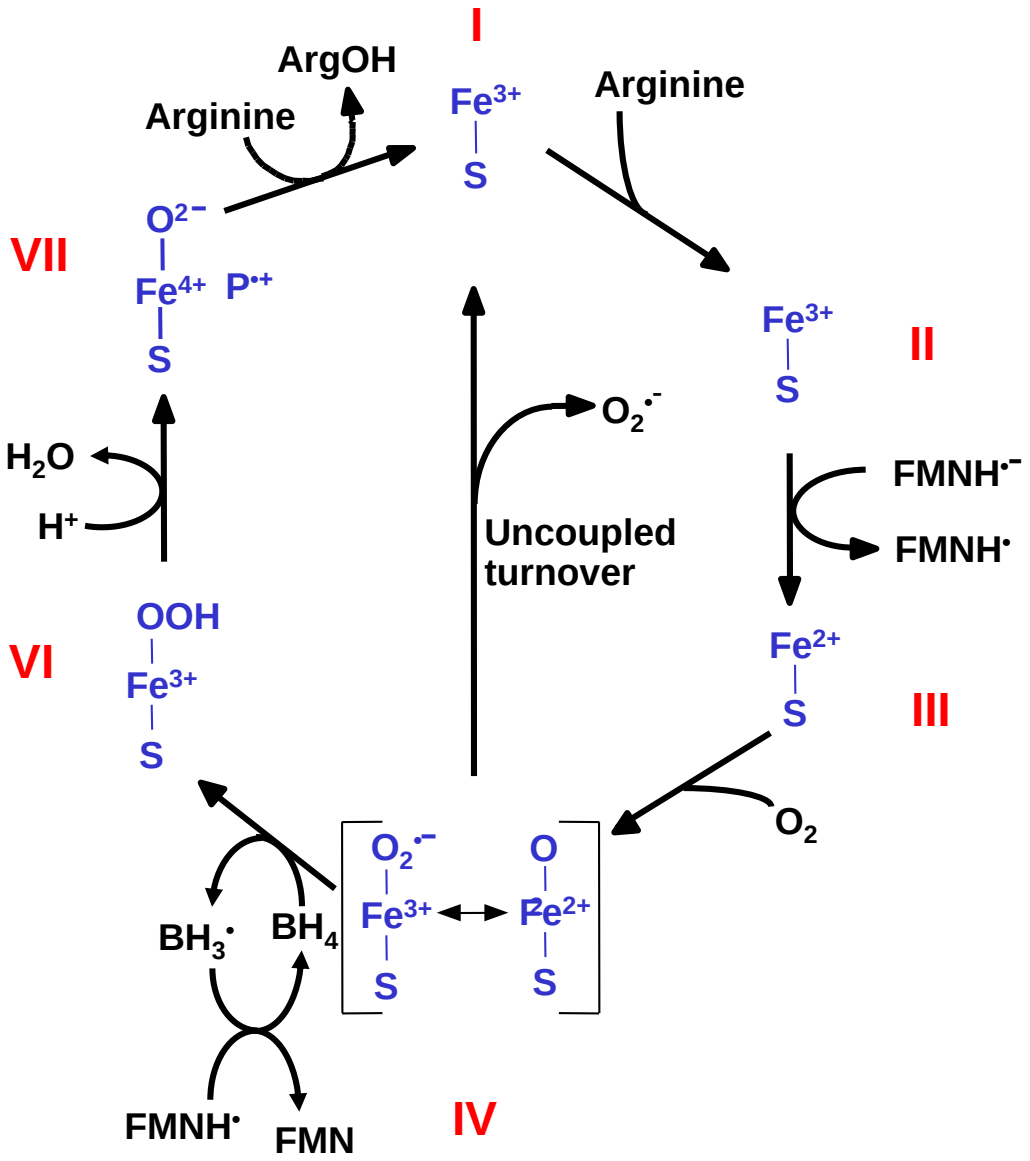
Chem. Rev. **2002**, *102*, 1191-1199.

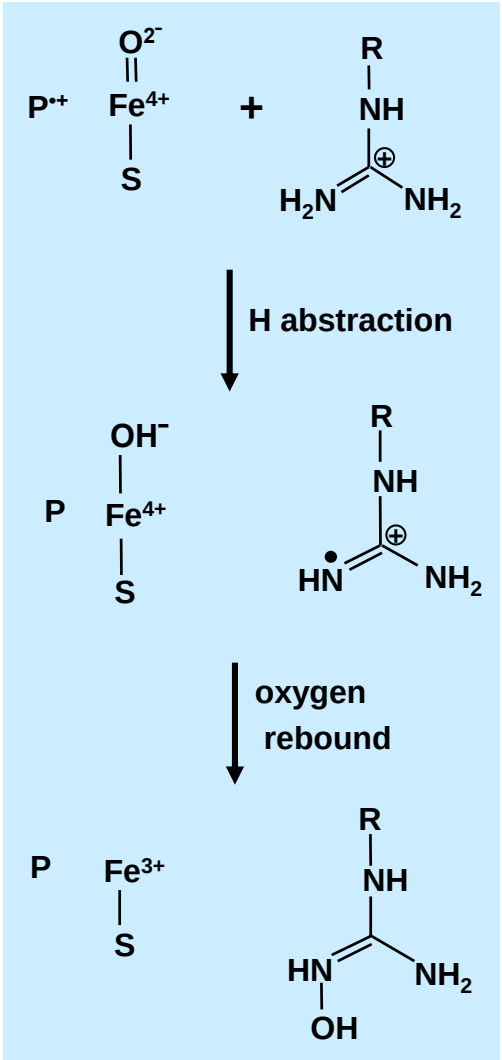
Reduction of Oxygen by Oxygenases



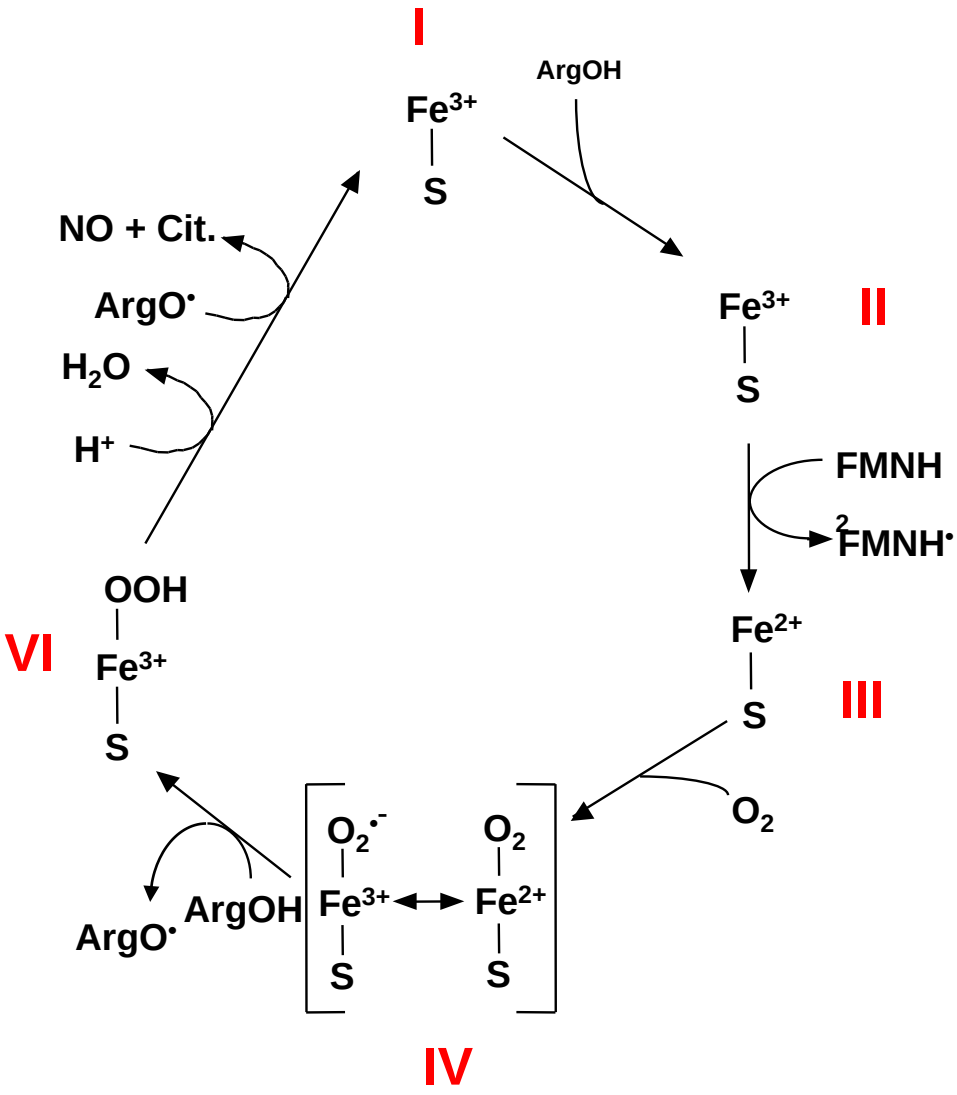
Chem. Rev. **2002**, 102, 1191-1199;
Curr. Op. in Chem. Bio. **1999**, **3**:131-137

Oxygenation of Arginine by Iron Enzymes



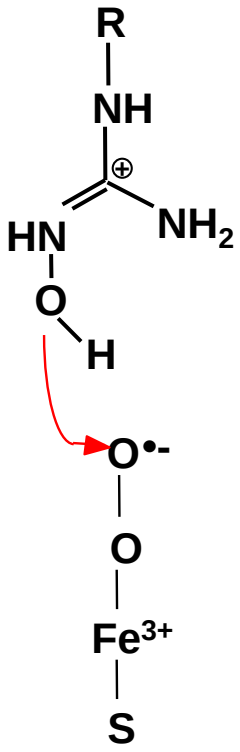


In the Absence of Donor Protein



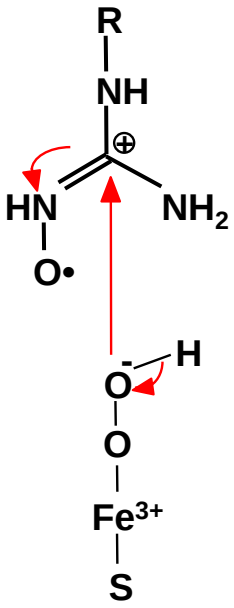
•NO Generation Detail of Mechanism

IV(oxy)

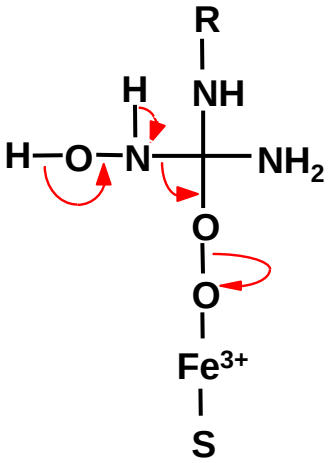


hydroxyarginine

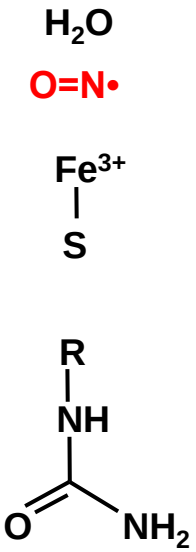
VI(hydroperoxy)



TETRAHEDRAL INTERMEDIATE



I(ferric)



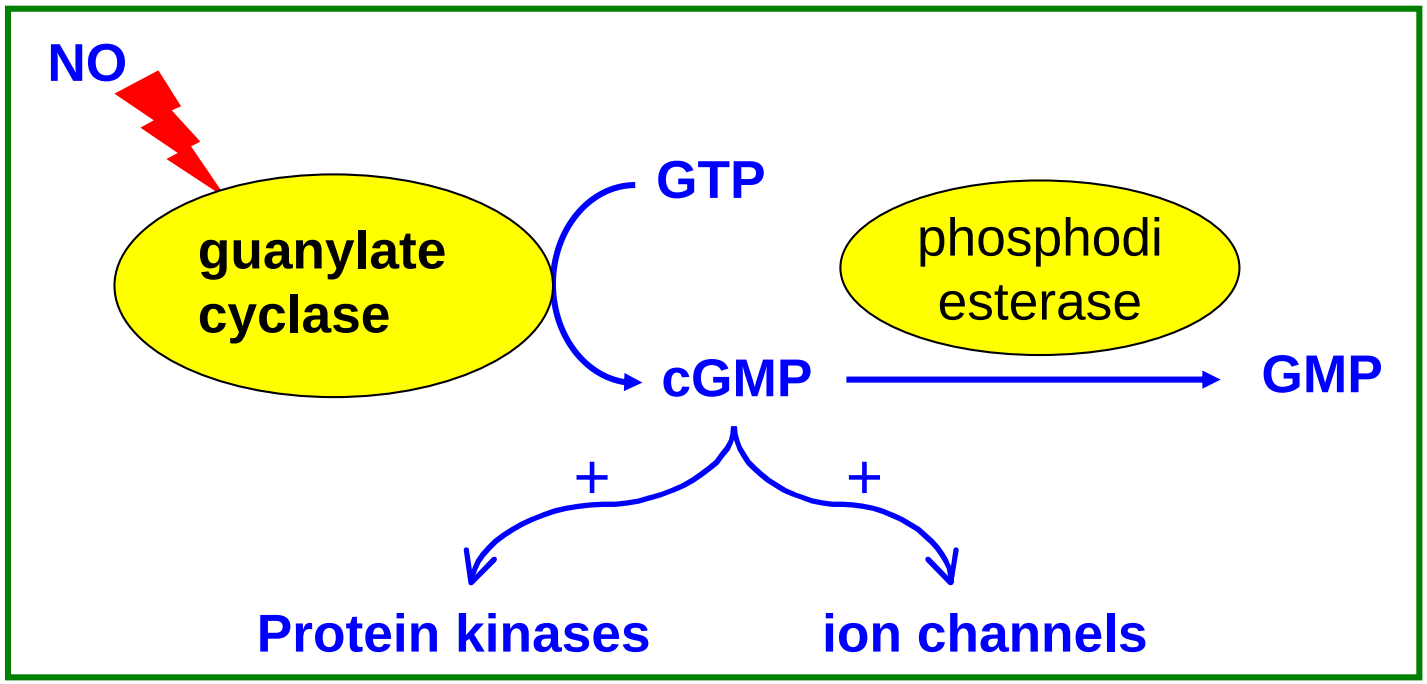
citrulline

Peroxynitrite

$$NO + O_2^{\bullet -} \rightarrow ONOO^-$$

Nitric oxide + superoxide → peroxynitrite

→ **nitrate tyrosine**
oxidise lipids, DNA



Peroxynitrite (PON) as a Free Radical Producer

Beckman and co-workers were the first to propose that when overproduced, •NO was likely to react with $O_2^{\bullet -}$ to form peroxynitrite (PON) (Beckman *et al., Proc Natl Acad Sci. USA* **87**:1620, 1990). This seminal paper attracted much attention because based on scanty chemical literature and on the oxidation of DMSO and deoxyribose, it proposed that (PON) could be a transition metal ion-independent route for hydroxyl radical production *in vivo.*

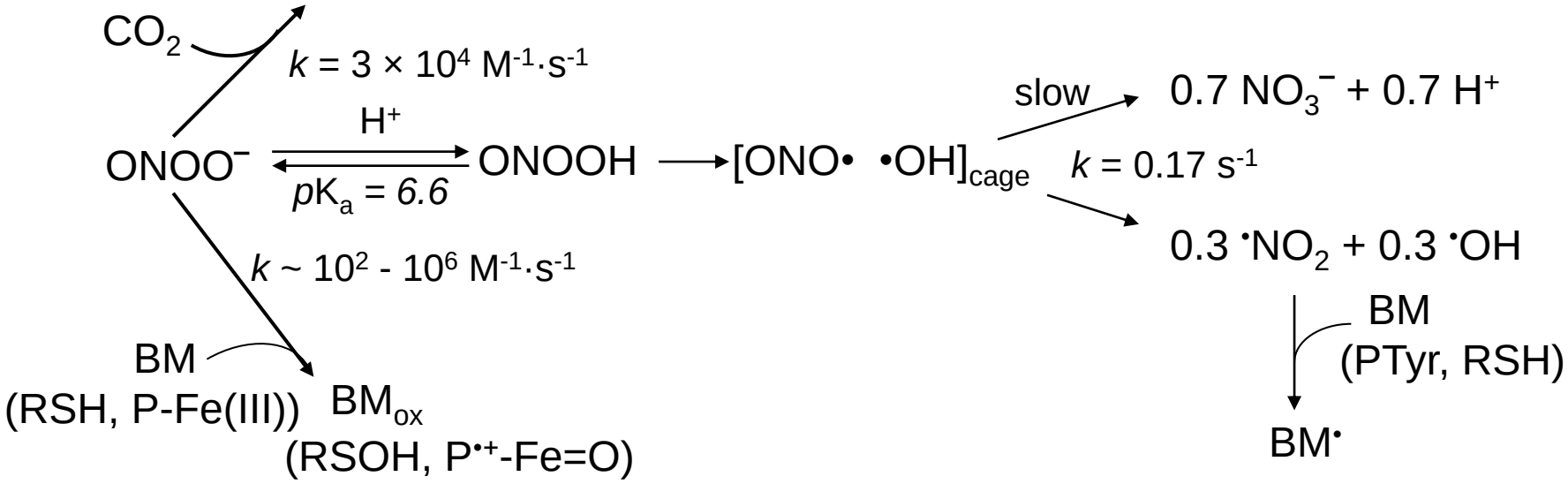
$$^{\bullet}NO + O_2^{\bullet -} \xrightarrow{k \sim 10^{10}\ M^{-1}\cdot s^{-1}} ONOO^- + H^+ \underset{}{\overset{pK_a = 6.6}{\rightleftharpoons}} ONOOH \longrightarrow [^{\bullet}NO_2 \quad {}^{\bullet}OH] \longrightarrow \text{(DMSO/ deoxyribose)}$$

This led to many papers and to a huge controversy about the possibility of free radical production from (PON) *in vitro*. The controversy was resolved and the oxidant is likely to act as free radical producer *in vivo.* Relevant is the rapid reaction of (PON) with the biologically ubiquitous CO_2 that increases $(PON)^-$ mediated one-electron oxidation and nitration of biomolecules. In some environments, reaction of (PON) with hemeproteins may also become important for biomolecule--derived radical production.

(Augusto *et al.*, *Free Rad Biol Med.* **32**:849, 2002).

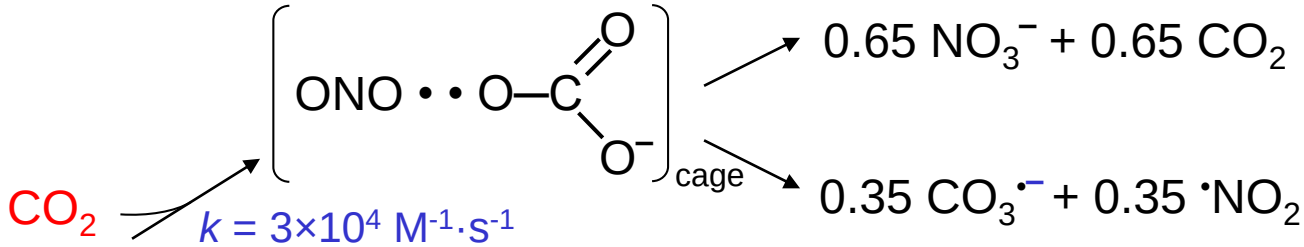
Proton-catalyzed Decomposition of Peroxinitrite

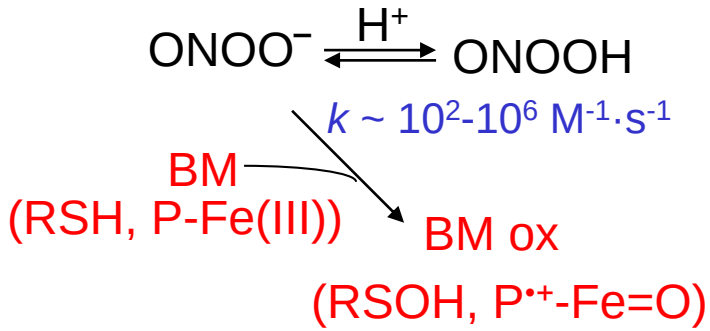
Peroxynitrite is stable at alkaline pH but upon protonation ($pK_a = 6.6$) it decomposes to yield 70% NO_3^- and 30% $^{\bullet}OH$ and $^{\bullet}NO_2$ that can oxidize biomolecules (BM) to the corresponding radicals. *In vivo,* however, these one-electron oxidations are likely to become relevant only at acid pH because at neutral pH the proton-catalyzed decay is too slow to compete with bio targets such as CO_2, bio thiols (RSH) and hemoproteins P-Fe(III) that react directly with peroxynitrite



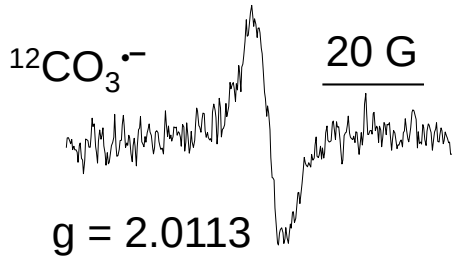
Peroxynitrite Reactions *in vitro:* Two- *versus* One-Electron Oxidations

RSH, P-Fe(III) and CO_2 are anticipated to be the most important peroxynitrite bio targets because of their high biological concentrations and rapid reaction rate with the oxidant ($k \sim 10^2$-10^6 $M^{-1}\cdot s^{-1}$). These reactions greatly reduce the half-life of peroxynitrite (from s to ms) and the targets are usually oxidized by two-electron mechanisms. An important exception is the reaction with the biologically ubiquitous CO_2 that produces 65% NO_3^- and 35% $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (Bonini *et al., J Biol Chem.* **274**:10802, 1999).





EPR detection of $CO_3^{\bullet-}$ during fast-flow mixing of $ONOO^-$ and HCO_3^-



Peroxynitrite as a biomolecule-derived radical producer

(i) Peroxynitrite is likely to act through its derived radicals ($CO_3^{\bullet -}$ and $^{\bullet}NO_2$) *in vivo* due to the high concentration of CO_2 in equilibrium with HCO_3^- in most biological fluids. In low pH environments, the peroxynitrite-derived $^{\bullet}OH$ radical can also become relevant.

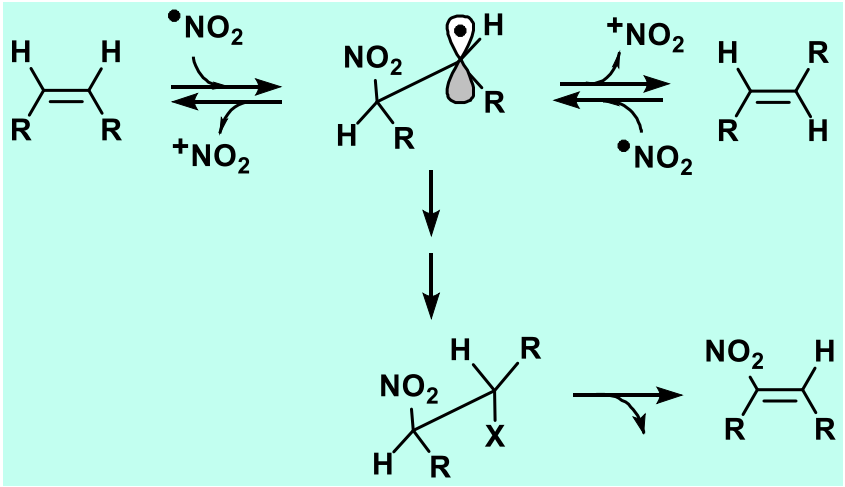
(ii) Both, $CO_3^{\bullet -}$ ($E°' = 1.8$ V; pH 7.0) and $^{\bullet}NO_2$ ($E°' = 0.9$ V, pH = 7.0) are oxidizing radicals that can oxidize several bio targets to their corresponding radicals. A flux of both radicals (as produced from peroxynitrite) is very efficient in nitrating tyrosine and guanine residues by combining the oxidizing power of $CO_3^{\bullet -}$ with the radical recombination reactions of $^{\bullet}NO_2$

(iii) The main biomolecules that are likely to be oxidized to their corresponding radicals by peroxynitrite-derived radicals *in vivo* are GSH, P-SH, P-Tyr, P-Trp, RNA-Gua and DNA-Gua.

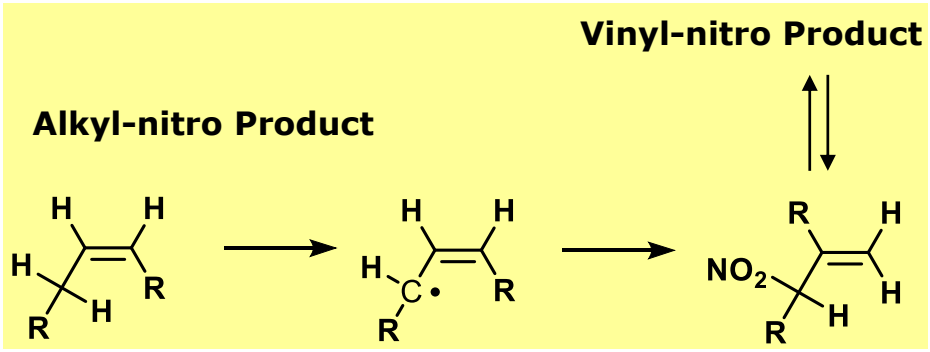
(iv) Oxidation of GSH to GS•, occurs even in environments where peroxynitrite reacts preferentially with hemoproteins rather than with CO_2. Then, oxidation of GSH to $GS^{\bullet}$ is likely to be a consequence of peroxynitrite production *in vivo*.

Cis-trans Isomerization

- $^{\bullet}NO_2$ can induce *cis-trans* isomerization of *cis* double bonds in unsaturated fatty acids

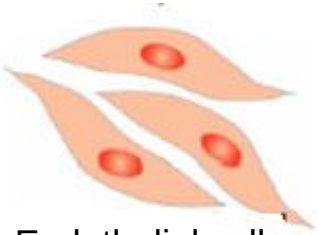


***Cis-trans* isomerization and vinyl-nitro products**



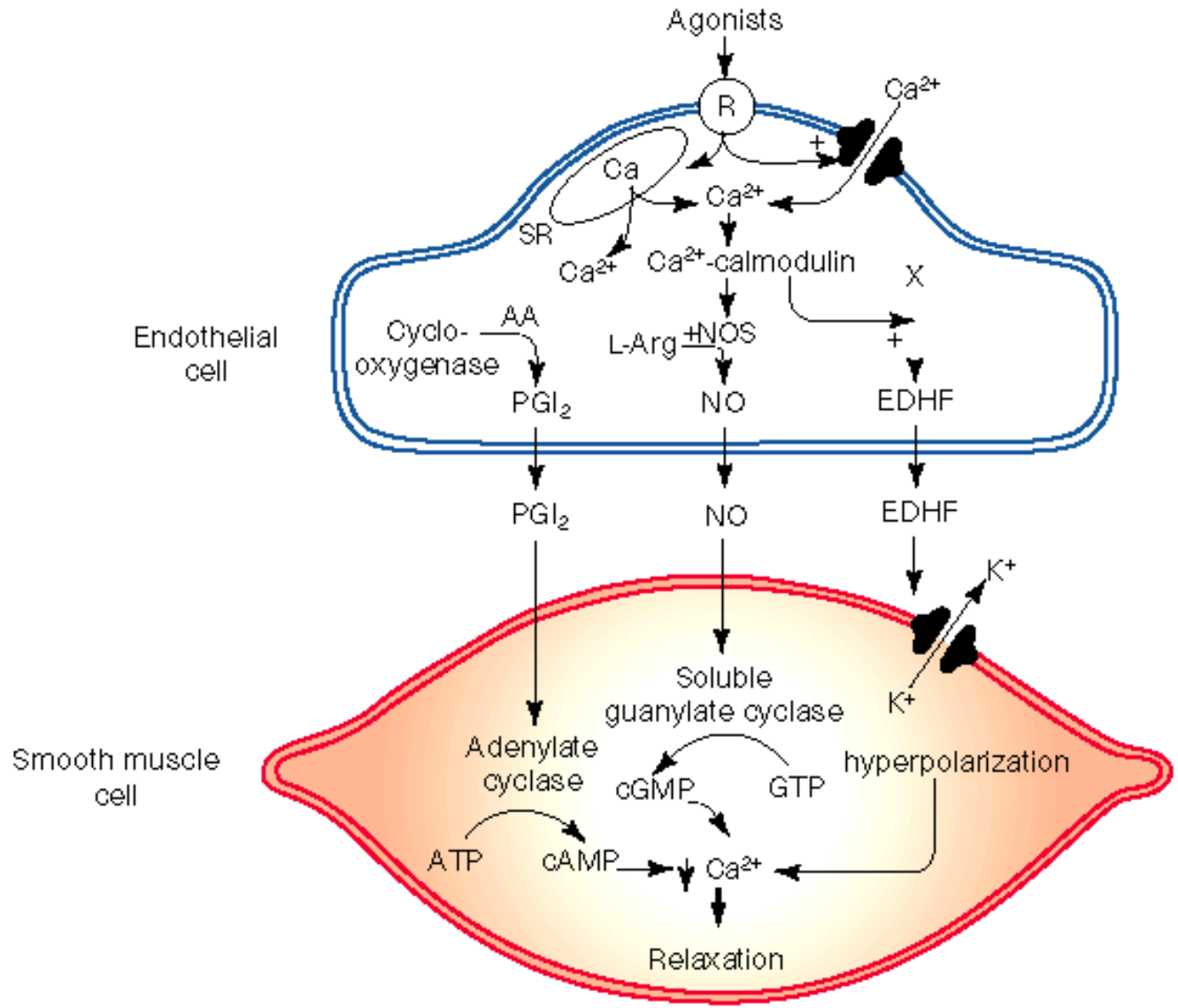
LNO_2: Lipid derived signaling mediators?

Nitric Oxide as Cellular Response Signal

Stimulation	Acetylcholine Bradykinin	Glutamate	Lipopolysaccharide Cytokline
Cell line			
	Endothelial cell	Neuron	Macrophage
	NO	NO	NO
Bioactivity	Endothelium-derived relaxing factor	Neurotransmitter	Antibacterial activity tumor cell killing
	Palmer et al. Nature 1988	Brenman et al. Cell 1996	Xie et al. Science 1992

Blood pressure control

NO• Signaling in Physiology



Nitric **O**xide **S**ynthase

$O_2^{\bullet-}$ → NO• → $ONOO^-$

Binds to heme moiety of guanylate cyclase

Conformational change of the enzyme

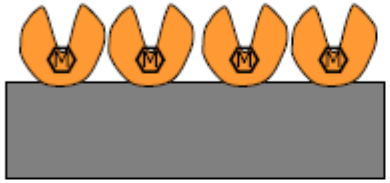
Increased activity (production of cGMP)

Modulation of activity of other proteins (protein kinases, phospho-diesterases, ion channels)

Physiological response (relaxation of smooth muscles, inhibition of platelet aggregation, etc.)

Electrochemical Nitric Oxide Sensor

Metalloprotein



Haruyama *et al.*, *Biosensors & Bioelectronics* **13** 763-769 (1998)
Lisdat *et al.*, *Electroanalysis* **12** 946-951 (2000)
Fan *et al.*, *Analytica Chimica Acta* **523** 225-228 (2004)

Metal complex

Pontie *et al.*, *Sensors & Actuators B-Chem.*, **56** 1-5 (1999)
Kashevskii *et al.*, *J. Electroanal. Chem.*, **531** 71-79 (2002)
Caro *et al.*, *J. Electrochem. Soc*, **150** 95-103 (2003)

Spin-trap reagent

Haruyama *et al.*, *Biosensors & Bioelectronics* **13** 763-769 (1998)
Haruyama *et al.*, *Japanese Sensor Newsletter* **12**, 4-7 (1998)

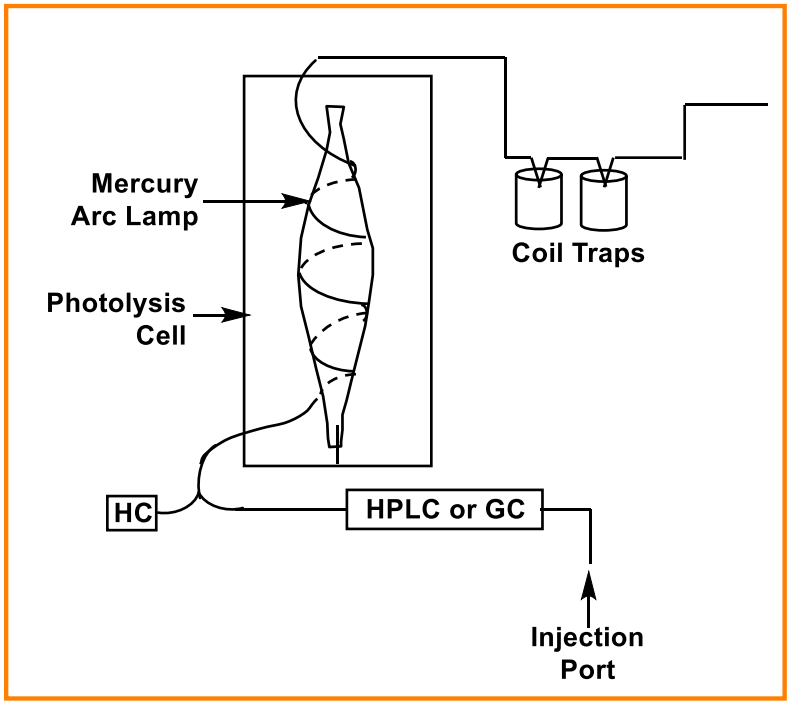
It is difficult to apply the known sensor material to cellular biosensing.

Novel sensor material for cellular biosensing is required!

Advantages and Disadvantages of Chemiluminescence Compared to Other Methods of NO Detection

- *Advantages*
  - High Sensitivity and Linearity over a Broad Concentration Range
  - Good Reproducibility and High Specificity for NO (few other gaseous substances (DMSO, ethylene) react with ozone)
  - Measurements Possible in Turbid or Colored Samples, Even at Extreme pH (in solution, in the headspace, in expired air)
  - Besides Mass Spectrometry the Only Other Method that Allows Quantification of Absolute Amounts of Nitroso Species
  - Moderate Running Costs
- *Disadvantages*
  - With Some Biological Samples Difficult to Extract NO into Gas Phase
  - Provides Limited Structural Information
  - Limited Sample Throughput, High Purchase Price for Detector

Photolysis/Chemiluminescence Approach for Detection of Nitroso Species



Reaction principle:

$$RSNO \xrightarrow{light} RS^{\bullet} + NO\cdot$$

$$R_2NNO \xrightarrow{light} R_2N\cdot + NO\cdot$$

Discrimination of RSNOs from other nitroso species and nitrite by measurement before and after $HgCl_2$ treatment, and with lamp ON and OFF

Stamler et al., 1992, Alpert et al., 1997

Sample Processing Using Redox-Active Reaction Mixtures - Many Choices, Many Pitfalls

Most techniques use Chemical Reactions to convert nitroso and nitrosyl species into NO, which is then detected by chemiluminescence

- Reducing mixtures differ largely in reducing strengths and reduction capacity

Iodine/iodide (I_3^-)	60 mM I^-/6-20 mM I_2/ 1M HCl, RT	Samouilov & Zweier, 1998
	56 mM I^-/ 2 mM I_2, 4mM CuCl, CH_3COOH, 68°C	Marley et al., 2000
	60 mM I^-/10 mM I_2, CH_3COOH, 60°C	Feelisch et al., 2002
Cysteine/CuCl	1 mM L-cysteine, 0.1 mM CuCl	Fang et al., 1998
Hydroquinone/Quinone	0.1/0.01 mM	Samouilov & Zweier, 1998
VCl_3/H^+	0.1 M in 2M HCl	Ewing et al., 1998

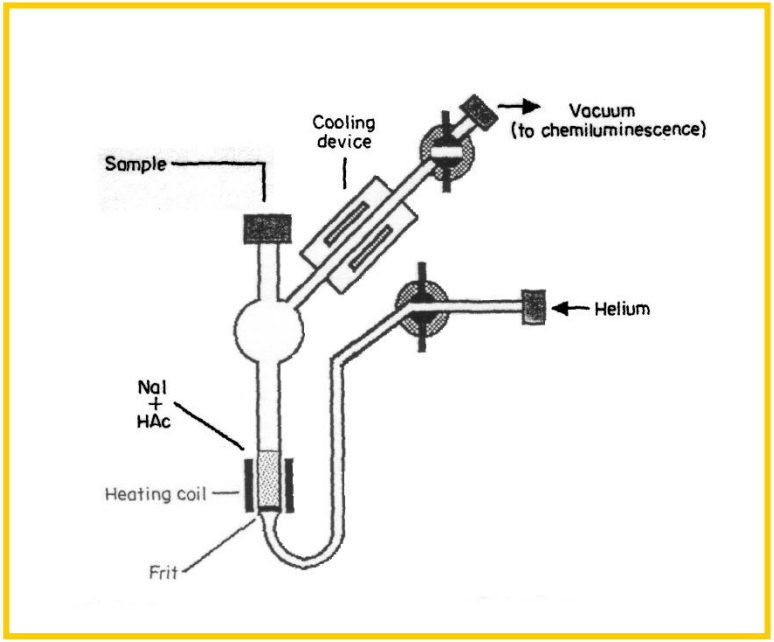
- Oxidizing mixture for determination of NO-hemes

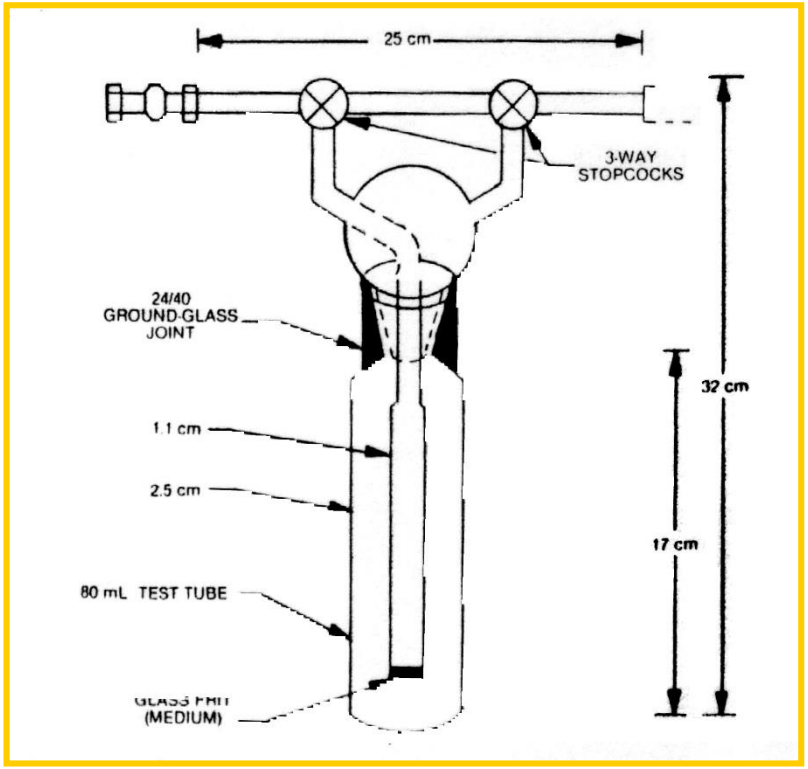
Ferricyanide	0.2 M in PBS pH 7.5	Gladwin et al., 2002 Bryan et al, 2004

General Problem:
Neither method is absolutely specific and bears the potential to produce false positive (nitrate, L-NitroArg, ...) or negative signals

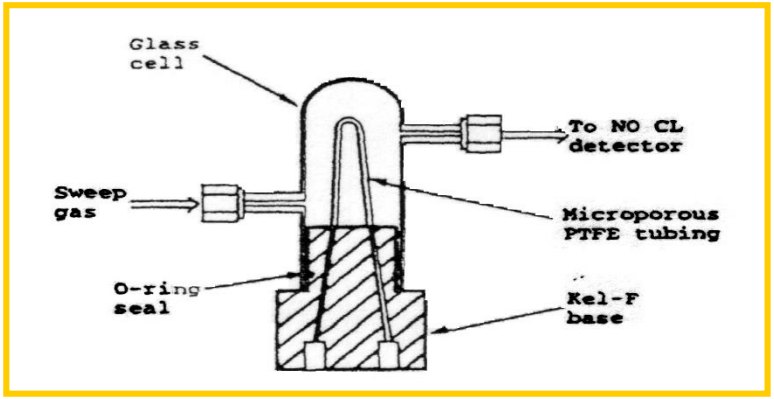
Reaction Chambers Come in Many Different Designs

Menon et al., 1991



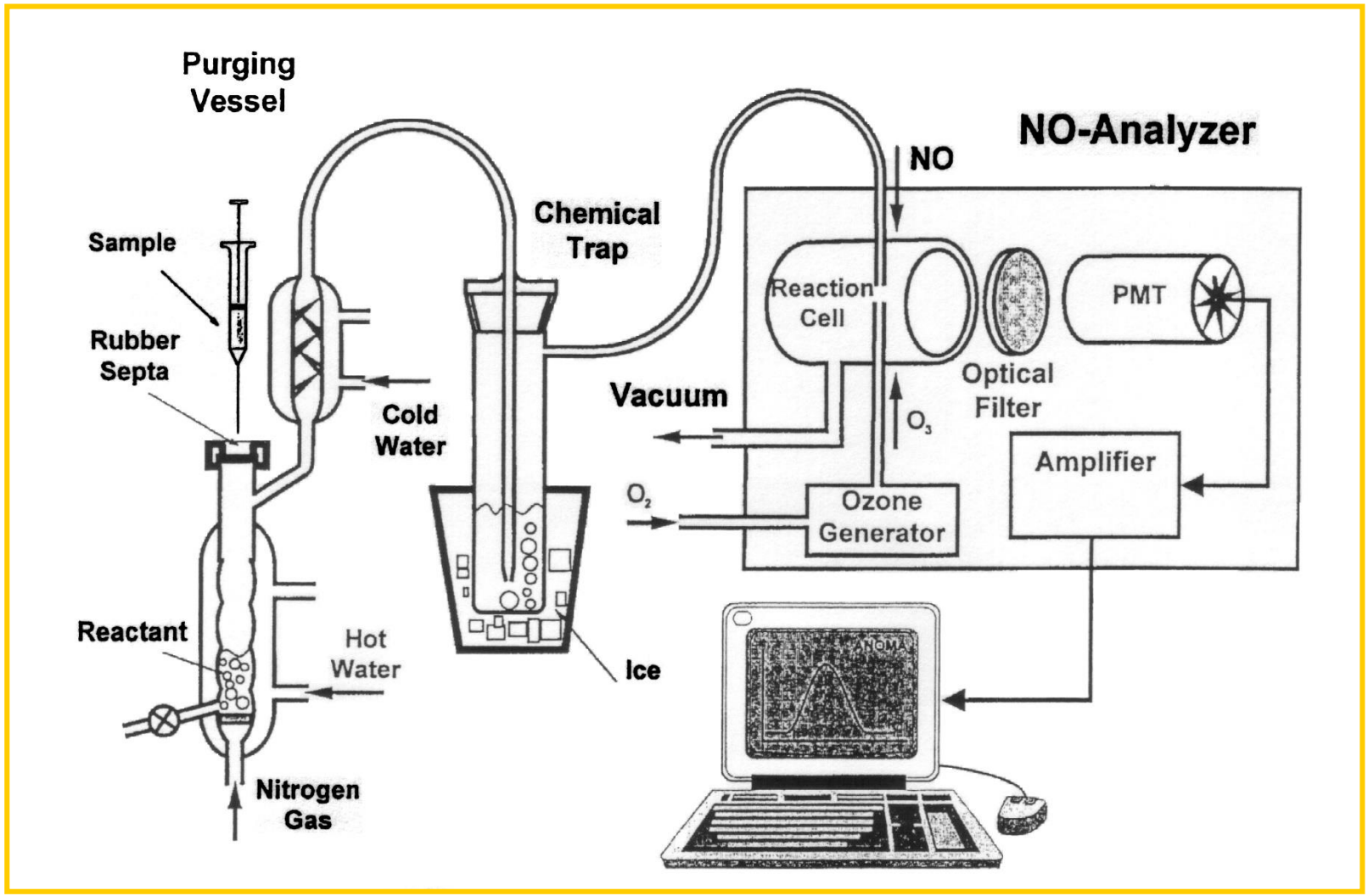


Cox & Frank, 1982



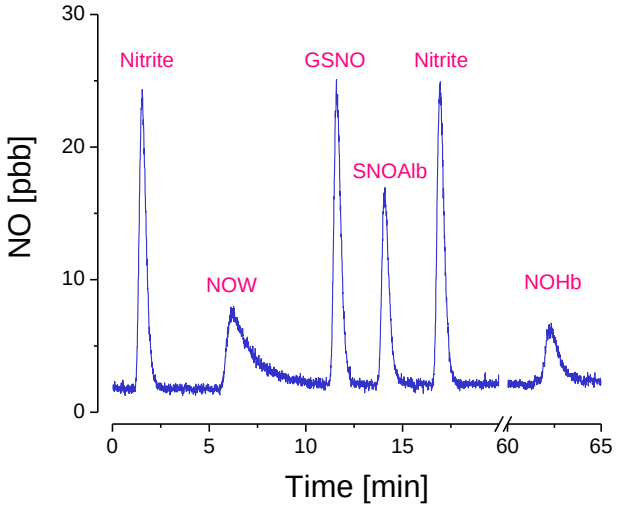
Dunham et al., 1995

The Most Frequently Used Type of Chemiluminescence Set-up



Which NO-Related Species are Detected and How can they be Discriminated from One Another?

- Without Reduction Step:
  - NO (direct injection into buffer or water)
- Upon Acidification:
  - NO_2^- (disproportionation of HNO_2)
  - RONO (acid-catalyzed decomposition)
- With Sample Reduction:
  - NO_2^-/NO_3^- (KI/CH_3COOH, RT for nitrite, VCl_3/H^+, 90°C for nitrate)
  - RSNO, RNNO, NO-Heme (I_3^-/CH_3COOH, 60°C)



detection limit: 1-50 nM, depending on flow and inj. volume
250 fmoles NO (at 50 µL injection vol.)

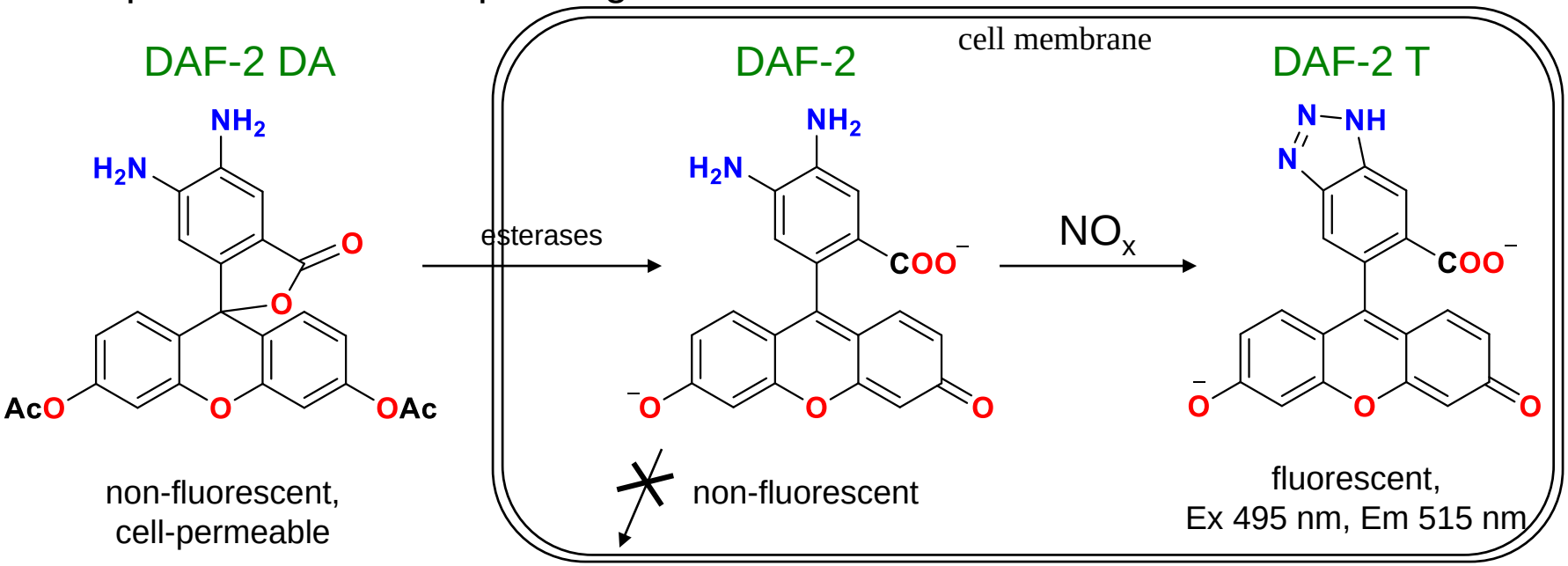
- Discrimination between different species:

Selective NO_2^- removal	$Sulfanilamide/H^+$
RSNOs from other Nitroso-Species	$HgCl_2$/sulfanilamide
Nitroso from Nitrosyl Species	Reducing vs. Oxidizing Reaction Mix

Bio-imaging of Nitric Oxide Using DAF-2

Detection principle:

Reaction of aromatic vicinal diamines with NO in the presence of oxygen to produce the corresponding triazenes



Advantages: Sensitivity for NO (5 nM *in vitro*) with high temporal and spatial resolution
No cross-reactivity to NO_2^-/NO_3^- and $ONOO^-$

Assay limitations: Possible interference by reducing agents and divalent cations, pH sensitive, subject to photo bleaching, requiring standardized illumination conditions

Recent Developments

- **Development** and commercial availability **of red fluorescent chromophores** (diamino-rhodamine-based; DAR-4M) increases flexibility for combinations with other green-fluorescent probes and shows reduced interference with tissue auto fluorescence, but is otherwise very similar to DAF-2

- **Difluoroboradiaza-s-indacene** based fluorophore (similar chemistry)

- **Detection of nitroso peptides and proteins on diaminofluoresceine gels** (standard SDS-PAGE followed by UV photolysis in the presence of DAF-2 or DAF-FM for detection of C-, O-, N- and S-nitrosated compounds) *(Mannick et al, 2005)*

- **Near-Infrared fluorescent probes for „NO“ detection** in isolated organs (tricarbocyanine as NIR fluorochrome coupled to *o*-phenylenediamine as NO sensor; NIR is potentially very interesting for *in vivo* imaging approaches as it allows deeper penetration of light into tissues and shows no interference with tissue autofluorescence; promising novel approach *(Nagano et al, 2005)*

- **Amplifier-coupled fluorescent NO indicator** with nanomolar sensitivity in living cells (genetically encoded fluorescent indicator based on the binding of NO to soluble guanyl cyclase and detection of formed cGMP by FRET; interesting, but potentially problematic cross-talk with cGMP generated by particulate GC and modulation of sensitivity by PDE activity) *(Sato et al, 2005)*