2008 Sunrise Free Radical School

Presentation by:

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What are free radicals?

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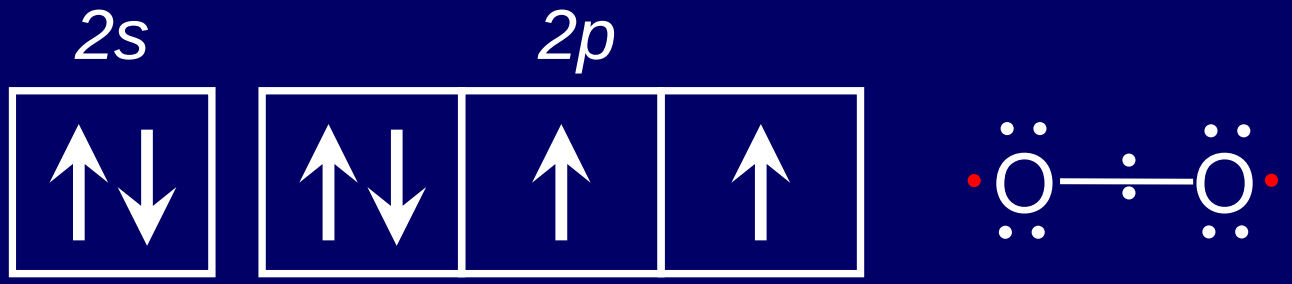
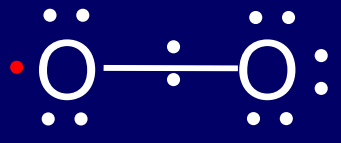
What is a Free Radical? _ Definition

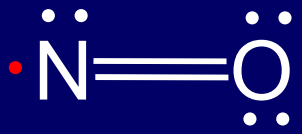
✓ *A free radical is defined as a species that contains one or more unpaired electrons occupying an atomic or molecular orbital by itself*

Molecular oxygen: a di-radical



Superoxide and nitric oxide radicals





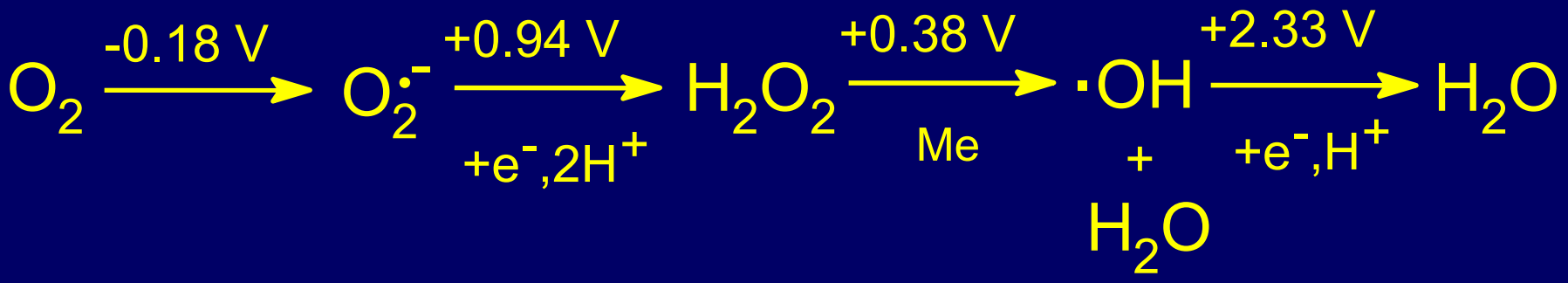
Common (and not necessarily correct) statements in free radical chemistry and biology:

- Radicals are always short-lived
- Radicals are always highly reactive
- Radicals are always oxidizing species
- Radicals are always formed as “undesirable” “side products

Other relevant points to consider:

- ✓ One- and two-electron oxidants
- ✓ Methodologies to study free radical chemistry
- ✓ Reactive “oxygen” and “nitrogen” species. What about “sulfur”?
- ✓ Radicals and intramolecular electron transfer
- ✓ “Stable” radicals in the active site of enzymes

Oxygen free radicals and reactive oxygen species



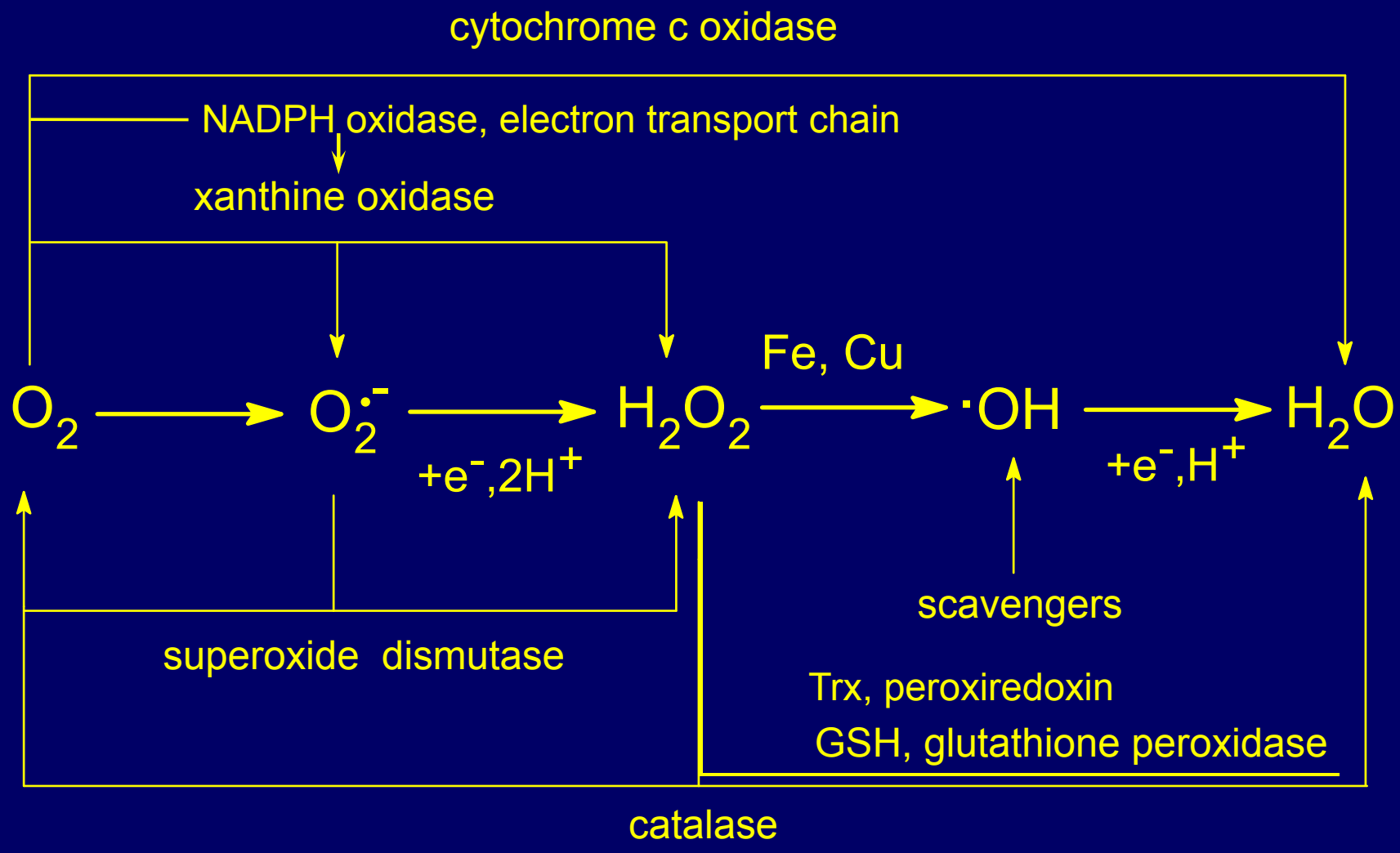
Superoxide radical anion

Hydrogen Peroxide

Hydroxyl Radical

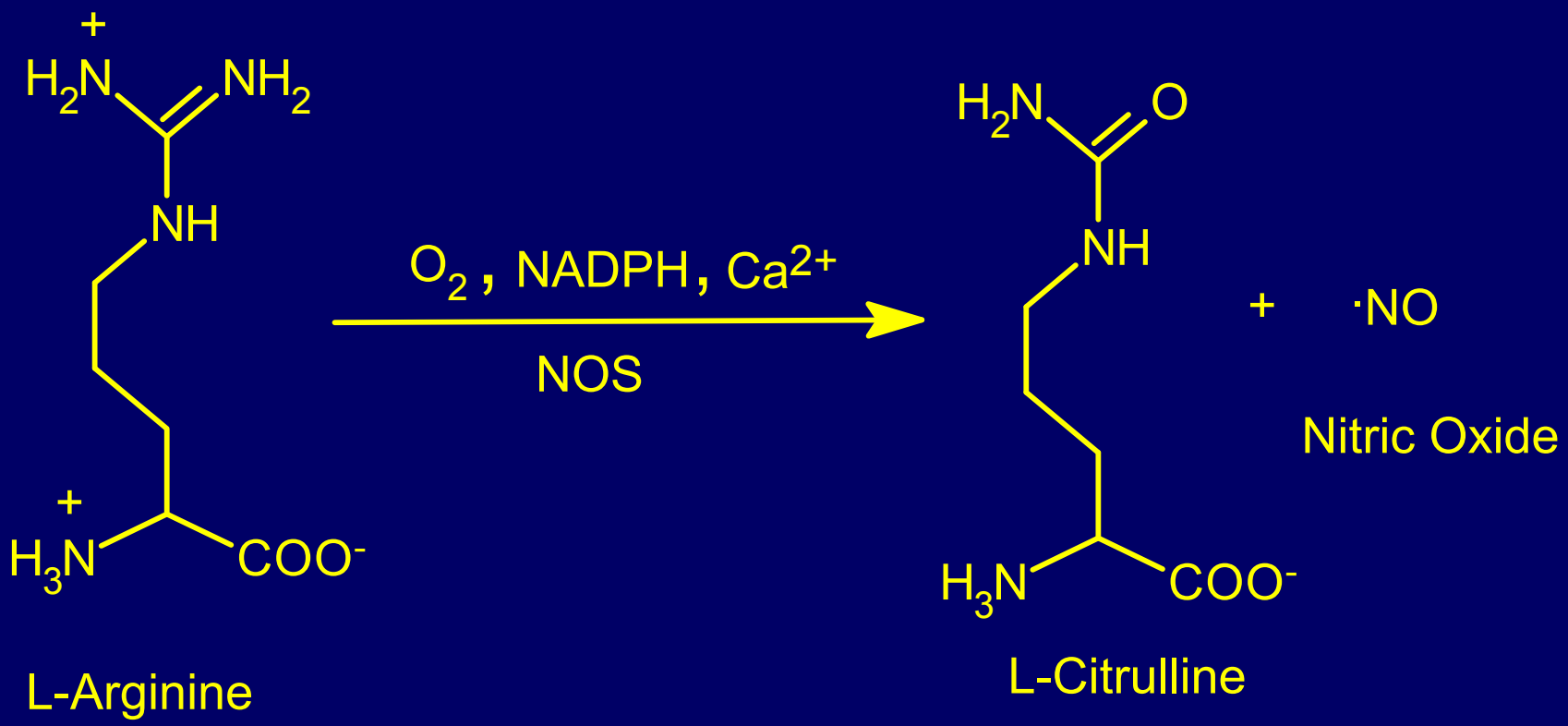
Me = transition metal center (*eg.* Fe)

Mechanisms of formation and detoxification of reactive oxygen species



Nitric oxide synthase reaction

An enzymic and highly controlled generation of a signal transducing free radical

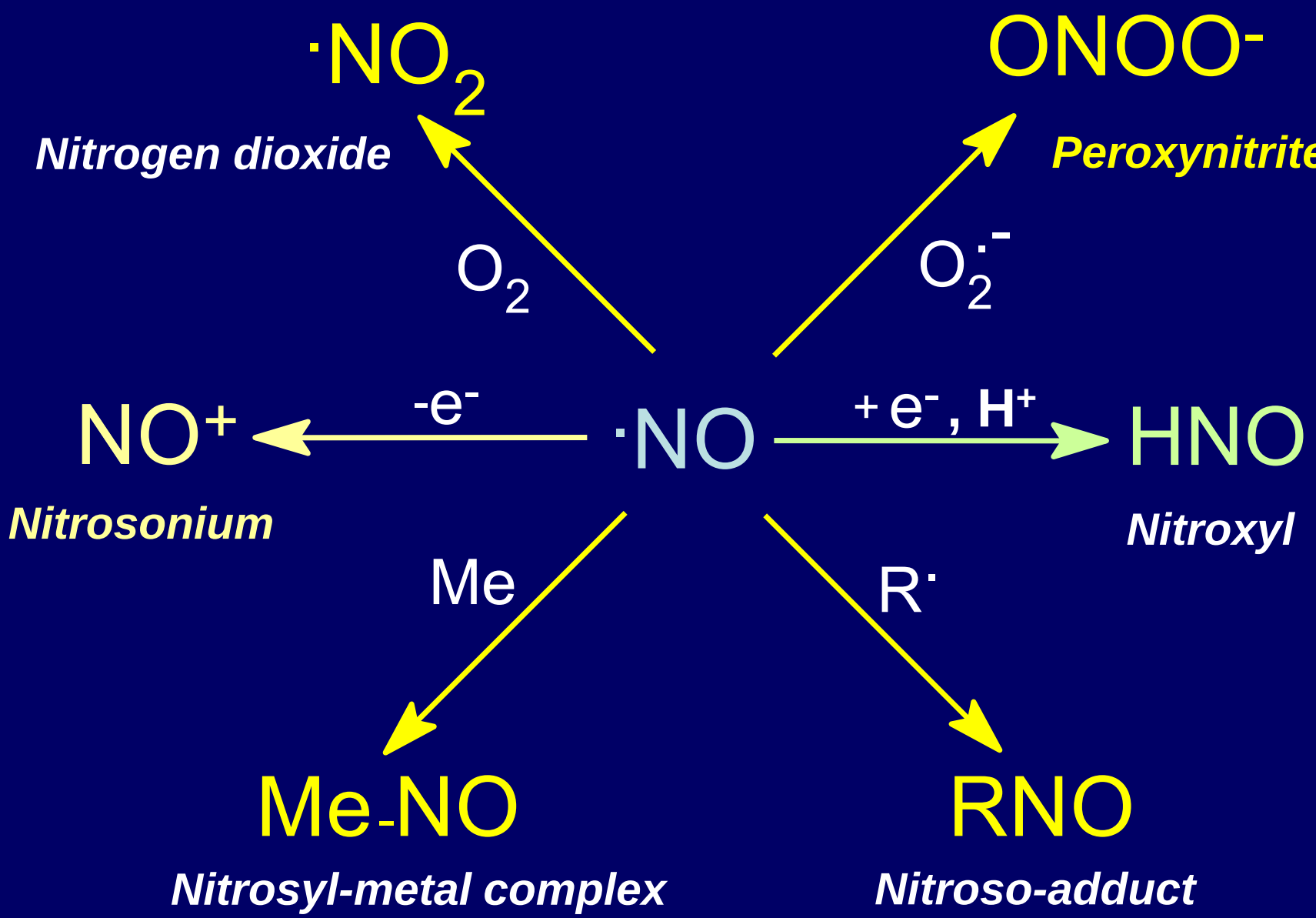


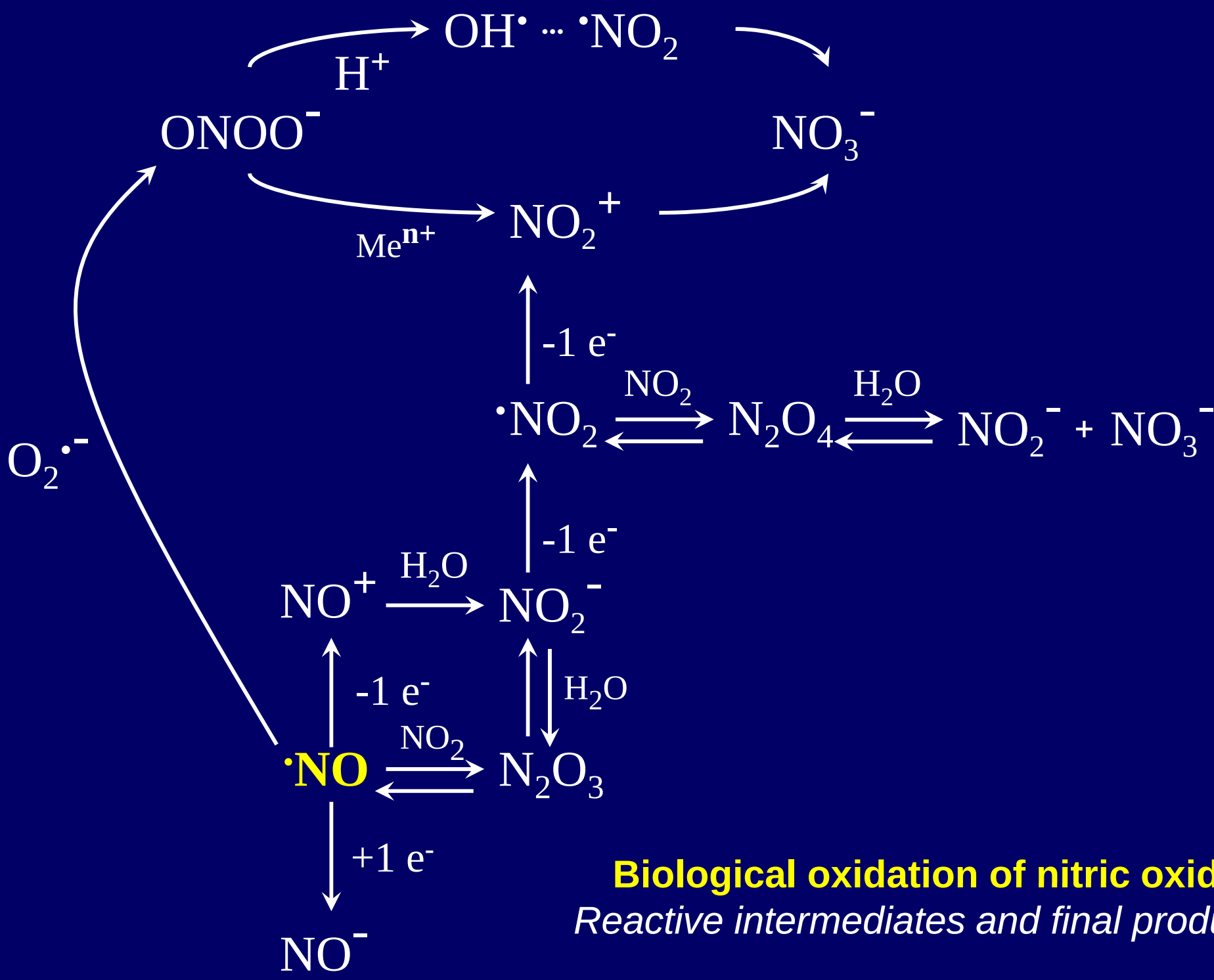
eNOS (endothelial) and nNOS (neuronal): *constitutive*

iNOS (inducible)

.

Main reactions of nitric oxide in biological systems



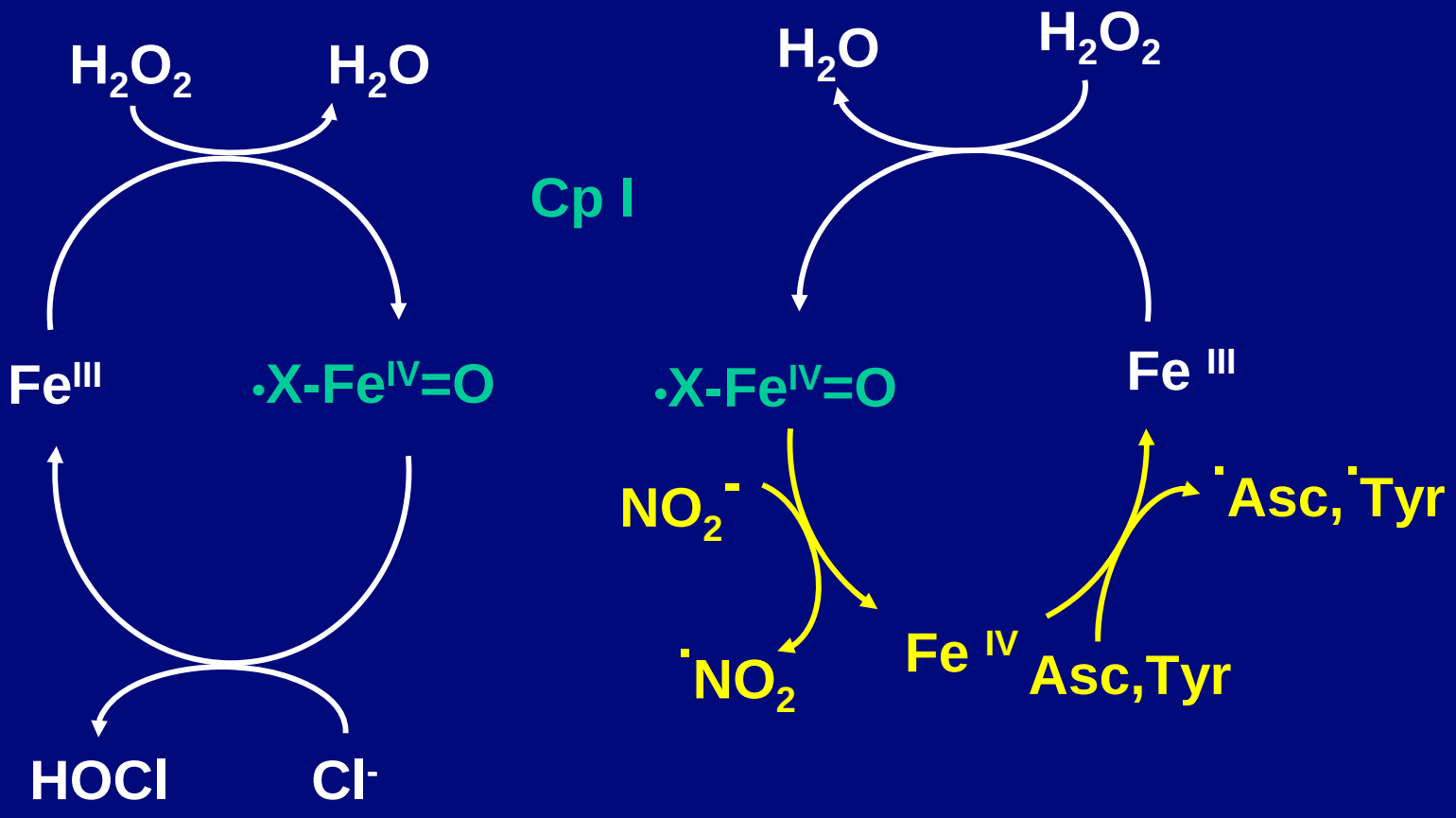


Biological oxidation of nitric oxide

Reactive intermediates and final products

Hemeperoxidase-catalyzed reactions

Two vs. one-electron oxidation steps



2-electron oxidation

1-electron oxidations

Biologically-relevant reducing radicals

Species	$E^{o'}$ (V)
$RSSR^{\bullet-}/RSSR$	-1.40
$NAD^{\bullet-}/NAD^{+}$	-0.91
$FADH^{\bullet}/FAD,H^{+}$	-0.31
$O_2^{\bullet-} / O_2$	-0.18

$$RSSR^{\bullet-} + O_2 \longrightarrow RSSR + O_2^{\bullet-}$$

$$O_2^{\bullet-} + \text{cyt } c^{3+} \longrightarrow O_2 + \text{cyt } c^{2+}$$

Iron and free radicals

- ***Haber-Weiss cycle***

$$Fe^{3+} + O_2^{\bullet -}\ (red) \longrightarrow Fe^{2+} + O_2\ (ox)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH$$ ***[Fenton reaction]***

- ***Iron storage proteins***

Ferritin, transferrin, hemeproteins, Fe-S proteins (aconitase, IRP-1)

- ***Low molecular weight, redox active iron complexes***

ATP, ADP, carboxylic acids

- ***Metal chelators***

Desferrioxamine, DTPA

Free Radical Reactions: *Initiation, Propagation, Termination*

Initiation

$A{:}A \rightarrow 2A\bullet$ homolysis

$B{:} + Me^{+n} \rightarrow B\bullet + Me^{+(n-1)}$ one-electron oxidation

$C + e^{-} \rightarrow C\bullet^{-}$ one-electron reduction

Propagation

$R\bullet + O_2 \rightarrow RO_2\bullet$ oxygen addition, peroxyl radical

$RO_2\bullet + R'H \rightarrow RO_2H + R'\bullet$ secondary radical formation

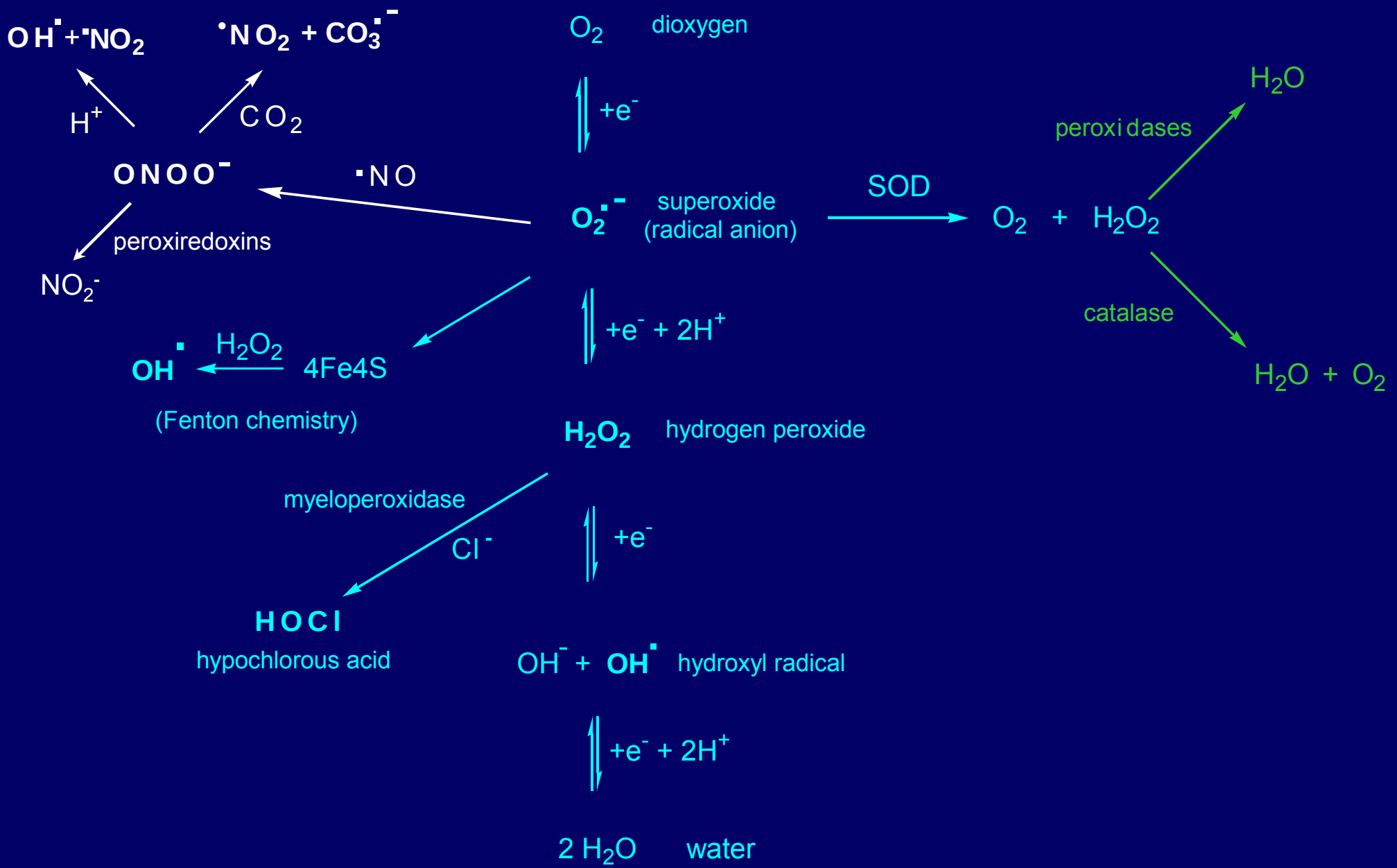
Termination

$R\bullet + R'\bullet \rightarrow R{:}R'$ dimerization, adduct formation

$2RH\bullet \rightarrow RH_2 + R$ dismutation

$R\bullet + AscH \rightarrow R{:}H + Asc\bullet$ formation of stable secondary radical

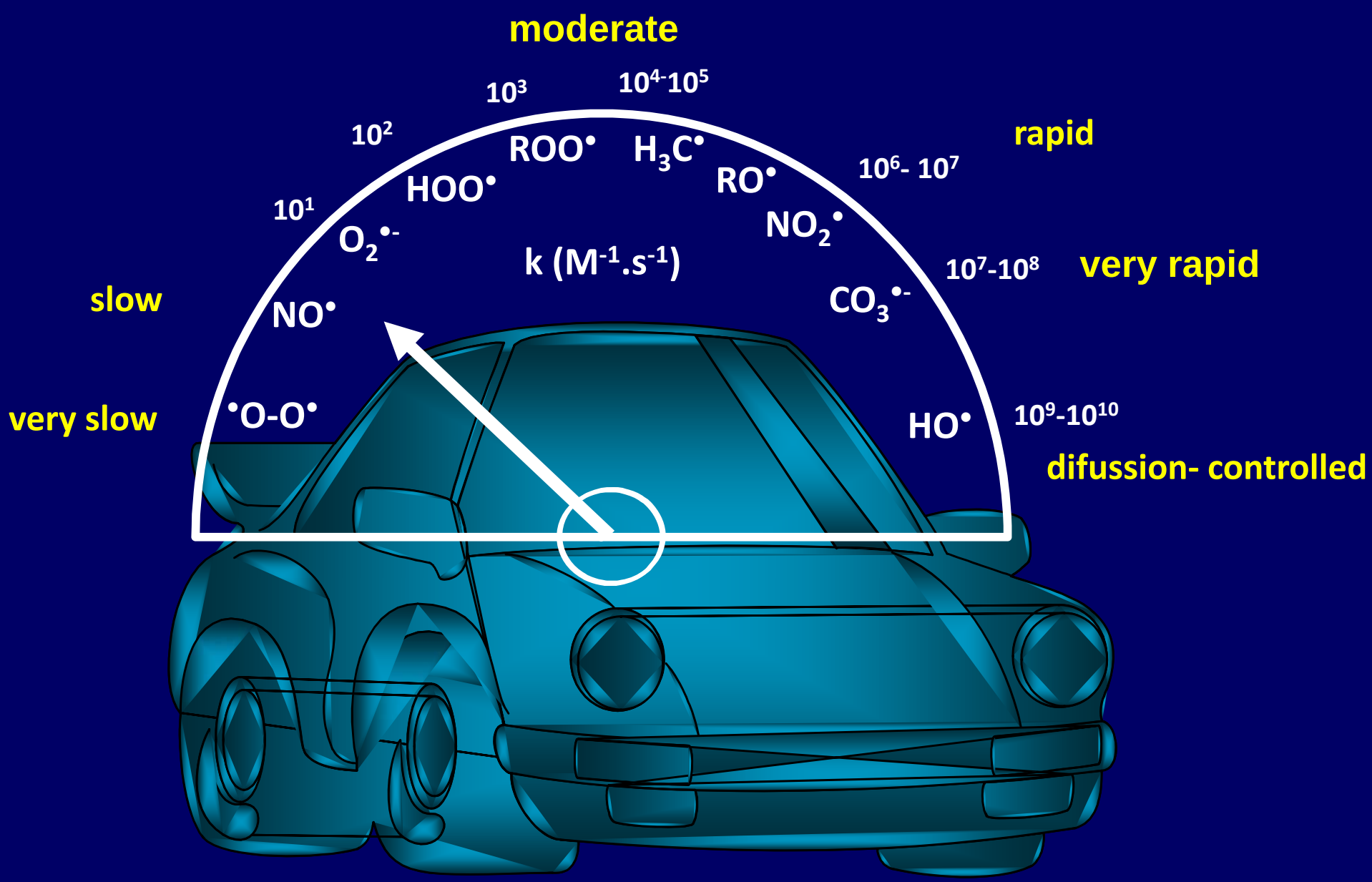
Radicals, oxidants and their catabolism in biological systems



Estimated half-lives of some radicals & oxidants in chemical and biological environments

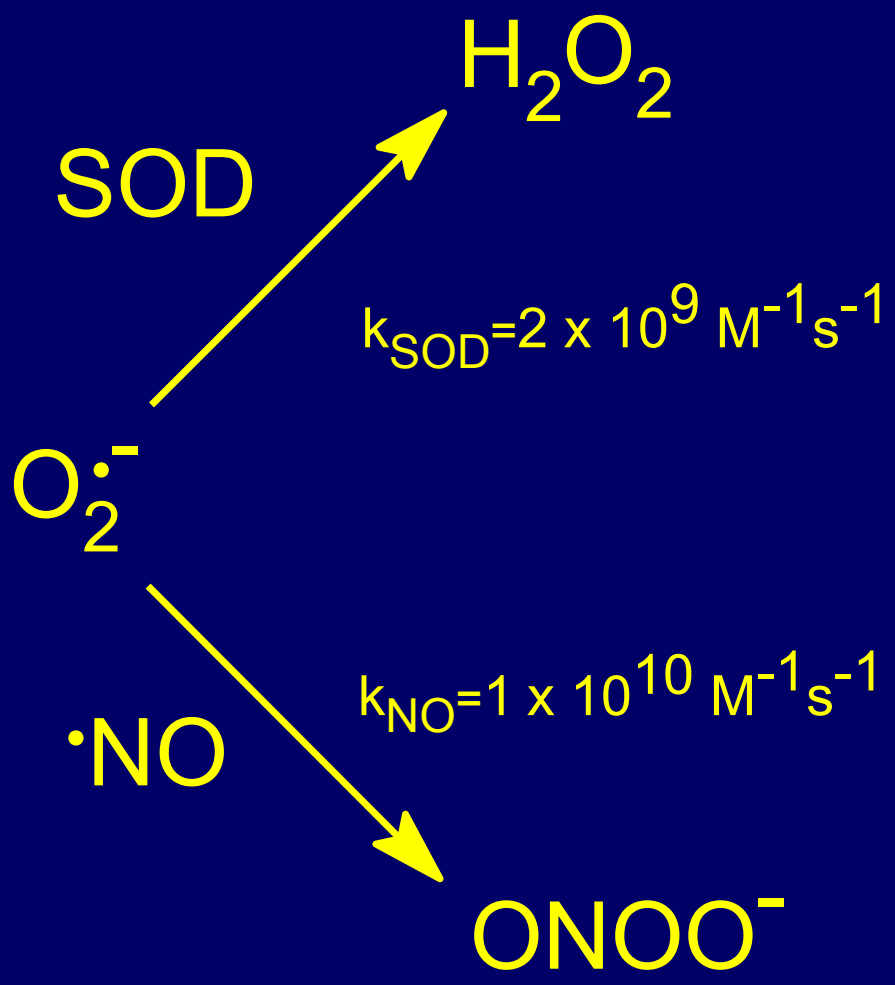
Species	$t_{1/}$ (seconds)
$HO^{\bullet}$	[2] $< 10^{-9}$
$RO^{\bullet}$	$\sim 10^{-6}$
$CO_3^{\bullet-}$	$\sim 10^{-6}$
$NO_2^{\bullet}$	$\sim 10^{-6}$
$ONOO^-$	10^{-2}
$^{\bullet}NO$	1- 10
$^1O_2$	10
$ROO^{\bullet}$	5-20
α-$TO^{\bullet}$	1-100
$Q^{\bullet-}$ (tar)	hours

Reactivity of Radicals with Biomolecules

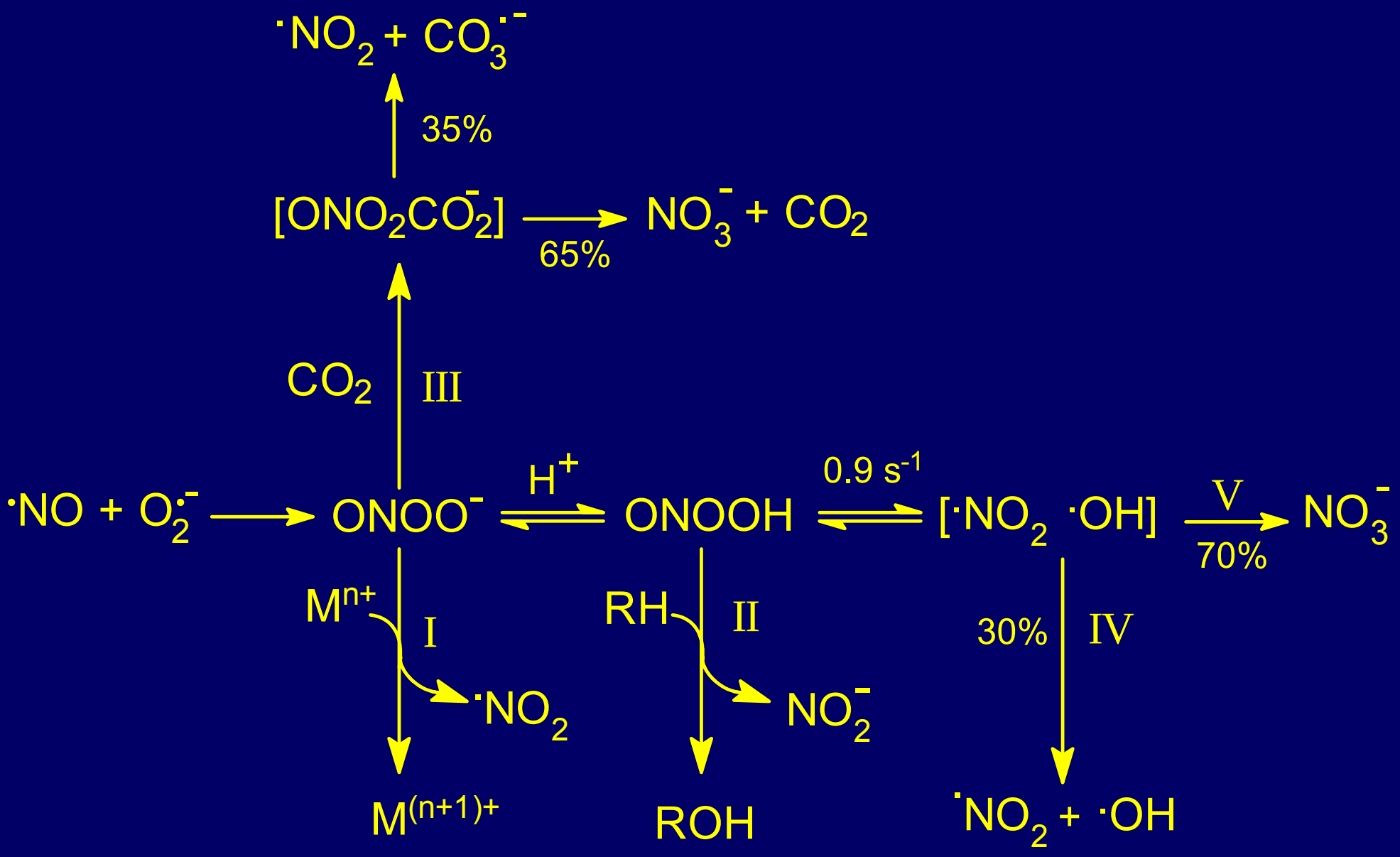


Adapted from O. Augusto

Competition between superoxide dismutation and peroxynitrite formation

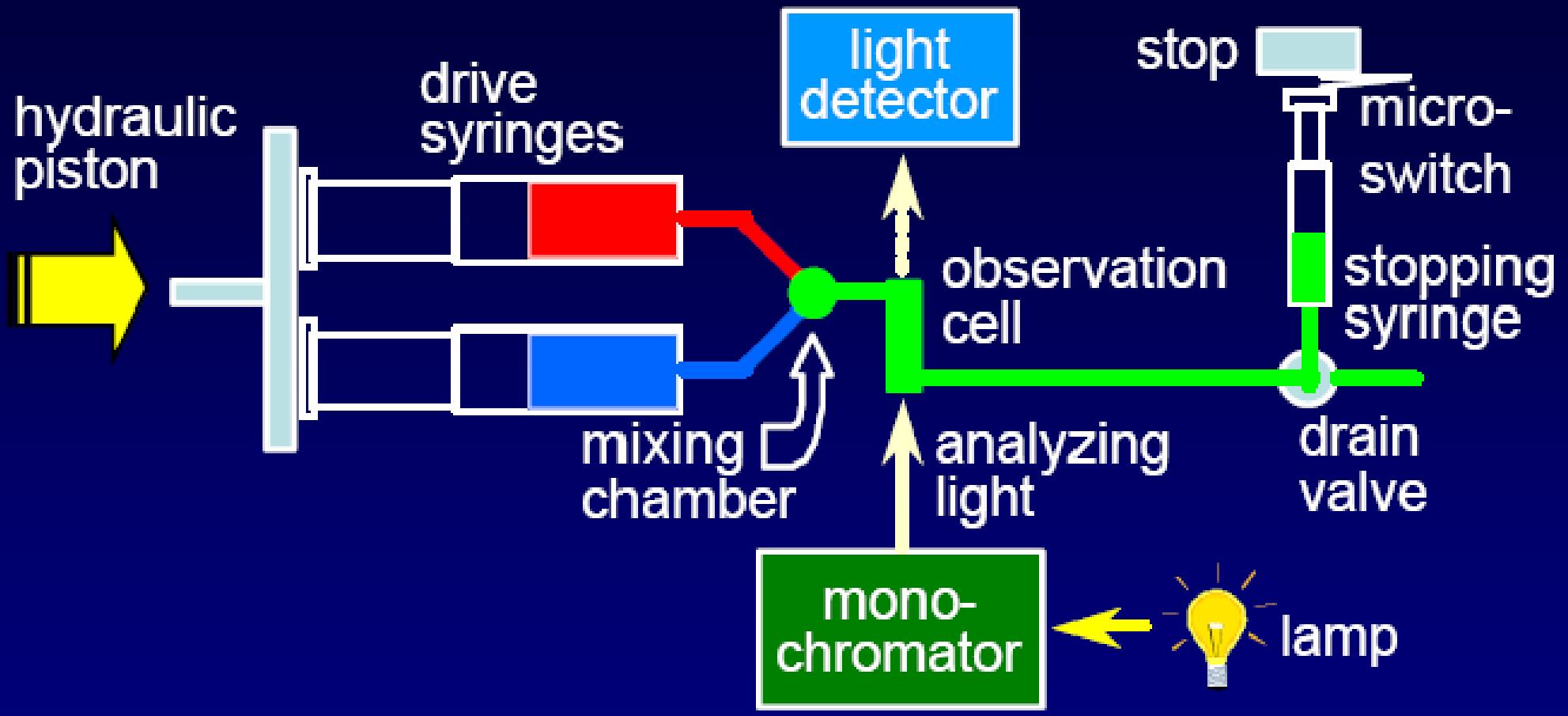


Formation and decomposition pathways of peroxynitrite



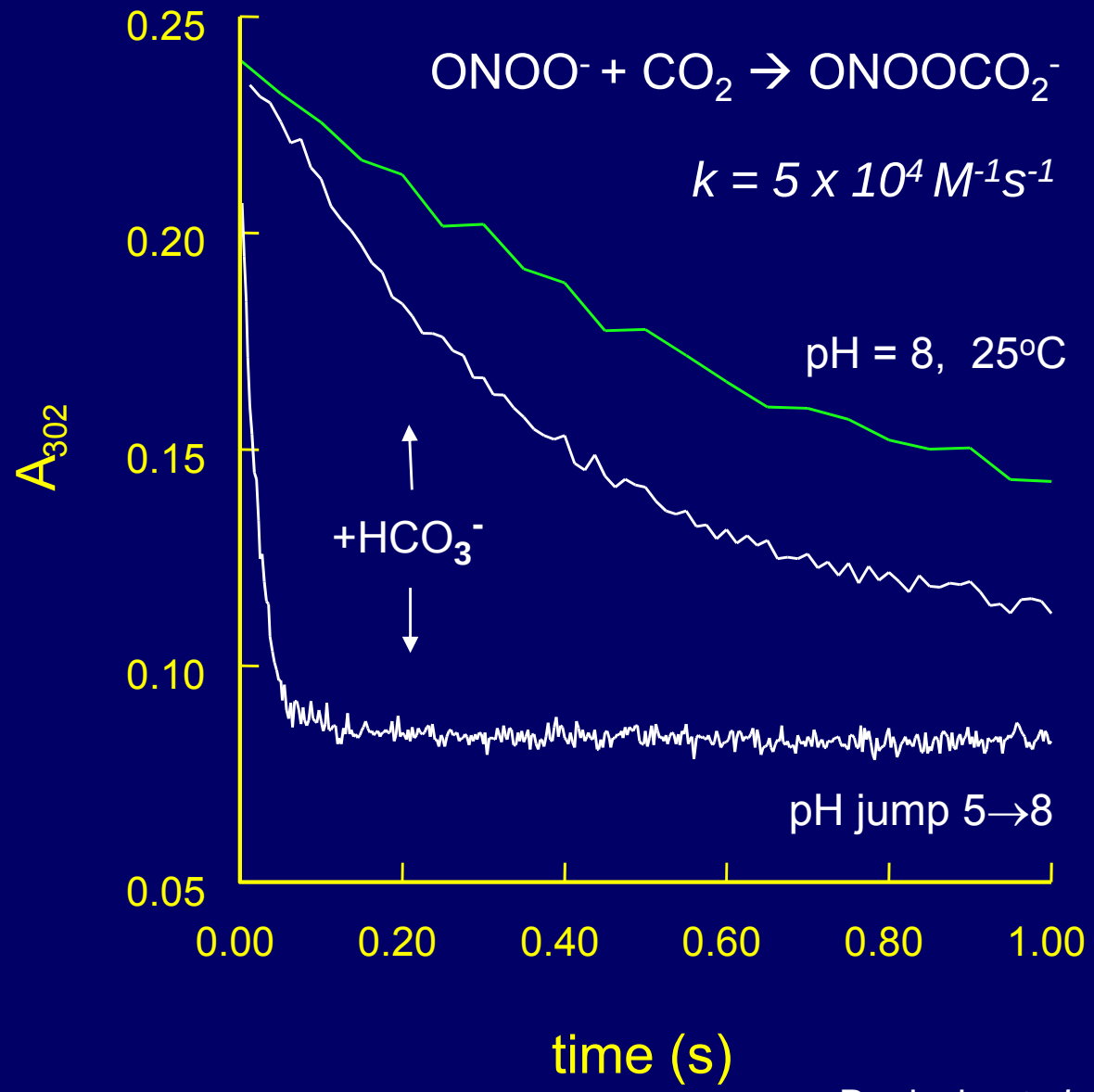
Radi R *et al*; FRBM, 2001

Stopped-flow rapid-mixing



- Time resolution limited to about 1 ms by interval between mixing and observation

Peroxynitrite reacts fast with carbon dioxide



Denicola *et al,* ABB 1996

See also, Lymar and Hurst, JACS 1995

EPR detection of free radicals in biology

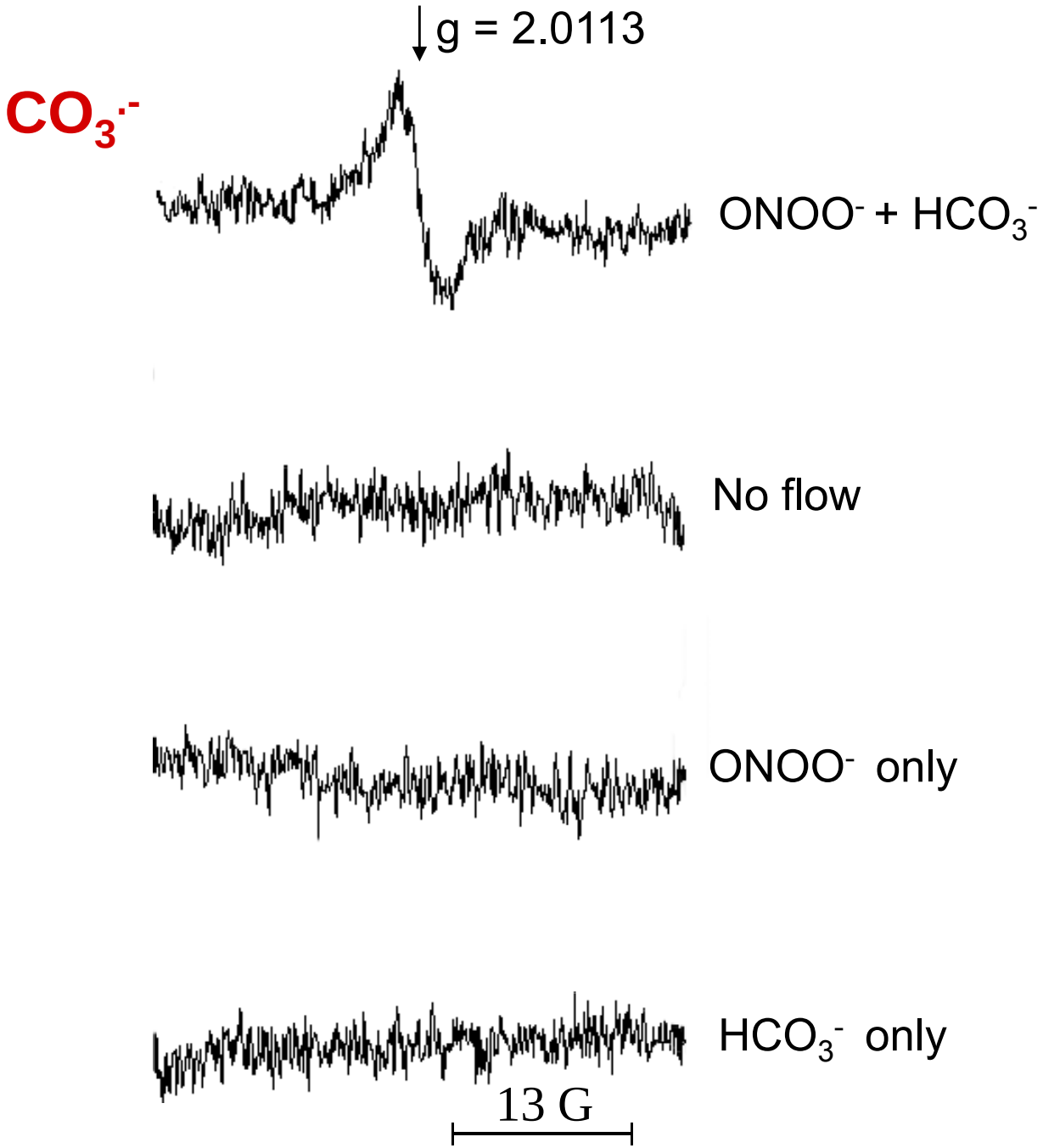
1. Direct EPR (solutions)
   a) Static : for long lived $R^{\cdot}$ ($t_{1/2}$ > min)
   b) Fast-flow : for short-lived $R^{\cdot}$, requires large samples
2. Direct EPR (frozen)
   Applicable to short-lived radicals, limited use for organic radicals due to anisotropy
3. Spin trapping
   Wide application but limited structural information

Modified from (Augusto, 2001)

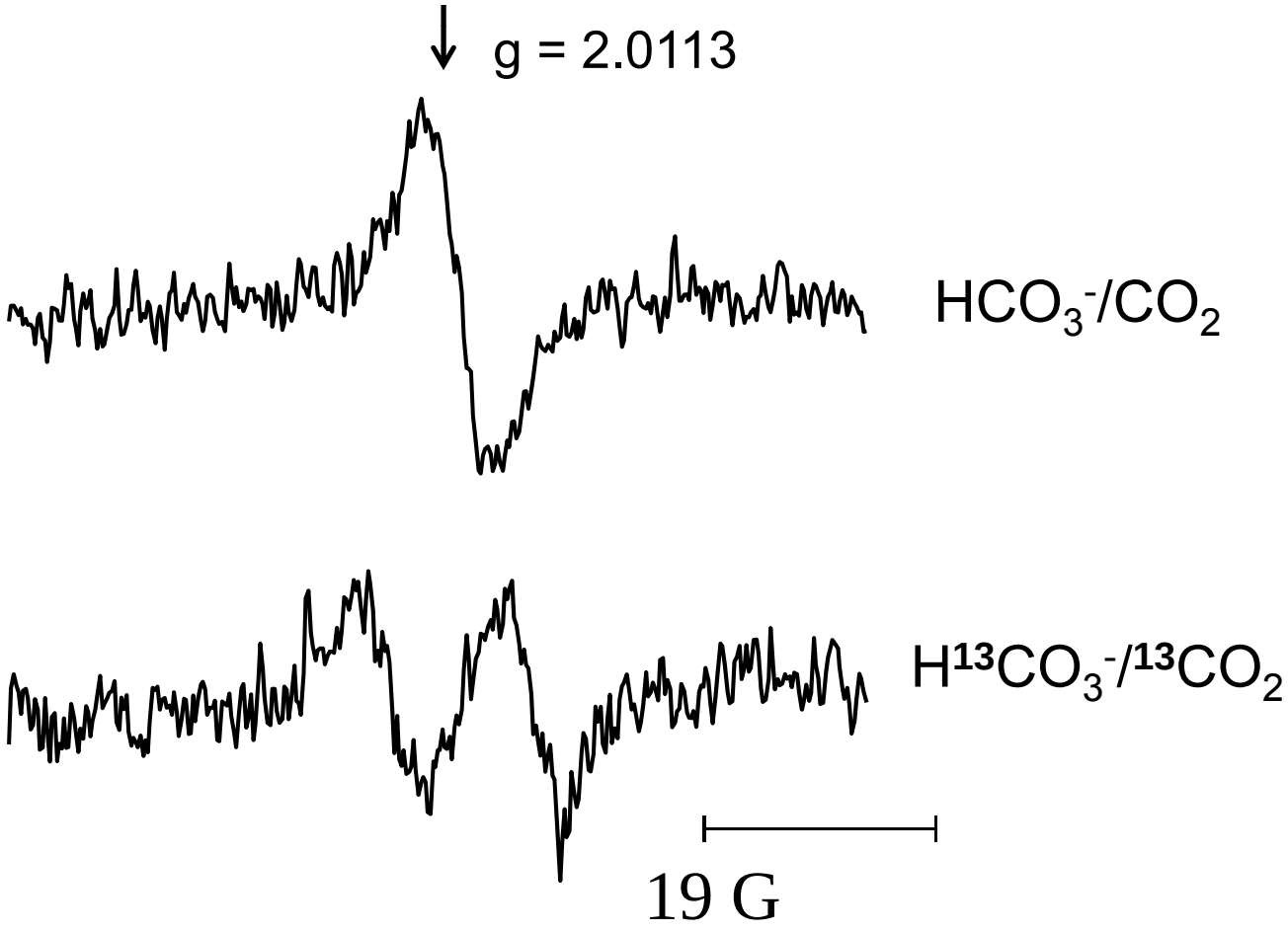
Carbonate radicals from the peroxynitrite-carbon dioxide reaction

Direct EPR studies

Bonini *et a*l, JBC 1999



+ $ONOO^-$
pH_f = 8.4

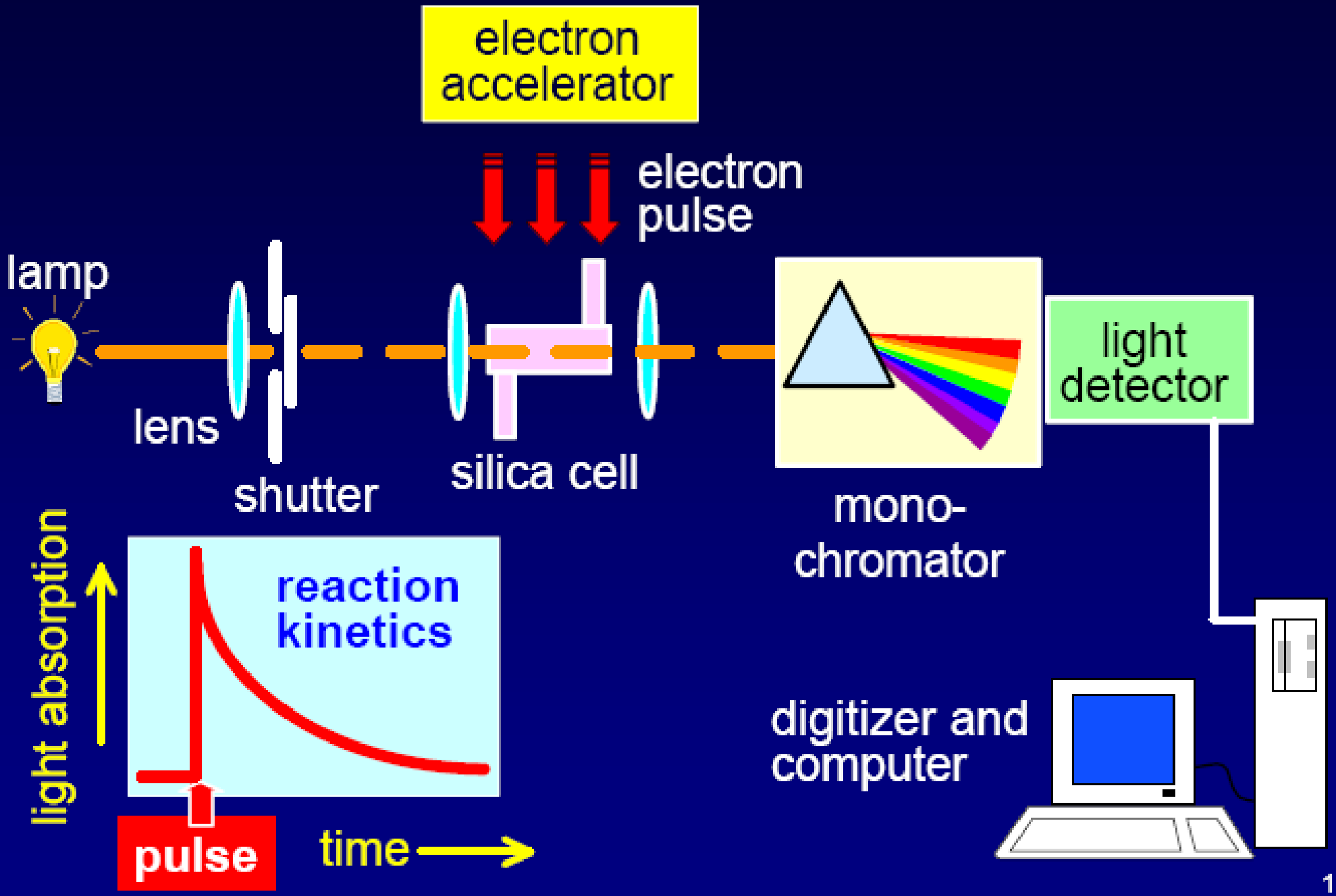


$CO_3^{\cdot-}$ from the $ONOO^-/CO_2$ reaction

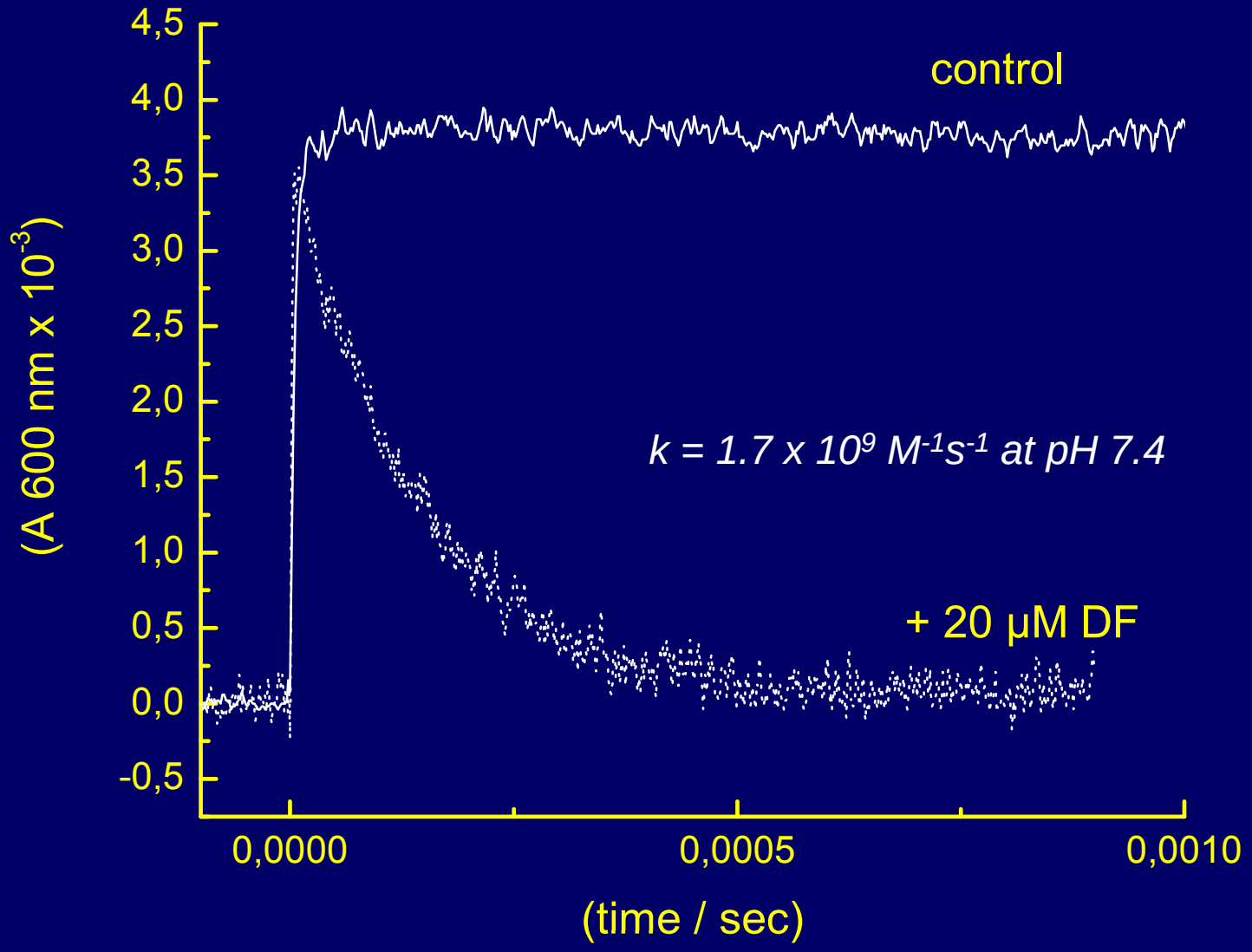
Bonini, Ferrer-Sueta, Radi, Augusto, JBC 1999

Pulse radiolysis

Courtesy, P. Wardman

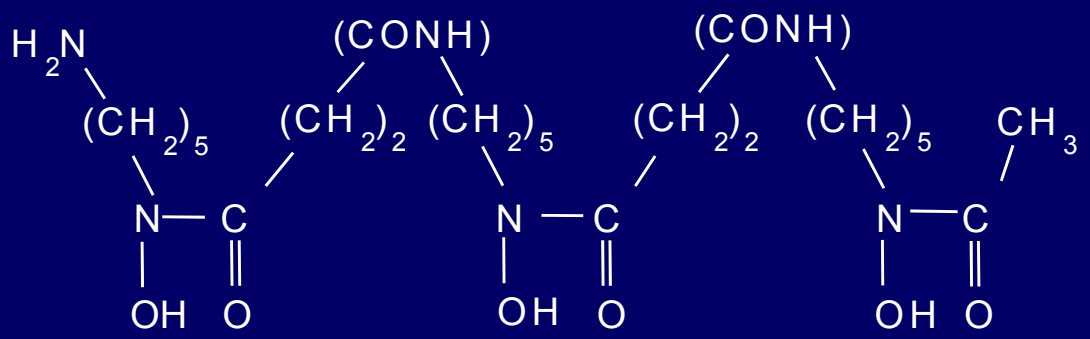


Carbonate radical reaction with desferrioxamine
P*ulse radiolysis studies*



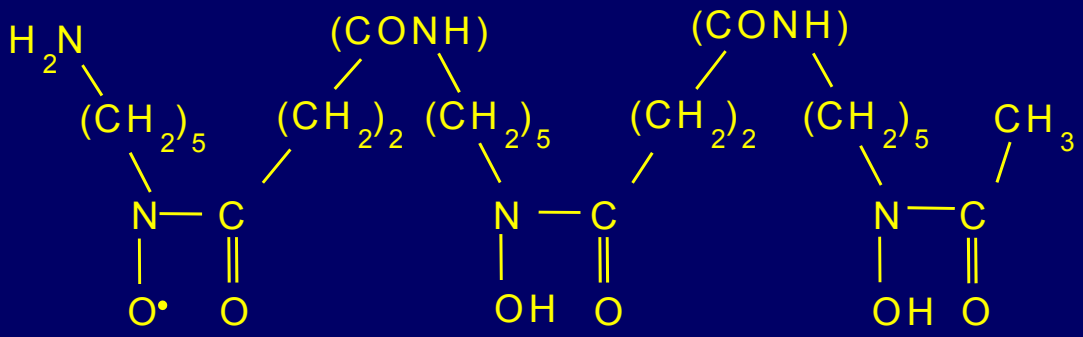
Bartesaghi *et al;* FRBM, 2004

One-electron oxidation of desferrioxamine by free radicals



Desferrioxamine

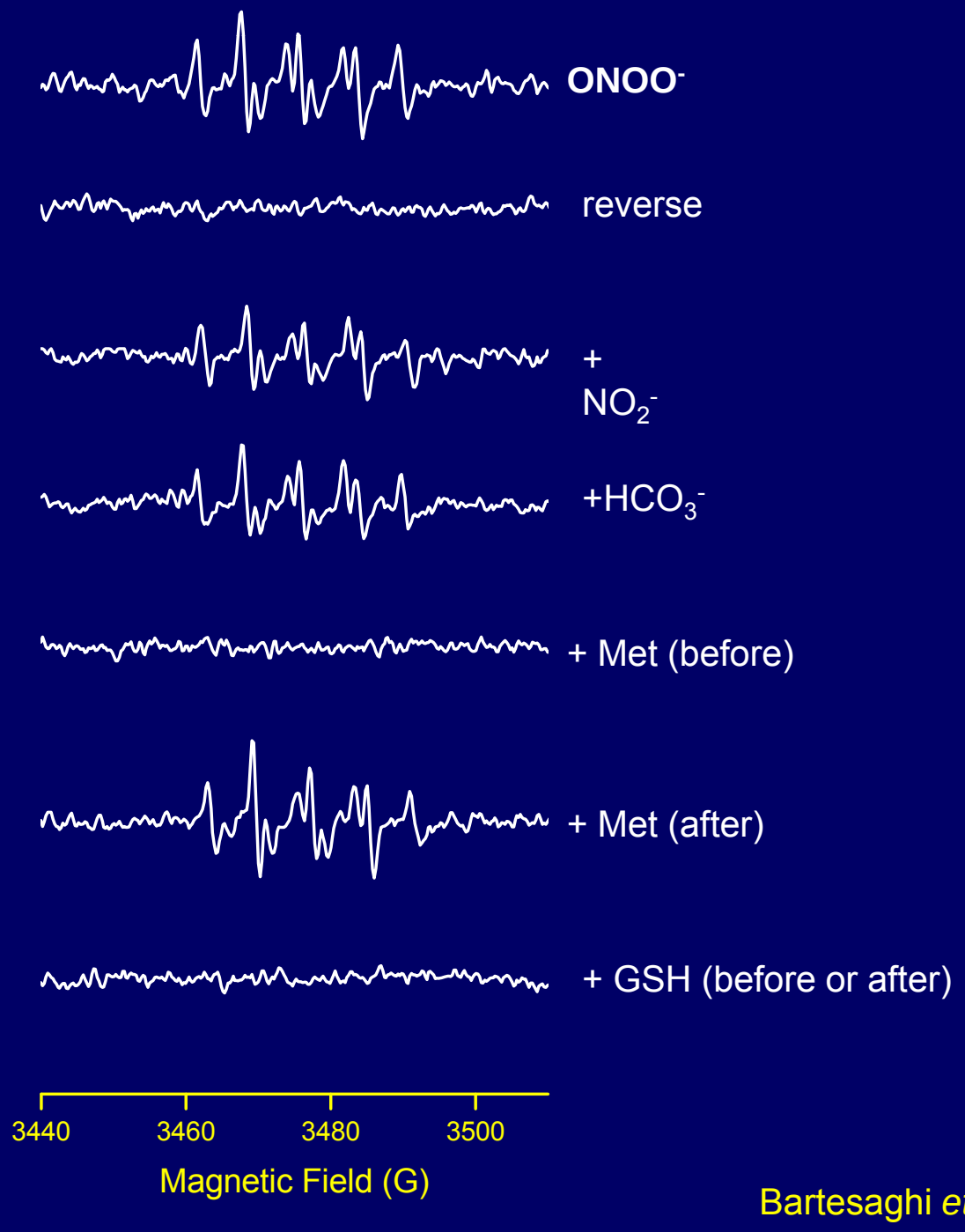
$\cdot OH, \cdot NO_2, CO_3^{\cdot -}$



Desferrioxamine

Nitroxide radical

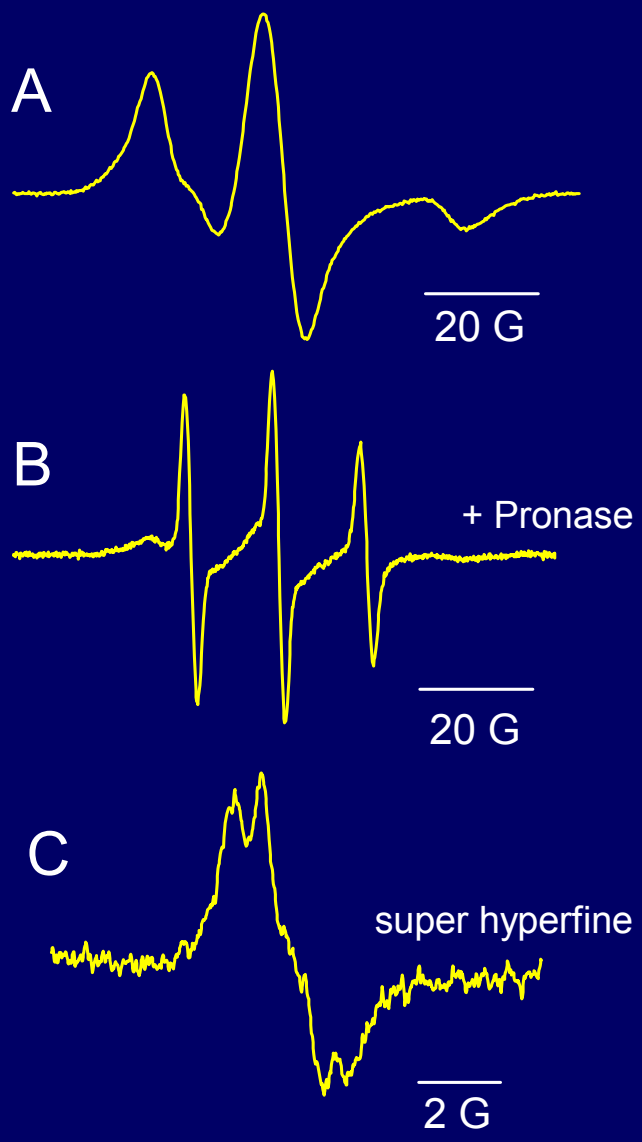
Direct EPR detection of desferrioxamine nitroxide radicals during reactions with peroxynitrite



Bartesaghi *et al;* FRBM 2004

EPR spectra of oxyHb reaction with peroxynitrite in the presence of MNP

EPR spin trapping of globin (protein-derived) radicals

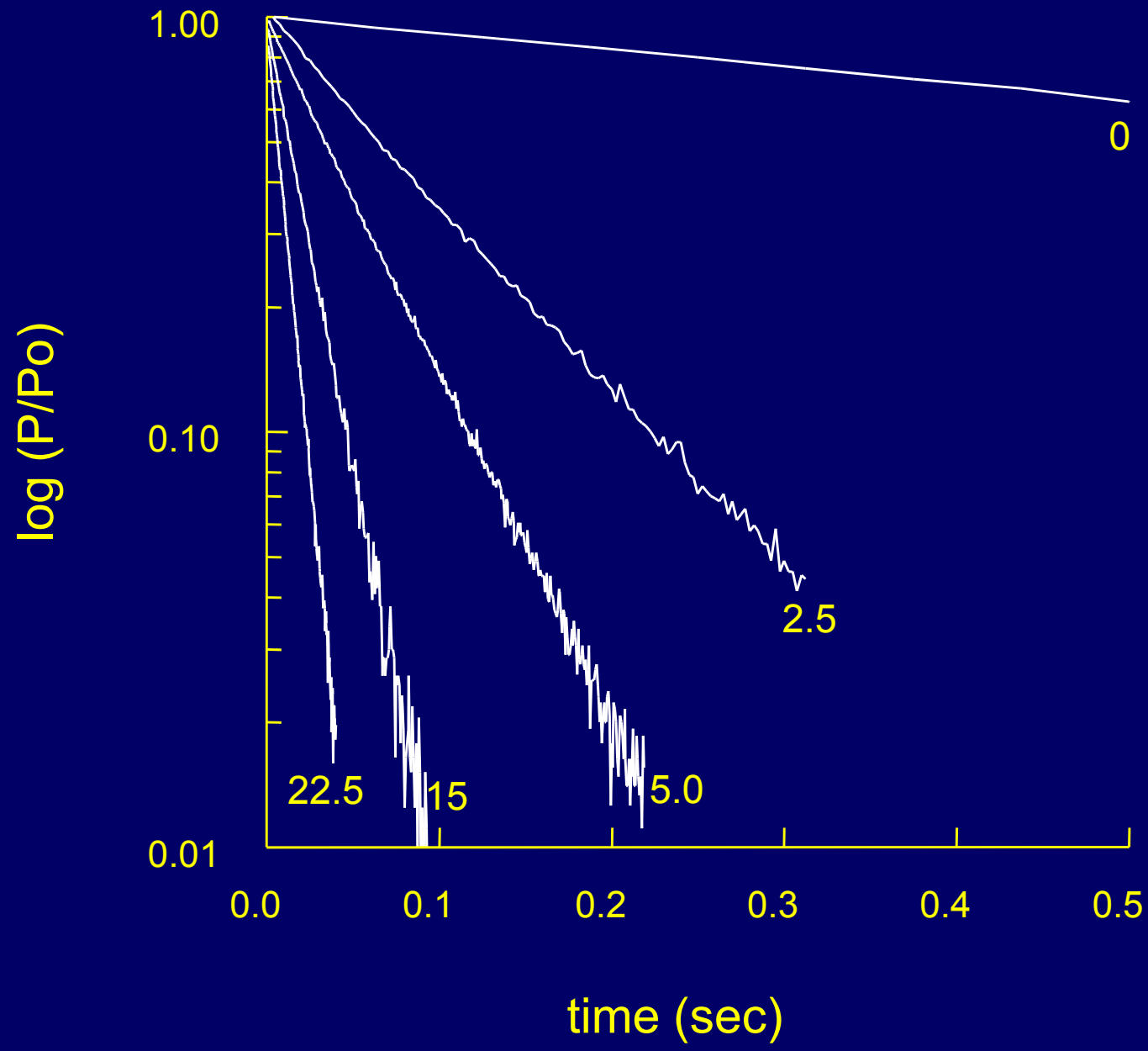


1 mM HbO_2
1 mM $ONOO^-$
20 mM MNP

Romero *et al*; JBC, 2003

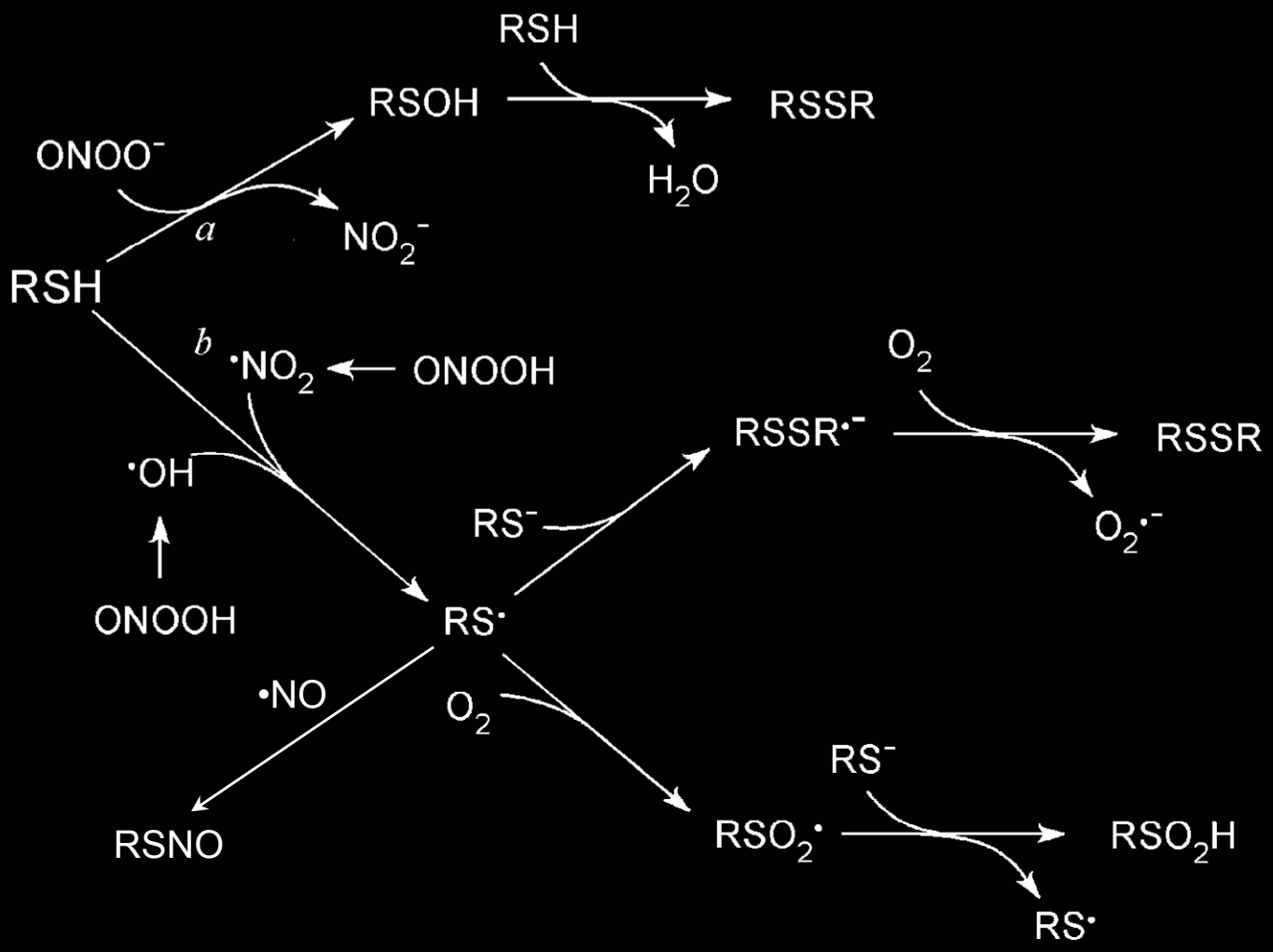
Cysteine oxidation by peroxynitrite

Stopped-flow studies



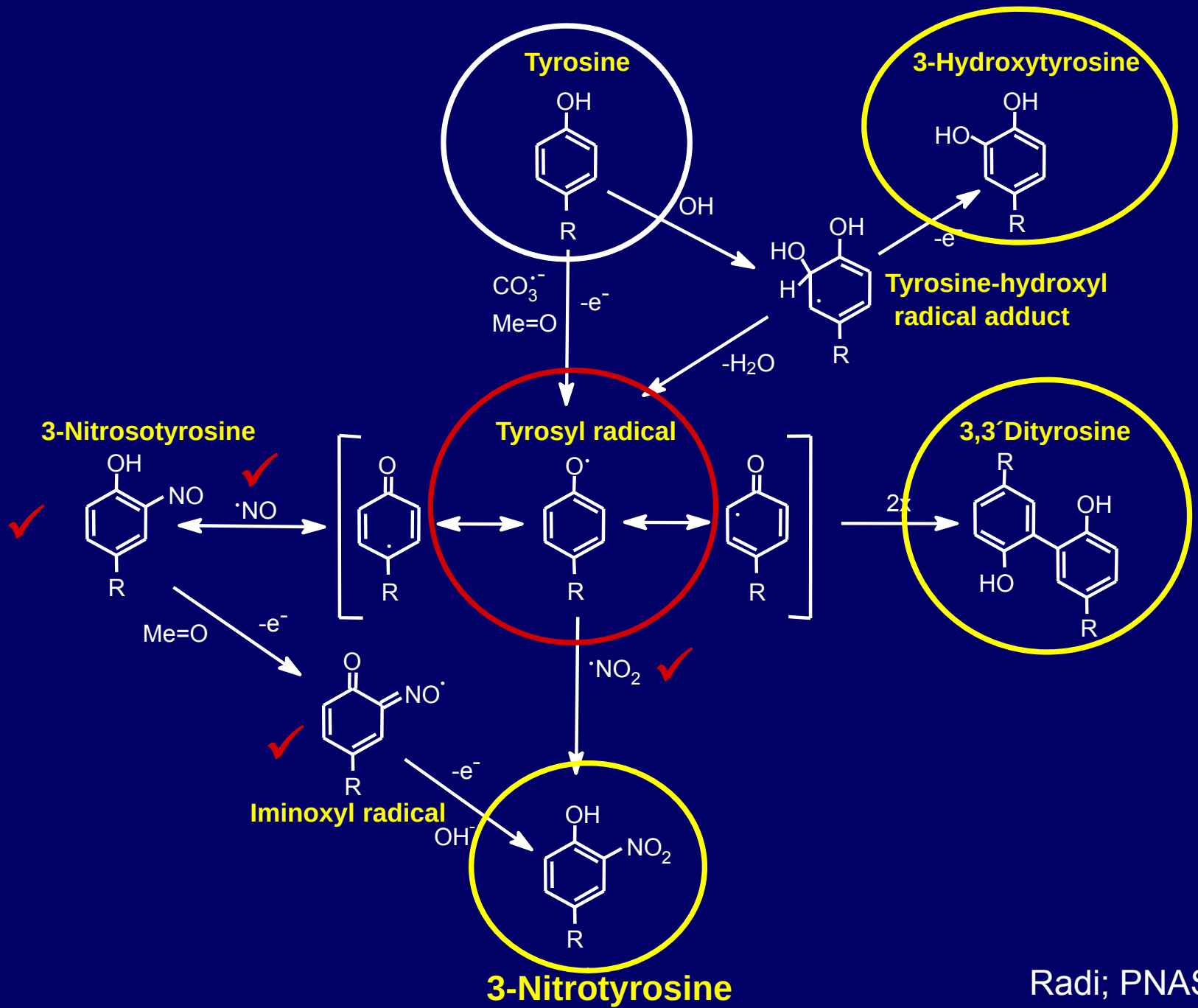
Radi, Beckman, Bush and Freeman; JBC, 1991

Peroxynitrite reactions with thiols:
Mechanisms, intermediates and products



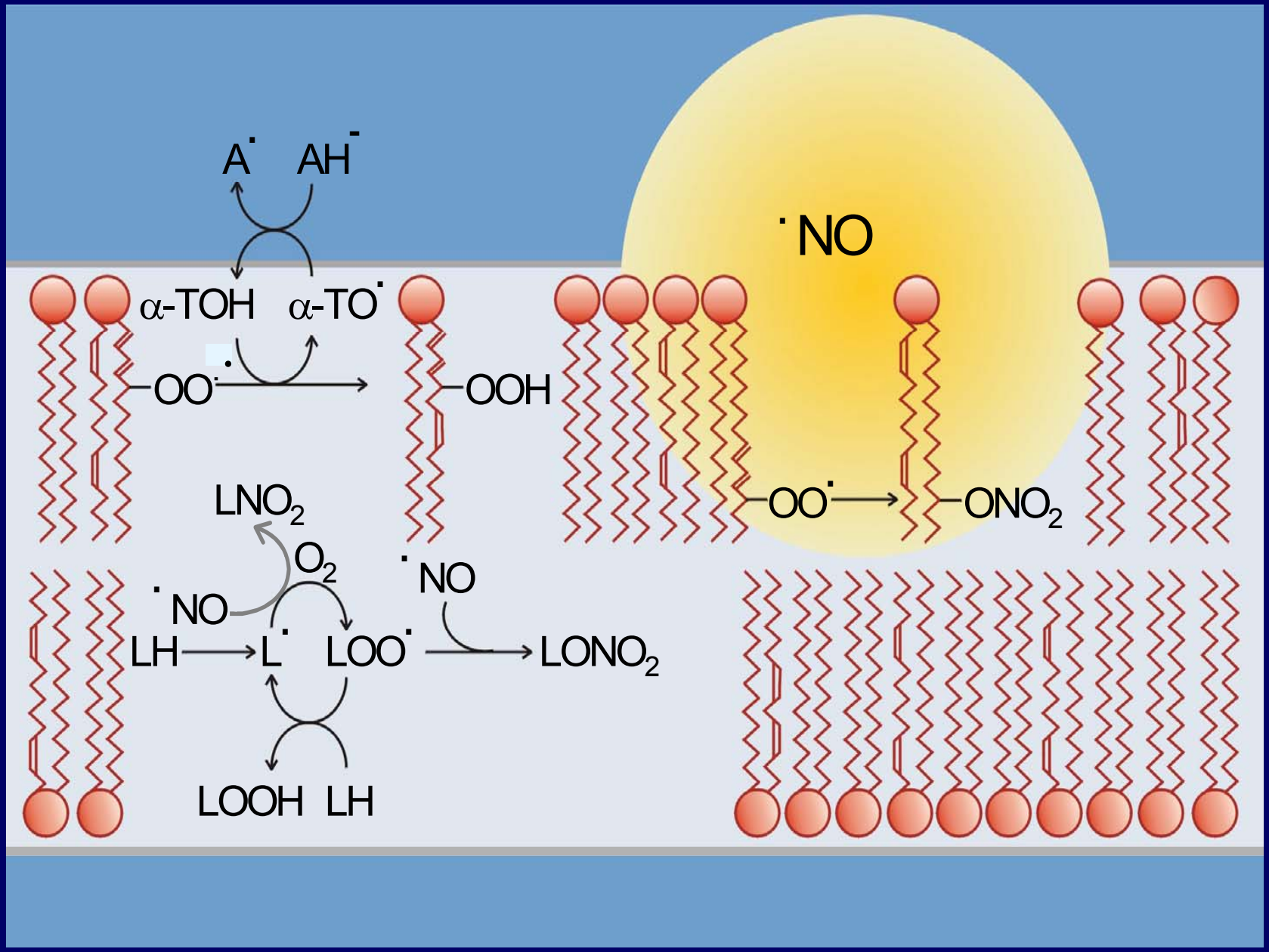
Modified from Radi *et al*
Nitric Oxide (Ignarro, L. Ed.); Academic Press 2000

Radical pathways of tyrosine nitration

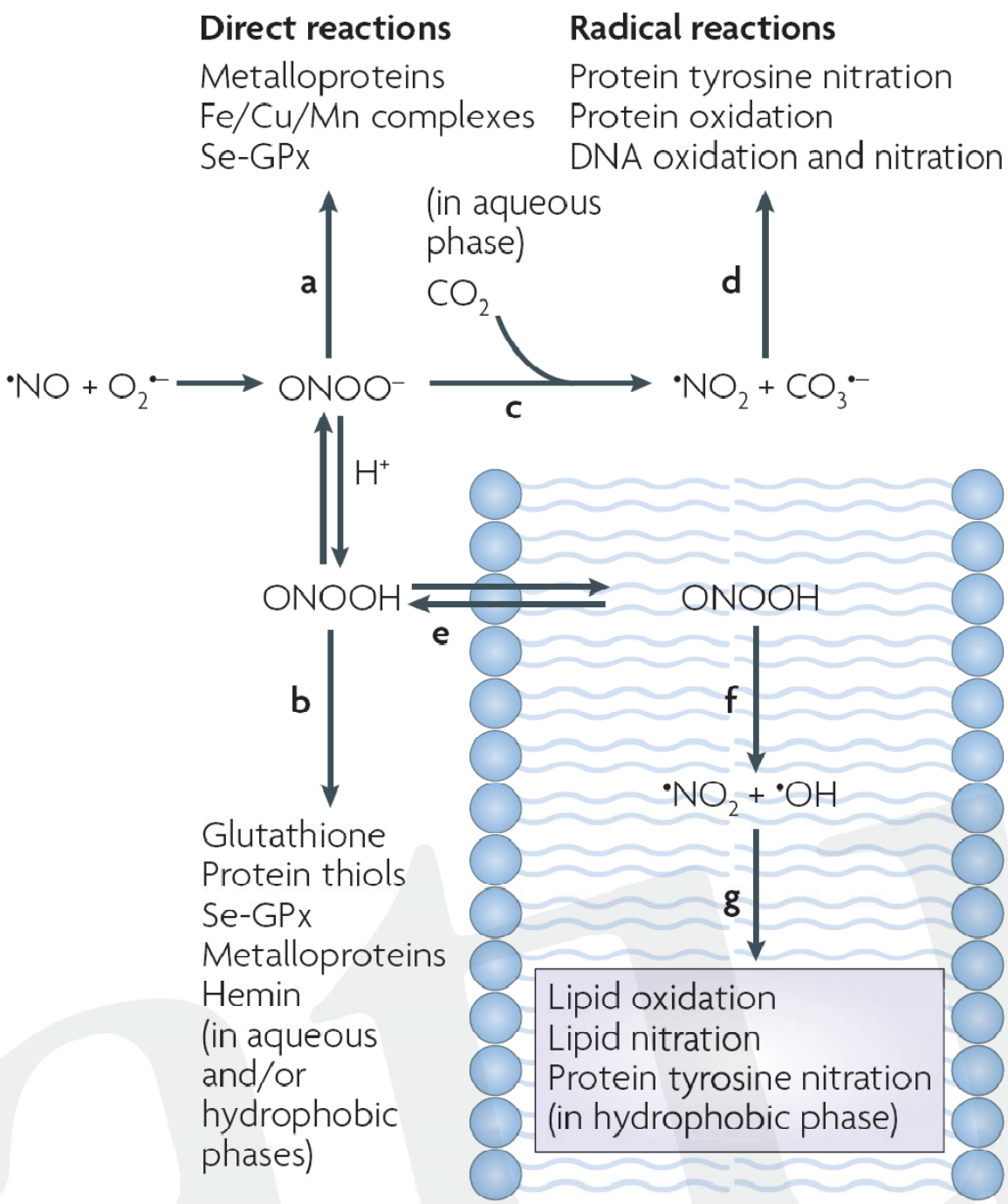


Radi; PNAS, 2004

Nitric oxide diffusion and interactions in lipid oxidation processes



Rubbo and Radi. *In*: Handbook of Antioxidants
(*Cadenas E and Packer L Eds.) Marcel Dekker Inc.*,2001



Szabo, Ischiropoulos and Radi
Nature Reviews (2007)

Chem. Rev. **1998,** *98,* 705–762

Protein Radicals in Enzyme Catalysis

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Received July 30, 1997 (Revised Manuscript Received November 25, 1997)

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Contents

I. Introduction

The interest in the involvement of protein radicals, stable and transient, in catalysis has skyrocketed since our initial reviews on this topic in 1988 and 1989.[1,2] This explosion of interest in the past few years has thus elicited a number of important reviews on this topic.[3−7] This review will focus on the general principles that have evolved governing the formation of protein radicals and their roles in catalysis. New experimental information that has emerged since 1988 will then be summarized for each system that has been thus far characterized. We will limit the scope of this review to enzymatic systems that utilize amino acid or modified amino acid based radicals that are covalently linked to the protein. Other enzymatic systems in which the involvement of non-amino acid based organic radicals have been proposed such as the cytochrome P450 enzyme family,[8] flavin and pyrroloquinoline quinone (PQQ) dependent enzymes,[9,10] heme peroxidases,[11] the pyridoxal phosphate dependent 2,3-lysine aminomutase,[3,12] pyruvate:ferredoxin oxidoreductase,[13] and enzymes

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Published on Web 03/19/1998

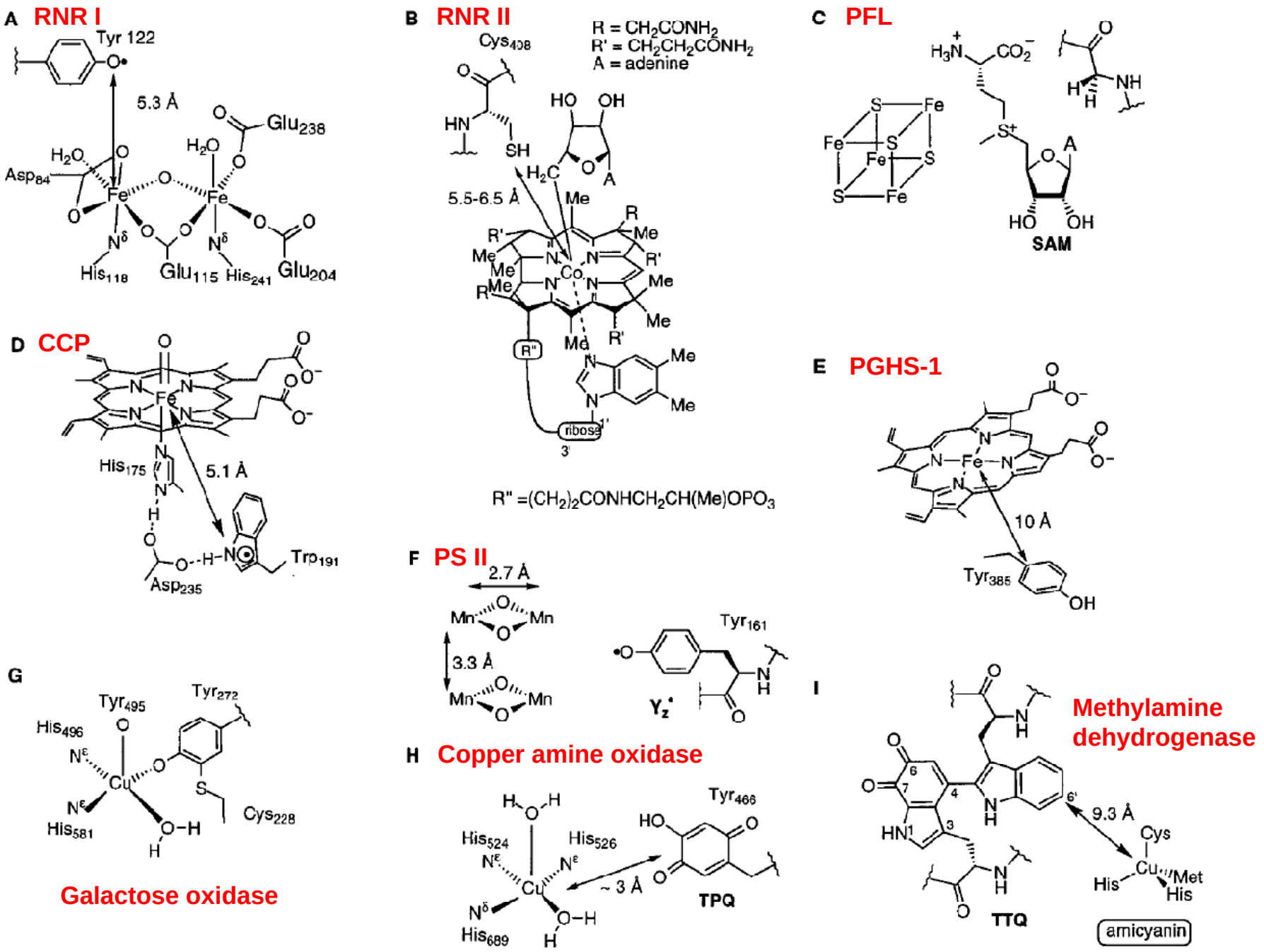

Figure 1. The structures of the metallocofactors and the (modified) amino acids that they oxidize.

Intramolecular transfer processes in ribonucleotide reductase
Participation of tyrosyl and cysteinyl radicals

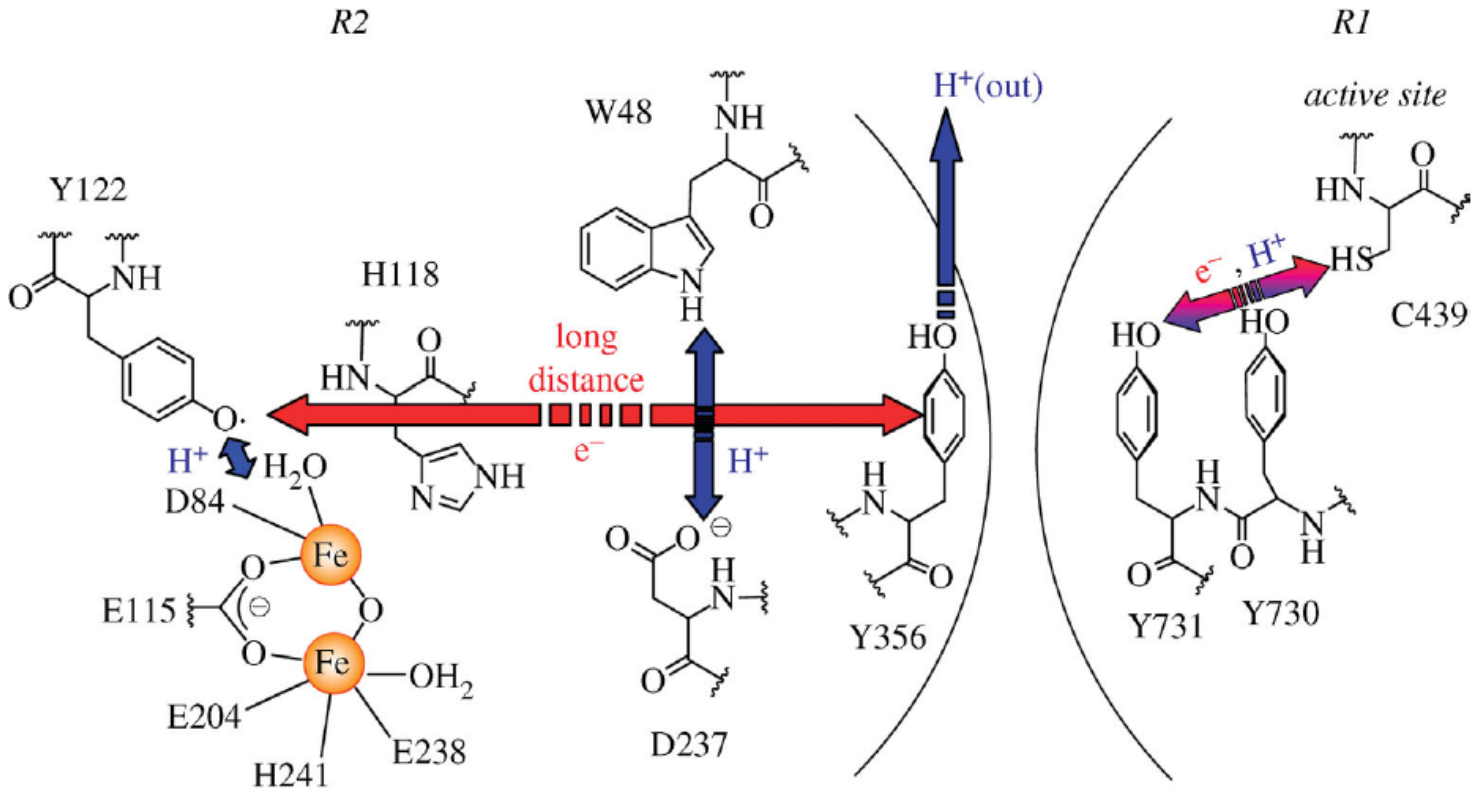

Figure 12. Proposed model for radical transport in RNR. The mode of transport at the interface (between Y356 and 731) is undefined.

See also: Kalyanaraman *et al*; J Biol Chem, 2005; Methods in Enzymology; Vol 440, 2008

the authors to distinguish denitrification from the temporary storage of nitrogen in biomass, but also allowed the rates of denitrification and biotic uptake to be assessed for the entire reach.

The authors found that measurable rates of nitrate removal occurred through biotic uptake and denitrification in most streams, and that absolute rates of removal generally increased as nitrate concentrations increased. But the efficiency of nitrate removal — the proportion of nitrate removed relative to the total amount present — decreased exponentially as nitrate concentrations increased. This pattern held across nitrate concentrations that differed by six orders of magnitude in eight different biomes. As we use land ever more intensively, the effectiveness of the receiving streams in removing the additional nitrate pollution is likely to continue to diminish. Thus, looking at the results of this study, the pressure on policy-makers to reduce the load of anthropogenic nitrogen entering rivers from terrestrial systems will grow.

Of course, small streams are only one component of the filigree of flowing waters that drain a landscape. Small streams connect to middling channels, which connect to larger rivers, all of which propel water and its dissolved and particulate constituents towards coastal systems. The length of all the channels in a river system is often extensive — those within the 30,000 km^2 watershed of the Potomac River in the eastern United States amount to a length of some 25,000 km, equivalent to about five times the distance across the United States from coast to coast. Unsurprisingly, therefore, there are ample opportunities for nitrates to be removed on their journey from a river's headwaters to the coast that are not covered by Mulholland and colleagues' study.

To address this point, the researchers use a model to scale up their results to an entire river network. The outcome underscores the importance of river channels of all sizes, as well as the distribution of nitrate loading within *al.*[2] across a range of stream sizes and throughout the annual cycle.

Denitrification occurs not only in rivers, but in almost all environments at some time and place: nearly 80% of all reactive nitrogen is disposed of before it reaches coastal waters. Soils are generally the first receptor of the large amounts of nitrogen that we add to terrestrial systems, and the amount of denitrification in soils can substantially exceed that removed in the stream network[4]. Groundwater, lakes and coastal systems are also important sites of denitrification. For all these environments we urgently need advances in methods and models[5] that will increase our understanding of how large amounts of nitrogen move and are removed as they journey from soils to the sea. ■

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1. Galloway, J. N. *et al. Biogeochemistry* **70**, 153–226 (2004).
2. Mulholland, P. J. *et al. Nature* **452**, 202–205 (2008).
3. Alexander, R. B., Smith, R. A. & Schwarz, G. E. *Nature* **403**, 758–761 (2000).
4. Seitzinger, S. *et al. Ecol. Appl.* **16**, 2064–2090 (2006).
5. Groffman, P. M. *et al. Ecol. Appl.* **16**, 2091–2122 (2006).

BIOCHEMISTRY

Radicals by reduction

Joseph T. Jarrett

Many enzymes convert their substrates into organic radicals to allow challenging reactions to occur. A microbial enzyme does so by simple electron transfer, casting fresh light on enzyme evolution.

The harmful effects of free radicals have been widely publicized over the past two decades. As a result, pharmacists are now well stocked with vitamins and nutritional supplements purported to combat the damage caused by these chemical bogeymen. Less attention has been given to the many beneficial uses of radicals in biology, where they are often generated by enzymes to overcome chemically difficult problems. On page 239, Kim *et al.*[1] report a remarkable example of this in certain anaerobic bacteria found in the human gut. These bacteria generate energy by breaking down the amino acid leucine; this is a particularly tough chemical process in the absence of oxygen. The authors show that a key enzyme for this process overcomes the reaction barriers

enzymes might use transient radicals as intermediates came with the discovery of vitamin B_{12}. The active cofactor form of this vitamin contains a weak carbon–cobalt bond that is broken to generate a carbon radical[3]; this radical transiently oxidizes other molecules by removing a hydrogen atom, a process that can often promote difficult chemical reactions (Fig. 1a, overleaf).

Around the same time as the discovery of vitamin B_{12}, organic radicals were shown to be essential for the activity of ribonucleotide reductase, an enzyme that controls the cellular concentrations of deoxyribonucleotides (the building-blocks of DNA)[4]. The active form of ribonucleotide reductase has a radical on the side chain of a tyrosine amino acid (Fig. 1b).

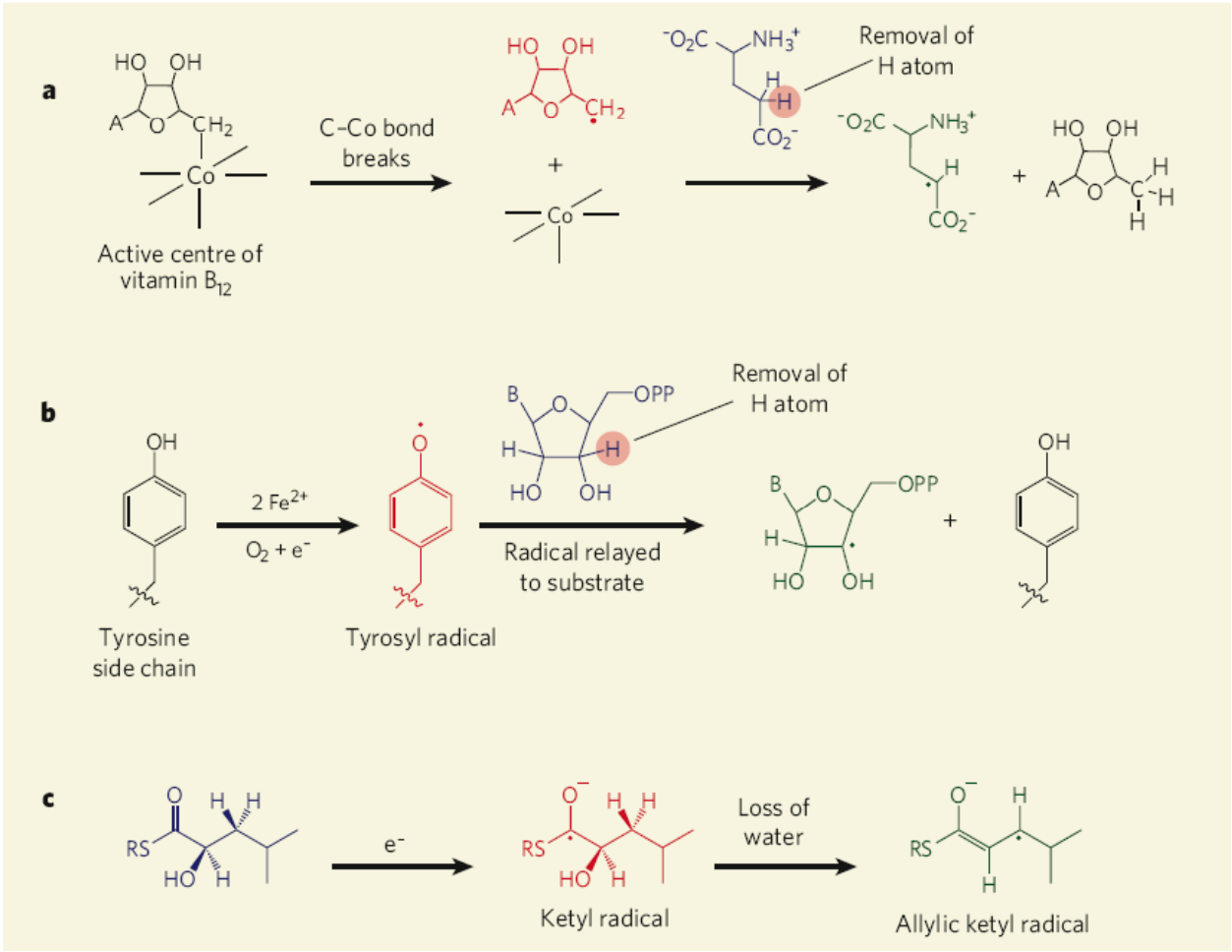

Figure 1 | Enzymatic conversion of organic molecules into radicals. Enzyme mechanisms that involve radicals are relatively rare. **a**, Some enzymes require vitamin B_{12} as a cofactor. Vitamin B_{12} contains a weak carbon–cobalt bond that breaks apart to form an organic radical (red). This in turn converts the enzyme substrate (blue) into a radical (green) by removing a hydrogen atom, as in this example. Co represents a cobalt atom; A is the DNA base adenine. **b**, In the enzyme ribonucleotide reductase, the side chain of a tyrosine amino acid is converted into a tyrosyl radical (red) in a reaction involving oxygen and electrons (e^-) from two iron ions (Fe^{2+}). The radical is relayed to the substrate, which is oxidized to another radical (green) by the removal of a hydrogen atom. B represents an RNA base; OPP is a diphosphate group. **c**, Kim *et al.*[1] report a previously undiscovered radical mechanism in the enzyme 2-hydroxyisocaproyl-CoA dehydratase. The enzyme adds an electron directly to the substrate (blue) in a reduction process, forming a ketyl radical (red). The ketyl radical then loses hydrogen and oxygen atoms as a water molecule, forming an allylic ketyl radical (green). This is the first example of a radical enzyme mechanism in which the substrate is reduced, rather than oxidized. R represents coenzyme A, a common cofactor.

Summary

✓ Free radicals in biology represent a large variety of species which range from very reactive and short-lived to stable and long-lived molecular species

✓ While many radicals tend to be oxidizing species, there are several biological examples of radicals that behave as good one-electron reductants

✓ Radicals and oxidants are not quite the same; moreover, important biological oxidants such as hydrogen peroxide, peroxynitrite and hypochlorous acid are not radicals and participate in two-electron oxidation reactions

✓ Some radicals such as nitric oxide are mainly formed through strictly regulated enzyme-catalyzed pathways and play roles in signal transduction processes

✓ Radicals in proteins can also assist in enzyme catalysis steps

✓ Radicals participate in chain propagation reactions and also in intramolecular electron transfer processes, therefore transmitting effects from the initial site of formation to relatively distant sites

✓ The diffusion of radicals in biological environments largely depend on molecular size and charge, hydrophobicity and reactivity; some like nitric oxide are highly diffusible; others like superoxide and hydroxyl radicals are not.

The Montevideo Bay, Uruguay