Methods to Quantify Nitric Oxide *en vivo*:

Concepts and Considerations

LSU
Health Sciences Center

HOT ΣΤΥΦΦ!
INSTITUTE FOR
LOUISIANA STATE
FREE RADICAL
UNIVERSITY
BIOLOGY AND MEDICINE
12 fl oz (355 mL)

Atmospheric Nitrogen Oxides

Nitric Oxide

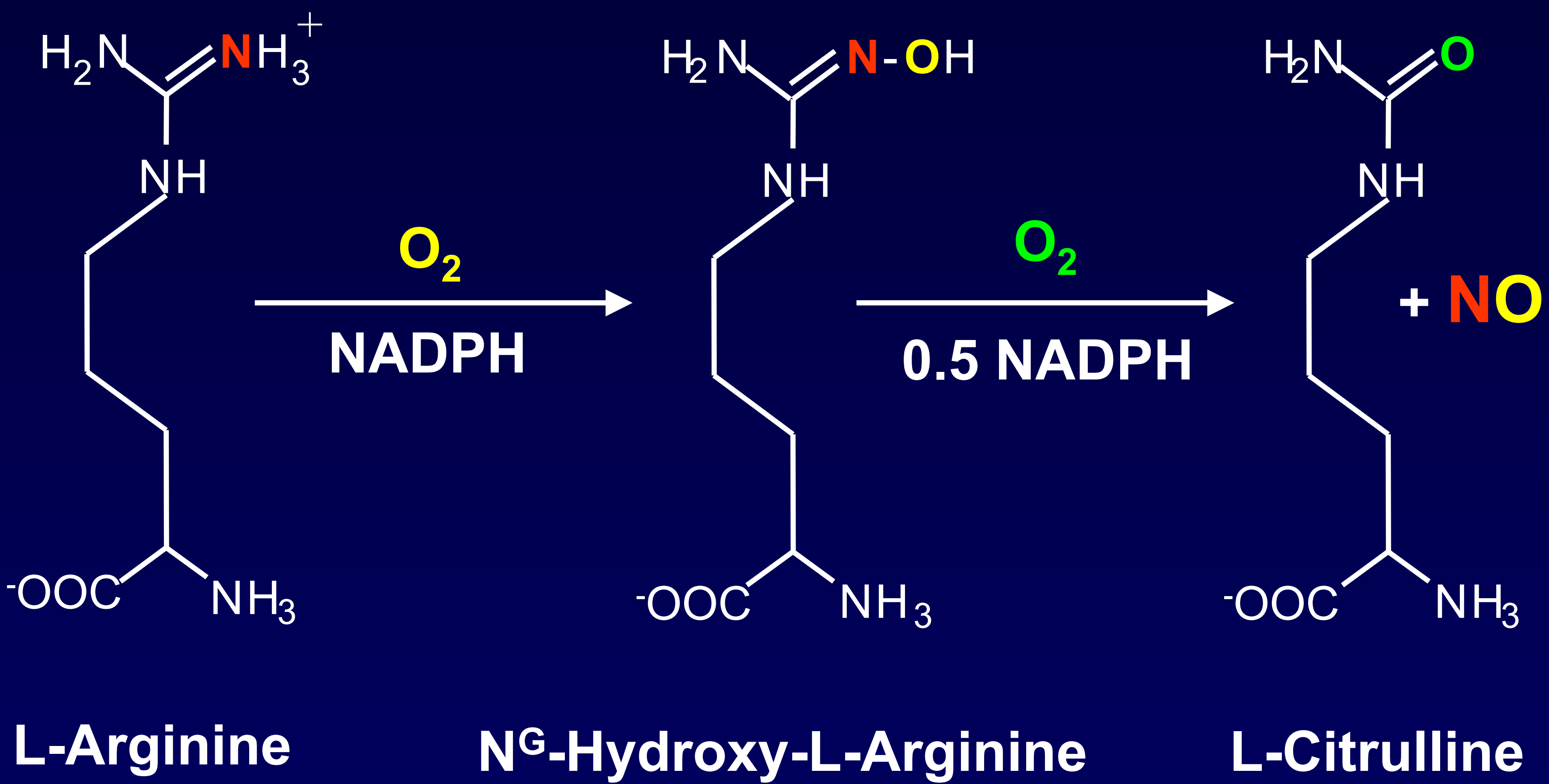
$\cdot N \equiv O$

Colorless Gas

Free Radical

Potent Vasodilator (EDRF)

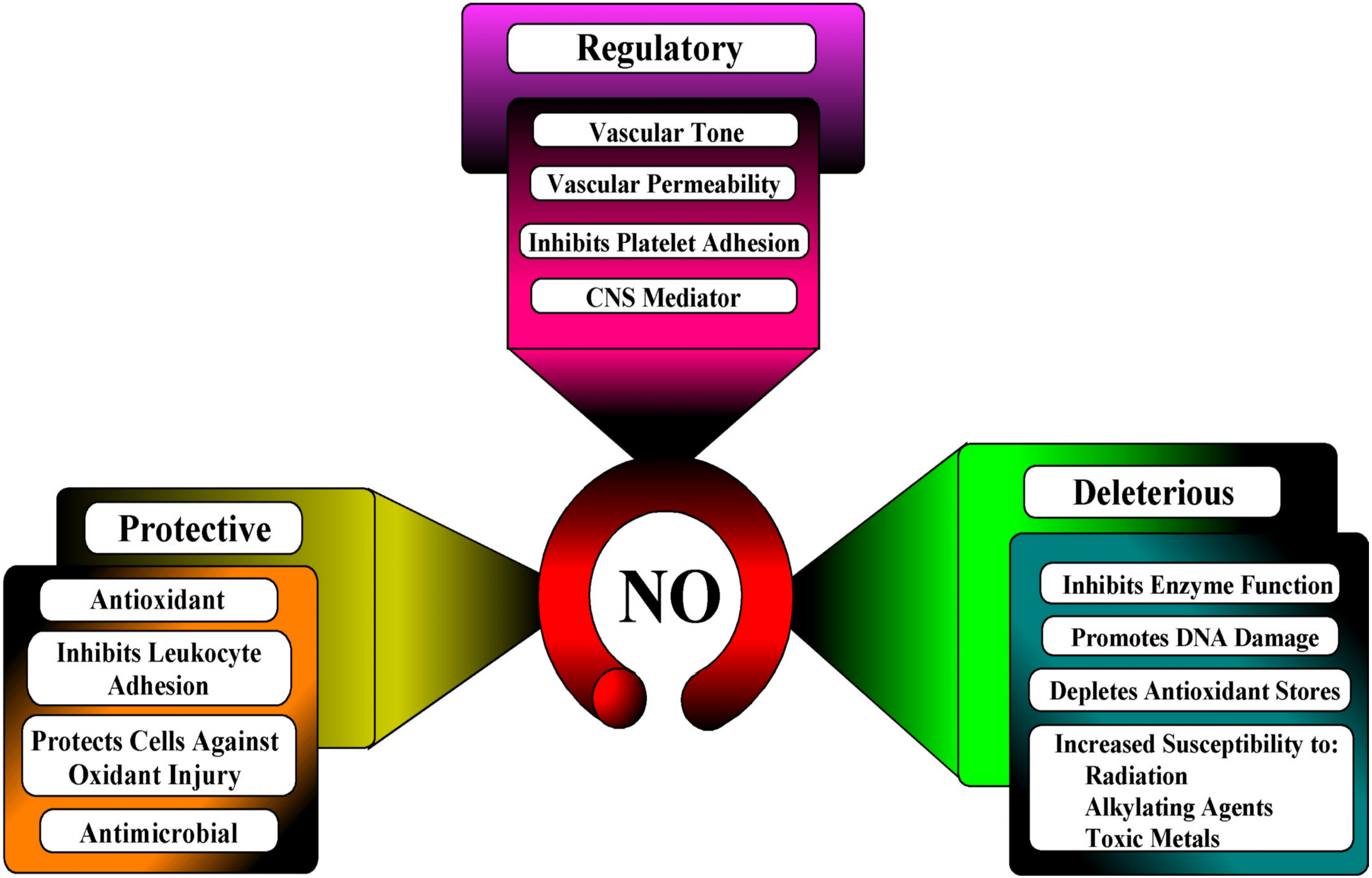
NO Production by Nitric Oxide Synthase

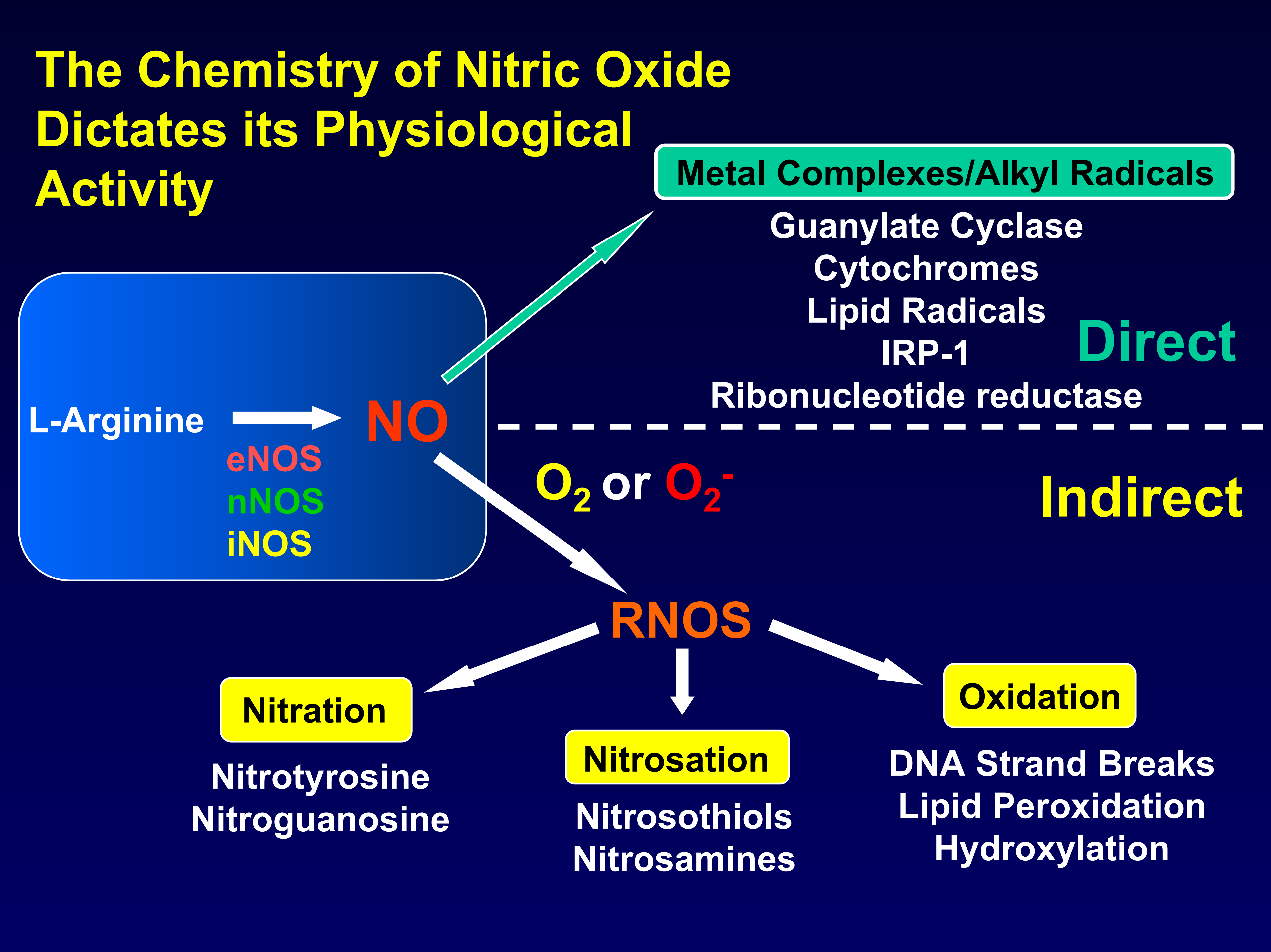


Nitric Oxide Synthase (NOS) Isoenzymes

- NOS
  - Constitutive
    - endothelial NOS (eNOS; NOS 3)
    - neuronal NOS (nNOS; NOS 1)
  - Inducible
    - inducible NOS (iNOS; NOS 2)

Nitric Oxide is a Pleiotropic Regulator of the Immune, Cardiovascular and Nervous Systems




The Chemistry of Nitric Oxide Dictates its Physiological Activity
Metal Complexes/Alkyl Radicals
Guanylate Cyclase
Cytochromes
Lipid Radicals
IRP-1
Ribonucleotide reductase
Direct
L-Arginine
NO
eNOS
nNOS
iNOS
O_2 or O_2^-
Indirect
RNOS
Nitration
Nitrotyrosine
Nitroguanosine
Nitrosation
Nitrosothiols
Nitrosamines
Oxidation
DNA Strand Breaks
Lipid Peroxidation
Hydroxylation

Methods to Quantify NO and its Metabolites in Extracellular Fluids and/or Tissue

- Gas Phase Chemiluminescence (NO)

- Spectrophotometric Assays for Oxidized Metabolites of NO (Nitrite/Nitrate)
  - Griess Assay
  - Fluorescence Assay
  - DAF-2 Bio-imaging

- Spectrophotometric Assays for RSNOs
  - Saville Assay
  - Modified Saville Assay

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NO Detection by Gas Phase Chemiluminescence

Detection Principle:

NO is purged from an aqueous solution using an innert gas such as Ar or He and transferred to a mixing chamber where it reacts with O_3 under reduced pressure.

$$NO + O_3 \longrightarrow NO_2^* + O_2$$

$$NO_2^* \longrightarrow NO_2 + h \cdot \upsilon$$

The light emitted by excited NO_2 upon returning to the ground state is measured by photon counting *(fmol-pmol).* Not very useful when attempting to quantify NO in physiological fluids such as serum, plasma or urine. *Why?*

Autoxidation of NO

$$2NO + O_2 \longrightarrow 2NO_2$$

$$2NO + 2NO_2 \longrightarrow 2N_2O_3$$

$$2N_2O_3 + H_2O \longrightarrow 4NO_2^- + 2H^+$$

$$4NO + O_2 + 2H_2O \longrightarrow 4NO_2^- + 4H^+$$

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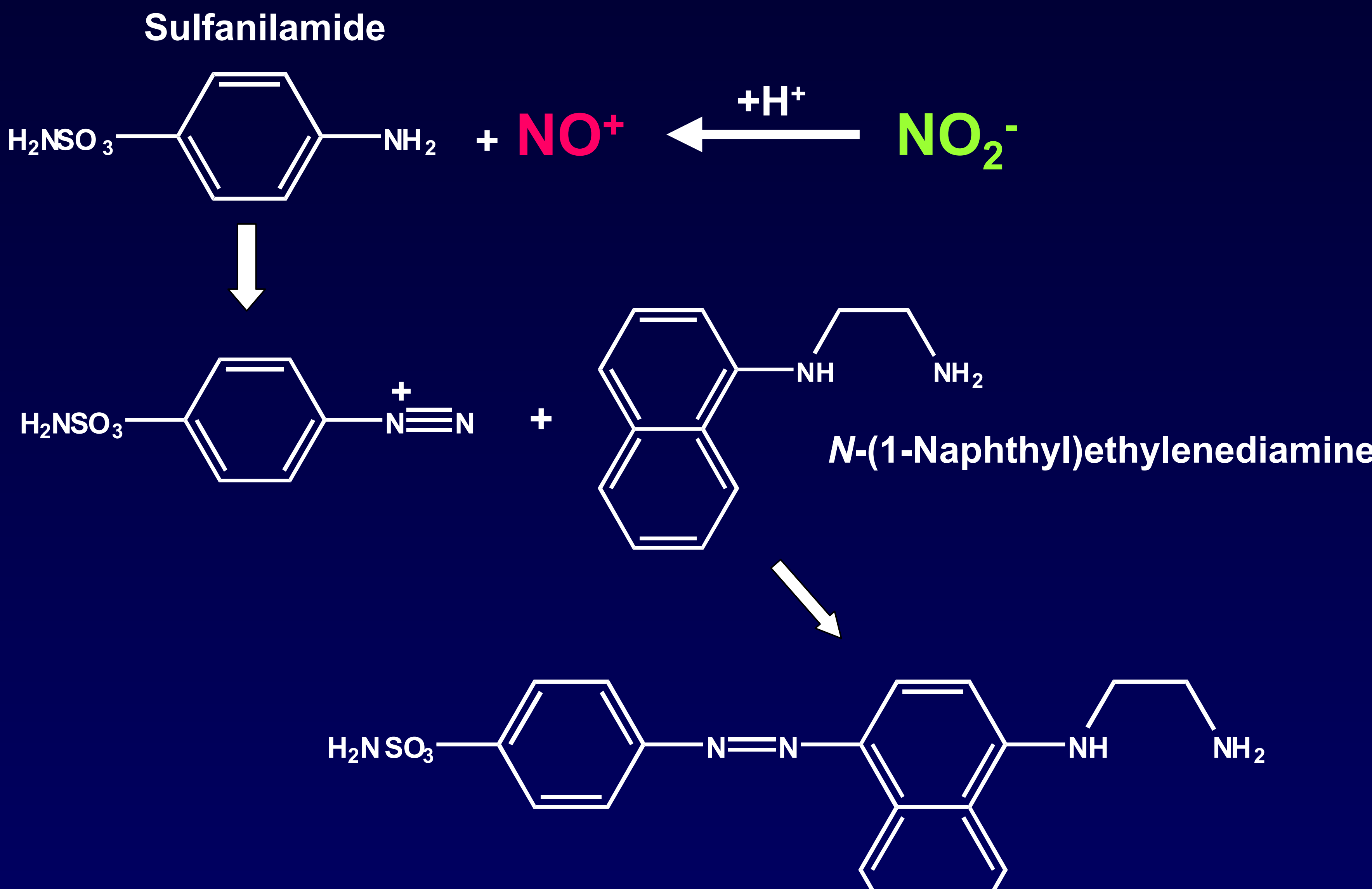
Griess Reaction



Azo Dye (λ_{max} = 540 nm)

Although *Nitrite* is Produced from NO Autoxidation, *Nitrate* is the Major NO-Derived Metabolite in Plasma and Urine:

Role of *Hemoglobin*

$$2Hb\text{-}Fe^{+2}O_2 + 3NO_2^- + 2H^+ \longrightarrow 2Hb\text{-}Fe^{+3} + 3NO_3^- + H_2O$$

$$4Hb\text{-}Fe^{+2}O_2 + 4NO_2^- + 4H^+ \longrightarrow 4Hb\text{-}Fe^{+3} + 4NO_3^- + O_2 + 2H_2O$$

$$Hb\text{-}Fe^{+2}O_2 + NO \longrightarrow Hb\text{-}Fe^{+3} + NO_3^-$$

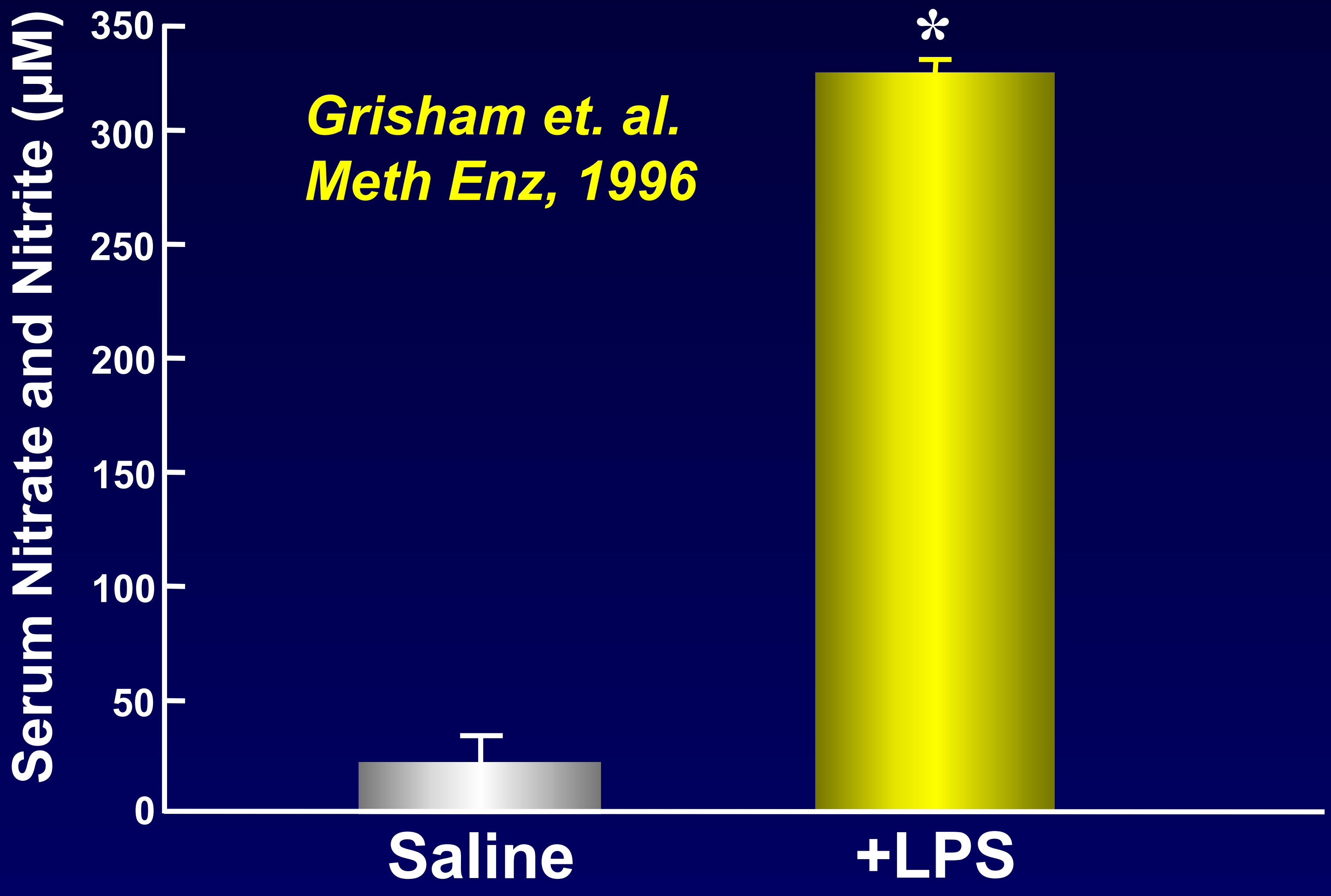
Quantification of NO-Derived NO_3^- and NO_2^- in Extracellular Fluids

$$NO_3^- + FAD + NADPH \xrightarrow{NR} NO_2^- + NADP^+$$

- Nitrate Reductase (NR) Converts all NO_3^- to NO_2^-
- All *unreacted* NADPH must be oxidized
- NO_2^- quantified by Griess Reaction

Grisham, M.B. et.al Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol* 1996 268:237-246.

LPS Induces Nitric Oxide Production *In Vivo*



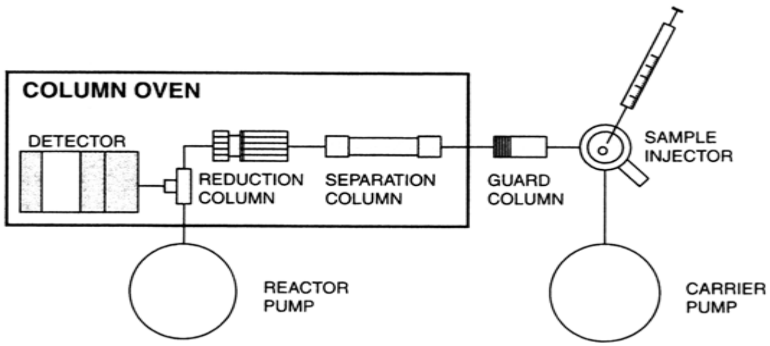
Measurement of NO_2^-/NO_3^- in Plasma Using the Griess Reagent: *Problems and Considerations*

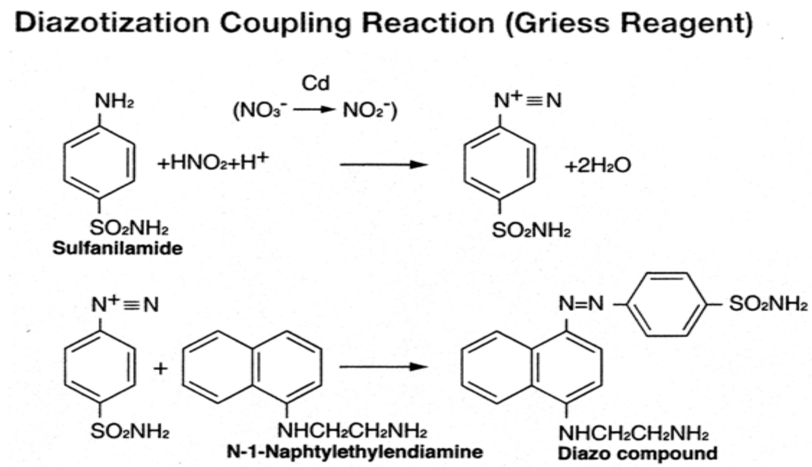
- ***Heparin*** may produce precipitant upon addition of Griess Reagent. Addition of protamine sulfate will remove heparin (*Suggestion: Use serum or calcium chelators such as EDTA or citrate*).

- NO_2^-/NO_3^- may be underestimated in ***hemolyzed plasma or Serum*** due to Hb-catalyzed oxidation of NO_2^- (*Suggestion: Ultrafilterplasma or serum to obtain a low molecular weight fraction; < 15,000).*

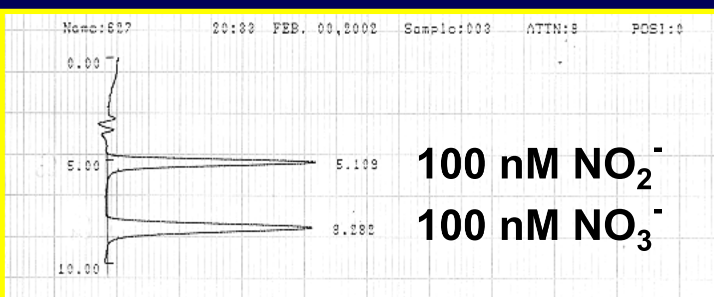
- The presence of some ***plasma or serum-associated proteins*** may inhibit nitrate reductase (*Suggestion: Ultrafilter plasma or serum to obtain a low molecular weight fraction*).

Simultaneous Quantification of Nitrite and Nitrate by HPLC Using the Griess Reaction





Rodriguez and Feelisch
PNAS 100:336, 2003



<u>Sensitivity:</u>

1 pmol/ml for either anion (injection volume 100µl)

No interference by proteins or colored species

Sources of NO_2^-/NO_3^- in Extracellular Fluids (Blood, Lymph, Urine, Saliva):

- NO
- Bacteria (Enteric; Oral)
- Diet *(need for fasting or nitrate/ nitrite-free chow)*

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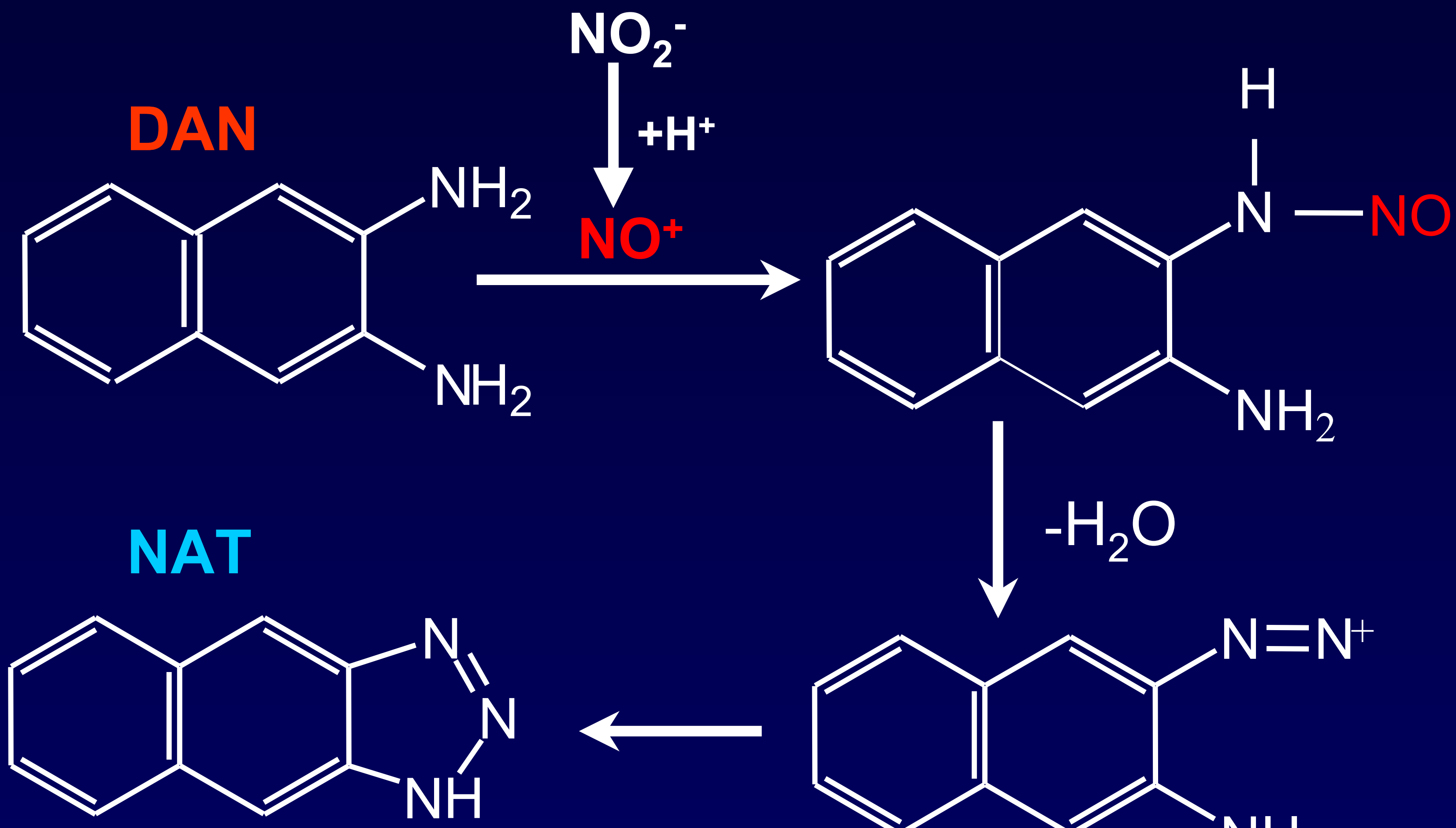
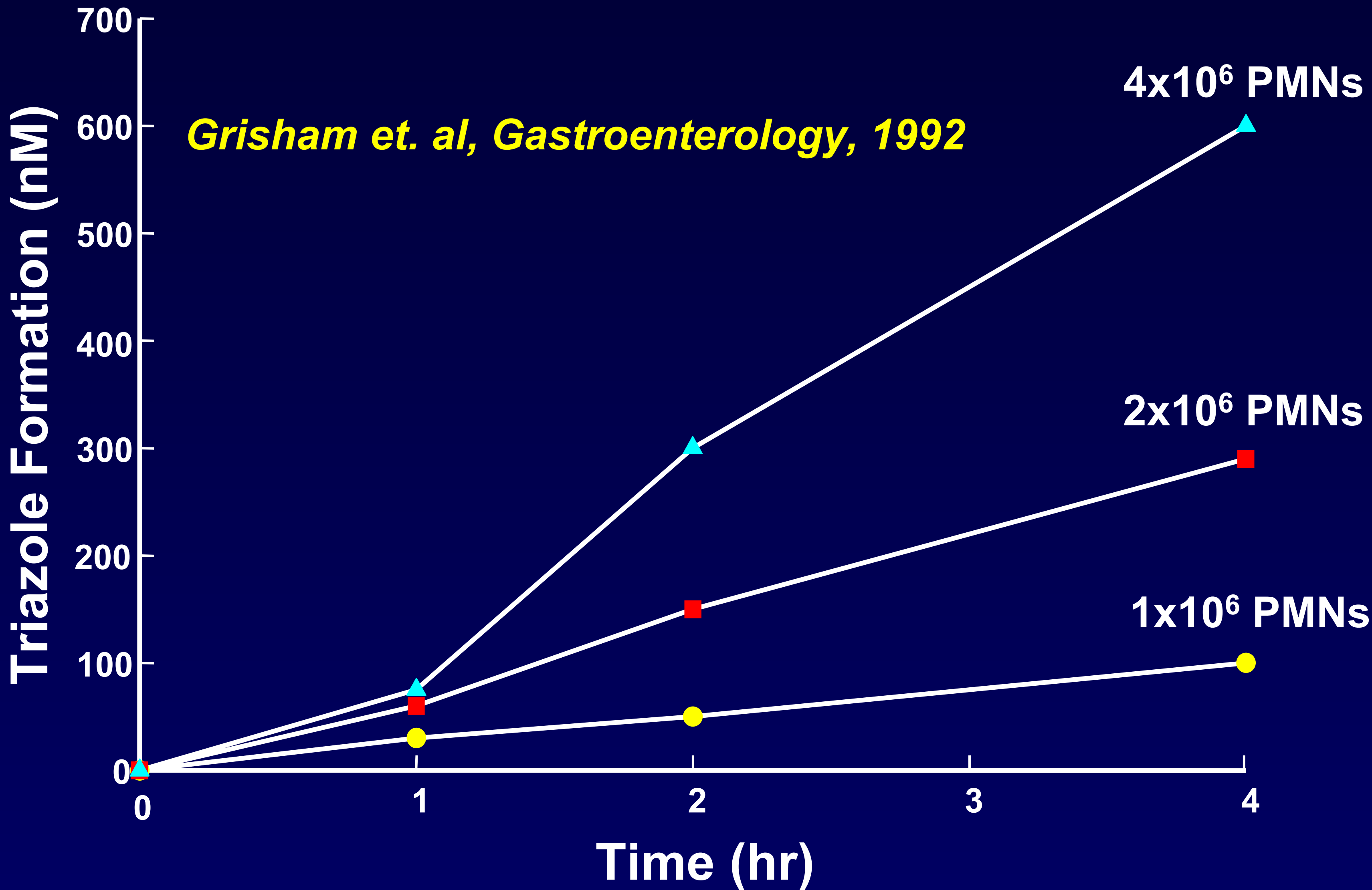
N-nitrosation of 2,3 diaminonaphthalene (DAN) to yield 2,3-naphthotriazole (NAT)



λ_{ex} = 375nm
λ_{em} = 415nm

N-Nitrosation of DAN By Extravasated PMNs



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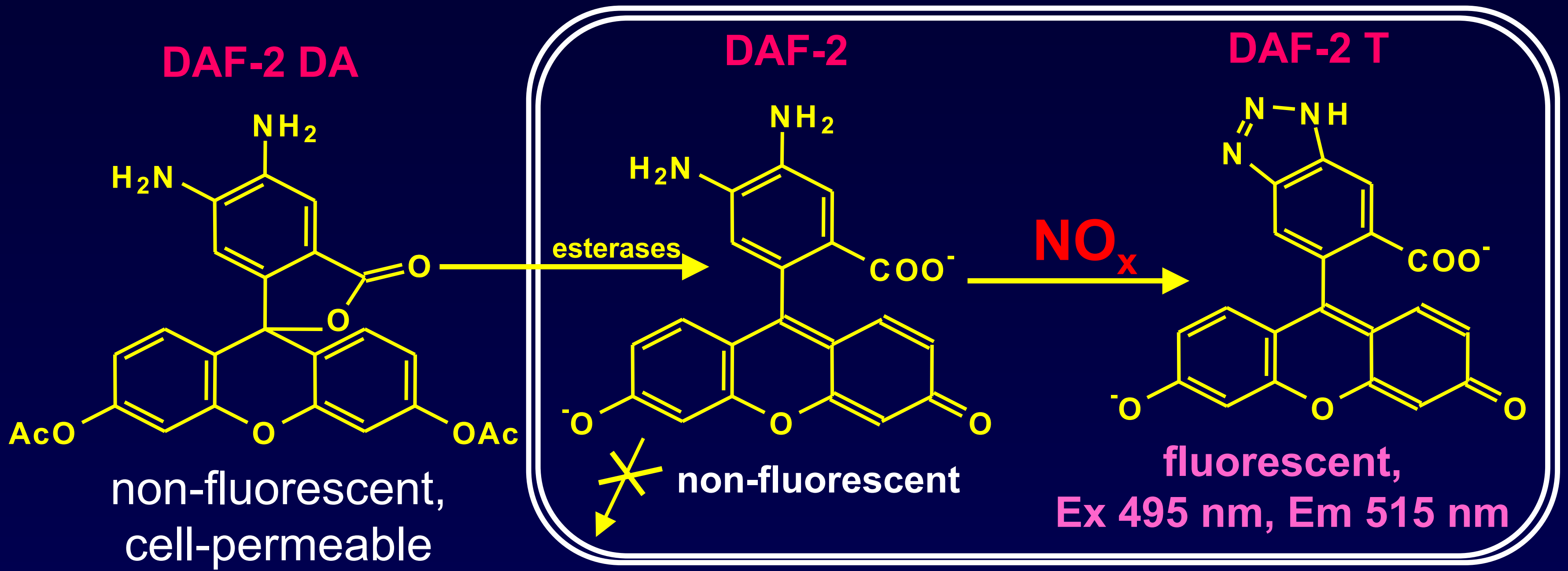
Bioimaging of Nitric Oxide Using Diaminofluoresceine-2 (DAF-2)

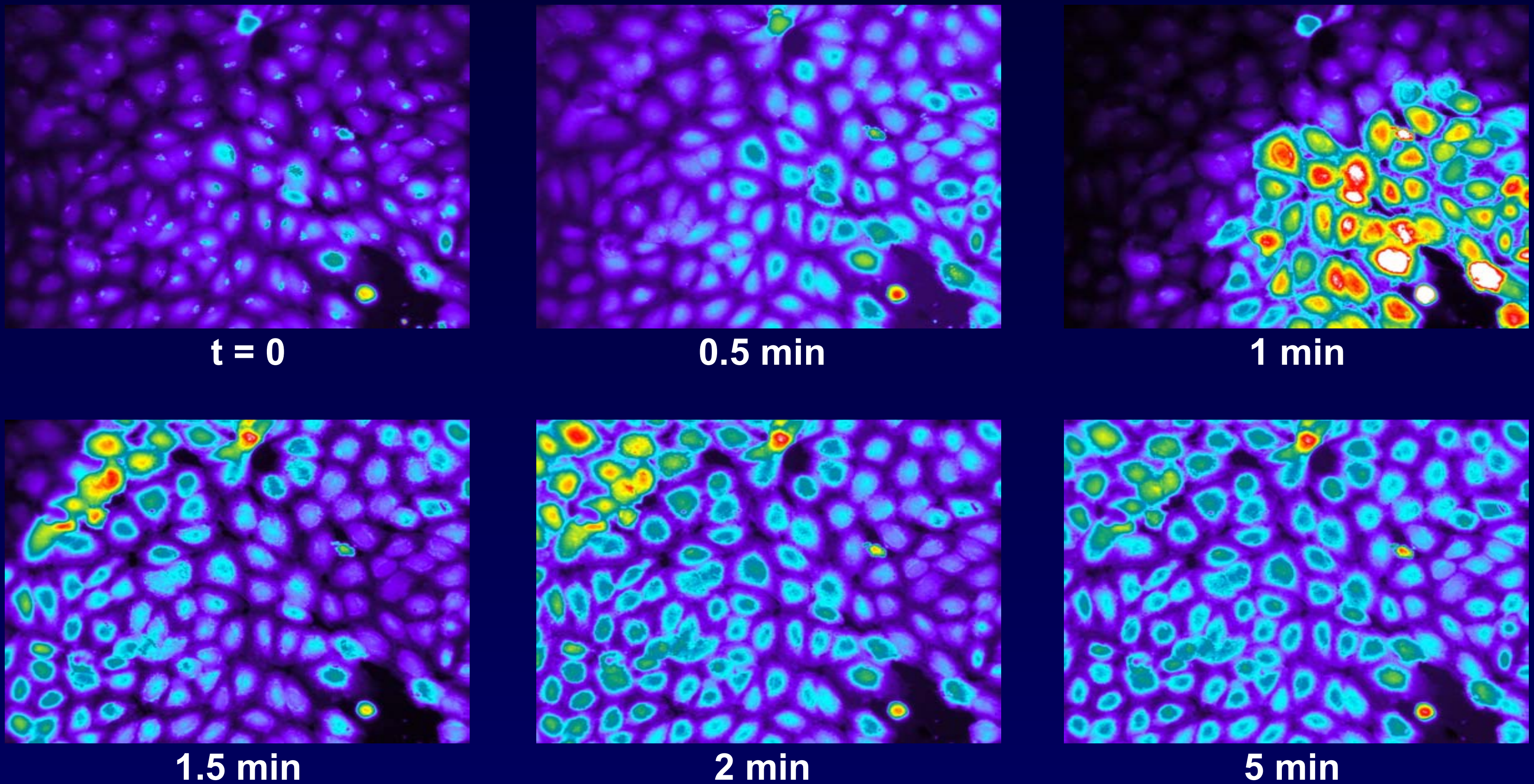


Advantages: **Sensitivity for NO (5 nM in vitro) with high temporal and spatial resolution.**
No cross-reactivity to NO_2^-/NO_3^- and $ONOO^-$

Kojima et al., Biol.Pharm. Bull. (1997)

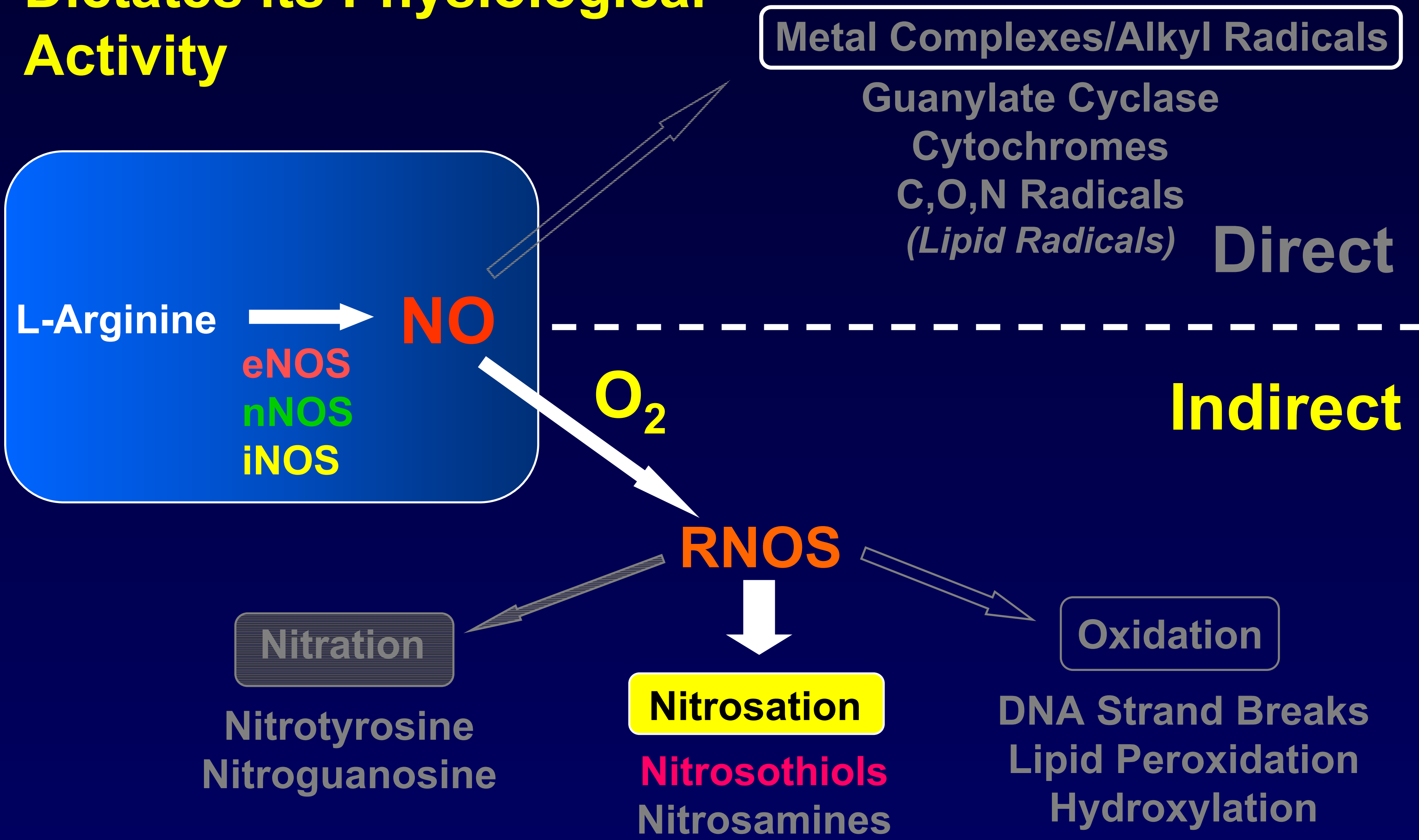
Assay limitations: **Possible interference by reducing agents and divalent cations, requires standardized illumination conditions**

Propagation of NO Wave during Stimulation of Endothelial Cells with the Calcium Ionophor, A23187 (1 μM)



Feelisch et. al. Unpublished Observations

The Chemistry of Nitric Oxide Dictates its Physiological Activity



Physiological Roles of Nitrosothiols (RSNOs)

RSNOs
S-Nitrosoglutathione (GSNO)
S-Nitrosoalbumin (AlbSNO)
S-Nitrosohemoglobin (HbSNO)

- ☑ Potent vasorelaxants.
- ☑ Antiplatelet activity.
- ☑ Antimicrobial activity.
- ☑ Regulation of vasodilation/ oxygenation (hemoglobin).
- ☑ Intermediates in the metabolism of organic nitrites and nitrates.
- ☑ Regulation of cell signaling/ protein functions.

NO-Dependent Formation of S-Nitrosothiols (RSNOs): *Large Amounts of NO*

$$2\,NO + O_2 \longrightarrow 2\,NO_2$$

$$2\,NO_2 + 2\,NO \longrightarrow 2\,N_2O_3$$

$$2\,N_2O_3 + 2\,RSH \longrightarrow 2\,\underline{\mathit{RSNO}} + 2\,NO_2^-$$

RSNOs

S-Nitrosohemoglobin (SNOHb)
S-Nitrosoglutathione (SNOGSH)
S-Nitrosoalbumin (SNOAlb)

NO-Dependent Formation of S-Nitrosothiols (RSNOs): *Physiological Levels of Oxygen, NO and RSH*

$$2\,NO + O_2 \longrightarrow 2\,NO_2$$

$$2\,NO_2 + 2\,RSH \longrightarrow 2\,NO_2^- + 2\,RS\bullet + 2H^+$$

$$2\,NO + 2\,RS\bullet \longrightarrow 2\,RSNO$$

RSNOs

S-Nitrosohemoglobin (SNOHb)

S-Nitrosoglutathione (SNOGSH)

S-Nitrosoalbumin (SNOAlb)

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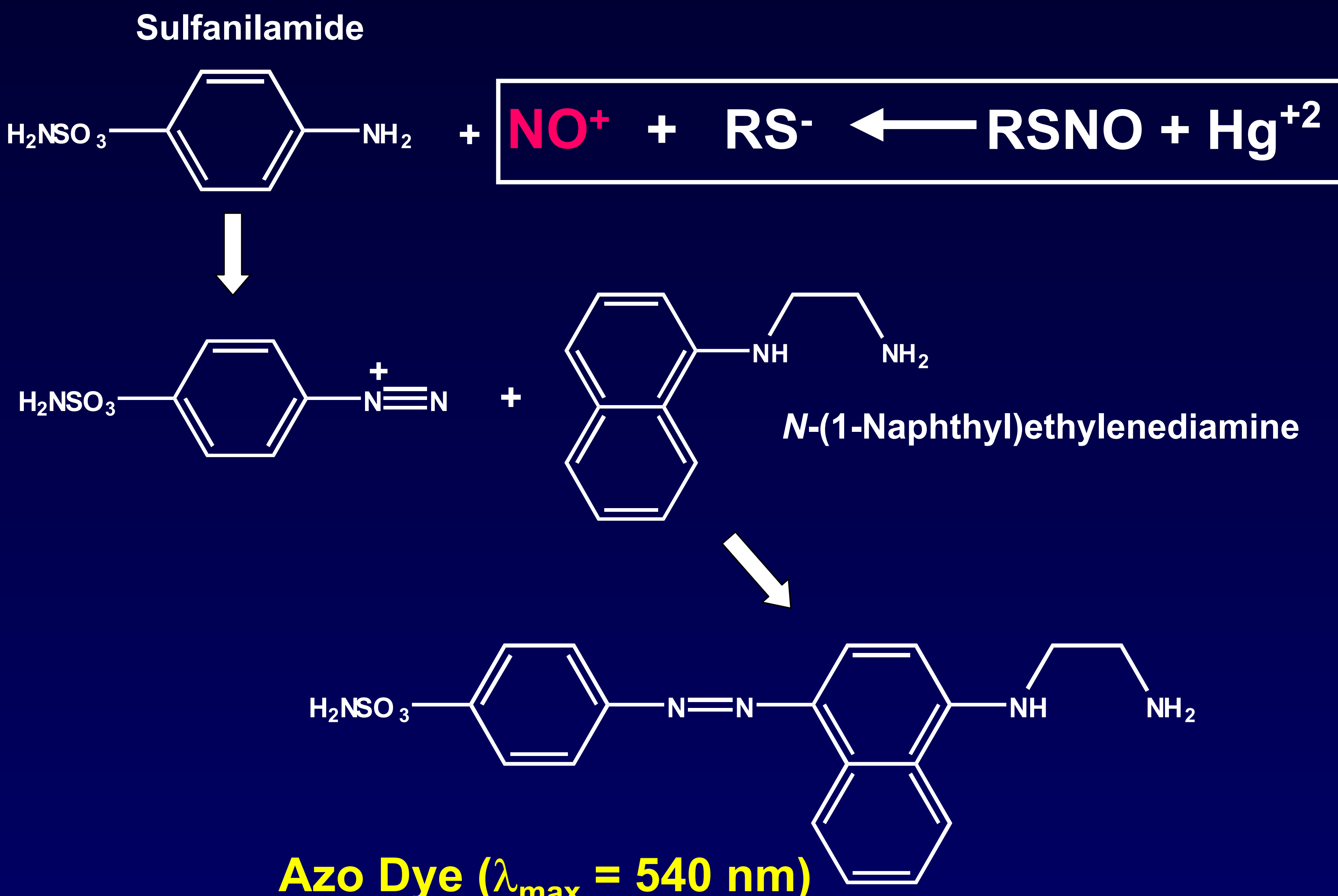
Saville Reaction

$$RSNO + Hg^{+2} \longrightarrow RS^{-} + Hg^{+3} + NO^{+}$$

Saville/Griess Reaction



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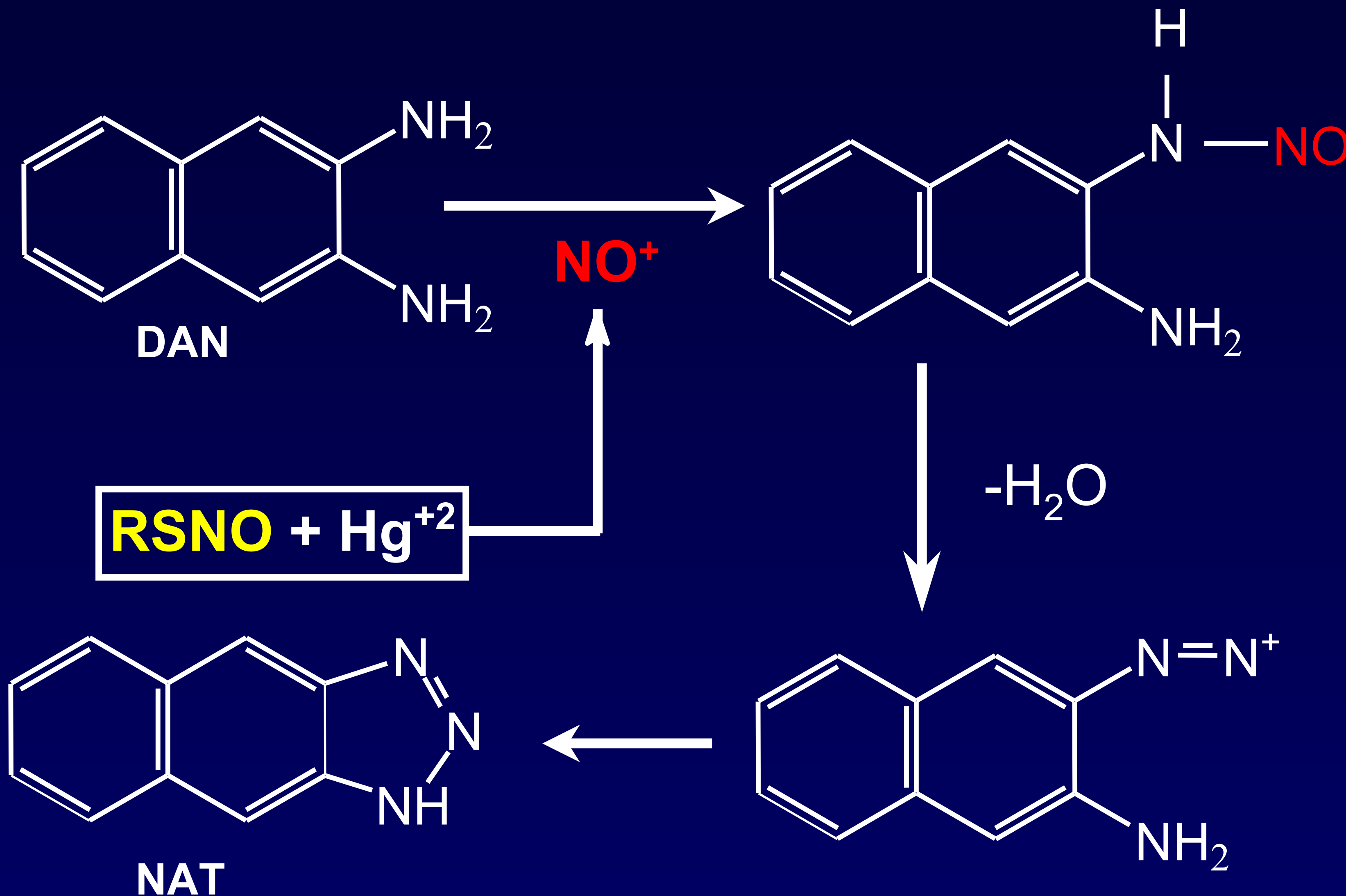
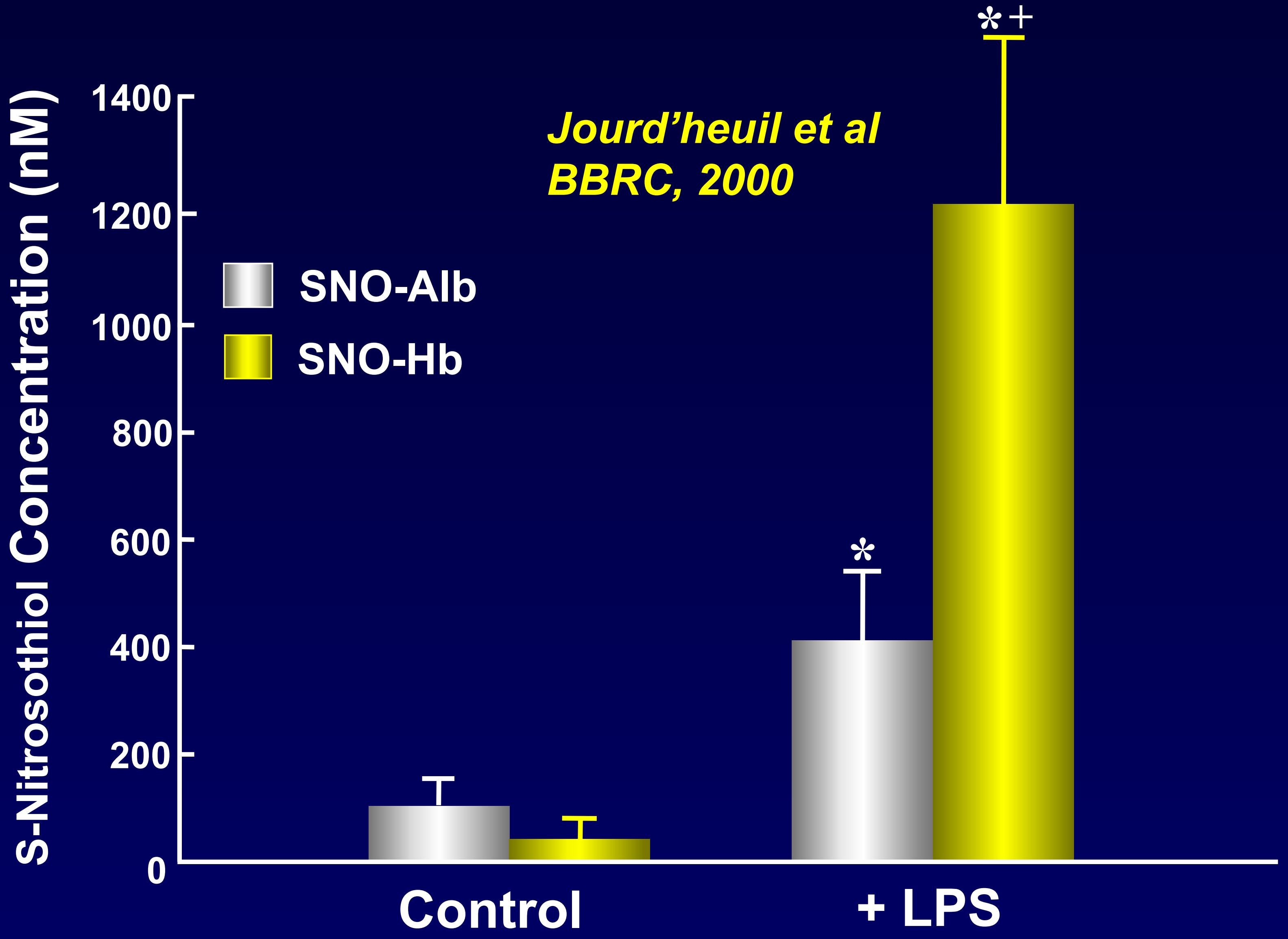
Fluorometric Determination of S-Nitrosothiols (RSNO)



S-Nitrosothiol Formation in Blood of LPS-Treated Rats



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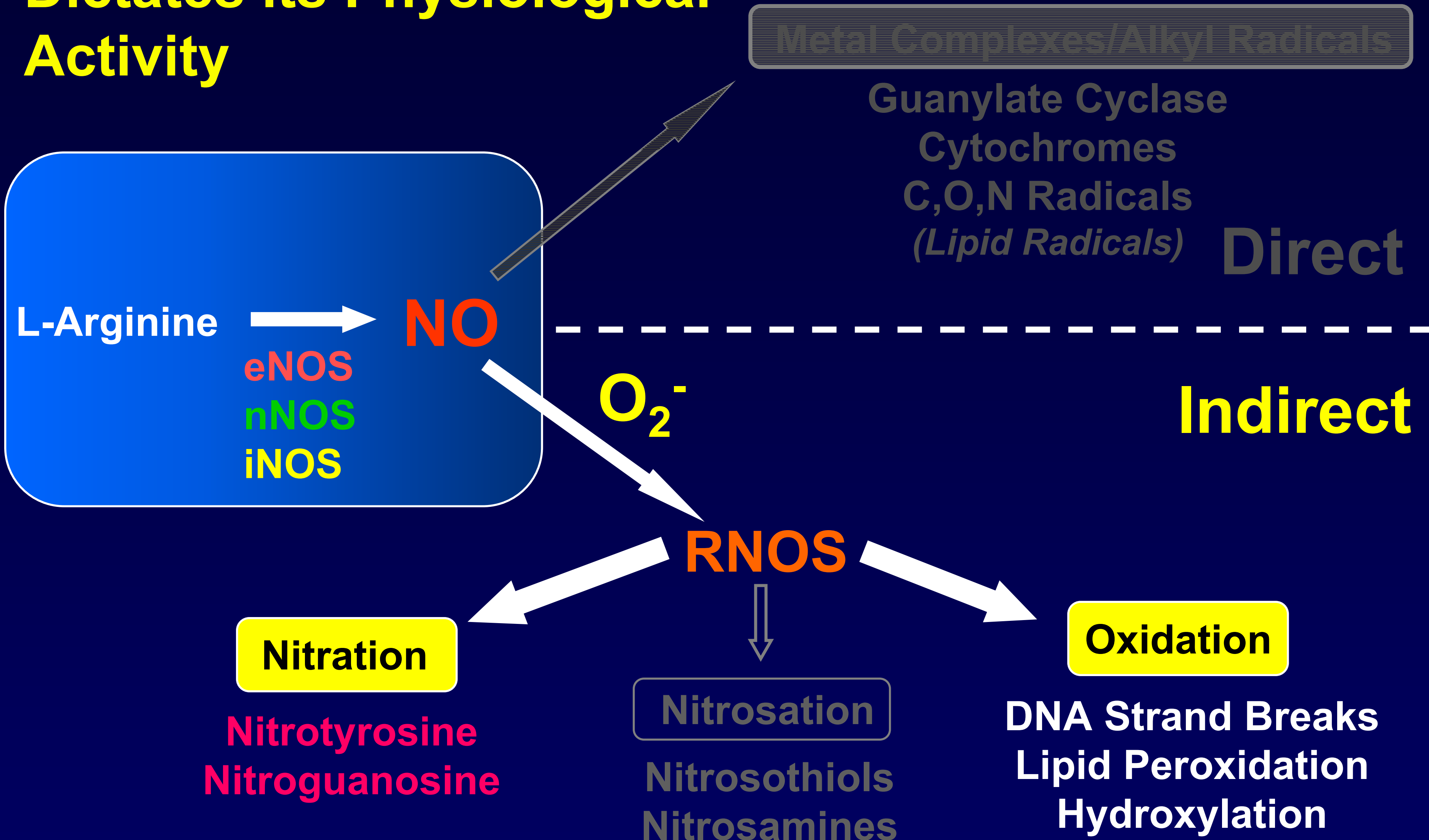


Interaction Between Superoxide and Nitric Oxide: Formation of Peroxynitrite/Peroxynitrous Acid

$$O_2^- + NO \overset{+\,H^+}{\rightleftharpoons} ONOO^-$$

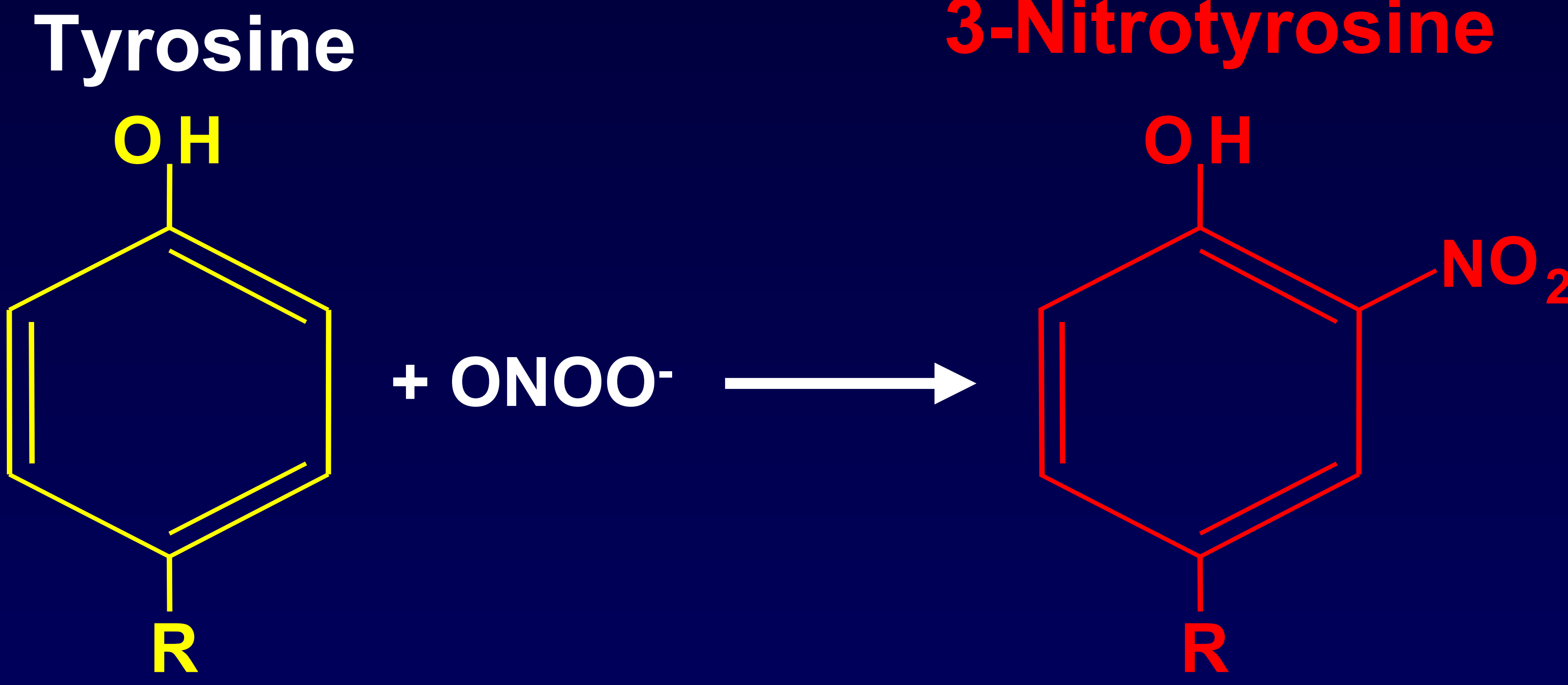
$$ONOO^- + H^+ \longrightarrow ONOOH$$

$$ONOOH \longrightarrow ONOOH^* \text{ (“}OH\cdot + NO_2\cdot\text{”)}$$

$$ONOOH^* \longrightarrow NO_3^- + H^+$$

Sum: $O_2^- + NO \longrightarrow NO_3^-$

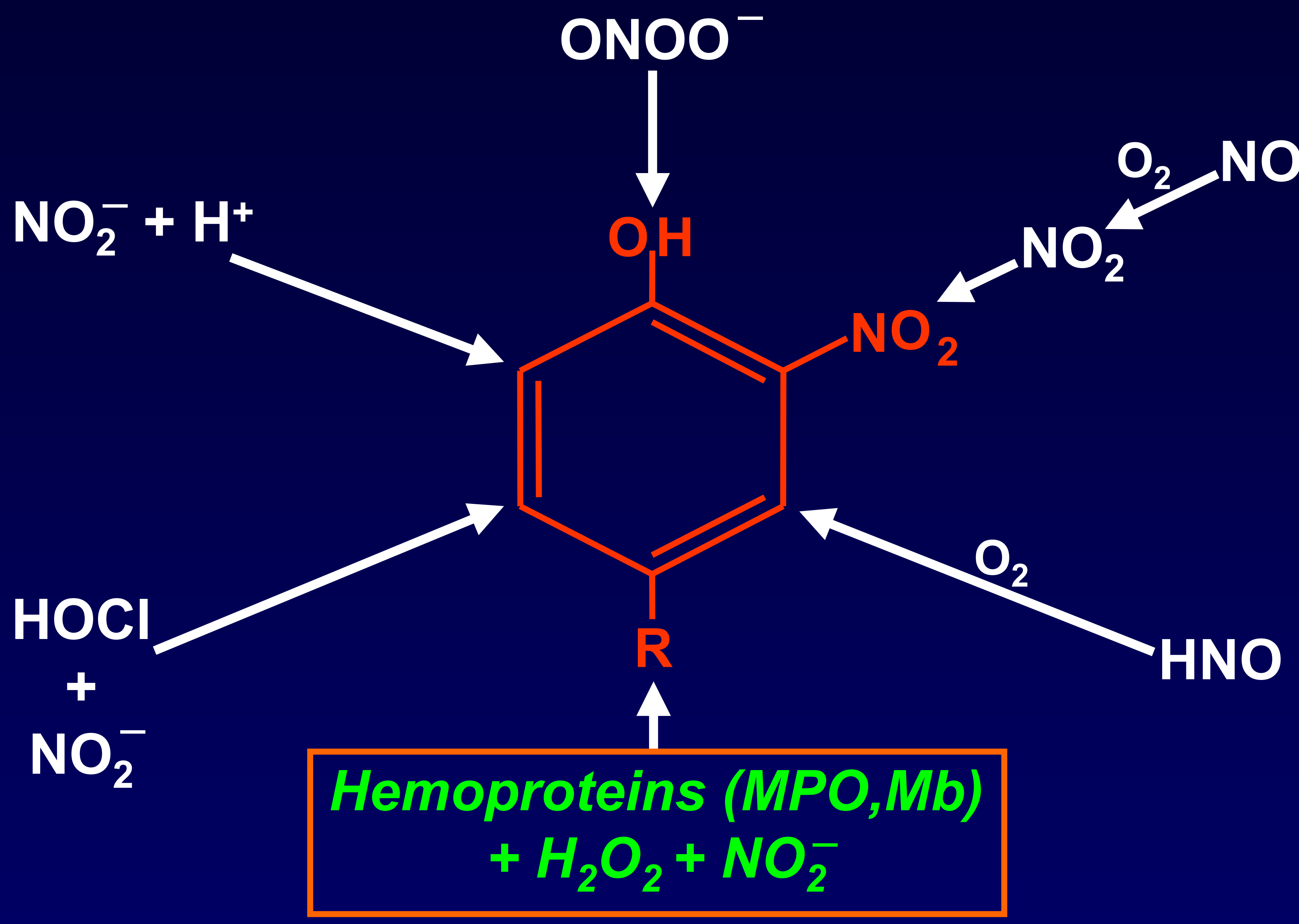
Peroxynitrite Nitrates Tyrosine to Yield 3-Nitrotyrosine



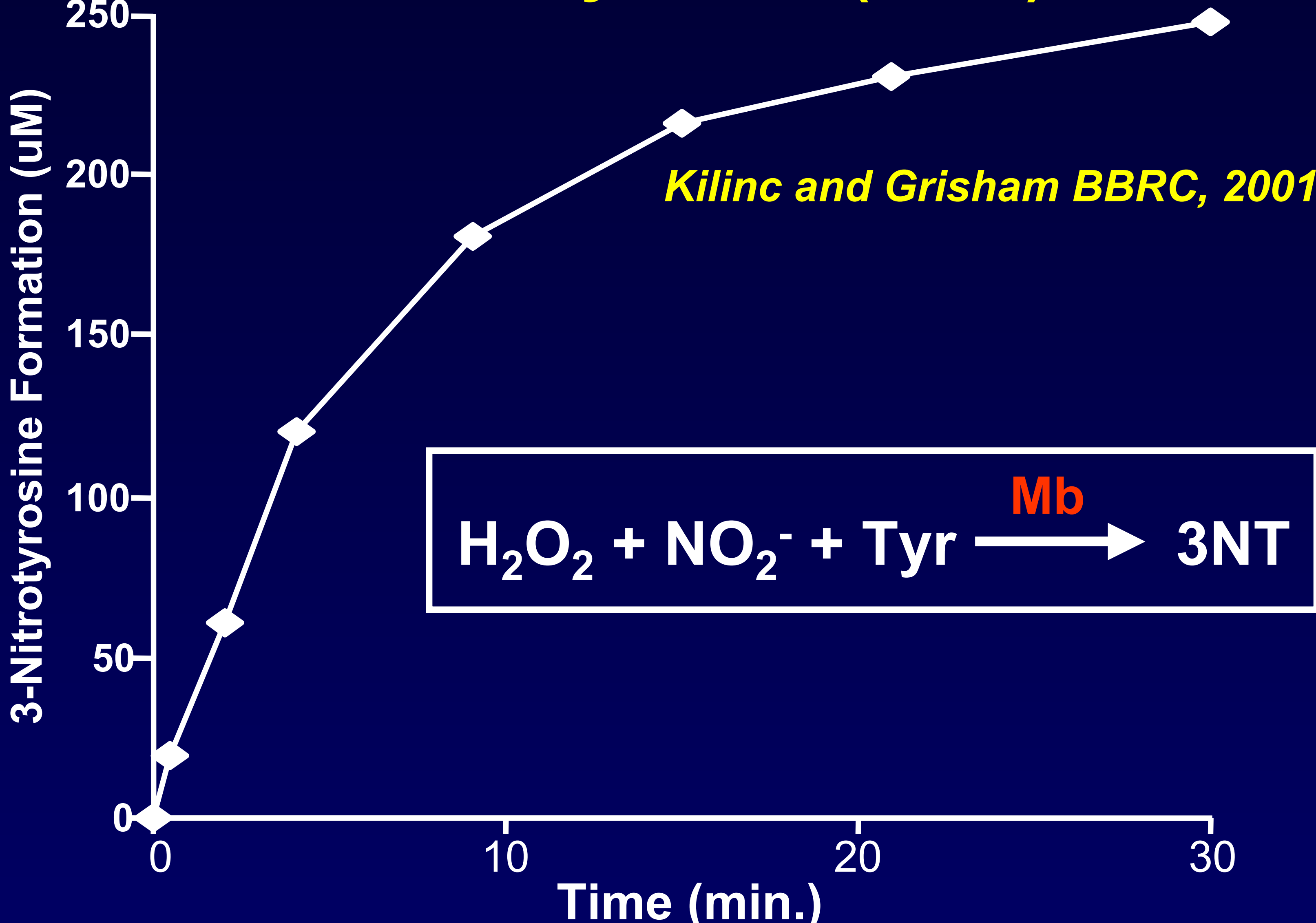
3-Nitrotyrosine Formation <u>in vivo</u>:

Specific Footprint for Peroxynitrite?

Generation of 3-Nitrotyrosine



Myoglobin-Catalyzed Formation of 3-Nitrotyrosine (3-NT)



LSU Health Sciences Center

Martin Feelisch (NitroMed)
Steve Laroux (Harvard)
Kamer Kilinc (Ankara Univ)

Albany College of Medicine

David Jourd'heuil

Grambling State University

Allen M. Miles

National Cancer Institute

David A. Wink
Mike Espey
Katrina Miranda (Univ Arizona)